別添１

厚生労働行政推進調査事業費補助金

化学物質リスク研究事業

OECDプロジェクトでの成果物を
厚生労働行政に反映させるための研究
(21KD2003)

令和３年度　総括・分担研究報告書

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令和４(2022)年３月

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令和３年度総括研究報告書

OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

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研究要旨

本研究の目的は、化学物質やその混合物の安全性を評価するための国際的な合意を推進する経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）の試験法ガイドライン（TG: Test Guideline）プログラム各国調整官作業グループ（WNT: Working Group of National Co-ordinators of the TGs programme）において、日本で開発された種々の TG やガイダンス文書（GD: Guidance Document）、毒性発現経路（AOP : Adverse Outcome Pathway）などの世界各国が必要とする成果物を公定化させるとともに、他国が提案する OECD 大型プロジェクトに関与し、その成果物に日本の主張を反映させ、これらから得られた成果を化学物質の審査及び製造等の規制に関する法律（化審法）や毒物及び劇物取締法（毒劇法）などの我が国の厚生労働行政に反映させることを目的とする。

過去の研究班の成果として、我が国で開発された腐食性試験代替法、皮膚感作性試験代替法、光毒性試験代替法、内分泌かく乱性スクリーニング法などに関する TG の成立、免疫毒性の AOP 成立などに寄与し、非遺伝毒性発がんの試験の実施と評価のための戦略的統合方式（IATA: Integrated Approaches to Testing and Assessment）や皮膚感作性の確定方式（DA: Defined Approach）の開発に協力してきた。

本研究班では、これまでの成果を生かし、免疫毒性や生殖毒性試験の総説（DRP: Detailed Review Paper）及び非遺伝毒性発がんや光毒性の IATA などの GD の公定化に尽力する。本年度、AOP に関しては、「カルシニューリン阻害による T 細胞依存的抗体産生抑制：AOP154」が OECD にて正式に承認された。TG に関しては、既存の TG である皮膚感作性試験代替法 ADRA（Amino acid Derivative Reactivity Assay）を含む TG442C の改定をなすことができた。同時に承認された Defined Approach for Skin Sensitisation ガイドライン 497 の開発にも寄与した。

上記した OECD で検討されている皮膚感作性の確定方式（Defined Approach）や発達神経毒性に関する大型プロジェクト等に参画して、成果物に日本の意見や結果を反映させた。この目的を果たすため、TG や AOP それらに必要な補足実験データを取得するとともに、日本から OECD に提出する資料を事前に相互確認し、また、OECD からの提案資料への意見募集に適切な意見を返した。

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A. 研究目的

本研究の目的は、化学物質やその混合物の安全性を評価するための国際的な合意を推進する経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）の試験法ガイドライン（TG：Test Guideline）プログラム各国調整官作業グループ（WNT: Working Group of National Co-ordinators of the TGs programme）において、日本で開発された種々のTGやガイダンス文書（GD：Guidance Document）、毒性発現経路（AOP : Adverse Outcome Pathway）などの世界各国が必要とする成果物を公定化させるとともに、他国が提案するOECD 大型プロジェクトに関与し、その成果物に日本の主張を反映させ、これらから得られた成果を化学物質の審査及び製造等の規制に関する法律（化審法）や毒物及び劇物取締法（毒劇法）などの我が国の厚生労働行政に反映させることを目的とする。

B. 研究方法

B.1. AOP の開発

分担研究者の小島は、EAGMST（Extended Advisory Group on Molecular Screening and Toxicogenomics）で行われているOECDのAOP開発プロジェクトの進捗に合わせ、班員を支援した。

B.1.1. 免疫毒性の AOP

研究分担者の小島と足利は、日本免疫毒性学会会員をメンバーとする同学会試験法委員会、AOP 検討小委員会に免疫毒性 AOP154, AOP313, AOP314, AOP315 およびAOP277の開発を委託している。文献調査の結果に基づいて、MIE（Molecular Initiating Event）、AO（Adverse Outcome）及

びその間に介在するKE (Key Event)を定めて、OECD に指定された外部(または scientific)評価者及びコーチの指摘事項に対応することで開発を進めた。

B.1.2. 発がん性の AOP

研究分担者小川は、研究協力者西川の協力を得て、ホルムアルデヒド誘発鼻腔発がん機序に関する論文に引き続き、化学物質暴露による鼻腔発がん全般の AOP について論文化に必要な情報を収集し、解析した。ラット、マウス、ハムスターに鼻腔腫瘍を誘発する化学物質について、PubMed の文献に加えて、NTP(National Toxicology Program)、IARC(International Agency for Research on Cancer)、及び、日本バイオアッセイ研究センターのデータベースを使用して抽出し、誘発された鼻腔腫瘍について、動物種、投与経路、組織型などを分類した。また、関連する非腫瘍性病変及び遺伝毒性のデータについても抽出し、腫瘍発生経路の推定を行った。

B.1.3. 光毒性の AOP

研究分担者の尾上は、開発中の光毒性 AOP を AOP wiki に入力した。

B.2. TG 及び DRP の開発

平林と小島は、OECDのTGの開発プロジェクトWNTの進捗に合わせ、班員を支援した。

B.2.1. 皮膚感作性試験

小島は、協力研究者の笠原とともに、皮膚感作性試験代替法 *In Chemico* Skin Sensitisation、ADRA(Amino acid Derivative Reactivity Assay)の追加バリデーション報告書及び TG442C の改定案を作成した。

また、小島は協力研究者の相場とともに、IL-8 Luc assay　TG442E の改定案を作成した。

小島と足利は、Defined Approach for Skin Sensitisation のプロジェクトに参加し、ガイドラインの成立に協力した。

B.2.2. 免疫毒性試験

小島は、相場及び国際的な専門家とともに、*in vitro* 免疫毒性に関する DRP (Detailed Review Paper)を作成した。

DRP の承認を待って提出することを予定している IL-2 を指標とした免疫毒性試験の TG 案を作成した。

B.2.3. 生殖毒性試験の DRP

小島は、国際的な専門家とともに、*in vitro* 生殖発生毒性に関する DRP を作成した。

B.2.4. 発達神経毒性に起因する行動解析に関する情報収集

研究分担者の斎藤は、これまでの国内外における発達神経毒性評価の現状について情報収集を行うとともに課題を抽出した。今年度は、発達神経毒性試験における動向を知るために、米国環境保護庁(EPA: Environmental Protection Agency)及び OECD の発達神経毒性(DNT : Developmental neurotoxicity)に関する TG における、行動解析に関する情報について内容を確認し、比較を行った。加えて、PubMed を用い、発達神経毒性評価の現状について文献調査を行った。

また、JaCVAM 発達神経毒性(DNT)資料編纂委員会にオブザーバーとして参画するとともに、公定化に向けて進行中の *in vitro* DNT の DRP に対して、OECD からの

意見募集に適切な意見を返した。

B.3. IATA の開発

B.3.1. 非遺伝毒性発がん性の IATA 開発への協力

1)発がん性 IATA 開発

小川と西川は、非遺伝毒性発がん性IATA開発専門委員会のweb会議に参加し、開発方針に関する議論及び最新の評価方法に関するwebinarに参加した。全体会合に加えて、当該IATAにおける13のAssay Blockの内2つまたは3つを分担し、そのサブグループ会議にも参加し、現存の試験法の利用に関する考え方などに関する論文化に向けて検討した。

2) Bhas42 細胞形質転換試験法の TG 開発

Bhas42 細胞形質転換試験法（Bhas42CTA）は、化学物質の非遺伝毒性発がん性を遺伝毒性発がん性と区別して検出できる OECD 唯一の試験法（GD231）である。OECD では、非遺伝毒性発がん性検出を目的とした IATA 開発が 2016 年から行われている。専門委員会では mode of action（MoA）が議論され、それに基づき IATA 構築の方針が国際合意され、2020 年は専門委員会として総説論文を公表した。MoA を構成する各 KE 及びそれらに対応した 13 の Assay Block において、各種試験法の選出やその利用に関する考え方の作成及び評価を行った。

Step 1 では試験法毎にその利用に関する詳細な情報をとりまとめた考え方を作成し、Step 2 では他のメンバーが試験法の利用に関する考え方の評価案を作成した。Step 2 の評価案をもとに、Assay Block のメンバー全体で協議し、合意したものを Assay Block からの提案試験法とその評価結果としてグループ全体に提案した。

分担研究者の大森は、Block 3 の“Cell Transformation”と Block 4 の“Gap Junction”を担当し、Block 3 では Bhas 42 CTA のアッセイの利用に関する考え方を作成し、Balb 3T3 CTA の評価を担当した。また、Block 4 では、Dye Transfer Assay と Inhibition of Metabolic Co-operation Assay を提案するとともに、Metabolic Cooperation Assay with（HGPRT+&-）V79 Cells（using 6-TG）の利用に関する考え方の作成及び代謝活性化に関与しないアッセイについて評価案を作成した。

B.3.2. 光毒性 IATA

尾上と小島は、JaCVAM 資料編纂委員会の協力を得て、光毒性 IATA を作成した。

B.4. AOP 及び TG の実験データ支援

B.4.1. 光毒性

1) ROS アッセイ

尾上らが既に公表している ROS アッセイ推奨プロトコルに基づき、acridine（ACD）、furosemide（FSM）、hexachlorophene（HCP）、8-methoxypsoralen（MOP）、norfloxacin（NFX）及び promethazine（PMZ）について ROS アッセイを行った。

2) *In vitro* 皮膚内動態実験

上記 5 種の被験物質について、フランツ型拡散セルを用いて人工膜 Strat-M における *in vitro* 皮膚透過性試験を実施した。ドナー側に被験物質（各 1 mg/mL）を入れ、経時的に皮膚を透過したレセプター液中の被験物質の量を UPLC/ESI-MS にてモニタリングし、*in vitro* 皮膚透過性のデータを得た。得られたデータを基に定常状態における各被験物質の皮膚内濃度（C_{ss}）を算出した。得られた C_{ss} の値と光化学的特性データを併せて考慮することで光毒性予測を実施した。

3) ラット *in vivo* 光毒性試験

前日に腹部を剃毛した雄性ラットに各被験物質（10 mg/site）を塗布し、塗布後 3 時間で black light にて UVA（30 J/cm^2）を照射した。照射終了後 24 時間 に色差計にて皮膚表面の色調を計測し、光毒性の指標とした。

B.4.2. *In vivo* と相関性のある *in vitro* 毒性評価系による AOP 及び TG の実験データ支援

分担研究者の中江らは以下に示す研究を実施した。

B.4.2.1. 表皮組織における代替法の検討：ヒト 3D 再構成系表皮及びヒト表皮角化細胞単層培養系を用いた folpet に対する経皮毒性

ヒト 3D 再構成表皮系としては、LabCyte EPI 24 モデル（株式会社ジャパン・ティッシュ・エンジニアリング）を、当該モデルに添付の培養液と共に用いた。単層培養系としては、ヒト表皮角化細胞株 HaCaT 細胞を適宜継代して用いた。

被験物質としては、皮膚刺激性試験で陰性を示すものの 21 日間反復投与毒性を示す folpet（フタルイミド系殺菌剤）を使用した。

ヒト 3D 再構成表皮系を用いた実験は、OECD TG439 に従った皮膚刺激性試験（15 分曝露・42 時間後培養）と、24 時間曝露の 2 条件で、同じ濃度の folpet（0、100、300、1000、2000 μg/mL）による細胞毒性を（3-(4,5-di-methylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide: MTT）アッセイで比較評価した。また、24 時間曝露時における各種炎症性サイトカイン・バリア機能関連遺伝子発現を qPCR にて解析した。

HaCaT 細胞を用いた実験は、folpet を 0、1、3、10、30、40、60、80、100、120 μg /mL の濃度で 24 時間曝露し、WST-8 アッセイで細胞毒性を評価した。

B.4.2.2. 肝臓における代替法の検討：正常または腫瘍肝細胞における脂肪毒性に対する検討

ラット正常肝細胞株 Clone 9 と、ヒト肝癌由来細胞株 HepG2 を用い、各種脂肪酸（パルミチン酸、オレイン酸、エライジン酸、リノール酸、アラキドン酸、EPA、DHA）を 1-1600 μM の濃度で 24 時間曝露し、WST-8 アッセイで細胞毒性を評価した。

B.4.2.3. 腸管由来組織における代替法の検討

1) 腸管上皮由来 Caco-2 細胞を用いた平面培養による検討

DSS（Dioctyl Sodium Sulfosuccinate: MP Biomedicals）を通常培地（DMEM Low Glu、10% 胎仔ウシ血清（FBS）、1% 非必須アミノ酸溶液（NEAA）、1% ペニシリン・ストレプトマイシン）に後述する濃度%（w/v）で混じ、Caco-2 に 24 時間曝露した。

1-1) 遺伝子発現解析

2 枚の 12-well plate の各 well に Caco-2 を 4 ×10^4 cell/mL で播種して 2 日毎に培地交換を行い、コンフルエントになってから 5 日後に DSS をそれぞれの plate の 3well ずつに 1、3、5%及び 5、10、15%で曝露した。

DSS 10、15%においては RNA 量、cDNA 量共に測定できたにもかかわらず、内部標準遺伝子発現が僅かで、DSS 15%では内部因子を含めその他のターゲット遺伝子すべてに発現が見られなかった。従って DSS 10%以上の曝露は、評価に適さな

い状況にあるものと考えられた。

1-2) 蛍光免疫組織学的染色

Cell culture slide（4-well タイプ）の各 well に Caco-2 を 1.3 × 10^5 cell/mL で播種した。培地は 400 µL/well とした。2 日毎に培地交換を行い、約 2 ヶ月後に DSS を 1、3、5%の濃度で曝露した。DSS 処理後に 3%パラホルムアルデヒドで固定し、0.2% Triton-X100 で透過処理を行い、更に 1% FBS でブロッキング後、E-Cadherin Rabbit Polyclonal Antibody（ProteinTech）を添加し一晩処理し、Donkey Anti-Rabbit IgG (H+L) Highly Cross Adsorbed Secondary Antibody、Alexa Fluor 555（Thermo Fisher Scientific）を反応させ、4',6-diamidino-2-phenylindole（DAPI）添加剤にて封入した。

1-3) 生細胞数測定（WST-8 アッセイ）

96-well plate に Caco-2 を 8,000 cell/well で播種して 2 日毎に培地交換し、コンフルエントになってから 5 日後に 5、10、15、20、25、30%の濃度の DSS を曝露した後、Cell Counting Kit-8（同人化学:富士フィルム和光純薬株式会社）を添加してインキュベートした。WST-8 試薬添加後のインキュベート時間も考慮して、1 時間経過ごとに 4 時間まで、マルチモードプレートリーダー（バイオテックジャパン）にて 450 µm の吸光度測定を行った。

2) マウス空腸由来のオルガノイドを用いた検討

正常 C57BL/6J マウス由来の空腸オルガノイドに後述する濃度の DSS をインジェクションし、別のウェルでは培地に同濃度の DSS を添加し比較解析した。それぞれ経時的に撮影し、DSS のオルガノイドに対する影響を検討した。

2-1) 培地添加による曝露

5、10% DSS（in phosphate-buffered saline (PBS)）を調製し、24-well plate に 0.5、1%濃度になるよう培地に添加した。Control 群においては PBS を 1%になるように添加した。培地容量は、PBS、 DSS 共に、400 µL/well とした。

2-2) インジェクションによる曝露

15、30% DSS（in PBS）を調製し、オルガノイドにインジェクションした。オルガノイドの体積とインジェクションする液量の条件から、投与液は 30 倍希釈されることが明らかとなっている。そのため、インジェクション後オルガノイド中の DSS 濃度は、0.5、1%となった。Control 群には、PBS をインジェクションした。

B.4.3. 発がん性試験における AOP 及び TG の実験データ支援

分担研究者の豊田は、令和 3 年度に検索する新規被験物質として、腎発がん物質 5 種[1]を、6 週齢の雄 F344 ラットに 28 日間混餌（HCBD、 ADBAQ、 DMN）または飲水（EHEN、 AOM）投与した（各群 5 匹）。各物質の投与濃度は短期試験における最大耐量として、300 ppm HCBD、 10,000 ppm ADBAQ、 500 ppm DMN、 1,000 ppm EHEN 及び 40 ppm AOM に設定した。ただし EHEN 投与群については、顕著な体重増加抑制がみられたため、3 週目以降投与濃度を 500 ppm に変更した。

投与期間終了時に解剖し、腎臓及び肝臓

[1] Hexachlorobutadiene（HCBD）、1-Amino-2,4-dibromoanthraquinone（ADBAQ）、Dimethylnitrosamine（DMN）、*N*-Ethyl-*N*-hydroxy-ethylnitrosamine (EHEN)、Azoxymethane（AOM）

の重量を測定した。腎臓の病理組織学的検索を実施するとともに、免疫組織化学的手法による γ-H2AX 形成の定量解析を実施した。右腎横断面から、皮質及び髄質外帯外層の特定部位を顕微鏡下(x400)でそれぞれ 4 か所撮影し、尿細管上皮細胞の総数ならびに γ-H2AX 陽性細胞から陽性細胞率を計測した。

B.4.4. 遺伝毒性の AOP 開発

分担研究者の堀端は、発がん性の AOP への組み込みを想定し、遺伝毒性初期応答反応の早期検出システムを構築するため、クロマチン免疫沈降法(Chromatin immunoprecipitation; ChIP)及び定量的 PCR を用いた DNA 損傷応答の分子生物学的解析を実施した。なお、一般的なコーディング DNA 領域と比べて、リボソーム DNA (ribosomal DNA; rDNA)は 1 細胞あたりヒトでは 200〜700 コピーから成るクラスターを形成しており、また、DNA 代謝反応である転写のメカニズムについての知見も豊富であることから、rDNA を ChIP 及び定量的 PCR を利用した DNA 損傷応答解析の標的領域とした。

DNA 損傷の定量化が可能な紫外線照射による DNA 損傷を誘導した Flp-In 293 細胞を用いて、rDNA 上の転写装置である RNA polymerase I (RNAPI)の構成サブユニット RPA194、DNA 損傷応答マーカーであるヒストンバリアント γH2AX、または DNA 損傷応答タンパク質の一つとして知られる Ku80 を標的とした ChIP を実施し、RNAPI、γH2AX または Ku80 が局在する DNA 画分をそれぞれ調製した。これらの DNA 画分を鋳型 DNA とし、rDNA unit を転写領域及び非転写領域を含む領域に分けてそれらを標的とした 9 つのプライマーセット(H1〜H4. 9)を用いた定量的 PCR により、DNA 損傷誘導時における RNAPI、γH2AX または Ku80 の rDNA 上での位置的相対量変化を解析することで、DNA 上で直接的に生じている DNA 損傷応答の定量・定性的検出を試みた。

B.4.5. 腎障害・線維化の分子メカニズムに関する研究

分担研究者の松下は、腎障害・腎線維化の AOP 作製に資するデータを得るため、障害機序の異なる 3 種類の腎毒性物質：アロプリノール(APL)、バンコマイシン(VAN)及びピューロマイシン(PAN)を用いて腎線維化モデルラットの作出を試みた。

実験 1：6 週齢の雄性 SD ラットを 3 群に配し(n=5)、媒体である 0.5%メチルセルロースもしくは APL を 100 及び 150 mg/kg 体重(5 mL/kg 体重)の用量で 1 日 1 回、28 日間反復強制経口投与した。体重測定を週に 1 回行い、最新体重に基づいて投与容量を算出した。最終投与 1 日後にイソフルラン深麻酔下において腹大動脈から採血した後、放血により安楽死させて剖検を行った。剖検時に腎臓を摘出して重量を測定した後、一部を 10%中性緩衝ホルマリンにて固定し、残りの組織は液体窒素にて瞬間凍結もしくは OCT コンパウンドにて凍結ブロックを作製した後、-80℃にて保存した。得られた血液サンプルを常温下で遠心(3,000 rpm、15 分)して血清を分離し、尿素窒素(BUN)及びクレアチニン(sCre)の値を測定した。また、10%中性緩衝ホルマリンで固定した腎臓組織を用いて定法に従いパラフィン包埋、薄切し、HE 染色及び膠原線維を赤色に染色するシリウスレッド染色を施して病理組織学的

検索を行った。さらに免疫組織学的解析のため、組織標本を抗原賦活化処置としてクエン酸緩衝液(pH6.0)に浸漬してオートクレーブ処置し(121℃、15 分)、3% H_2O_2/メタノールにて内因性ペルオキシダーゼを除去した。引き続き、非特異反応を除去するため 10%正常ヤギ血清を用いてブロッキング処置を施した後、抗 CD44 抗体(ポリクローナル、x10,000、Abcam)を 4℃にて一晩インキュベートし、二次抗体(ポリマー法：ヒストファインシンプルステイン)を室温下で 30 分インキュベートした。ジアミノベンジジンにて反応を可視化し、ヘマトキシリンにより核染色を行った。統計学的解析として、体重、血液生化学的検査及び腎重量のデータについて一元配置分散分析(ANOVA)を実施した後に Dunnett 法による多重検定を行った。有意水準は 0.05 に設定した。

実験 2：6 週齢の雄性 SD ラットを 3 群に配し(n=5)、媒体である生理食塩水もしくは VAN を 200 及び 400 mg/kg 体重(10 mL/kg 体重)の用量で 1 日 1 回、28 日間反復腹腔内投与し、実験 1 と同様の測定及び解析を実施した。

実験 3：6 週齢の雄性 SD ラットを 3 群に配し(n=5)、媒体である生理食塩水もしくは PAN を 8 及び 12 mg/kg 体重(5mL/kg 体重)の用量で 1 日 1 回、28 日間反復静脈内投与し、実験 1 及び 2 と同様の測定及び解析を行った。

B.5. OECD に提出する資料の事前確認と OECD からの意見募集への対応

平林は、WNT の Bureau として OECD 活動に協力するとともに、研究班内に文書検討グループを組織し、日本から OECD に提出する資料を事前に相互確認し、また、OECD からの提案資料への意見募集に適切な意見を返した。

B.6. 毒性等情報収集調査

分担研究者の山田は、平成 30 年度開始の厚生労働科学研究化学物質リスク研究事業［公募型］計 4 課題の令和 2 年度報告書を入手して*1、毒性エンドポイントと解析対象の化学物質、評価系の構築状況ならびに試験結果を精査して Excel 形式で整理した。さらに、各研究課題の分担研究について、AOP の構築と TG 化へ向けた位置づけを整理し、俯瞰する図を PowerPoint 形式でまとめた。調査対象は以下の通りである。

1) 化学物質の動物個体レベルの免疫毒性データ集積とそれに基づく Multi-ImmunoTox assay(MITA)による予測性試験法の確立と国際標準化
2) 家庭用品化学物質が周産期の中枢神経系に及ぼす遅発性毒性の評価系作出に資する研究
3) 生体影響予測を基盤としたナノマテリアルの統合的健康影響評価方法の提案
4) 血液中の核酸をバイオマーカーに用いた化学物質の高感度な有害性評価に資する研究

さらに、AOP の IATA への活用に関する調査を行った。主に、OECD IATA Case Studies Project における事例研究から学んだ留意事項をまとめた公開資料*2 を使用した。

*1https://mhlw-grants.niph.go.jp/

*2http://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm

（倫理面への配慮）

本研究は動物福祉の3Rs（Replacement、Reduction、 Refinement）に配慮して、各施設における動物実験委員会の承認のもとに基本指針を遵守して実施し、動物使用数や動物に与える苦痛は最小限に留めた。

腸管組織における検討においては、マウス回腸由来のオルガノイド培養組織を実施するにあたりマウスを使用した。ただし、その使用は最少匹数に留め、東京農業大学動物実験委員会より承認を受けた申請内容に則り実施した。

国立医薬品食品衛生研究所の実験は、動物の数は最小限にとどめ、実験動物取扱い規定に基づき、動物の苦痛を最小限とするよう配慮して行った。

ボランティア及びヒト組織は使用しなかった。これらのことから、倫理的問題は無いと考える。

C. 研究結果

C.1. AOP の開発

C.1.1. 免疫毒性の AOP

「AOP154: カルシニューリン阻害による T 細胞依存的抗体産生抑制」については、WNT/WPHA（Working Party of Hazard Assessment）に提出したのち、ドイツからコメントが提示されたという連絡があり、AOP Wiki の改訂作業を行った。ドイツからの主な指摘は、TDAR（T cell Dependent Antibody Response）アッセイの方法をガイドラインごとに詳細に記載すること、本 AOP のみで免疫毒性試験が免除されることはなく、本 AOP は IATA 開発に利用されるべきであること、本 AOP の EU 地域での規制上の重要性についても記載することなどであった。指摘事項に対応し、OECD 事務局に改訂完了の連絡と著者回答ファイルを提出したところ、OECD 事務局から、本 AOP が WNT/WPHA で承認された。その後 OECD 事務局に著作権譲渡に関する著者全員の署名書類を提出し、令和 3（2021）年 10 月 15 日に OECD Library において公開された。

「AOP313: TLR (Toll-like receptor) 7/8 への結合による乾癬様皮膚疾患の増悪」については、コーチとの web ミーティングの指摘事項（測定結果だけでなく測定対象及び方法を記載すること、Taxonomic Applicability は AOP が環境毒性からきているためにある項目であり、サポートする evidence を KE や KER に記載すること、IL-23 について構造だけでなく機能も記載すること、IL-17 及び Th17 に focus するのであればそのことを明記することなど）に対応中である。

「AOP314: 免疫細胞に存在する ER（Estrogen Receptor）の活性化による全身性リテマトーデス（SLE）の増悪」については、AO 及び KE の置き換えなど大幅な見直しを検討していることをコーチに連絡した。

「AOP315: JAK3 の阻害による T 細胞依存的抗体産生抑制」については、コーチに改訂完了の連絡と著者回答ファイルを提出したところ、これに対するコーチコメントを受領した。対する AOP Wiki 改訂作業を行い、再びコーチに改訂版を提出した。コーチからの主な指摘は、本 AOP の AO が AOP154 の AO と類似しているので同じイベントの場合は AOP154 の AO（KE984）にリンクさせ、別のイベントを作成したい場合は全体を修正すること、KER3 の定量的考察はデキサメタゾンがどのように STAT5 の DNA への結合を阻害するかをより詳細に説明すること、“biological plausibility of KERs”、 “essentiality of KEs”

及び“empirical support for KERs”についてハンドブックに従って要約表を加えることなどであった。OECD より、外部評価に入れる AOP があればチェックリストを提出するよう要請を受けたコーチが、2021 年 11 月 8 日にチェックリストを OECD に提出したことから、今後、外部評価に進むことが予想される。AOP154 の場合、外部評価が決まってから OECD に承認されるまで約 1 年半かかっており、AOP315 も同程度の期間内の承認を目指す。

「AOP277: IL-1 receptor 結合阻害」については、AOP 開発の引継ぎに関する web 会議を実施し、元の AOP 開発者である東北大学の相場節也先生、木村裕先生から説明を受けた。コーチとのweb会議が行われ、評価結果について説明を受けた。その後 OECD から scientific review report を受領した。その主な推奨事項は、IL-1R シグナルを阻害するストレッサーに特異抗体だけでなく化合物/医薬品を加えること、AP-1 など NF-kB が関与しない経路も考慮すること、T cell のタイプを明確にすること、増加する感染のタイプを明確にすることなどであった。現在これらの推奨事項に対応案を作成中である。

C.1.2.　発がん性の AOP

網羅的に情報収集した鼻腔発がん物質のうち 40 種の吸入暴露による発がん物質（ラット 38 物質、マウス 11 物質、ハムスター5 物質）及び 38 種の非吸入暴露による発がん物質（ラット 36 物質、マウス 5 物質、ハムスター17 物質）について、誘発された鼻腔腫瘍を INHAND に基づいて分類した結果、扁平上乳頭腫、扁平上皮癌、腺腫、腺癌、腺扁平上皮癌、神経上皮癌、未分化癌、非特異的な癌、線維肉腫、血管腫、血管肉腫、粘表皮腫、横紋筋腫、横紋筋肉腫が報告されていた。最も高頻度の鼻腔腫瘍は扁平上皮癌であり、投与経路に関係なく認められ、その前駆病変として、扁平上皮化生及び/または扁平上皮乳頭腫と呼吸上皮過形成が示唆された。 2 番目に多いのは腺癌であり、その前駆病変として主に嗅上皮過形成が示唆された。一方、腺腫の前駆病変は呼吸上皮病変と考えられた。これらの経路はげっ歯類間で共通していると考えられるが、マウスまたはハムスターのデータは限定的であった。

C.1.3. 光毒性の AOP

令和元（2019）年 6 月に OECD TG495 としてガイドライン化された ROS アッセイを主軸として、新たに光安全性評価のための AOP 構築を進めている。既に OECD の専門家会議で意見を求め、光刺激性に絞った AOP 作成を進めることで同意を得た。

C.2. TG 及び DRP の開発

C.2.1. 皮膚感作性試験

小島は、協力研究者の笠原とともに、昨年度から検討を続けてきた *In Chemico* Skin Sensitisation、 ADRA の改定に尽力した結果、令和 3（2021）年 6 月に TG の改定が承認された。引き続き、OECD の専門家から要請を受け、ADRA の国内施設の協力を得て実施した追加バリデーションの報告書及びTG442Cの最終改定案を作成し、7 月に OECD に提出した。11 月の専門家会議を経て、改定 TG 案を OECD に提出した。

また、小島は、協力研究者の相場とともに、IL-8 Luc assay　TG442E の改定案を作成し、7 月に OECD に提出した。11 月

の専門家会議後に反論がでてきたこともあり、来年、再協議を行うことになっている。

小島と足利は、昨年度から専門家委員会で検討を続けてきた Defined Approach for Skin Sensitisation の承認に寄与した結果、令和 3(2021)年 6 月にガイドライン 497 が承認された。

本ガイドラインは新しいタイプの OECD ガイドラインである。DA では化学物質の物性および *in vitro* 試験データで検証された OECD の組み合わせを使用している。その一つとして、DA では、化学物質規制に *in silico* データを受け入れることを可能にした最初の事例である。

このガイドラインには以下に示す画期的な点が複数含まれている。

1 初めて試験法の結果を組み合わせて評価する手法が公定化された。
2 初めて *in silico* の利用が組み合わせ評価に利用された。

ヒトの感作性が予測できる初のガイドラインである。

C.2.2. 免疫毒性試験

小島は、相場及び国際的な専門家とともに、昨年度、*in vitro* 免疫毒性に関する DRP を作成した。OECD が集めた意見に対応する改定版を 9 月に提出したところ、2 次募集において追加意見が集まった。現在、この意見に対応する改定を検討している。

DRP の承認を待って提出する予定の IL-2 を指標とした免疫毒性試験の TG 案を作成し、3 月に OECD に提出した。

C.2.3. 生殖毒性試験の DRP

国際的な専門家とともに、*in vitro* 生殖発生毒性に関する DRP の作成をこの一年継続して実施している。本来は夏休み前に適切な論文に投稿する予定であったが、著者の中に EPA や企業の専門家が含まれていることから、公表にあたり EPA や企業の同意を得ることに手間取ったこともあり、令和 4(2022)年 2 月に Current Research Toxicology に投稿し、3 月に改訂の指示を受けた。

C.2.4. 発達神経毒性に起因する行動解析に関する情報収集

EPA 及び OECD の DNT に関する TG を確認し、げっ歯類に対する行動解析に関する内容を中心に比較を行った結果、発達神経毒性に関連する OECD TG426 は、全般に EPA ガイドライン(OPPTS 876. 6300)に沿った内容であり、EPA のガイドラインに比べ詳細に記載されていた。試験項目の内容に関しては、大きく分けて「行動発達」、「自発運動量」、「運動及び感覚機能」、「学習及び記憶」における推奨検査項目や実験条件について述べられていた。しかしながら、実施する行動評価の選択については具体的な記載はなく、明記されているのは評価の対象となる機能及び試験を行う際の推奨日齢のみであった。また、脳高次機能に関する試験項目としては「学習及び記憶」が該当するが、試験方法の記述は曖昧な表現にとどまっていた。さらに、行動試験を行うにあたり、解析環境の記載については、具体的な説明が乏しいことや、特定の試験を組み合わせた評価系を構築するなど、標準化(統一化)されたプロトコルではないことも明らかとなった。

PubMed を用い、OECD TG426 が制定された平成 19(2007)年から令和 3(2021)年ま

での範囲で文献数調査を行ったところ、一例として、検索キーワード「developmental neurotoxicity」のみの検索条件では総文献数が 995 件にのぼり、その報告数は年々増加していることから、発達神経毒性研究に対する関心が伺えた。一方で、「"developmental neurotoxicity" AND "OECD test guideline 426"」の検索条件では、わずか 2 件の該当であった。文献検索については、検索条件を変更しながら、学術ベースとガイドラインベース（OECD あるいは EPA のガイドラインに準拠した試験）で、ガイドラインの使用実績を含めて引き続き調査を進め、得られた文献情報についてとりまとめる予定である。

JaCVAM 発達神経毒性資料編纂委員会のオブザーバーとして委員会に参画するとともに、*in vitro* DNT ガイダンス文書（Guidance on the Interpretation of Data from the Developmental Neurotoxicity (DNT) *In-Vitro* Testing Assays for Use in Integrated Approaches for Testing and Assessment (IATA)）及び Case Study に関して、OECD 事務局に提出するコメント募集に応じた。神経行動毒性の評価系、特に *in vivo* 試験を行っている立場から、本ガイダンスの改善点・懸念点について以下のコメントを行った。

1. 提案されている *in vitro* 試験バッテリー（Developmental Neurotoxicity *In Vitro* Battery (DNT IVB)）により in vivo 発達神経毒性を予測するにあたって、化学物質影響の種間差、性差に関する言及がないこと。
2. 行動毒性においては、*in vitro* 試験の複数のアッセイを組み合わせたとしても、予測は困難であり、現在の内容に加えて、さらに考察を追加する必要があること。
3. *in vivo* 試験で観察される神経回路の機能変化や、顕在化する行動影響に関して、*in vitro* 試験において得られたデータはどこまで対応しうるのか、あるいは妥当性があるのか、具体的な言及を追加すること。
4. 被験物質の複合曝露による、相加・相乗的影響についての懸念。また、げっ歯類の代替としてのゼブラフィッシュによる解析を含めて、検出系の施設間差、試験プロトコルの統一性（個体数や分析法）についての議論をすること。
5. 今後、DNT IVB で種々の化学物質について検討する際に、*in vitro* 試験で確認されなかった影響が *in vivo* 試験のみで確認された場合の解釈について言及すること。
6. *in vivo* 脳高次機能（学習や記憶）の評価は現状難しいと考えられ、その際に、最終的に *in vivo* アッセイで補強すべき役割について言及すること。

C.3. IATA の開発

C.3.1.非遺伝毒性発がん性 IATA 開発への協力

1)発がん性 IATA

小川研究分担者は cell proliferation 及び resistance to apoptotic cell death のサブグループに、西川研究協力者は cell transformation、indicator of oxidative stress 及び resistance of apoptosis cell death のサブグループに参画し、Assay Block の評価を行った。cell proliferation においては、細胞増殖の評価に関する *in vitro/ex-vivo*/short term *in vivo* assay の非遺伝毒性発がん性の KE としての網羅的文献検索、オミックス解析の利用の可能性、規制への応用の可能性などを内容とする論文作成について議論された。

新規評価法としては、米国 EPA の Dr. Chris Corton による eSTAR (Emerging Systems Toxicology for the Assessment of Risk)に関する webinar があり、肝発がんにおいては、AhR、CAR、ER、PPARa の活性及び cytotoxicity が非遺伝毒性発がん性の MIE として利用可能との紹介があった（Corton JC, et al. Toxicol Sci 177; 11-26 2020）。また、オランダ ライデン大学の Dr. Bob van de Water による weighted gene co-expression network analysis（WGCNA）に関する webinar があり、TXG-MAPr webtool（https://txg-mapr.eu/WGCNA_PHH/TGGATEs_PHH/）を用いた初代ヒト肝細胞のストレス応答遺伝子共発現分析について紹介があった（Callegro G, et al. Archive Tox 95; 3745-75 2021）。非遺伝毒性発がん物質と遺伝毒性発がん物質で誘導される遺伝子発現パターンの相違について、これらのデータを比較することも有用と考えられた。

2) Bhas42 細胞形質転換試験の TG 開発

大森は、Bhas42 細胞形質転換試験（Bhas 42 Cell Transformation Assay: Bhas 42 CTA）が含まれる“Cell Transformation”（Block 3）及び、約 40 年前からわが国で非遺伝毒性発がん物質の検出法として有用性評価が行われてきた“Gap Junction”（Block 4）を担当した。

Block 3 の“Cell Transformation”では、SHE Cell Transformation Assay（SHE CTA）、Bhas 42 CTA 、Balb 3T3 CTA、 Balb c/3T3 transformics assay、Clonogenic soft agar Assay の計 5 種の assay が評価候補となった。IATA の全 assay 候補の中で OECD の GD として掲載されている試験法は、SHE CTA 及び Bhas 42 CTA のみである。この 2 つの試験法については、各種性能及び再現性等の検証データに基づき利用に関する考え方を作成し、各評価項目において A 評価を得た結果、Block 3 メンバーの全会一致でランク A の合意を得た。一方、Clonogenic soft agar Assay はランク B であった。Balb 3T3 CTA については、2-step assay と 1-step assay が開発されていることから、いずれのプロトコルを利用に関する考え方に記載し評価を行うかについて議論されたが、かつて ECVAM から OECD に提案され評価が中断された 1-step assay を Block 3 での評価プロトコルとすることになった。同プロトコルに基づく Balb c/3T3 transformics assay も Balb 3T3 CTA とは別に利用に関する考え方が作成されたが、最終的には Balb 3T3 CTA の optional assay として Balb 3T3 CTA の利用に関する考え方に組み込まれ、Balb 3T3 CTA として評価が行われた。Balb 3T3 CTA では、対象化合物での明確な陽性結果が報告されていないことに加え、性能評価及び再現性検証の化合物数も限定的であることなどから、各評価項目は A 評価と B 評価とが混在した。ランキングについて Block メンバー内で議論され、Balb 3T3 CTA（Balb c/3T3 transformics assay 含む）はランク A で合意された。それら 3 種の Cell transformation assay 評価結果は、10 月の全体会議での報告に至った。

Block 4 の“Gap Junction”では、大森から提案した、Dye Transfer Assay と Inhibition of Metabolic Co-operation Assay について、該当する 3 種 assay（Metabolic Cooperation Assay with (HGPRT+&-) V79 Cells (using 6-TG)、Gap junction dye transfer assay–combined、Gap Junction Multiparametric Scrape Loading-Dye Transfer assay (mSLDT)）を選出した。Metabolic Cooperation Assay

with (HGPRT+&-) V79 Cells (using 6-TG)については、467 化合物で結果と 166 化合物での性能評価について総説（ATLA）が我が国から報告されており、それをもとに大森が利用に関する考え方の作成を行った。他の 2 種の assay は、他の 2 名のメンバーが利用に関する考え方を作成した。Metabolic Cooperation Assay with (HGPRT+&-) V79 Cells (using 6-TG) は、感度（49%）及び特異性（63%）が十分でなく施設間再現性評価が未報告のためランク B となった。Gap junction dye transfer assay–combined は、ECVAM のプロトコルではあるが、17 化合物のデータのみで検証データが無いためランク C であった。Gap Junction Multiparametric Scrape Loading-Dye Transfer assay（mSLDT）は、328 化合物での結果と IARC グループ 1 及び 2 の 72 化合物での性能評価が報告されており、感度は 75%以上であるが、特異性が 45%と低値であり、施設間再現性評価が未実施であることからランク B となった。これら Block 4 の 3 種 Gap junction assay の評価結果についても、10 月の全体会議での報告に至った。

C.3.2. 光毒性 IATA

令和元（2019）年 6 月に OECD TG495 としてガイドライン化された ROS assay を主軸として、新たに光安全性評価のための IATA 構築を進めている。既に OECD の専門家会議にて共有し、専門家より修正に資する重要なコメントを頂いている。ICH S10 において推奨されているストレテジーをベースとし、(i) 被験物質の光化学的特性評価、(ii) 光生物化学的特性評価、そして (iii) 皮膚や眼への移行性・滞留性等体内動態評価の 3 段階のスクリーニングによる tiered approach を案として提示すべく準備を進めている。

C.4. AOP 及び TG の実験データ支援

C.4.1. 光安全性評価ツールの予測精度

被験物質である ACD、FSM、HCP、MOP、NFX および PMZ の光毒性リスクを予測し、本評価系の適用可能性を精査した。Strat-M® を二層膜モデル、30 min の間 methanol 処置した Strat-M® を単層膜モデルとして用い、レセプター液中に到達した被験物質量を経時的に測定することにより各被験物質のそれぞれの膜に対する透過性を評価した。本試験では、 Strat-M® の薄さを鑑みて試験開始から 8 h で膜透過速度定常状態に達すると考えた。全被験物質の透過量について、単層膜モデルの方が二層膜モデルと比較して高かった。二層膜および単層膜モデルのどちらにおいてもレセプター液中 PMZ 量は全被験物質中最も高く、試験開始 8 h 後における値はそれぞれ 0.4 および 1.3 μg/cm^2 であった。被験物質の累積量プロファイルに基づき、P_{TLM} は 5.0×10^{-5} (ACD)、5.0×10^{-5} (FSM)、6.0×10^{-5} (HCP)、4.0×10^{-5} (MOP)、2.0×10^{-5} (NFX) および 1.0×10^{-4} (PMZ) cm/s であり、P_{OLM} は 4.0×10^{-4} (ACD)、1.7×10^{-4} (FSM)、1.8×10^{-4} (HCP)、1.1×10^{-4} (MOP)、1.2×10^{-4} (NFX) および 3.9×10^{-4} (PMZ) cm/s であった。全被験物質の P_{OLM} は P_{TLM} と比較して高く、その差は 2.9 から 7.4 倍であった。二層膜モデルに対する透過性は Clog *P* 値が -0.78 と被験物質中で唯一負の値を持つ NFX が最も低かった。HCP および PMZ の Clog *P* 値はそれぞれ 7.0 および 4.6 であり、Clog *P* を基準とした脂溶性は HCP が最も高い一方で、PMZ の P_{TLM} は HCP より高く全被験物質中最も高値であった。これら Strat-M® に対する各被

験物質の透過性を基に Css を算出したところ、ACD、FSM、HCP、MOP、NFX および PMZ の C_{ss} はそれぞれ 23.4、19.6、20.5、9.4、5.9 および 38.1 μg/mL であった。すなわち、PMZ が最も高く、次いで ACD、HCP および FSM の順であった。MOP および NFX のCss は他の被験物質と比較し低かった。

UV/VIS 吸収スペクトル測定および ROS assay を 6 種被験物質に対して実施することで、それらの光化学的特性を評価した。全被験物質は UV 領域に強い吸収を持ち、その範囲で吸収が極大となる波長での MEC 値は 19,500 (ACD)、14,900 (FSM)、11,200 (HCP)、24,900 (MOP)、24,900 (NFX) と 6,600 (PMZ) $M^{-1}\cdot cm^{-1}$ であった。つまり、6 種類の被験物質は高い光励起性を持つと判断できる。ROS assay において、HCP を除く全被験物質が擬似太陽光照射により産生した singlet oxygen および superoxide anion はガイドラインによって定められた criteria を超えていた。HCP は singlet oxygen の産生量のみ criteria を超え、その産生量は全被験物質中最も多かった。したがって、6 種の被験物質全てが criteria を超えており、光反応性を持つことが明らかになった。特に、ACD は singlet oxygen および superoxide anion のどちらの産生量も非常に多く、被験物質中で最も高い光反応性を有していた。

提案した光安全性評価系の適用可能性の検証には、本評価系により予測した結果と実際に生体内で起こる光毒性反応との関連性を評価する必要がある。したがって、各被験物質の *in vivo* 光毒性を評価すべく、ラットを用いた *in vivo* 光毒性試験を行った。一般に、露光部に分布した光毒性陽性化合物から産生された ROS および光励起された化合物自身の一方もしくは両方が細胞膜障害を引き起こし、発赤や紅斑を主徴とする光刺激反応を呈する。本試験では、UVA 照射前後のラット腹部の皮膚表面における色調変化を客観的に評価することで光毒性、特に光刺激性の強さを評価した。全被験物質は UVA 照射により有意に皮膚表面の色調が変化した。つまり、6 種の被験物質が光毒性反応を誘発した。UVA 照射群では、全 6 種被験物質で Δa 値が正の変化を示した。この結果は塗布部の赤みが UVA 照射により増したこと示しており、単回投与により紅斑反応を誘発することを認めた。つまり、全被験物質は光刺激性を有することが明らかとなった。特に、ACD および HCP 投与群の色調変化が他の 4 種被験物質と比較して大きかった。

以上、被験物質の *in vitro* 皮膚滞留性および光反応性の統合的な解析により予測した光毒性リスクおよび *in vivo* 光毒性の強さを以下に示す。

Predicted phototoxic risk:

ACD＞HCP＞PMZ＞FSM＞MOP＞NFX

Observed phototoxicity:

ACD≒HCP＞PMZ＞MOP＞FSM＞NFX

予測した光毒性リスクと *in vivo* 光毒性の順は良い対応を示した。人工膜を用いた統合的光安全性評価法は精度良く被験物質の光毒性リスク予測ができ、実験動物を用いずとも光安全性評価が可能であることを示唆した。

C.4.2. *In vivo* と相関性のある *in vitro* 毒性評価系による AOP 及び TG の実験データ支援

C.4.2.1. 表皮組織における代替法の検討

ヒト 3D 皮膚再構成系を用いた folpet に

対する経皮毒性評価において、OECD TG439 に従った皮膚刺激性試験では 2000 μg/mL の最高濃度まで陰性であったのに対し、24 時間曝露では同条件の対照群と比較して 2000 μg/mL より細胞毒性がみられた。24 時間曝露における qPCR では、炎症性サイトカインの mRNA 発現が 1000 μg/mL で有意に増加した。また、濃度依存による検討では、3 時間あるいは 8 時間の曝露より炎症性サイトカインの上昇がみられた。HaCaT 細胞を用いた 24 時間曝露では、60 μg/mL 以上で細胞毒性を示した。

C.4.2.2. 肝臓における代替法の検討

Clone 9、HepG2、Hepa1-6 において、パルミチン酸やリノール酸に比してオレイン酸やオレイン酸のトランス異性体であるエライジン酸の細胞毒性は弱かった。多価不飽和脂肪酸は、Clone 9 と比較して HepG2 と Hepa1-6 において、低濃度曝露で細胞毒性を示した。

C.4.2.3. 腸管由来組織における代替法の検討

1) DSS 曝露によるマウス腸管への影響

DSS 群の小腸では、病理組織学的に明らかな変化がなかったが、細胞接着関連因子(TJP-1)及び炎症関連因子(IL-1β)の遺伝子発現の減少傾向が見られた。DSS 群の大腸では、軽度の炎症性病変が誘発された。

2) 腸管上皮由来 Caco-2 細胞を用いた平面培養による検討

2-1) Caco-2 の遺伝子発現解析

DSS 添加により control と比較し 1%濃度以上で、TJP-1 並びに IL-1βの遺伝子発現が減少傾向を示した。DSS 5%濃度においては、e-cadherin と IL-1β の発現に加え、ZO-1、IL-6、TNF-α についても control と比較し、発現がやや低下する傾向が認められた。DSS 10、15%濃度においては、内部標準遺伝子の発現自体が少なく、PCR の結果が得られなかった。このことから DSS 10%濃度以上では、細胞の死滅が生じているものと考えられた。

2-2) Caco-2 の蛍光免疫組織学的染色及び遺伝子発現解析

2 週間培養し 24 時間 DSS を曝露したところ、10%では明らかな細胞の脱落・離開が見られた。5%では細胞培養の状態に control と差が見られなかったが、e-cadherin の免疫組織化学では細胞間の陽性部位の減弱が認められた。DSS 1 及び 3%濃度では、明らかな差が見られなかった。

2-3) Caco-2 の生細胞数測定 (WST-8)

WST-8 試薬添加後のインキュベート 1 時間後の評価において、control に比較し、DSS 5%濃度以上で、細胞が死滅し毒性が生じているものと考えられた。

3) マウス空腸由来オルガノイド

3-1)マウス空腸由来オルガノイドの培地中曝露

添加後 1 時間で、DSS 0.5、1%濃度培地で死滅しているオルガノイドが見られたことから、1 時間足らずでオルガノイドへの影響が確認できなくなり、マトリゲルが破壊され緩くなることから、さらに低濃度による検討を要することが明らかとなった。

3-2) マウス空腸由来オルガノイドのインジェクションによる曝露

インジェクション後に DSS 1%濃度でもオルガノイドは死滅せず、大型化・変形した。

TJP-1 の遺伝子発現は 0.5%濃度以上で一過性の顕著な増加を示したが、24 時間後には、control と差はなく 1%濃度では一転し発現が低下した。さらに IL-1βの遺伝子発現は減少傾向を示した。

C.4.3. 発がん性試験における AOP 及び TG の実験データ支援

投与期間終了時点で、HCBD・DMN・EHEN・AOM 投与群において、有意な体重増加抑制が認められた。摂餌量は HCBD・DMN・EHEN・AOM 投与群、飲水量は EHEN・AOM 投与群で低値の傾向を示した。

HCBD・ADBAQ 投与群では腎絶対・相対重量の有意な増加が、DMN・EHEN・AOM 投与群では腎絶対重量の減少及び相対重量の増加が認められた。また、ADBAQ 投与群で肝絶対・相対重量の増加、HCBD 投与群で肝相対重量の増加、DMN・EHEN・AOM 投与群では肝絶対重量の減少がみられた。

腎臓の病理組織学的検索の結果、HCBD・ADBAQ 投与群では再生尿細管の出現及び尿細管上皮への好酸性顆粒沈着増加等が、DMN・EHEN・AOM 投与群では間質における炎症性細胞浸潤、尿細管上皮細胞の変性及び核の大型化等が種々の程度で認められた。

腎尿細管上皮細胞における γ-H2AX 形成を免疫組織化学的に検討した結果、対照群では陽性細胞は稀であったのに対し、被験物質を投与した各群ではいずれも γ-H2AX 陽性細胞の増加が認められた。

C.4.4. 遺伝毒性の AOP 開発

遺伝毒性初期応答反応の早期検出システム構築に用いた rDNA unit 上のプライマーセット H1～H42. 9 の内、H1～H13 は転写領域、H18～H42. 9 は非転写領域を検出することができる。DNA 損傷を誘発しない条件において、RNAPI 共沈 DNA 中には H1～H13 の転写領域の DNA のみが均一に存在する一方で、紫外線 DNA 損傷誘発時の RNAPI 共沈 DNA 中には、転写領域の中でも H1 と H4 の DNA 領域が特に多く存在することが示された。一方で、DNA 損傷を誘発しない条件において、γH2AX 及び Ku80 共沈 DNA 中には rDNA unit 全領域の H1～H42。9 がわずかに、かつ、均一に存在しているが、紫外線 DNA 損傷誘発時の γH2AX 及び Ku80 共沈 DNA 中には H1～H42 がさらに多く存在し、その中でも特に H18 及び H27 の DNA 領域が多く存在することが示された。

C.4.5. 腎障害・線維化の分子メカニズムに関する研究

実験 1：APL 投与群では実験期間を通して体重増加抑制が認められ、APL 100 mg/kg 群では実験開始 1 から 2 週後に、APL 150 mg/kg 群では実験開始 1 から 4 週後にかけて対照群と比較して有意な低値を示した。血清生化学的検査では、BUN 及び sCre ともに APL 100 mg/kg 群から増加傾向を示し、APL 150 mg/kg 群では有意に増加していた。腎重量の測定では、APL 100 及び 150 mg/kg 群ともに絶対及び相対重量が用量依存性を伴って有意に増加した。病理組織標本を用いたシリウスレッド染色による線維化の評価では、APL 100 及び 150 mg/kg 群ともに間質の膠原線維の明らかな増加を認めた。膠原線維の増加した領域では尿細管は拡張あるいは萎縮しており、これらの尿細管は免疫染色にて CD44 陽性を示した。

実験 2：体重測定において VAN 投与の影

響は認められなかった。血清生化学的検査では、BUN の有意な高値が VAN 200 及び 400 mg/kg 群に、sCre の有意な高値が VAN 400 mg/kg 群に認められた。腎重量測定では、VAN 400 mg/kg 群において絶対及び相対重量の有意な増加が認められた。シリウスレッド染色では VAN 200 及び 400 mg/kg 群ともに軽度な膠原線維の増加が認められ、膠原線維の増加した領域における尿細管は拡張あるいは萎縮しており、免疫染色にて CD44 陽性を示した。
実験 3：体重測定において PAN 投与の影響は認められなかった。血清生化学的検査は現在実施中である。腎重量測定では、VAN 12 mg/kg 群において相対重量の有意な増加が認められた。VAN 8 及び 12 mg/kg 群の腎臓では糸球体及び尿細管障害を示唆する変化がみられたものの、間質の膠原線維の明らかな増加は認められず、拡張／委縮尿細管も観察されなかった。CD44 の免疫染色においても陽性を示す尿細管は認められなかった。

C.5. OECD に提出する資料の事前確認と OECD からの意見募集への対応

本年 11 月に日本から以下の SPSF を提出した。提出にあたり、厚生労働省とも内容を調整した。

1) 皮膚感作性試験　Epidermal Sensitization Assay (EpiSensA): An In Vitro Method for Identifying the Skin Sensitisation Potential of Chemicals
2) 免疫毒性試験　Use of an interleukin-2 luciferase lymphotoxicity test for identifying the immunotoxic potential of chemicals that is caused by anti-proliferative effects

上記した活動に加え、本年度、以下の意見募集が OECD よりあり、コメントを提出した。

- ✓ Draft TG on physical-chemical properties Particle Size and Size Distribution of Manufactured Nanomaterials (PSD)
- ✓ Draft TG on Volume Specific Surface Area of Manufactured Nanomaterials (VSSA)
- ✓ Draft updated Test Guideline No. 488 on Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays
- ✓ Draft Test Guideline on the Mammalian Erythrocyte Pig-a Gene Mutation Assay
- ✓ Draft Test Guideline No. 442C: Draft updated Appendix II on In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)
- ✓ Draft updated Performance Standards for the assessment of proposed similar or modified in vitro skin sensitisation DPRA and ADRA test methods
- ✓ Draft Test Guideline No. 442E updated with a new Annex: Annex IV on Genomic allergen rapid detection for assessment of skin sensitisiters (GARD™skin)
- ✓ Draft Test Guideline for Defined Approaches for Serious Eye Damage and Eye Irritation
- ✓ Draft Supporting Document for Defined Approaches for Serious Eye Damage and Eye irritation
- ✓ Draft Test Guideline No. 492B on Reconstructed Human Cornea-like Epithelium (RHCE) test method for eye hazard identification

- ✓ Draft Performance Standards for Test Guideline No. 498 on RhE Phototoxicity
- ✓ Draft Detailed Review Paper on the miniaturised versions of the bacterial reverse gene mutation test
- ✓ Guidance Notes on the Adaptation of the *In Vitro* micronucleus assay

C.6. 毒性等情報収集調査

C.6.1. 各研究事業における新規有害性評価系の開発状況と AOP 開発へ向けた課題

各研究事業の分担研究ごとに、各分担研究課題、目的、研究対象物質、材料と方法、結論、の項目を設定し情報を整理した。さらにそれらを総合して AOP 開発へ向けた位置づけを整理し、課題を考察した。

1)“化学物質の動物個体レベルの免疫毒性データ集積とそれに基づく Multi-ImmunoTox assay(MITA)による予測性試験法の確立と国際標準化”においては、化学物質の免疫毒性評価法を 2 つ作出した。IL-2 Luc assay に関しては validation、peer review を経て、令和 2(2020)年 11 月に SPSF を OECD に提出した。IL-1 Luc assay も validation を行い、report を作成した。また、上記の評価法の確立の過程で得た免疫毒性データを収集しデータベースを構築した。なお、本 assay と免疫毒性を関連付ける AOP は、“OECD プログラムにおいて TG と DA を開発するための AOP に関する研究”で開発を進めた。

2)“家庭用品化学物質が周産期の中枢神経系に及ぼす遅発性毒性の評価系作出に資する研究”では、家庭用品に含まれる化学物質について、妊婦(胎児)や小児を対象に、低用量暴露による遅発性の中枢神経系への影響を検討した。令和 2(2020)年度は、アセフェート、塩化トリブチルスズ、ビスフェノール類などによる成熟後の行動、中枢神経系、脳回路機能への影響などを体系的に解析したほか、影響されるノンコーディング RNA を探索した。また、液性因子、DNA メチル化などの評価手法の構築を進めた。さらに、行動影響と海馬の遺伝子発現様式についてデータベース化を行ったほか、ガイドライン作出に向け、OECD や JaCVAM の協力のもと、標準プロジェクト化への調整を行った。

3)“生体影響予測を基盤としたナノマテリアルの統合的健康影響評価方法の提案”では、二酸化チタンのナノ粒子を被験物質として、(1)ナノマテリアルの *in vitro* 安全性評価法の高度化として評価準備を行った。また、(2) AOP の確立に向けて、新規評価項目を見出した。(3)毒性試験データベースの構築に向けては、試験データや *in silico* 予測に用いる情報項目の性差を行った。さらに、(4) *in silico* 生体影響予測を組合せたナノマテリアルの統合的健康影響評価方法の構築を目指し、機械学習に用いる実測データの条件を決定した。

4)“血液中の核酸をバイオマーカーに用いた化学物質の高感度な有害性評価に資する研究”では化学物質の「次世代型」有害性評価による迅速化、高度化および標準化に向け、化学物質ばく露後のマウスの血液中のエクソソーム RNA の網羅的解析により、標的臓器を特定し、更に毒性発現機序の解明を目指した。昨年度までに確立した標準化プロトコルを用いて、四塩化炭素投与による肝毒性を検出する新規バイオマーカーとして、エクソソー

ム中の small RNA の網羅的スクリーニングを行い、1318 個の新規バイオマーカー候補を得た。

また、5 種類のベンゾトリアゾール類それぞれに特異的なバイオマーカー候補となる 476 個の新規 small RNA を単離し、クラスタリングを行った結果、ベンゾトリアゾール 5 種を 2 群に層別できた。

今回、エクソソーム RNA を指標とした次世代型安全性評価法により、迅速かつ高感度に肝臓毒性を検出でき、評価法の有効性が確かめられた。

いずれの研究課題も毒性がよく知られている化合物(AOP に作用する stressor に相当)を用い、分子レベル、細胞レベル、個体レベルでの解析(KE に相当)がバランスよく配置されていた。しかし、共通の課題として、*in vivo* 毒性との関連付けと、分子開始イベント(MIE)情報の不足が挙げられる。MIE は化学物質と生体分子との相互作用により、毒性発現に至る最初の引き金となる反応である。OECD での AOP 開発においては必須の情報であり、例えば”Histone deacetylase inhibition leading to testicular toxicity”(AOP212)のように、MIE に関する情報は AOP のタイトルに含められる。AOP 開発の促進のためには、毒性発現に寄与する標的分子を効率的に同定する研究手法の開発が求められる。

C.6.2. AOP ネットワークを用いた IATA の実例と課題

OECD IATA Case Studies Project では、AOP を全身毒性評価の IATA へ活用した事例の提案が増えつつある。AOP は、リードアクロスなどに毒性機序に基づく類似性仮説の構築に有用であることは、広く認識されている。AOP を構成する KE を測定する *in vitro* 試験は、毒性予測の不確実性を減少させ、信頼性を高める上で有効であると考えられている。

今回、令和元(2019)年に提出された「2-エチル酪酸の 90 日反復投与毒性予測」のケーススタディを題材として、AOP を活用する毒性予測の優位性と課題を調査した。本ケーススタディはリードアクロスにより 2-エチル酪酸の 90 日間経口曝露毒性を予測したもので、鎖長の異なる類似物質の毒性試験データと、提案中の AOP を統合した AOP ネットワークから選定した *in vitro* 試験と PBPK モデルの情報を総合的に判断して、肝臓の脂肪症リスクを導出している。使用された AOP は多因子の関与する複雑なネットワークを構成しており(図 1)、この内、幾つかの MIE と KE の試験を使用した。

OECD の専門家レビューでも AOP により高い信頼性で機序を決定できたことが指摘されており、MIE の活性化だけでは有害事象を引き起こすと断定できないことを提示し、さらなる根拠として KE の *in vitro* 試験による裏付けを行った点は高く評価された。

一方で、複雑な AOP から *in vitro* 試験を選定した根拠の明示、提案中で承認されていない AOP についての補足などが課題として指摘された。

また、予測の不確実性に寄与するものとして、毒性試験データのない類似物質や代謝物の毒性を無視したこと、エンドポイントの種差、AOP が未承認であること、*in vitro* 試験による不確実性が挙げられた。

AOP を活用した IATA 事例から、複雑な AOP ネットワークを成す事象を論じる場

合、全ての MIE と KE の測定は不可能かつ不要である可能性が指摘され、一方で、MIE だけでは経路全体が活性化されるか判断できないことが明らかとなった。選定した *in vitro* 試験が AOP をどの程度網羅しているかが不確実性につながるが、必須の KE の特定や AOP の根拠が示されることで低減できるとしている。また、未承認の AOP を用いたが、*in vitro* 試験により類似物質の実測データ（陽性/陰性）を正しく予測できたことで、その不確実性を低減できたと評価された。

このケーススタディでは、複数の AOP を結び付けて AOP ネットワークを形成すると、複雑なエンドポイントを評価するための共通の有害事象につながるさまざまな MIE/KE を検討でき、より適切な *in vitro* 試験を根拠とともに選定できることにより、高い信頼性で作用機序を決定することができたと考えられる。

AOP を IATA に活用していくためには、上記を含む様々な経験や情報を関係者が共有することが有用である。AOP の研究は日々進められており、IATA への活用事例も蓄積されつつある。これらの情報は、本研究で蓄積された AOP に関する知見や評価手法の実用化に貢献することと期待している。今後は IATA Case Studies Project のさまざまな事例の調査を進め、AOP による毒性評価の優位性や課題、ノウハウを明らかにしていく。

D. 考察

D.1. AOP の開発

D.1.1. 免疫毒性の AOP

開発中の AOP については、コーチ及び scientific reviewer のコメントに基づいて修正を行っている。IL-23 の機能の説明のように既存の情報を収集することで対応できるものもあるが、医薬品だけでなく化学物質のストレッサーを示すこと、など情報がないものについては調査したことを示して納得いただく必要がある。また、AO が疾患の憎悪である AOP については、行政活用の観点からその実用性に疑問が投げかけられており、すでに承認された AOP154 のように TDAR に変更するか、開発そのものを見直すことを検討中である。ただし、OECD に承認された AOP154 の KE の一つは IL-2 産生であり、本年度日本から別途 OECD に提案した免疫毒性スクリーニング試験 IL-2 Luc assay の TG 化の理論的基盤になるため、その意義は大きいと考える。

D.1.2.　発がん性の AOP

げっ歯類における化学物質誘発鼻腔発がんの網羅的解析結果は、各種鼻腔腫瘍の前駆病変は、遺伝毒性に関係なく、腫瘍タイプの化学物質誘発性の細胞毒性と一般的に関連している可能性があり、分子開始イベント後の経路は、遺伝毒性発がん物質と非遺伝毒性発がん物質の間で大きく重複している可能性が示唆された。

D.1.3. 光毒性の AOP

IATA の開発を基に、それに準じた AOP 案を wiki に入力し、外部評価に入れるよう尽力する。

D.2. TG 及び DRP の開発

もとより TG 及び DRP の開発は、提案してから承認まで数年かけて国際合意を取っていく作業である。免疫毒性や生殖毒性試験などの全身毒性に関する *in vitro* TG の開発は前例がなく、これまで以上に

時間を要しており、費用も嵩んでいる。即ち、OECD は、こうした前例のない TG を開発するために、まずは DRP の作成を求めており、数年掛かりで免疫毒性と生殖毒性の GD 作成を進めてきている。さらに、OECD では公定化にあたり、通常、2 回の意見募集を実施するが、これらに対しては、OECD 事務局も通例になく慎重を期している。この分野の先頭を行く日本としては、国際的な専門家の協力を得ながら、焦らず、ことを進めて行く所存である。

また、OECD からの意見募集（in vitro DNT の GD 案）に関しては、ガイダンス文書自体が、*in vitro* 試験の有用性を示す内容でまとめられていることに変わりはないが、本来げっ歯類を主とした動物試験を代替する評価手法として、現行の *in vivo* 試験との対応や、解決すべき課題についての言及が少ないように感じており、*in vivo* 試験と *in vitro* 試験で行われる評価手法と得られる結果のブリッジングの点で、さらに議論を深める必要があると考える。

D.3. IATA の開発

D.3.1. 非遺伝毒性発がん性の IATA 開発への協力

OECDの非遺伝毒性発がん性のIATA開発においては、新規アッセイ法の有用性について注視すると共に、アッセイ系の評価が適切になされるよう、引き続き協力を続ける必要があると考えられた。

Block 3（Cell transformation assay）及び Block 4（Gap junction assay）での assay の選出、利用の考え方の作成ならびに評価が完了し、Block3 は Bhas42 CTA を含む全てのランクで A と Expert working group の全体会議で報告された。Block4 では、2 種のアッセイがランク B、1 種がランク C であり、これらも全体会議で報告された。今後、13 の Block 間での評価の一貫性を確認し、整合性を図る工程が重要になると考えられた。

D.3.2. 光毒性 IATA

構築した IATA 案については今後当該領域のエキスパートから頂いたコメントを基に修正していく予定である。特に decision tree の構築を強く求められているので、draft を作成して関係者間で慎重な協議を進めている。専門家の意見を反映しつつ完成に向けて改訂作業を進めていく。

D.4. AOP 及び TG の実験データ支援

D.4.1. 光安全性評価ツールの予測精度

人工膜を用いた *in vitro* 皮膚透過性試験および ROS assay により構成された光安全性評価系は化合物の光毒性リスクを適切に予測可能であった．さらには予測結果のヒトへの外挿可能性においても良い知見が得られた．以上より，*in vivo* 試験に依存しない本光安全性評価系は将来的に光安全性の高い新規化合物創製に貢献すると期待する．

D.4.2. *In vivo* と相関性のある *in vitro* 毒性評価系による AOP 及び TG の実験データ支援

D.4.2.1. 皮膚組織における代替法の検討

ヒト 3D 皮膚再構成系を用いた評価において、folpet は、OECD TG439 による皮膚刺激性試験で陰性であったが、24 時間暴露条件下で濃度が高くなると細胞生存率が下降傾向を示し、炎症性サイトカインの mRNA 発現が 1000 µg/mL で有意に増加した。したがって、後者のプロトコルは、

OECD TG439による皮膚刺激性試験で陰性の化学物質の細胞傷害性や、in vivoの反復投与経皮毒性に相当する変化を検出できる可能性が示唆された。今後、表皮の毒性に鋭敏な因子の探索や、より簡便なスクリーニングを目指すための HaCaT 細胞を用いた検討を行う予定である。

D.4.2.2.肝臓における代替法の検討

正常または腫瘍肝細胞における脂肪毒性に対する評価において、本実験で使用した細胞株では、特に多価不飽和脂肪酸曝露に対する細胞毒性が異なることが明らかとなった。今後、正常または腫瘍肝細胞の種類を増やし、脂肪酸曝露による細胞毒性の差異を精査する予定である。

D.4.2.3. 腸管由来組織における代替法の検討

腸管由来組織における動物実験代替法の確立を目的として、今年度は、マウスへのDSSの投与、腸管由来の株化細胞 Caco-2 を用いた 2 次元培養による影響を検討し、マウス空腸由来のオルガノイドを用いた 3 次元培養による評価について検討した。いずれの実験系も陽性対照物質としてDSSを用いた。

DSS を投与したマウスにおいて、回腸に明らかな影響は認められなかった。遺伝子発現解析では、TJP-1 の発現が DSS 群において減少傾向にあった。その他 IL-1βなどの炎症促進に関与するサイトカインの遺伝子発現量も低下した。

2 次元培養において、遺伝子発現解析の結果では、DSS 10%濃度以上で細胞が離開し、毒性が生じているものと考えられた。また、WST-8 アッセイの結果、DSS 5%濃度以上で死滅が生じているものと考えられた。今後は、死細胞数測定(lactic acid dehydrogenase (LDH) assay)も行い、実際に細胞死について検討することで、２次元培養条件下における毒性兆候をより明らかにする予定である。

マウス空腸由来のオルガノイドを用いた DSS の評価では、培地中への曝露により DSS 0.5、1%といった低濃度からオルガノイド組織の死滅を示唆する変化が認められた。一方、オルガノイド管腔内にインジェクション曝露した場合には、オルガノイド自体に大型化など形態的変化を認めたものの、組織が死滅に至るような傷害性変化を観察しなかった。このことから、DSS を同濃度に設定して曝露したにもかかわらず、上記のように結果に差が生じたことから、腸管管腔側(インジェクション曝露)よりも基底膜側(培地内曝露)からの DSS 処理による影響が大きいことが明らかとなった。

0.5%濃度以上の DSS のインジェクションにより TJP-1 遺伝子発現の変化や IL-1β遺伝子発現の低下が見られた。

以上、腸管に関連したいずれの実験系においてもDSSの曝露により TJP-1 遺伝子発現への影響や IL-1β遺伝子発現低下が認められた。これは新たな腸管毒性評価における AOP となり得る可能性を示唆するものと考えた。

D.4.3. 発がん性試験における AOP 及び TG の実験データ支援

令和 3(2021)年度は、新規被験物質として腎発がん物質 5 種について、短期試験での最大耐量を用いたラット28日間反復経口投与試験を実施し、腎臓における病理組織学的検索及び γ-H2AX 形成の免疫組織化学的解析を行った。

その結果、被験物質投与群ではいずれも、腎臓における病理組織学的所見及び尿細管上皮細胞での γ-H2AX 形成が誘発されることが明らかとなった。これまでの結果を総合的すると、腎発がん物質 14 物質中 13 物質が γ-H2AX 陽性率の増加を引き起こした一方、非腎発がん物質 7 物質ではいずれも対照群と同じレベルに留まった。以上の結果から、γ-H2AX 免疫染色によって化学物質の腎発がん性を短期間かつ高い感度および特異度で検出可能であることが示唆された。

D.4.4. 遺伝毒性の AOP 開発

発がん性の AOP への組み込みを想定した遺伝毒性初期応答反応の早期検出システム構築の研究では、RNAPI、γH2AX または Ku80 を標的とした ChIP 及び rDNA unit 領域の定量的 PCR の結果から、通常時の RNAPI は rDNA unit 中の転写領域のみに満遍なく存在している一方で、紫外線 DNA 損傷誘導時には転写領域の上流に局在することが明らかになった。これらのことから、通常時の RNAPI は滞りなく rDNA を転写しているが、紫外線 DNA 損傷誘導時には DNA 損傷上で転写が阻害され、RNAPI が転写開始領域近辺に蓄積していることが示唆される。他方、γH2AX 及び Ku80 については、通常時には rDNA 領域にはわずかにしか存在せず、DNA 損傷誘導時に強く rDNA 領域に局在することが明らかになった。特に、これらの局在は rDNA の非転写領域の上流 H18 及び H27 に偏っており、この DNA 領域において γH2AX 及び Ku80 が修復に関わるような DNA 損傷である DNA 鎖切断が生じていることを示唆する。

本手法は ChIP に用いる抗体を改変することで解析の標的タンパク質を自在に設定することができる。今後は化学物質による DNA 損傷誘導時、そして、RPA194、γH2AX、または Ku80 以外の DNA 損傷応答タンパク質や DNA 修復・複製タンパク質等を標的とした同様の解析により、この試験法の発がん性・遺伝毒性 AOP 開発に対する高い有効性を示すことができる。

D.4.5. 腎障害・線維化の分子メカニズムに関する研究

APL 投与ラットにおいては広範な線維化病変が認められ、病変内の尿細管が拡張あるいは委縮していた。この線維化病変内の尿細管の形態学的な特徴は虚血再灌流障害による腎線維化ラットでみられたものと一致していた。この拡張／委縮尿細管は免疫染色において CD44 に明らかに陽性を示した。PNA 投与ラット腎臓においても軽度な線維化が認められた。APL 投与ラット腎臓と同様に、PAN 投与ラット腎臓においても線維化病変内の尿細管は拡張あるいは委縮しており、CD44 の発現が認められた。PAN 投与ラットの腎臓では、典型病変である糸球体障害およびそれに続発するとされる尿細管障害が認められたものの、間質の膠原線維の明らかな増加はみられず、拡張／委縮尿細管も認められなかった。これらの結果は、PAN 投与ラット腎臓では尿細管障害が生じているものの、その再生機構は破綻していないことを示唆していると考えられた、また、PAN 投与ラット腎臓では CD44 陽性の尿細管は認められなかった。

以上の結果は、CD44 は線維化病変内の再生機構の破綻した尿細管に発現することを示唆するものであり、CD44 の汎用性を支持するものと考えられた。

D.5. OECD に提出する資料の事前確認と OECD からの意見募集への対応

過去に日本の対応の不備により混乱を引き起こしたり、日本の SPSF の採択率が低いといった問題点に対処するため、事前確認を実施している。研究代表者の平林は WNT の Bureau であるとともに、安全性生物試験研究センター長でもあることから、厚生労働省の担当者とも連携を図り、引き続き、日本として適切な対応を心掛けていく。

D.6. 毒性等情報収集調査

厚生労働科学研究化学物質リスク研究事業の 4 課題を取り上げ、その進捗を整理し、AOP の構築に向けた課題を明らかにした。

OECD IATA Case Studies Project から AOP を用いた事例を取り上げ、その優位性や課題を整理した。この成果は AOP の確立と AOP を用いた毒性評価の行政導入に検討に資するものであると期待している。

E. 結論

AOP に関しては、「カルシニューリン阻害による T 細胞依存的抗体産生抑制：AOP154」が OECD にて正式に承認され、OECD iLibrary において公開された。日本で開発された初めての AOP である。

TG に関しては、皮膚感作性試験代替法 *In Chemico* Skin Sensitisation、 ADRA TG442C の改定案が令和 3（2021）年 6 月に承認された。また、同時に承認された Defined Approach for Skin Sensitisation の開発に寄与した。

本年 4 月の WNT にて、TG に関しては、*In Chemico* Skin Sensitisation、 ADRA TG442C の最終改定が承認される予定となった。DRP に関しては、*in vitro* 免疫毒性試験が合意される予定である。

F. 健康危険情報

なし

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厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

OECDにおけるAOP, TG及びGDの開発

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研究要旨

本研究班は、化学物質やその混合物の安全性を評価するための国際的な合意を推進する経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）の試験法ガイドライン（TG: Test Guideline）プログラム各国調整官作業グループ（WNT: Working Group of National Co-ordinators of the TGs programme）において、日本で開発された種々の TG やガイダンス文書（GD: Guidance Document）、毒性発現経路（AOP : Adverse Outcome Pathway）などの世界各国が必要とする成果物を公定化させることが目的の一つである。

本研究班の中で、私はこの AOP, TG および GD の公定化を担当した。昨年度からの継続した活動の中、日本人の開発した AOP として、「カルシニューリン阻害による T 細胞依存的抗体産生抑制：AOP154」が OECD にて正式に承認された。TG に関しては、既存の TG である皮膚感作性試験代替法 ADRA（Amino acid Derivative Reactivity Assay）を含む TG442C の改定をなすことができた。同時に承認された Defined Approach for Skin Sensitisation ガイドライン 497 の開発にも寄与した。

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　皮膚科学分野　助教

A. 研究目的

本研究班は、化学物質やその混合物の安全性を評価するための国際的な合意を推進する経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）の試験法ガイドライン（TG: Test Guideline）プログラム各国調整官作業グループ（WNT: Working Group of National Co-ordinators of the TGs programme）において、日本で開発された種々の TG やガイダンス文書（GD: Guidance Document）、毒性発現経路（AOP : Adverse Outcome Pathway）などの世界各国が必要とする成果物を公定化させるとともに、他国が提案する OECD 大型プロジェクトに関与し、その成果物に日本の主張を反映させ、これらから得られた成果を化学物質の審査及び製造等の規制に関する法律（化審法）や毒物及び劇物取締法（毒劇法）などの我が国の厚生労働行政に反映させることを目的としている。

私は、本研究班の中で目的の一つである AOP, TG 及び GD の開発を担当した。本年度、既存の TG である皮膚感作性試験代替法 ADRA や IL-8 Luc アッセイの改定、免疫毒性試験 MITA（Multi-Immuno Toxicity Assay)の TG を開発に努めた。一方で、皮膚感作性 DA（DASS: Defined Approach for Skin Sensitisation）の開発に関与することを通じて、IATA(Integrated Approaches to Testing and Assessment)や DA の国内での普及に務める。

B. 研究方法

B.1. AOP の開発

EAGMST（Extended Advisory Group on Molecular Screening and Toxicogenomics）で行われているOECDのAOP開発プロジェクトの進捗に合わせ、班員を支援した。

研究分担者の足利とともに、日本免疫毒性学会会員をメンバーとする同学会試験法委員会、AOP 検討小委員会に免疫毒性 AOP である「カルシニューリン阻害による T 細胞依存的抗体産生抑制 : AOP154」および「IL-1 receptor 結合阻害 : AOP277 の開発を委託している。文献調査の結果に基づいて、MIE（Molecular Initiating Event）、AO（Adverse Outcome）及びその間に介在する KE (Key Event）を定めて、OECD に指定された外部（または scientific）評価者及びコーチの指摘事項に対応することで開発を進めた。

B.2. TG 及び DRP の開発

研究代表者の平林とともに、OECDのTGの開発プロジェクトWNTの進捗に合わせ、班員を支援した。

B.2.1. 皮膚感作性試験

協力研究者の笠原とともに、皮膚感作性試験代替法 *In Chemico* Skin Sensitisation、ADRA（Amino acid Derivative Reactivity Assay）の追加バリデーション報告書及び TG442C の改定案を作成した。

また、協力研究者の相場とともに、IL-8 Luc assay TG442E の改定案を作成した。

足利とともに、Defined Approach for Skin Sensitisation のプロジェクトに参加し、ガイドラインの成立に協力した。

B.2.2. 免疫毒性試験

相場及び国際的な専門家とともに、*in vitro* 免疫毒性に関する DRP (Detailed Review Paper)を作成した。

DRP の承認を待って提出することを予定している IL-2 を指標とした免疫毒性試験の TG 案を作成した。

B.2.3. 生殖毒性試験の DRP

国際的な専門家とともに、*in vitro* 生殖発生毒性に関する DRP を作成した。

B.3. 光毒性 IATA

研究分担者の尾上及び JaCVAM 資料編纂委員会の協力を得て、光毒性 IATA 案を作成した。

（倫理面への配慮）

特になし

C. 研究結果

C.1.免疫毒性の AOP

「カルシニューリン阻害による T 細胞依存的抗体産生抑制：AOP154」については、WNT/WPHA（ Working Party of Hazard Assessment）に提出したのち、ドイツからコメントが提示されたという連絡があり、AOP Wiki の改訂作業を行った。ドイツからの主な指摘は、TDAR（T cell Dependent Antibody Response）アッセイの方法を詳細に記載すること、本 AOP のみで免疫毒性試験が免除されることはなく、本 AOP は IATA 開発に利用されるべきであること、本 AOP の EU 地域での規制上の重要性についても記載することなどであった。指摘事項に対応し、OECD 事務局に改訂完了の連絡と著者回答ファイルを提出したところ、OECD 事務局から、本 AOP が WNT/WPHA で承認された。その後 OECD 事務局に著作権譲渡に関する著者全員の署名書類を提出し、令和 3（2021）年 10 月 15 日に OECD Library において公開された。

「IL-1 receptor 結合阻害：AOP277」については、AOP 開発の引継ぎに関する web 会議を実施し、元の AOP 開発者である相場らとコーチとの web 会議が行われ、評価結果について説明を受けた。その後 OECD から scientific review report を受領した。その主な推奨事項は、IL-1R シグナルを阻害するストレッサーに特異抗体だけでなく化合物/医薬品を加えること、AP-1 など NF-kB が関与しない経路も考慮すること、T cell のタイプを明確にすること、増加する感染のタイプを明確にすることなどであった。現在これらの推奨事項に対応しており、来年度早々に修正 AOP を OECD に提出予定である。

C.2. TG 及び DRP の開発

C.2.1. 皮膚感作性試験

協力研究者の笠原とともに、昨年度から検討を続けてきた *In Chemico* Skin Sensitisation、 ADRA の改定に尽力した結果、2021 年 6 月に TG の改定が承認された。引き続き、OECD の専門家から要請を受け、ADRA の国内施設の協力を得て適用濃度を 1mM から 4mM に引き上げたプロトコルを用いた追加バリデーションの報告書及び TG442C の最終改定案を作成し、7 月に OECD に提出した。11 月の専門家会議を経て、改訂 TG 案を OECD に提出した。

また、協力研究者の相場とともに、IL-8 Luc assay TG442E の改定案を作成し、7 月に OECD に提出した。11 月に peer review panel からコメントを受け、まだ合意に至っていない。来年度、再協議を行うことになっている。

足利とともに、昨年度から専門家委員会で検討を続けてきた Defined Approach（DA）for Skin Sensitisation の承認に寄与した結果、令和 3（2021）年 6 月にガイドライン 497 が承認された。

本ガイドラインは新しいタイプの OECD ガイドラインである。DA では化学物質の物性および *in vitro* 試験データで検

証された OECD の組み合わせを使用している。その一つとして、 DA では、化学物質規制に *in silico* データを受け入れることを可能にした最初の事例である。

このガイドラインには以下に示す画期的な点が複数含まれている。

1　初めて試験法の結果を組み合わせて評価する手法が公定化された。
2　初めて *in silico* の利用が組み合わせ評価に利用された。
3　ヒトの感作性が予測できる初のガイドラインである。

C.2.2.　免疫毒性試験

相場及び国際的な専門家とともに、昨年度、*in vitro* 免疫毒性に関する DRP を作成した。OECD が集めた意見に対応する改定版を 9 月に提出したところ、2 次募集において追加意見が集まった。国際的な専門家の協力を得て DRP を改定し、2 月に OECD に提出した。

DRP の承認を待って提出する予定の IL-2 を指標とした免疫毒性試験の TG 案を作成し、3 月に OECD に提出した。

C.2.3. 生殖毒性試験の DRP

国際的な専門家とともに、*in vitro* 生殖発生毒性に関する DRP の作成をこの一年継続して実施している。本来は夏休み前に適切な論文に投稿する予定であったが、著者の中に EPA や企業の専門家が含まれていることから、公表にあたり EPA や企業の同意を得ることに手間取ったこともあり、2022 年 2 月に Current Research Toxicology に投稿し、3 月に改訂の指示を受けた。

C.3.　光毒性 IATA

令和元（2019）年 6 月に OECD TG495 としてガイドライン化された ROS assay を主軸として、新たに光安全性評価のための IATA 構築を進めている。既に OECD の専門家会議にて共有し、専門家より修正に資する重要なコメントを頂いている。ICH S10 において推奨されているストレテジーをベースとし、(i) 被験物質の光化学的特性評価、(ii) 光生物化学的特性評価、そして (iii) 皮膚や眼への移行性・滞留性等体内動態評価の　3 段階のスクリーニングによる tiered approach を案として光毒性を中心とした提示をし、各国の専門家と議論している。

D. 考察

D.1. 免疫毒性の AOP

開発中の AOP については、コーチ及び scientific reviewer のコメントに基づいて修正を行っている。IL-23 の機能の説明のように既存の情報を収集することで対応できるものもあるが、医薬品だけでなく化学物質のストレッサーを示すこと、など情報がないものについては調査したことを示して納得いただく必要がある。また、AO が疾患の憎悪である AOP については、行政活用の観点からその実用性に疑問が投げかけられており、すでに承認された AOP154 のように TDAR に変更するか、開発そのものを見直すことを検討中である。ただし、OECD に承認された AOP154 の KE の一つは IL-2 産生であり、本年度日本から別途 OECD に提案した免疫毒性スクリーニング試験 IL-2 Luc assay の TG 化の理論的基盤になるため、その意義は大きいと考える。

D.2. TG 及び DRP の開発

もとより TG 及び DRP の開発は、提案してから承認まで数年かけて国際合意を

取っていく作業である。免疫毒性や生殖毒性試験などの全身毒性に関する *in vitro* TG の開発は前例がなく、これまで以上に時間を要しており、費用も嵩んでいる。即ち、OECD は、こうした前例のない TG を開発するために、まずは DRP の作成を求めており、数年掛かりで免疫毒性と生殖毒性の GD 作成を進めてきている。さらに、OECD では公定化にあたり、通常、2 回の意見募集を実施するが、これらに対しては、OECD 事務局も通例になく慎重を期している。この分野の先頭を行く日本としては、国際的な専門家の協力を得ながら、焦らず、ことを進めて行く所存である。

D.3.2. 光毒性 IATA

構築した IATA 案については今後当該領域の専門家から頂いたコメントを基に修正していく予定である。特に decision tree の構築を強く求められているので、draft を作成して関係者間で慎重な協議を進めたい。

F. 添付資料

1. OECD Test Guideline 442C for the Testing Chemicals on in chemico skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on covalent binding to proteins (2021)
2. OECD Guideline No.497, Guideline on Defined Approaches for Skin Sensitisation (2021)

G. 研究発表

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4. Kojima H. 21st-Century Toxicology and Regulatory Testing: An Update from Japan, 11th World Congress on Alternatives and Animal Use in the Life Sciences (WC11)（2021.9.1, the Netherlands, On line）
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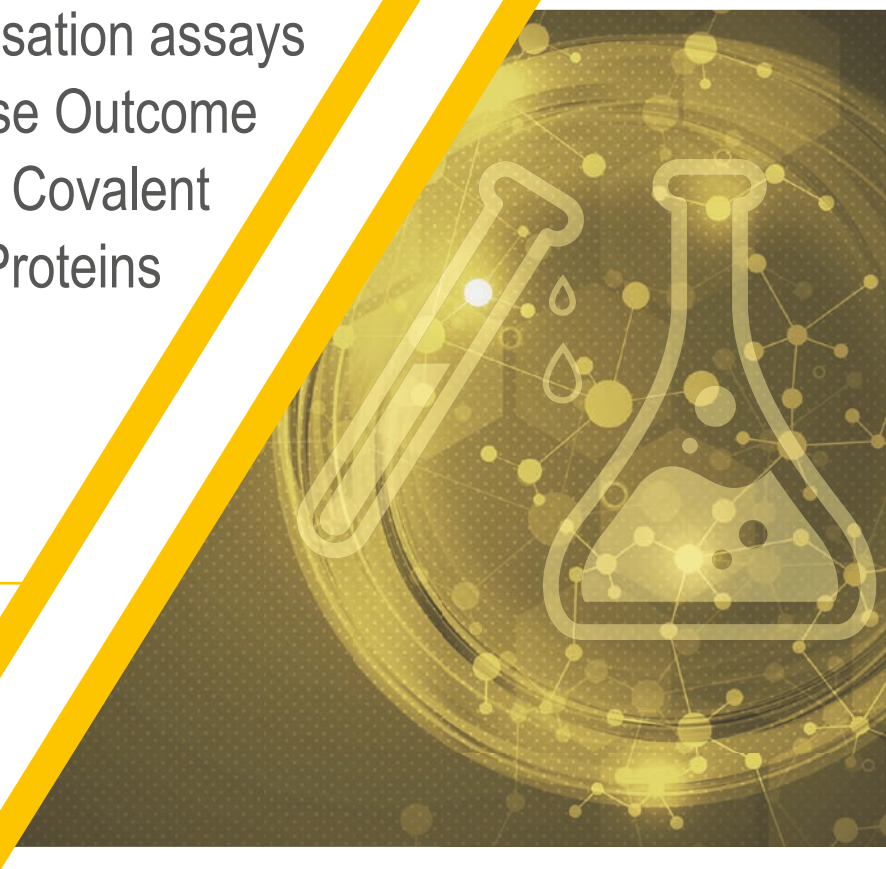
H. 知的所有権の取得状況

1. 特許取得
   特になし
2. 実用新案登録
   特になし
3. その他
   特になし

Section 4
Health effects

Test Guideline No. 442C

Key Event-Based Test Guideline for *in chemico* skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins



14 June 2021

OECD Guidelines for the Testing of Chemicals

OECD
BETTER POLICIES FOR BETTER LIVES

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Key Event-Based Test Guideline for in chemico skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins

INTRODUCTION

Covalent binding to proteins Key Event based Test Guideline.

1. A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). There is general agreement on the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised as an Adverse Outcome Pathway (AOP) (2) starting with a molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with amino-acid residues (i.e. cysteine or lysine) such as organic chemicals. In this instance, the molecular initiating event (i.e. the first key event), is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation.

2. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman and the Buehler Test (OECD TG 406) (11) assess both the induction and elicitation phases of skin sensitisation. The murine tests, such as the LLNA (OECD TG 429) (12) and its three non-radioactive modifications — LLNA:DA (OECD TG 442A) (13), LLNA:BrdU-ELISA, and BrdU-FCM (OECD TG 442B) (14) — all assess the induction response exclusively and have gained acceptance, since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitisation.

3. Mechanistically-based *in chemico* and *in vitro* test methods addressing the first three key events of the skin sensitisation AOP have been adopted for contributing to the evaluation of the skin sensitisation hazard potential of chemicals: the present Test Guideline assesses covalent binding to proteins, addressing the first key event; the OECD TG 442D assesses keratinocyte activation (15), the second key event and the OECD TG 442E addresses the activation of dendritic cells (16), the third key event of the skin sensitisation AOP. Finally, the fourth key event representing T-cell proliferation is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (12).

Background and principles of the test methods included in the Key Event based Test Guideline

4. This Test Guideline (TG) describes *in chemico* assays that address mechanisms described under the first key event of the AOP for skin sensitisation, namely covalent binding to proteins (2). The test methods currently included in this Test Guideline are:
    - The Direct Peptide Reactivity Assay (DPRA) (Appendix I),
    - The Amino Acid Derivative Reactivity Assay (ADRA) (Appendix II), and
    - The kinetic Direct Peptide Reactivity Assay (kDPRA) (Appendix III).
5. The test methods are based on *in chemico* covalent binding to proteins and are considered to be scientifically valid. The DPRA has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-lead validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) (3) (4) (5). The ADRA underwent a validation study coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6) (7) (8) (9) followed by an independent peer-review (10). The kDPRA underwent an industry-coordinated validation study followed by an independent peer-review (17).
6. The test methods included in this Test Guideline might differ with regard to the procedures used to generate the data but can each be used to address countries' requirements for test results on protein reactivity, while benefiting from the Mutual Acceptance of Data.
7. The correlation of protein reactivity with skin sensitisation potential is well established (18) (19) (20). Nevertheless, since protein reactivity represents only one key event of the skin sensitisation AOP (2) (21), information generated with test methods developed to address this specific key event may not be sufficient as stand-alone methods to conclude on the presence or absence of skin sensitisation potential of chemicals. Therefore data generated with the test methods described in this Test Guideline are proposed to be used within Integrated Approaches to Testing and Assessment (IATA), together with other relevant complementary information from *in vitro* assays addressing other key events of the skin sensitisation AOP as well as non-testing methods, including *in silico* modelling and read-across from chemical analogues (21). Examples on the use of data generated with these methods within Defined Approaches (DAs), i.e. approaches standardised both in relation to the set of information sources used and in the procedure applied to derive predictions, have been published (21) and can be employed as useful elements within IATA.
8. The DPRA and ADRA described in Appendixes I and II to this Test Guideline, respectively, support the discrimination of skin sensitisers (Category 1) from non-sensitisers. Depending on the regulatory framework, positive results generated with these methods may be used on their own to classify a chemical into UN GHS Category 1. However, these test methods do not allow on their own, the sub-categorisation of skin sensitisers into subcategories 1A and 1B (22), as defined by UN GHS (1) for authorities implementing these two optional subcategories, or potency prediction for safety assessment decisions.
9. In contrast, the kDPRA described in Appendix III of this Test Guideline, allows discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) i.e., subcategory 1B or no category (1) but does not allow to distinguish sensitisers (Category 1) from non-sensitisers. Depending on the regulatory framework, positive results generated with the kDPRA may be used on their own to classify a chemical into UN GHS subcategory 1A.
10. Definitions are provided in the Annex. Performance Standards for the assessment of proposed similar or modified *in vitro* skin sensitisation DPRA and ADRA test methods have been developed (23).

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ANNEX - DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (1). The formula used to derive accuracy is shown under "Calculation" of predictive capacity.

ADRA: Amino acid Derivative Reactivity Assay.

AOP (Adverse Outcome Pathway): sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an *in vivo* outcome of interest (2).

Calculation

Calculating depletion of either NAC or NAL

Depletion is calculated as follows:

$$\text{Percent NAC or NAL depletion} = \left[1-\left[\frac{\text{NAC or NAL peak area in replicate injection}}{\text{Mean NAC or NAL peak area in reference controls C}}\right]\right]\text{x}100$$

Calculating predictive capacity

Sensitivity, specificity and accuracy are calculated based on the true positive (TP), true negative (TN), false negative (FN), and false positive (FP) values as follows:

$$\text{Sensitivity} = \frac{\text{Number of true positives (TP)}}{\text{Number of all positive chemicals (TP+FN)}}$$

$$\text{Specificity} = \frac{\text{Number of true negatives (TN)}}{\text{Number of all negative chemicals (TN+FP)}}$$

$$\text{Accuracy} = \frac{\text{Number of correct predictions (TP+TN))}}{\text{Number of all chemicals (TP+FN+TN+FP)}}$$

Calibration curve: The relationship between the experimental response value and the analytical concentration (also called standard curve) of a known substance.

Coefficient of variation: a measure of variability that is calculated for a group of replicate data by dividing the standard deviation by the mean. It can be multiplied by 100 for expression as a percentage.

Defined Approach (DA): a DA consists of a fixed data interpretation procedure (e.g. statistical, mathematical models) applied to data (e.g. *in silico* predictions, *in chemico*, *in vitro* data) generated with a defined set of information sources to derive a prediction.

DPRA: Direct Peptide Reactivity Assay.

EDTA: Ethylenediaminetetraacetic acid.

EURL ECVAM: the European Union Reference Laboratory for Alternatives to Animal Testing.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

IATA (Integrated Approach to Testing and Assessment): A structured approach used for hazard identification (potential), hazard characterisation (potency), and/or safety assessment (potential/potency

and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazards, risks, and the need for further targeted and therefore minimal testing.

JaCVAM: Japanese Center for the Validation of Alternative Methods.

kDPRA: kinetic Direct Peptide Reactivity Assay.

$\mathbf{k_{max}}$: is the maximum rate constant (in $s^{-1}M^{-1}$) determined from the reaction kinetics for a tested substance in the kDPRA (see Appendix III, paragraph 24).

LLNA: murine Local Lymph Node Assay issued as OECD TG 429 in 2010.

Molecular Initiating Event: Chemical-induced perturbation of a biological system at the molecular level identified to be the starting event in the adverse outcome pathway.

Mixture: A solid or liquid comprising two or more substances which do not react chemically (3).

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent comprises at least 80% (w/w) of the whole.

Multi-constituent substance: A substance, defined by its quantitative composition, in which two or more main constituents are present in concentrations ≥ 10% (w/w) and < 80% (w/w). Multi-constituent substances are the result of a manufacturing process. The difference between a mixture and a multi-constituent substance is that a mixture comprises two or more substances which do not react chemically, whereas a multi-constituent substance comprises two or more substances that do react chemically.

NAC: N-(2-(1-naphthyl)acetyl)-L-cysteine (4) (5) (6).

NAL: α-N-(2-(1-naphthyl)acetyl)-L-lysine (4) (5) (6).

Positive control: A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Pre-haptens: chemicals which become sensitisers through abiotic transformation.

Pro-haptens: chemicals requiring enzymatic activation to exert skin sensitisation potential.

Reference control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent negative control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (1).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (1).

Reproducibility: The concordance of results obtained from testing the same substance using the same test protocol (see reliability). (1)

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important

consideration in assessing the relevance of a test method (1). The formula used to derive sensitivity is shown under ”Calculation” of predictive capacity.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (1). The formula used to derive specificity is shown under “Calculation” of predictive capacity.

Substance: Chemical elements and their compounds in the natural state or resulting from a manufacturing process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process, but excluding solvents that may be separated without affecting the stability of the substance or changing its composition (3).

System suitability: Determination of instrument performance (e.g. sensitivity) by analysis of reference standards prior to running the analytical run (7).

Test chemical: The term test chemical is used to refer to the substance being tested.

TFA: Trifluoroacetic acid.

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (3).

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (1).

Literature for definitions

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APPENDIX I

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The DPRA is proposed to address the molecular initiating event of the skin sensitisation AOP, namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine (1). Cysteine and lysine percent peptide depletion values are then used to categorise a substance in one of four classes of reactivity for supporting the discrimination between skin sensitisers and non-sensitisers (2).

2. The DPRA test method proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 85% within laboratories and 80% between laboratories (3). Results generated in the validation study (4) and published studies (5) overall indicate that the accuracy of the DPRA in discriminating sensitisers (i.e. UN GHS Category 1) from non-sensitisers is 80% (N=157) with a sensitivity of 80% (88/109) and specificity of 77% (37/48) when compared to LLNA results. The DPRA is more likely to under predict chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (4) (5). However, the accuracy values given here for the DPRA as a stand-alone test method are only indicative since the test method should be considered in combination with other sources of information in the context of an IATA or a DA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in the species of interest, i.e. humans. On the basis of the overall data available, the DPRA was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physico-chemical properties (1) (2) (3) (5). Taken together, this information indicates the usefulness of the DPRA to contribute to the identification of skin sensitisation hazard.

3. The term "test chemical" is used in this Test Guideline to refer to what is being tested[1] and is not related to the applicability of the DPRA to the testing of substances and/or mixtures (see a summary of the known limitations of the DPRA in Annex 1 of this Appendix). This test method is not applicable for the testing of metal compounds since they are known to react with proteins with mechanisms other than covalent binding. A test chemical should be soluble in an appropriate solvent at a final concentration of 100 mM (see paragraph 10).

[1] In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term “test chemical” describing what is being tested should now be applied in new and updated Test Guidelines.

However, test chemicals that are not soluble at this concentration may still be tested at lower soluble concentrations. In such a case, a positive result could still be used to support the identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result. Limited information is currently available on the applicability of the DPRA to mixtures of known composition (4) (5). The DPRA is nevertheless considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition (see paragraph 4 and 10). When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Appendix of the Test Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. The current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances) due to the defined molar ratio of test chemical and peptide. For this purpose a new prediction model based on a gravimetric approach will need to be developed. In cases where evidence can be demonstrated on the non-applicability of the test method to other specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

4. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be detected by the test method. Chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) are reported to be in most cases correctly detected by the test method (4) (9) (10). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA or a DA. Test chemicals that do not covalently bind to the peptide but promote its oxidation (i.e. cysteine dimerisation) could lead to a potential over estimation of peptide depletion, resulting in possible false positive predictions and/or assignment to a higher reactivity class (see paragraphs 21 and 22).

5. As described, the DPRA assay supports the discrimination between skin sensitisers and non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency (6) (11) when used in integrated approaches such as IATA or DA (12). However further work, preferably based on human data, is required to determine how DPRA results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

6. The DPRA is an *in chemico* method which quantifies the remaining concentration of cysteine- or lysine-containing peptide following 24 hours incubation with the test chemical at 22.5-30°C. The synthetic peptides contain phenylalanine to aid in the detection. Relative peptide concentration is measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm. Cysteine- and lysine peptide percent depletion values are then calculated and used in a prediction model (see paragraph 21) which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitisers and non-sensitisers.

7. Prior to routine use of the method described in this Appendix, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 2.

PROCEDURE

8. This test method is based on the DPRA DB-ALM protocol n° 154 (7) which represents the protocol used for the EURL ECVAM-coordinated validation study. It is recommended that this protocol is used when implementing and using the method in the laboratory. The following is a description of the main components and procedures for the DPRA. If an alternative HPLC set-up is used, its equivalence to the validated set-up described in the DB-ALM protocol should be demonstrated (e.g. by testing the proficiency substances in Annex 2).

Preparation of the cysteine or lysine-containing peptides

9. Stock solutions of cysteine (Ac-RFAA**C**AA-COOH) and lysine (Ac-RFAA**K**AA-COOH) containing synthetic peptides of purity higher than 85% and preferably > 90%, should be freshly prepared just before their incubation with the test chemical. The final concentration of the cysteine peptide should be 0.667 mM in pH 7.5 phosphate buffer whereas the final concentration of the lysine peptide should be 0.667 mM in pH 10.2 ammonium acetate buffer. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this test method, up to 26 analysis samples (which include the test chemical, the positive control and the appropriate number of solvent controls based on the number of individual solvents used in the test, each tested in triplicate), can be accommodated in a single HPLC run. All of the replicates analysed in the same run should use the identical cysteine and lysine peptide stock solutions. It is recommended to prove individual peptide batches for proper solubility prior to their use.

Preparation of the test chemical

10. Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay following the solubilisation procedure described in the DPRA DB-ALM protocol (7). An appropriate solvent will dissolve the test chemical completely. Since in the DPRA the test chemical is incubated in large excess with either the cysteine or the lysine peptides, visual inspection of the forming of a clear solution is considered sufficient to ascertain that the test chemical (and all of its components in the case of testing a multi-constituent substance or a mixture) is dissolved. Suitable solvents are, acetonitrile, water, 1:1 mixture water:acetonitrile, isopropanol, acetone or 1:1 mixture acetone:acetonitrile. Other solvents can be used as long as they do not have an impact on the stability of the peptide as monitored with reference controls C (i.e. samples constituted by the peptide alone dissolved in the appropriate solvent; see Annex 3). If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts. It is important to note that DMSO may lead to peptide dimerisation and as a result, it may be more difficult to meet the acceptance criteria. If DMSO is chosen, attempts should be made to first solubilise the test chemical in 300 µL of DMSO and dilute the resulting solution with 2700 µL of acetonitrile. If the test chemical is not soluble in this mixture, attempts should be made to solubilise the same amount of test chemicals in 1500 µL of DMSO and dilute the resulting solution with 1500 µL of acetonitrile. The test chemical should be pre-weighed into glass vials and dissolved immediately before testing in an appropriate solvent to prepare a 100 mM solution. For mixtures and multi-constituent substances of known composition, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight should be determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight should then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution. For polymers for which a predominant molecular weight cannot be determined, the molecular weight of the monomer (or the apparent molecular weight of the various monomers constituting the polymer) may be considered to

prepare a 100 mM solution. However, when testing mixtures, multi-constituent substances or polymers of known composition, it should be considered to also test the neat chemical. For liquids, the neat chemical should be tested as such without any prior dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. For solids, the test chemical should be dissolved to its maximum soluble concentration in the same solvent used to prepare the apparent 100 mM solution. It should then be tested as such without any further dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. Concordant results (reactive or non-reactive) between the apparent 100 mM solution and the neat chemical should allow for a firm conclusion on the result.

Preparation of the positive control, reference controls and coelution controls

11. Cinnamic aldehyde (CAS 104-55-2; ≥95% food-grade purity) should be used as positive control (PC) at a concentration of 100 mM in acetonitrile. Other suitable positive controls providing mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition reference controls (i.e. samples containing only the peptide dissolved in the appropriate solvent) should also be included in the HPLC run sequence and these are used to verify the HPLC system suitability prior to the analysis (reference controls A), the stability of the reference controls over time (reference control B) and to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion (reference control C) (see Annex 3). The appropriate reference control for each substance is used to calculate the percent peptide depletion for that substance (see paragraph 18). In addition, a co-elution control constituted by the test chemical alone for each of the test chemicals analysed should be included in the run sequence to detect possible co-elution of the test chemical with either the lysine or the cysteine peptide.

Incubation of the test chemical with the cysteine and lysine peptide solutions

12. Cysteine and lysine peptide solutions should be incubated in glass autosampler vials with the test chemical at 1:10 and 1:50 ratio respectively. If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, one cannot be sure how much test chemical remained in the solution to react with the peptide. Therefore, in such a case, a positive result could still be used, but a negative result is uncertain and should be interpreted with due care (see also provisions in paragraph 10 for the testing of chemicals not soluble up to a concentration of 100 mM). The reaction solution should be left in the dark at 22.5-30°C for 24±2 hours before running the HPLC analysis. Each test chemical should be analysed in triplicate for both peptides. Samples have to be visually inspected prior to HPLC analysis. If a precipitate or phase separation is observed, samples may be centrifuged at low speed (100-400xg) to force precipitate to the bottom of the vial as a precaution since large amounts of precipitate may clog the HPLC tubing or columns. If a precipitation or phase separation is observed after the incubation period, peptide depletion may be underestimated and a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result.

Preparation of the HPLC standard calibration curve

13. A standard calibration curve should be generated for both the cysteine and the lysine peptides. Peptide standards should be prepared in a solution of 20% or 25% acetonitrile:buffer using phosphate buffer (pH 7.5) for the cysteine peptide and ammonium acetate buffer (pH 10.2) for the lysine peptide. Using serial dilution standards of the peptide stock solution (0.667 mM), 6 calibration solutions should be prepared to cover the range from 0.534 to 0.0167 mM. A blank of the dilution buffer should also be included in the standard calibration curve. Suitable calibration curves should have an $r^2 > 0.99$.

HPLC preparation and analysis

14. The suitability of the HPLC system should be verified before conducting the analysis. Peptide depletion is monitored by HPLC coupled with an UV detector (photodiode array detector or fixed wavelength absorbance detector with 220 nm signal). The appropriate column is installed in the HPLC system. The HPLC set-up described in the validated protocol uses a Zorbax SB-C-18 2.1 mm x 100 mm x 3.5 micron as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated at 30°C with 50% phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for at least 2 hours before running. The HPLC analysis should be performed using a flow rate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 minutes, followed by a rapid increase to 90% acetonitrile to remove other materials. Equal volumes of each standard, sample and control should be injected. The column should be re-equilibrated under initial conditions for 7 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the cysteine and lysine peptides, including the injection volume, which may vary according to the system used (typically in the range from 3-10 µL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated (e.g. by testing the proficiency substances in Annex 2). Absorbance is monitored at 220 nm. If a photodiode array detector is used, absorbance at 258 nm should also be recorded. It should be noted that some supplies of acetonitrile could have a negative impact on peptide stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 220 peak area and the 258 peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90%<mean[2] area ratio of control samples<100% would give a good indication that co-elution has not occurred.

15. There may be test chemicals which could promote the oxidation of the cysteine peptide. The peak of the dimerised cysteine peptide may be visually monitored. If dimerisation appears to have occurred, this should be noted as percent peptide depletion may be over-estimated leading to false positive predictions and/or assignment to a higher reactivity class (see paragraphs 21 and 22).

16. The HPLC analysis should be timed to assure that the injection of the first sample starts 22 to 26 hours after the test chemical was mixed with the peptide solution. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this test method, up to 26 analysis samples can be accommodated in a single HPLC run (see also paragraph 9). An example of HPLC analysis sequence is provided in Annex 3.

DATA AND REPORTING

Data evaluation

17. The concentration of cysteine or lysine peptide is photometrically determined at 220 nm in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of peptide using the linear calibration curve derived from the standards.

18. The percent peptide depletion is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant reference controls C (see Annex 3) according to the formula described below.

[2] For mean it is meant arithmetic mean throughout the document.

$$\text{Percent peptide depletion} = \left[1 - \left(\frac{\text{Peptide peak area in replicate injection}}{\text{Mean peptide peak area in reference controls } C}\right)\right] \times 100$$

Acceptance criteria

19. The following criteria should be met for a run to be considered valid:

a) the standard calibration curve should have an $r^2 > 0.99$,

b) the mean percent peptide depletion value of the three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69.0% for the lysine peptide (for other positive controls a reference range needs to be established) and the maximum standard deviation (SD) for the positive control replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion and

c) the mean peptide concentration of reference controls A should be 0.50±0.05 mM and the coefficient of variation (CV) of peptide peak areas for the nine reference controls B and C in acetonitrile should be <15.0%.

If one or more of these criteria is not met the run should be repeated.

20. The following criteria should be met for a test chemical's results to be considered valid:

a) the maximum standard deviation for the test chemical replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion,

b) the mean peptide concentration of the three reference controls C in the appropriate solvent should be 0.50±0.05 mM.

If these criteria are not met the data should be rejected and the run should be repeated for that specific test chemical.

Prediction model

21. The mean percent cysteine and percent lysine depletion value is calculated for each test chemical. Negative depletion is considered as "0" when calculating the mean. By using the cysteine 1:10/lysine 1:50 prediction model shown in Table 1, the threshold of 6.38% average peptide depletion should be used to support the discrimination between skin sensitisers and non-sensitisers in the framework of an IATA or DA. Application of the prediction model for assigning a test chemical to a reactivity class (i.e. low, moderate and high reactivity) may perhaps prove useful to inform potency assessment within the framework of an IATA or DA.

Table 1: Cysteine 1:10/lysine 1:50 prediction model[1]

Mean of cysteine and lysine % depletion	Reactivity Class	DPRA Prediction[2]
0% ≤ mean % depletion ≤ 6.38%	No or minimal reactivity	Negative
6.38% < mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < mean % depletion ≤ 100%	High reactivity	

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement (2).
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

22. There might be cases where the test chemical (the substance or one or several of the components of a multi-constituent substance or a mixture) absorbs significantly at 220 nm and has the same retention time of the peptide (co-elution). Co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and the peptide. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated (e.g. by testing the proficiency substances in Annex 2). When co-elution occurs the peak of the peptide cannot be integrated and the calculation of the percent peptide depletion is not possible. If co-elution of such test chemicals occurs with both the cysteine and the lysine peptides, or with the cysteine peptide only, then the analysis should be reported as “inconclusive”. In cases where co-elution occurs only with the lysine peptide, then the cysteine 1:10 prediction model reported in Table 2 can be used.

Table 2: Cysteine 1:10 prediction model[1]

Cysteine (Cys) % depletion	Reactivity class	DPRA prediction[2]
0% ≤ Cys % depletion ≤ 13.89%	No or minimal reactivity	Negative
13.89% < Cys % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < Cys % depletion ≤ 98.24%	Moderate reactivity	
98.24% < Cys % depletion ≤ 100%	High reactivity	

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

23. There might be other cases where the overlap in retention time between the test chemical and either of the peptides is incomplete. In such cases percent peptide depletion values can be estimated and used in the cysteine 1:10/lysine 1:50 prediction model, however assignment of the test chemical to a reactivity class cannot be made with accuracy.

24. A single HPLC analysis for both the cysteine and the lysine peptide should be sufficient for a test chemical when the result is unequivocal. However, in cases of results close to the threshold used to discriminate between positive and negative results (i.e. mean percent depletion falls in the range of 3% to 10% for the cysteine 1:10/lysine 1:50 prediction model or cysteine percent depletion falls in the range of 9% to 17% for the cysteine 1:10

prediction model), additional testing is recommended. In particular, in case of negative results in these ranges (i.e. 3% to 6.38% for the cysteine 1:10/lysine 1:50 prediction model or 9% to 13.89% for the cysteine 1:10 prediction model), a second run should be conducted, as well as a third one in case of discordant results between the first two runs.

Test report

25. The test report should include the following information

Test chemical and Controls (positive control and solvent/vehicle)

- Mono-constituent substance (test and control chemicals)
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physicochemical properties such as physical state, appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
    - Treatment prior to testing, if applicable (e.g. warming, grinding);
    - Concentration(s) tested;
    - Storage conditions and stability to the extent available.
- Multi-constituent substance, UVCB and mixture:
    - Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
    - Physical appearance, water solubility and additional relevant physicochemical properties, to the extent available;
    - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
    - Treatment prior to testing, if applicable (e.g. warming, grinding);
    - Concentration(s) tested;
    - Storage conditions and stability to the extent available.
- Additional information for positive control
    - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Additional information for solvent/vehicle control
    - Solvent/vehicle used and ratio of its constituents, if applicable;
    - Justification for choice of solvent for each test chemical;
    - For acetonitrile, results of test of impact on peptide stability.

Peptides

- Supplier, lot, purity

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, detector, autosampler;
- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient.

System suitability

- Peptide peak area at 220 nm of each standard and reference control A replicate;
- Linear calibration curve graphically represented and the r^2 reported;
- Peptide concentration of each reference control A replicate;
- Mean peptide concentration (mM) of the three reference controls A, SD and CV;
- Peptide concentration of reference controls A and C.

Analysis sequence

- For reference controls:
    - Peptide peak area at 220 nm of each B and C replicate;
    - Mean peptide peak area at 220 nm of the nine reference controls B and C in acetonitrile, SD an CV (for stability of reference controls over analysis time);
    - For each solvent used, the mean peptide peak area at 220 nm of the three appropriate reference controls C (for the calculation of percent peptide depletion);
    - For each solvent used, the peptide concentration (mM) of the three appropriate reference controls C;
    - For each solvent used, the mean peptide concentration (mM) of the three appropriate reference controls C, SD and CV.
- For positive control:
    - Peptide peak area at 220 nm of each replicate;
    - Percent peptide depletion of each replicate;
    - Mean percent peptide depletion of the three replicates, SD and CV.
- For each test chemical:
    - Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;
    - Presence of co-elution;
    - Description of any other relevant observations, if applicable;
    - Peptide peak area at 220 nm of each replicate;
    - Percent peptide depletion of each replicate;
    - Mean of percent peptide depletion of the three replicate, SD and CV;
    - Mean of percent cysteine and percent lysine depletion values;
    - Prediction model used and DPRA prediction.

Proficiency testing

- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals.

Discussion of the results

- Description of any unintended modifications to the test procedure.
- Discussion of the results obtained with the DPRA test method and if it is within the ranges described in paragraph 24.

Conclusion

LITERATURE FOR APPENDIX I

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APPENDIX I, ANNEX 1

KNOWN LIMITATIONS OF THE DIRECT PEPTIDE REACTIVITY ASSAY

The table below provides a summary of the known limitations of the DPRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Pro-haptens	Test Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential; cannot be detected by the test method unless activation is caused by auto-oxidation to a similar degree as in vivo /in humans. It will however normally not be known whether this will be the case	May lead to false negatives. Negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA or a DA	Diethylenetriamine; 111-40-0 (1A chez l'homme, LLNA n/a)
Pre-haptens	Chemicals that become sensitisers after abiotic transformation are reported to be in most cases correctly detected by the test method		Linalool: 78-70-6
Test chemicals absorbing significantly at 220 nm and having the same retention time of the peptides (co-elution)	When co-elution occurs the peak of the peptide cannot be integrated and the calculation of the percent peptide depletion is not possible	If co-elution of such test chemicals occurs with both the cysteine and the lysine peptides, or with the cysteine peptide only, then the analysis should be reported as "inconclusive" and alternative HPLC set up should be considered (see paragraph 22). In cases where co-elution occurs only with the lysine peptide, then the cysteine 1:10 prediction model reported in Table 2 can be used.	Salicylic acid: 69-72-7
Complex mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials	This is due to the need for defined molar ratio of test chemical and peptide	n/a	UVCBs, chemical emissions, products or formulations with variable or not fully known composition
Test chemicals which cannot be dissolved in an appropriate solvent at a final concentration of 100 mM	Not sure if sufficient exposure can be achieved	Test chemicals that are not soluble at this concentration may still be tested at lower soluble concentrations. In such a case, a positive result could be used to support the identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.	n/a
Chemicals which precipitate in reaction solution	Not sure if sufficient exposure can be achieved	A conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result	Isopropyl myristate CAS: 110-27-0

Test chemicals that do not covalently bind to the cysteine-peptide but promote its oxidation (i.e. cysteine dimerisation)	Could lead to a potential over-estimation of cysteine-peptide depletion, resulting in possible false positive predictions.		DMSO Oxidant
Test chemicals that are only soluble in DMSO	DMSO causes excessive peptide depletion due to cysteine dimerization resulting in high background cysteine depletion.	May lead to false negative results	n/a

APPENDIX I, ANNEX 2

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay

Prior to routine use of the test method described in this test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected DPRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining cysteine and lysine depletion values that fall within the respective reference range for 8 out of the 10 proficiency substances for each peptide. These proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the DPRA are available, and that they were used in the EURL ECVAM-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Table 1: Recommended proficiency substances for demonstrating technical proficiency with the Direct Peptide Reactivity Assay

Proficiency substances	CASRN	Physical state	*In vivo* prediction[1]	DPRA prediction[2]	Range[3] of % cysteine peptide depletion	Range[3] of % lysine peptide depletion
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Positive	90-100	15-45
Oxazolone	15646-46-5	Solid	Sensitiser (extreme)	Positive	60-80	10-55
Formaldehyde	50-00-0	Liquid	Sensitiser (strong)	Positive	30-60	≤ 24
Benzylideneacetone	122-57-6	Solid	Sensitiser (moderate)	Positive	80-100	≤ 7
Farnesal	19317-11-4	Liquid	Sensitiser (weak)	Positive	15-55	≤ 25
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	Positive	60-100	10-45
1-Butanol	71-36-3	Liquid	Non-sensitiser	Negative	≤ 7	≤ 5.5
6-Methylcoumarin	92-48-8	Solid	Non-sensitiser	Negative	≤ 7	≤ 5.5
Lactic Acid	50-21-5	Liquid	Non-sensitiser	Negative	≤ 7	≤ 5.5
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	Negative	≤ 7	≤ 5.5

[1]The *in vivo* hazard and (potency) predictions are based on LLNA data (5). The *in vivo* potency is derived using the criteria proposed by ECETOC (8).

[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

[3] Ranges determined on the basis of at least 10 depletion values generated by 6 independent laboratories.

APPENDIX I, ANNEX 3

EXAMPLES OF ANALYSIS SEQUENCE

Calibration standards and reference controls	STD1 STD2 STD3 STD4 STD5 STD6 Dilution buffer Reference control A, rep 1 Reference control A, rep 2 Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1 Reference control B, rep 2 Reference control B, rep 3
First set of replicates	Reference control C, rep 1 Cinnamic aldehyde, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Cinnamic aldehyde, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Cinnamic aldehyde, rep 3 Sample 1, rep 3 Sample 2, rep 3
Reference controls	Reference control B, rep 4 Reference control B, rep 5 Reference control B, rep 6

Three sets of reference controls (i.e. samples constituted only by the peptide dissolved in the appropriate solvent) should be included in the analysis sequence:

Reference control A: used to verify the suitability of the HPLC system.

Reference control B: included at the beginning and at the end of the analysis sequence to verify stability of reference controls over the analysis time.

Reference control C: included in the analysis sequence to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion.

APPENDIX II

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The ADRA is proposed to address the molecular initiating event of the skin sensitisation AOP—namely, protein reactivity—by quantifying the reactivity of test chemicals towards model synthetic amino acid derivatives containing either lysine or cysteine (1) (2) (3). Depletion values of cysteine and lysine derivatives are then used to support the discrimination between skin sensitisers and non-sensitisers (1) (2) (3).

2. The ADRA proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis. ADRA's WLR was 100% (10/10), 100% (7/7), 90% (9/10), and 100% (10/10) in four participating laboratories. BLR for 40 test chemicals calculated based the results from three participating laboratories was 91.9% (4). For the 40 chemicals tested in the validation study in four laboratories, the cumulative accuracy was 86.9% (139/160), sensitivity was 81.5% (88/108), and specificity was 98.1% (51/52) (4) (5). Results from the validation study (4) (5) as well as from other published studies (3) indicate that ADRA identified sensitisers and non-sensitisers with an accuracy of 79% (98/124) (124 compounds that fall within ADRA's applicability domain), a sensitivity of 74% (65/88), and a specificity of 92% (33/36) relative to LLNA results (6). In addition, the prediction of human skin sensitisation for 73 compounds that fall within ADRA's applicability domain has an accuracy of 86% (63/73), a sensitivity of 85% (44/52), and a specificity of 90% (19/21) (6). However, the accuracy values given here for ADRA as a stand-alone test method are for reference only, since it is recommended that the test method be used in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General Introduction. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in the species of interest, which is humans. On the basis of the overall data available, ADRA's applicability domain was shown to include a variety of organic functional groups, reaction mechanisms, skin sensitisation potencies (as determined in *in vivo* studies), and physicochemical properties (1) (2) (3) (4). Following an independent peer review, the ADRA validation study was considered to demonstrate that this method should be acceptable as part of an integrated testing strategy for the predictive identification of skin sensitisation hazard (6) (7).

3. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the ADRA to the testing of substances and/or mixtures (see a summary of the known limitations of the ADRA in Annex 1 of this Appendix). This test method is not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be detected by the test method. Chemicals

that become sensitisers after abiotic transformation (i.e. pre-haptens) are reported to be in some cases correctly detected by the test method (1) (2) (3) (4). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA. Test chemicals that promote the oxidation of the *N*-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) reagent (i.e. cysteine dimerisation) could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions (see paragraphs 27 and 28); it may be possible to detect and quantify any NAC dimer formed by HPLC, thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and covalent bonding to the test item substance(s).

4. The ADRA test method allows testing of poorly soluble chemicals (8). To be tested, a test chemical should be soluble in an appropriate solvent at a final concentration of 1 mM (see paragraph 14). Test chemicals that are not soluble at this concentration may still be tested at lower concentrations. In such cases, a positive result could still be used to support identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.

5. In general, many organic compounds absorb UV in the range of 220 nm. In the case of co-elution of the nucleophilic reagent and the test chemical, this might result in false negative prediction. This may happen with the DPRA which specifies that quantification of the peptide-based nucleophilic reagents has to be performed at 220 nm. In contrast to this, the nucleophilic reagents used in ADRA are quantified at 281 nm. The substances that absorb UV in this range of the spectrum are generally limited to those having conjugated double bonds, which significantly lowers the potential for co-elution (9).

6. The current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products, or biological materials (UVCB substances) due to the need for defined molar ratio of test chemical and nucleophilic reagents. Limited information is currently available on the applicability of the ADRA to mixtures (10) (11). A new protocol has to be developed for multi-constituent substances and mixtures to be used with test methods like ADRA, which utilise HPLC analysis to quantify the depletion of nucleophilic reagents (10) (11). Thus, although it is impossible to define fixed methods in this guideline, which can evaluate multi-constituent substances and mixtures, paragraph 15 describes an evaluation method that is considered to be applicable at the present time for multi-constituent substances or mixtures of known composition (10). Such substances were however not tested during the validation studies. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically.

7. ADRA can be used to support the discrimination between skin sensitisers and non-sensitisers. Further work, preferably based on human data, is necessary to determine whether ADRA results can contribute to potency assessment when considered in combination with other information sources (6).

PRINCIPLE OF THE TEST

8. ADRA is an *in chemico* test method that quantifies residual concentrations of the cysteine derivative *N*-(2-(1-naphthyl)acetyl)-*L*-cysteine (CAS. 32668-00-1), which is known as NAC, and the lysine derivative α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine (CAS. 397841-92-8), known as NAL, following a 24±1 hour incubation at 25±1ºC in the presence of a test chemical. Both these derivatives include a naphthalene ring that is introduced to their *N*-terminal in order to facilitate UV detection. The relative concentrations of NAC and NAL are measured by high-performance liquid chromatography (HPLC) with gradient elution and

UV detection at 281 nm. Percent depletion values are then calculated for both NAC and NAL and compared to a prediction model (see paragraph 26).

9. Prior to routine use of the method described in this test method, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 2 of this Appendix.

PROCEDURE

10. This test method is based on the protocol (12) used for the JaCVAM-coordinated ADRA validation study and is recommended for use when implementing ADRA at a laboratory. The main components and procedures for the ADRA are described below. Before using an alternative HPLC set-up, its equivalence to the validated set-up described in the protocol should be demonstrated, preferably by testing the proficiency substances in Annex 2 of this Appendix.

Quality of NAC and NAL

11. The Nucleophilic Reagents can be obtained as an ADRA Kit for Skin Sensitisation Test, from FUJIFILM Wako Pure Chemical Corporation, Catalogue No. 296-80901. The use of NAC/NAL as reagent for detecting sensitisation is patented in Japan only, by Fujifilm Corporation. Therefore, in other countries, NAC/NAL can be used without permission. In case other manufacturer's NAC/NAL are used, these should satisfy three quality criteria described below. Quality checks can be obviated and ADRA testing can be performed without delay by purchasing NAC and NAL that have been manufactured specifically to satisfy these quality criteria.

Quality required for NAC and NAL:

1) Purity: Both NAC and NAL are to be at least 98% pure.

2) Stability: Using NAC and NAL stock solution, prepare a reference control free of any test chemical and quantify the residual levels of NAC and NAL both immediately after preparation (0 hours) and after a 24 hour incubation. Residual levels of NAC and NAL are to be a minimum of 90% in either case (12). The residual level of NAC is calculated as a percentage of the sum of NAC and the residual level of NAC dimers.

3) Reactivity: NAC and NAL are to be evaluated for reactivity with the ten proficiency substances given in Annex 2 and should satisfy the requirement given therein.

Preparation of the NAC and NAL stock solution

12. The solubility of individual NAC and NAL batches should be verified prior to use. NAC stock solution should be prepared to a concentration of 2 mM in 100 mM of pH 8.0 phosphate buffer, including 0.333 µM of EDTA, as well as NAL stock solution to a concentration of 2 mM in 100 mM of pH 10.2 phosphate buffer. These two stock solutions are then diluted in buffer to prepare 6.667 µM stock solutions. Both NAC and NAL stock solutions should be used as soon as possible after preparation (3). In the event that they are to be stored, these stock solutions may be frozen and stored for up to twelve months time at less than -75°C prior to use. The final concentration of the NAC solution is 5 µM in pH 8.0 phosphate buffer, and the final concentration of the NAL solution is 5 µM in pH 10.2 phosphate buffer.

Preparation of the test chemical solution

13. Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay in accordance with the solubilisation procedure described in the ADRA JaCVAM protocol (12). An appropriate solvent should dissolve the test chemical completely. Since the ADRA protocol stipulates that either NAC or NAL are incubated in an excess volume of the test chemical, visual inspection of the clear test chemical solution is considered sufficient to confirm that the test chemical (and all its constituents, if testing a multi-constituent substance or a mixture) is dissolved. Suitable solvents are distilled water, acetonitrile and acetone. If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts. It is important to note that DMSO may lead to dimerisation of the nucleophilic reagent NAC (13) and as a result, it may be more difficult to meet the acceptance criteria. If a DMSO-acetonitrile solvent is chosen (5% DMSO in acetonitrile), the test chemical should be dissolved at 20 mM in DMSO, and then this solution should be diluted 20-fold with acetonitrile to prepare a 1 mM test chemical solution. In case the use of DMSO leads to increased dimerisation of the NAC reagent, this can be checked analytically as the NAC dimer can be detected by HPLC. The test chemical should be pre-weighed into a disposable polypropylene tube and dissolved immediately before testing in an appropriate solvent to prepare a 1 mM solution.

14. Mono-constituent substances of unknown molecular weight may be tested in a test chemical solution at a concentration of 0.5 mg/mL rather than 1 mM (10). Polymers which are well characterised should also be tested at a concentration of 1 mM based on the mean number average molecular weight, in a manner analogous to the procedure for mono-constituent compounds.

15. Mixtures and multi constituent substances, of known composition are to be tested as follows:

1) Liquids: Generally, tested as an undiluted mixture. In cases where low solubility of the test item prevents formation of reaction solution, i.e. undissolved material, clouding, and/or precipitation is observed, a positive result may still be used in the assessment, whereas a negative result is uncertain and should be interpreted with due care. Insofar as results could be false positives, however, predictions should be interpreted with due care.

2) Solids: The test chemical should be dissolved to maximum soluble concentration in the same solvent used to prepare the 1 mM test chemical solution. The test chemical solution of the highest concentration possible is then tested as an undiluted mixture. In cases where low solubility of the test item prevents formation of reaction solution, i.e. undissolved material, clouding, and/or precipitation is observed, a positive result may still be used in the assessment, whereas a negative result is uncertain and should be interpreted with due care. Insofar as results could be false positives, however, predictions should be interpreted with due care.

Moreover, since mixtures consisting of multiple components cannot be evaluated for sensitisation for each chemical, positive result could still be used to support identification of the test chemical as a skin sensitiser but no firm conclusion should be drawn from a negative result.

Preparation of the positive control, reference controls and co-elution controls

16. Either phenylacetaldehyde (CAS 122-78-1, purity ≥ 90%) or squaric acid diethyl ester (CAS 5231-87-8, purity > 95%) should be used as the positive control (PC) at a concentration of 1 mM in acetonitrile. Phenylacetaldehyde should not be stored for a long time after opening since it may be polymerised and oxidised; it is recommended that it is

purchased as appropriate while paying attention to the NAC and NAL depletion. Squaric acid diethyl ester should be stored away from high temperature or humidity, since it may be hydrolysed. Other suitable positive controls that provide mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition, reference controls comprising only NAC or only NAL dissolved in the appropriate solvent should also be included in the HPLC run sequence, so they can be used to verify the HPLC system suitability prior to analysis (Reference Control A), the stability of the reference controls over time (Reference Control B), and any effects of the solvent used on depletion of NAC or NAL (Reference Control C) (See Annex 3). The percent NAC and NAL depletion for a test chemical is calculated using an appropriate reference control for that test chemical (see paragraph 23). Also, a co-elution control comprising only the test chemical should be included in the run sequence to detect possible co-elution of the test chemical with either the NAC or NAL.

Incubation of the test chemical with the NAC and NAL solutions

17. Both the NAC and the NAL solutions should be incubated with the test chemical at 1:50 ratio in a 96-well microplate. The observation of precipitate immediately upon addition of the test chemical solution to the NAC and the NAL solutions is an indication of poor solubility, which means that there is no way to know exactly how much test chemical is contained in the solution. Thus, although positive results can be used with confidence, negative results are uncertain and should be interpreted with due care (see also paragraph 4 regarding the testing of chemicals not soluble at concentrations as high as 1 mM). The reaction solution should be incubated in the dark at 25±1ºC for 24±1 hours before performing HPLC analysis. After incubation, trifluoroacetic acid (TFA) (≥ 98%) should be added as a fixing solution to stop the reaction (3).

HPLC preparation and analysis

18. Each test chemical should be analysed in triplicate to determine percent depletion for both NAC and NAL. Although adding the fixing solution does stop the reaction, measurement of the reaction solution is to be performed as soon as possible and in any case within three days after adding the fixing solution. For example, when HPLC analysis of NAC and NAL are performed separately using two 96-well microplates, up to 34 samples may be analysed at one time, including the test chemical, the positive control, and the appropriate number of solvent controls based on the number of individual solvents used in the test, each in triplicate. All of the replicates analysed in a single run should use identical batches of NAC and NAL stock solution. Test chemical and control solutions are to be visually inspected prior to HPLC analysis and may be centrifuged at low speed (100–400 × g) to force any precipitate to the bottom of the vial as a precaution against large amounts of precipitate clogging the HPLC tubing or columns. Observation of precipitation or phase separation after the incubation period is an indication that NAC and NAL depletion could be misleading, and negative results in that case are uncertain and should be interpreted with due care, as well as for any precipitate observed at the beginning of the incubation period (see above).

19. A standard calibration curve should be generated for both NAC and NAL. Standard solutions of both NAC and NAL should be prepared in 20% acetonitrile in buffer and containing 0.5% trifluoroacetic acid. For NAC, a phosphate buffer at pH 8.0, and for NAL, a phosphate buffer at pH 10.2 should be used. Serial dilution of the NAC and NAL stock solutions (5.0 µM) will be used to prepare six calibration solutions in concentrations from 5.0 to 0.156 µM as well as a blank of the dilution buffer. Suitable calibration curves should have an $R^2 > 0.990$.

20. The suitability of the HPLC system should be verified before conducting the analysis. Both NAC and NAL depletion is monitored by HPLC coupled with an UV detector (photodiode array detector or fixed wavelength absorbance detector with 281 nm signal). The appropriate column is installed in the HPLC system. The recommended HPLC set-up

described in the validated protocol uses a column (Base particle: core-shell type silica gel, Particle size: 2.5~2.7 µm, column size: 3.0 × 150 mm) as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated for at least 30 minutes at 40ºC with 50% phase A (0.1% (v/v) trifluoroacetic acid in water), 50% phase B (0.1% (v/v) trifluoroacetic acid in acetonitrile) before use. Then, the column is conditioned by running the gradient at least twice before actual use. The HPLC analysis should be performed using a flow rate of 0.30 mL/min and a linear gradient from 30% to 55% acetonitrile for NAC and from 25% to 45% acetonitrile for NAL within 10 minutes, followed by a rapid increase to 100% acetonitrile to remove other materials. Equal volumes of the standard solutions, test chemical solutions, and control solutions should be injected. The column should be re-equilibrated under initial conditions for 6.5 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the NAC and NAL, including the injection volume, which may vary according to the system used (typically in the range from 10–20 µL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated, preferably by testing the proficiency substances in Annex 2. Absorbance is monitored at 281 nm. If a photodiode array detector is used, absorbance at 291 nm should also be recorded. It should be noted that some batches of acetonitrile could have a negative impact on NAC and NAL stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 281 nm peak area and the 291 nm peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90% < mean area ratio of control samples < 100% would give a good indication that co-elution has not occurred. An example of HPLC analysis sequence is provided in Annex 3.

21. There are some test chemicals that could potentially promote oxidation of NAC. The peak of the dimerised NAC may be monitored visually. Any apparent dimerisation should be noted, since overestimation of NAC depletion could result in false-positive predictions (See paragraphs 26 and 27).

DATA AND REPORTING

Data evaluation

22. The concentration of both NAC and NAL is photometrically determined at 281 nm in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of both NAC and NAL using the linear calibration curve derived from the standards.

23. The percent depletion for both NAC and NAL is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant Reference Controls C (See Annex 3) according to the formula described below.

$$\text{Percent NAC or NAL depletion} = \left[1-\left[\frac{\text{NAC or NAL peak area in replicate injection}}{\text{Mean NAC or NAL peak area in reference controls C}}\right]\right]x100$$

Acceptance criteria

24. The following criteria should be met:

a) the standard calibration curve should have an $R^2 > 0.990$,

b) the mean percent NAC and NAL depletion value and the maximum standard deviation (SD) of the three replicates for the positive control (phenylacetaldehyde or squaric acid diethyl ester) should meet the following criteria:

- NAC depletion

Phenylacetaldehyde: 6 - 30%; Squaric acid diethyl ester: 15 - 40 %

- NAL depletion

Phenylacetaldehyde: 75 - 100%; Squaric acid diethyl ester: 40 - 85 %

- Maximum standard deviation (SD)

Both phenylacetaldehyde and squaric acid diethyl ester: < 10%, for both NAC and NAL depletion,

c) the mean NAC and NAL concentration of both Reference Controls A and C should be 3.2–4.4 µM and the coefficient of variation (CV) of NAC and NAL peak areas for the nine Reference Controls B and C in acetonitrile should be < 10%.

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated.

25. The following criteria should be satisfied for a test chemical's results to be accepted as valid:

a) the maximum standard deviation for the test chemical replicates should be < 10% for the percent depletion of both NAC and NAL,

b) the mean NAC and NAL concentration of the three Reference Controls C in the appropriate solvent should be 3.2–4.4 µM. The permissible range of the mean NAC concentration of Reference Control C when 5% DMSO in acetonitrile is used as a solvent is 2.8 to 4.0 µM (14).

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated.

Prediction model

26. The mean percent depletion of NAC and NAL is calculated for each test chemical. Negative depletion is considered to be "0" when calculating the mean. By using the NAC/NAL prediction model shown in Table 1, the threshold of 4.9% mean depletion should be used to support the discrimination between skin sensitisers and non-sensitiser in the framework of an IATA or a DA. The 4.9% of cut-off value for the mean percent depletion of NAC and NAL was set by using 2 class classification model so that the sensitizer and non-sensitizer could be predicted most appropriately.

Table 1: NAC/NAL prediction model[1]

Mean NAC and NAL percent depletion	ADRA prediction[2]
Less than 4.9%	Negative
4.9% or higher	Positive

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.

[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

27. Co-elution occurs when the test chemical (the substance or one or several of the constituents of a multi-constituent substance or a mixture) absorbs significantly at 281 nm and has the same retention time as NAC or NAL. Co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and NAC or NAL. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated, preferably by testing the proficiency substances in Annex 2. When co-elution occurs, it is not possible to integrate the peak of the NAC or NAL, thereby preventing calculation of the percent depletion of NAC or NAL. If co-elution of test chemicals occurs with both the NAC and NAL, or with the NAC only, and separation of elution time is not feasible, then the analysis should be reported to be inconclusive. In cases where co-elution occurs only with NAL and separation of elution time is not feasible, the NAC-only prediction model (See Table 2) can be used to make a prediction. The 5.6% cut-off value for the percent depletion of NAC was set by using 2 class classification model so that the sensitizer and non-sensitizer could be predicted most appropriately.

Table 2: NAC-only prediction model[1]

Mean NAC percent depletion	ADRA prediction[2]
Less than 5.6%	Negative
5.6% or higher	Positive

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.

[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

28. When a result is unequivocal, a single HPLC analysis for both NAC and NAL should be sufficient for a test chemical. However, in case of results close to the threshold used to discriminate between positive and negative results (i.e. in the range of 3% to 10% for NAC/NAL prediction model or NAC percent depletion falls in the range of 4% to 11% for NAC-only prediction model), additional testing is recommended. In particular, in case of negative results in these ranges (i.e. 3% to 4.9% for NAC/NAL prediction model or 4 % to 5.6% for NAC-only prediction model), a second run should be conducted, as well as a third one in case of discordant results between the first two runs.

Test report

29. The test report should include the following information:

Test chemical and Controls (positive control and solvent/vehicle)

- For all mono-constituent substance (test and control chemicals)

    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers
    - Physicochemical properties such as physical state, appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc.
    - Treatment prior to testing, if applicable (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability to the extent available
- Multi-constituent substance, UVCB, and mixtures
    - Characterisation by chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available
    - Physical appearance, water solubility, and additional relevant physicochemical properties, to the extent available
    - Molecular weight (or apparent molecular weight) for mixtures or polymers of known composition, or other information relevant to the study
    - Treatment prior to testing, if applicable (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability, to the extent available.
- Additional information for positive control
    - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Additional information for solvent/vehicle control
    - Solvent used and ratio of its constituents, if applicable
    - Justification for choice of solvent for each test chemical
    - Impact on NAC and NAL stability when using acetonitrile

Preparation of NAC and NAL, positive control and test chemical solution

- Characterisation of NAC and NAL solutions (supplier, lot, exact weight of NAC and NAL, volume added for the stock solution)
- Characterisation of positive control solutions (exact weight of positive control reagent, volume added for the control solution)
- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test chemical solution)

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, detector, autosampler
- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient

System suitability

- NAC and NAL peak area at 281 nm of each standard and reference control A replicate

- Linear calibration curve graphically represented and the R2 reported
- NAC and NAL concentration of each Reference Control A replicate
- Mean NAC and NAL concentration (µM) of the three reference controls A, SD and CV
- NAC and NAL concentration of Reference Controls A and C.

Analysis sequence

- For Reference Controls
    - NAC and NAL peak area at 281 nm of each replicate of Reference Controls B and C
    - Mean NAC and NAL peak area at 281 nm of the nine Reference Controls B and C in acetonitrile, SD and CV (for stability of reference controls over analysis time)
    - For each solvent used, the mean NAC and NAL peak area at 281 nm of the three appropriate Reference Controls C (for the calculation of percent NAC and NAL depletion)
    - For each solvent used, the NAC and NAL concentration (µM) of the three appropriate Reference Controls C
    - For each solvent used, the mean NAC and NAL concentration (µM) of the three appropriate Reference Controls C, SD and CV.
- For positive controls
    - NAC and NAL peak area at 281 nm of each replicate
    - Percent NAC and NAL depletion of each replicate
    - Mean percent NAC and NAL depletion of the three replicates, SD and CV.
- For each test chemical
    - Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;
    - Presence of co-elution
    - Description of any other relevant observations, if applicable
    - NAC and NAL peak area at 281 nm of each replicate
    - Percent NAC and NAL depletion of each replicate
    - Mean of percent NAC and NAL depletion of the three replicate, SD and CV
    - Mean of percent NAC and percent NAL depletion values
    - Prediction model used and ADRA prediction

Proficiency testing

- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals

Discussion of the results

- Description of any unintended modifications to the test procedure.
- Discussion of the results obtained with the ADRA test method and if it is within the ranges described in paragraph 28.

Conclusion

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APPENDIX II, ANNEX 1

KNOWN LIMITATIONS OF THE AMINO ACID DERIVATIVE REACTIVITY ASSAY

The table below provides a summary of the known limitations of the ADRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Pro-haptens	Test Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential ; cannot be detected by the test method unless activation is caused by auto-oxidation to a similar degree as in vivo /in humans. It will however normally not be known whether this will be the case	May lead to false negatives. Negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA	Diethylenetriamine; 111-40-0 (human 1A, LLNA n/a)
Pre-haptens	Chemicals that become sensitisers after abiotic transformation are reported to be in some cases correctly detected by the test method		Linalool: 78-70-6
Test chemicals absorbing significantly at 281 nm and having the same retention time of the NAC or NAL (co-elution)	When co-elution occurs the peak of the NAC or NAL cannot be integrated and the calculation of the percent NAC or NAL depletion is not possible.	The substances that absorb UV in this range of the spectrum are generally limited to those having conjugated double bonds, which significantly lowers the potential for co-elution. If co-elution of such test chemicals occurs with both the NAC and the NAL or with the NAC only, then the analysis should be reported as “inconclusive” and alternative HPLC set up should be considered (see paragraph 27). In cases where co-elution occurs only with the NAL, then the NAC-only prediction model reported in Table 2 can be used.”	Safranal; 116-26-7
Complex mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials	This is due to the need for defined molar ratio of test chemical and nucleophilic reagent. Limited information is currently available on the applicability of the ADRA	n/a	UVCBs, chemical emissions, products or formulations with variable or not fully known composition
Test chemicals which cannot be dissolved in an appropriate solvent at a final concentration of 1 mM (although it is unlikely to happen since test chemical	Not sure if sufficient exposure can be achieved	The ADRA test method allows testing of poorly soluble chemicals. Test chemicals that are not soluble at this concentration though may still be tested at lower soluble concentrations. In such a case, a positive result could be used to support the	n/a

solution in ADRA is prepared at a low concentration (1 mM))		identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.	
Chemicals which precipitate in reaction solution (although the test chemical is hardly precipitated when the test chemical solution is added to the reaction solution since ADRA is dissolved in a solvent at a low concentration (1 mM))	Not sure if sufficient exposure can be achieved	Test chemicals that precipitate in the reaction solution even if dissolved in the solvent may still be tested at lower soluble concentrations. In such a case, a positive result could still be used to support the identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.	Isopropyl myristate CAS: 110-27-0
Test chemicals that do not covalently bind to the NAC but promote its oxidation (i.e. NAC dimerisation)	Could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions.	It may be possible to detect and quantify any NAC dimer formed by HPLC, thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and covalent bonding to the test item substance(s) Therefore, ADRA may prevent erroneous judgement due to the oxidizing action of the test chemical.	DMSO Oxidant
Test chemicals that are only soluble in DMSO	DMSO causes excessive NAC depletion due to NAC dimerization resulting in high background NAC depletion.	DMSO is allowed to be contained in the test chemical solution up to 5%. If DMSO is chosen, attempts should be made to solubilise the test chemical in a 1:20 mixture of DMSO and acetonitrile (5% DMSO in acetonitrile).	n/a

APPENDIX II, ANNEX 2

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

Prior to routine use of the test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected ADRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining NAC and NAL depletion values that fall within the respective reference ranges for 8 out of the 10 proficiency substances. These proficiency substances were selected to represent the full range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality ADRA data are available, and that they were used during the JaCVAM-coordinated validation study to demonstrate successful implementation.

Table 1. Recommended chemicals for demonstrating technical proficiency with ADRA

No.	Test chemicals	CAS No.	Physical state	Molecular weight	*In vivo* Prediction[1]	ADRA prediction[2]	Range of % depletion	
							NAC[3]	NAL[3]
1	*p*-Benzoquinone	106-51-4	Solid	108.09	Sensitiser (extreme)	Positive	90-100	40-70
2	Diphenylcyclopropenone	4886-38-4	Solid	206.24	Sensitiser (strong)	Positive	15-45	≤ 10
3	2-Methyl-2H-isothiazol-3-one	2682-20-4	Solid	115.15	Sensitiser (moderate)	Positive	80-100	≤ 7
4	Palmitoyl Chloride	112-67-4	Liquid	274.87	Sensitiser (moderate)	Positive	≤ 10	50-100
5	Imidazolidinyl urea	39236-46-9	Solid	388.29	Sensitiser (weak)	Positive	10-45	≤ 10
6	Farnesal	19317-11-4	Liquid	220.35	Sensitiser (weak)	Positive	20-40	≤ 15
7	Glycerol	56-81-5	Liquid	92.09	Non-sensitiser	Negative	≤ 7	≤ 7
8	Isopropanol	67-63-0	Liquid	60.10	Non-sensitiser	Negative	≤ 7	≤ 7
9	Dimethyl isophthalate	1459-93-4	Solid	194.19	Non-sensitiser	Negative	≤ 7	≤ 7
10	Propyl paraben	94-13-3	Solid	180.20	Non-sensitiser	Negative	≤ 7	≤ 7

[1]The *in vivo* hazard (and potency) predictions are based on LLNA data. ((15) (16) (17). The *in vivo* potency is derived using the criteria proposed by ECETOC (18).

[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

[3] Ranges determined on the basis of at least 10 depletion values generated by 5 independent laboratories.

APPENDIX II, ANNEX 3

EXAMPLES OF ANALYSIS SEQUENCE

Each sample of HPLC analysis should be analysed in number order below. Refer to the table showing Examples of HPLC Sample Analysis Sequences for more practical sequences about HPLC analysis.

1. Start to analyse calibration standards and Reference Control A (N = 3).

2. The co-elution Control does not need to be analysed by turns if it is analysed after analysis of standard solution and Reference Control A.

3. Reference Control B should be analysed three times (total six times) before and after the analysis of sample, Reference Control C and Positive Control.

4. The Reference Control C, Positive Control and Test chemical solutions are analysed. (After the first set of replicates of each sample is analysed, the second set of replicates of each should be analysed).

Calibration standards and reference controls	STD1 STD2 STD3 STD4 STD5 STD6 Dilution buffer Reference control A, rep 1 Reference control A, rep 2 Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1 Reference control B, rep 2 Reference control B, rep 3
First set of replicates	Reference control C, rep 1 Positive control, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Positive control, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Positive control, rep 3 Sample 1, rep 3 Sample 2, rep 3
Reference controls	Reference control B, rep 4 Reference control B, rep 5 Reference control B, rep 6

Three sets of reference controls (NAC or NAL dissolved in the appropriate solvent) should be included in the analysis sequence:

Reference control A: Control for verifying validity of the HPLC system. Reference Control A is used to verify concentration of NAC and NAL from each calibration curve after addition of acetonitrile rather than test chemical.

Reference control B: Control for verifying stability of reaction solution under analysis. Reference Control B is used to verify variability (CV) of each three NAC/NAL peak areas in the solution after addition of acetonitrile rather than test chemical at the start of analysis and at the end of analysis.

Reference control C: Control for calculating NAC/NAL depletion of each test chemical solution. To calculate depletion of NAC/NAL, measure three Reference Controls C after addition of solvent instead of test chemical. Prepare reference Control C for all solvents used to dissolve the test chemicals.

APPENDIX III

In Chemico Skin Sensitisation: kinetic Direct Peptide Reactivity Assay (kDPRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The kDPRA is proposed to address the molecular initiating event of the skin sensitisation AOP - namely, protein reactivity - by quantifying the reactivity of test chemicals towards a synthetic model peptide containing cysteine in a time- and concentration dependent manner (1) (2). Kinetic rate constants are calculated and the logarithm of the maximum rate constant (log k_{max} value in $s^{-1}M^{-1}$) for a tested substance is then used to support the discrimination of UN GHS subcategory 1A skin sensitisers (subcategory 1A) from those not categorised as subcategory 1A (non-subcategory 1A) i.e., subcategory 1B or no category according to UN GHS (3). Based on theoretical consideration, the rate constant of the reaction between a test chemical and skin proteins will determine the amount of epitope formed from a given amount of chemical or, vice-versa, determine the dose needed to form the amount of epitope needed for induction of sensitization to occur and it is thus a rate limiting and potency determining step. Based on empirical evidence when evaluating 180 chemicals, the rate constant was shown to be the strongest determinant of potency among all evaluated parameters measured in OECD 442C, 442D and 442E (3).
2. The kDPRA proved to be transferable to laboratories without hands-on training (4). For the 24 test chemicals tested during the validation study, the overall within-laboratory reproducibility of kDPRA for assigning UN GHS subcategory 1A was 96% and the average between-laboratory reproducibility was 88% (4). Results from the validation study (4) as well as from other published studies (3) encompassing 180 test chemicals that fall within kDPRA's applicability domain indicate that kDPRA allows to discriminate UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) according to UN GHS with a balanced accuracy of 85%, a sensitivity of 84% (38/45), and a specificity of 86% (116/135) relative to LLNA results (3). Similar performances were obtained when comparing kDPRA outcomes with the OECD LLNA database compiled within the context of the Test Guideline on Defined Approaches for Skin Sensitization (15)[3]. In addition, the prediction for 123 test chemicals (out of the 180) having human skin sensitisation data (5) (6) has a balanced accuracy of 76%, a sensitivity of 64% (21/33), and a specificity of 89% (80/90) (3), although the human reference data are subject to a significant uncertainty[4]. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the

[3] A balanced accuracy of 85%, a sensitivity of 82% (31/38), and a specificity of 88% (102/116) were found relative to LLNA dataset compiled within the context of the Test Guideline on Defined Approaches for Skin Sensitization (15).

[4] A balanced accuracy of 67%, a sensitivity of 53% (9/17), and a specificity of 81% (25/31) were found relative to human skin sensitisation dataset compiled within the context of the Test Guideline on Defined Approaches for Skin Sensitization (15).

LLNA test as well as other animal tests may not fully reflect the situation in the species of interest, which is humans. For comparison, based on a data set of 123 chemicals used to evaluate the kDPRA vs. human sensitising potential, the LLNA showed a 73% balanced accuracy, a 55% (18/33) sensitivity and a 91% (82/90) specificity for the identification of UN GHS subcategory 1A. On the basis of the overall data available, kDPRA's applicability domain was shown to include a variety of organic functional groups, reaction mechanisms, skin sensitisation potencies (as determined in *in vivo* studies), and physicochemical properties (3). Following an independent peer review (16), the kDPRA was considered to be scientifically valid to discriminate UN GHS subcategory 1A skin sensitisers from those not categorised as 1A (non-subcategory 1A) according to UN GHS (7). The kDPRA can therefore be used (i) as a follow-up test method for sub-categorisation of chemicals identified as UN GHS Category 1 skin sensitisers, or (ii) on its own by using positive results for direct classification of a chemical into UN GHS subcategory 1A, depending on the regulatory framework.

3. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the kDPRA to the testing of substances and/or mixtures. This test method is not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding. Furthermore, kDPRA only measures reactivity with the cysteine peptide, so that strong sensitisers having an exclusive lysine-reactivity, such as some acyl-halides, phenol-esters or aldehydes are outside of the applicability domain of kDPRA. However, only few UN GHS subcategory 1A skin sensitisers are known currently to react exclusively with lysine residues. In addition, considering exclusive strong Lysine-reactivity from the DPRA or ADRA in a tiered strategy may reduce this uncertainty. Test chemicals that do not covalently bind to the peptide but promote its oxidation (i.e. cysteine dimerisation) could lead to a potential over estimation of peptide depletion, resulting in possible false positive predictions and/or assignment to a higher reactivity class. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Reactivity of chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be reliably detected by the test method. However, the limitation for detecting pro-haptens was found to be less pronounced when identifying strong sensitisers as compared to the identification of weak sensitisers (3). The majority of chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) were reported to be correctly detected by *in chemico* test methods (8) (9). However, spontaneously rapidly oxidizing pre-haptens may be under-predicted by kDPRA (as in any *in vitro* skin sensitisation assay) due to a lag-phase for oxidation which reduces the overall reaction rate. In the light of the above, results obtained with the test method that do not lead to subcategory1A categorisation should be interpreted in the context of the currently known limitations (see also Annex 1 of this Appendix), i.e.:

   - aromatic amines, catechols or hydroquinones may require further data to confirm their weak reactivity even under oxidizing conditions, and
   - acyl-halides, phenol-esters or aldehydes specifically reacting with Lysine-residue according to e.g. the DPRA or ADRA, may require further data to confirm their weak reactivity.

4. To be tested, a test chemical should be soluble in an appropriate solvent at a final concentration of 20 mM (see paragraphs 12-13). Test chemicals that are not soluble at this concentration may still be tested at lower concentrations as long as a k_{max} value (i.e., the maximum rate constant (in $s^{-1}M^{-1}$) determined from the reaction kinetics for a tested substance in the kDPRA (see paragraph 24)), can be derived. In such a case, a positive result leading to a UN GHS subcategory 1A skin sensitization prediction (i.e. log $k_{max} \geq -2.0$) could still be used, but no firm conclusion should be drawn from a negative result (i.e., non-reactive or log $k_{max} < -2.0$ outcome).

5. The kDPRA uses a fluorescence readout which requires attention for potential test chemical autofluorescence, fluorescence quenching or interaction with the reagent (monobromobimane). In particular, it is important to include the respective test chemical controls as described in paragraph 16 and to assess the incubation time dependence of the determined peptide depletion. Furthermore, test chemicals with primary SH-group (thiols) cannot be tested with the kDPRA as the thiol group can interact with the monobromobimane (see paragraph 8) leading to enhanced fluorescence. Finally, chemicals decomposing under the conditions of the assay (neutral, aqueous conditions) and releasing a free SH-group will be prone to the same limitations.

6. The kDPRA is considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition, although such substances were not tested during the validation studies. In this case, a single purity may be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight may be determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 20 mM solution. Results obtained with mixtures and multi-constituent substances of known composition can lead to a non-linear behaviour, so that the provisions described in paragraph 27(ii) should be used. Regarding mixtures and substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances), the current model cannot be used due to the need for defined molar ratios. In any case, when considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. Finally, in cases where evidence can be demonstrated on the non-applicability of the test method to specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

7. The kDPRA can be used for the discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory1A) according to UN GHS (3). As for any key-event based test method, the performance of kDPRA will have to be further assessed when used in combination with other assays such as DPRA or ADRA, and within integrated approaches such as IATA or DA for a more comprehensive analysis of skin sensitisation (3) (10).

PRINCIPLE OF THE TEST

8. The kDPRA is a modification of the *in chemico* test method DPRA (described in Appendix I of this Test Guideline). The kDPRA uses the cysteine peptide (Ac-RFAA**C**AA-COOH) also used in the DPRA, while it does not use a lysine containing peptide. The final concentration of the test peptide (0.5 mM) and the reaction medium (25% acetonitrile in phosphate buffer) is identical in the kDPRA and in the DPRA. While the DPRA measures only at one concentration of the test chemical (5 mM for the cysteine peptide) and at one time point (≥ 24 h), the kDPRA performs parallel reactions at five concentrations (5, 2.5, 1.25, 0.625 and 0.3125 mM) and at six time-points (10, 30, 90, 150, 210 and 1440 min) at 25±2.5ºC. Residual concentration of the cysteine peptide after the respective reaction time is measured after stopping the reaction by the addition of monobromobimane (mBrB; CAS 74235-78-2). The highly reactive and non-fluorescent mBrB rapidly reacts with unbound cysteine moieties of the model peptide to form a fluorescent complex which is measured in order to quantify the non-depleted peptide concentration. If the depletion of the highest concentration surpasses the threshold of 13.89% (cut-off used in the DPRA for positivity in the cysteine only prediction model) and this depletion is statistically significant vs. controls with peptide

only, further calculations are performed (otherwise the test chemical is considered to be non-reactive according to the prediction model shown in paragraph 28). The natural logarithm of the non-depleted peptide concentrations is plotted vs. the concentration of the test chemical at each time point. If a linear relationship is observed (correlation coefficient > 0.90), the slope of this curve is determined and divided by the incubation time to calculate the rate constant in [$min^{-1}mM^{-1}$]. This value is transformed to the rate constant in [$s^{-1}M^{-1}$] and the logarithm is calculated. The maximum value observed at any time point is taken as the log k_{max}, and this maximum rate constant is the primary read-out of the test. It gives a quantification of the maximum kinetic rate of the reaction of the test chemical with the test peptide. Kinetic reaction rates of the cysteine peptide depletion are then used to discriminate UN GHS subcategory 1A skin sensitisers from those not categorised as 1A (non-subcategory 1A) according to UN GHS. Chemicals with a log $k_{max} \geq -2.0$ are predicted as UN GHS subcategory 1A. The kinetic rate constant may be further used in integrated approaches such as IATA or DA to assess the skin sensitisation potency of a test chemical in a continuous scale as needed for risk assessment (3) (10).

9. Prior to routine use of this test method, laboratories should demonstrate technical proficiency, using the nine proficiency substances listed in Annex 2 of this Appendix.

PROCEDURE

10. This test method is based on the kDPRA DB-ALM protocol no 217 (11) which represents the protocol used for the industry-coordinated validation study. It is recommended that this protocol is used when implementing and using the method in a laboratory. The main components and procedures for the kDPRA are described below.

Preparation of the cysteine-peptide

11. The stock solution of the cysteine containing synthetic peptide (Ac-RFAA**C**AA-COOH) of purity equal to or higher than 95% should be freshly prepared just before the incubation with the test chemical. The final concentration of the cysteine peptide should be 0.667 mM in pH 7.5 phosphate buffer for test chemical soluble in acetonitrile and 1.0 mM for chemicals soluble in pH 7.5 phosphate buffer.

Preparation of the test chemical

12. Solubility of the test chemical in an appropriate vehicle should be assessed before performing the assay. A non-reactive, water-miscible vehicle able to completely dissolve the test chemical should be used. Solubility is checked by visual inspection where the forming of a clear solution is considered sufficient to ascertain that the test chemical is dissolved. The preferred vehicle is acetonitrile. When a substance is not soluble in acetonitrile, solubilisation in pH 7.5 phosphate buffer should be assessed. Further vehicles have not been tested yet but may be used if it is demonstrated that the vehicle does not interfere with the assay, e.g. all controls should be prepared using the same vehicle, and the reaction rates obtained for the positive control and for the proficiency chemicals should fall within the ranges described in paragraph 26 and Annex 2 of this Appendix, respectively. It is important to note that use of DMSO as a vehicle should be avoided as it may lead to peptide dimerisation.

13. The test chemical should be pre-weighed into glass vials and dissolved immediately before testing to prepare a 20 mM solution using the appropriate vehicle as described in paragraph 12. Test chemical dilutions are prepared by serial dilution to obtain concentrations of 20, 10, 5, 2.5 and 1.25 mM.

Preparation of controls

14. Cinnamic aldehyde (CAS 104-55-2; ≥95% food-grade purity) should be used as positive control (PC). It is dissolved at a concentration of 20 mM in acetonitrile immediately before testing. Serial dilutions are then prepared to obtain PC concentrations of 20, 10, 5, 2.5 and 1.25 mM. Use of other positive controls is not recommended since in this assay an exact reaction rate is measured and consistent use of the positive control allows quantitative comparison between laboratories, with validation study data and as intra-laboratory historical control.

15. A vehicle control (VC), considered as the negative control, includes the peptide dissolved in buffer and vehicle respectively but no test chemical nor PC. The peptide-depletion of test chemical or PC incubated samples is calculated relative to the respective VC.

16. The assay also includes test chemical controls at the respective test chemical concentration in the vehicle and buffer but without peptide. This set of controls is used for the identification of interference of the test chemical with the fluorescence measurement (autofluorescence and quenching) to assess e.g., interference with monobromobimane and as a background measurement.

17. A blank control (BC) is used as a background measurement and is prepared with vehicle and buffer but without test chemical, PC, or peptide.

Incubation of the test chemical with the cysteine peptide solution

18. Serial dilutions of the test chemical and PC are prepared in a 96-well microtiter plate referred to as the application plate. Further, a 96-well black assay plate for each exposure time is prepared, referred to as the assay plates, by adding the relevant reagents (i.e., peptide stock solution, vehicle and buffer solution) according to a predefined plate layout such as recommended within the kDPRA protocol (11). Each test chemical concentration should be analysed in triplicate. The reaction is started by adding the test chemical and PC dilutions from the application plates to the assay plates. If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, one cannot be sure how much test chemical remained in the solution to react with the peptide. In such a case, a positive result (i.e. log k_{max} ≥ -2.0) could still be used, but a negative result (i.e., non-reactive or log k_{max} < -2.0 outcome) should be interpreted with due care (see also provisions in paragraph 4 for the testing of chemicals not soluble up to a concentration of 20 mM in the kDPRA). After adding the test chemical and PC, plates are sealed with gas-tight adhesive foil and shaken at least 200 rpm for 5 min. Assay plates solution should be incubated in the dark at 25 ± 2.5° C for several incubation (exposure) times, i.e. 10, 30, 90, 150, 210, and 1440 min before addition of mBrB solution. Incubation times may be adapted to investigate the most relevant time points for a specific chemical (e.g., shorter incubation times might be more suitable for fast reacting chemicals). However, 1440 min should always be tested, as it corresponds to the incubation time of the DPRA. The incubation (exposure) time is the time interval from the application of the test chemical and PC dilutions to the assay plate until the addition of mBrB.

Fluorescence measurement

19. When the desired incubation (exposure) time is reached, freshly prepared mBrB solution (3 mM in acetonitrile) is added rapidly to the wells of the assay plates (one per exposure time) in the dark. Plates are sealed with gas-tight adhesive foil and shaken at least 200 rpm for 5 min. Fluorescence intensity is then determined using an excitation filter of 390 nm and an emission filter of 480 nm.

DATA AND REPORTING

Data evaluation

20. An automated Excel-evaluation spreadsheet is available with the DB-ALM protocol and should be used for data evaluation. Detailed instructions are provided in the DB-ALM protocol no. 217 (11).

21. For each incubation (exposure) time 't' the following parameters are calculated:

    - The arithmetic mean and standard deviation of the fluorescence intensity of the 12 blank controls (BC);
    - The arithmetic mean and standard deviation of the fluorescence intensity of the 12 vehicle controls (VC);
    - The mean BC value is subtracted from the VCs to obtain corrected VC values.
    - For each test chemical and PC concentration, the respective test chemical control value is subtracted from their obtained values to calculate corrected test chemical or PC values.

22. To determine the relative peptide depletion in % for each test chemical concentration per exposure time, the following calculation is performed:

$$relative\ peptide\ depletion\ [\%] \quad = \left[1 - \left(\frac{corrected\ test\ chemical\ or\ PC\ value}{mean\ of\ corrected\ VC}\right)\right] x100\%$$

23. For each test chemical concentration, the arithmetic mean and standard deviation of the three replicates is calculated (per exposure time). A student's t-test is performed to test whether the peptide concentrations measured in the three replicates is statistically significantly lower as compared to the concentration in the 12 VC wells.

24. In the kDPRA, reaction kinetic rate constants are determined as explained below if (i) a peptide depletion of ≥ 13.89% is observed at the highest test chemical concentration (final test chemical concentration 5 mM) at a given time and if (ii) the difference is statistically different from the VC. This 'positivity criterion' is based on the 'positive' criterion for peptide reactivity in the cysteine only prediction model of the DPRA described in Appendix I of this test guideline. If the positive criterion is not met, the test chemical is considered to be non-reactive according to the prediction model shown in paragraph 28.

    The natural logarithm of the non-depleted peptide concentrations (100-relative peptide depletion (%)) is plotted vs. the concentration of the test chemical at each time point. If a linear relationship is observed (correlation coefficient > 0.90), the slope of this curve is determined. The absolute value of this negative slope corresponds to the observed reaction kinetic constant (pseudo first order rate constants $k_{observed}$ in mM^{-1}). From the $k_{observed}$ value for each exposure time, the reaction kinetic constant (k_t) per concentration and incubation (exposure) time 't' is calculated as follows:

$$k_t\ [M^{-1}s^{-1}] = k_{observed} \cdot \frac{1000}{60 \cdot t}$$

    with 't' being the exposure time in minutes. If no linear relationship is observed (i.e., correlation coefficient < 0.90), the recommendations within paragraph 27.ii should be followed.

25. For each exposure time 't' with a correlation > 0.90, the decimal logarithm (log k_t) is calculated and the highest value is determined as log k_{max}.

Acceptance criteria

26. The following criteria should be met for a run to be considered valid. If one or more of these criteria is not met the run should be repeated.

    a. PC: the log k of the PC at 90 min (log $k_{90\ min}$) should be within the following range: -1.75 to -1.40 $M^{-1}s^{-1}$. If no log $k_{90\ min}$ is obtained in case of e.g., reactivity is not yet statistically significant, the value at 150 min (log $k_{150\ min}$) can be taken into account and should lie in the following range: -1.90 to -1.45 $M^{-1}s^{-1}$.

    b. VC: The coefficient of variance of the 12 VC values of a plate should be < 12.5% for at least 5 of the 6 exposure times.

27. The data obtained for the test chemical are further assessed to check for possible conditions which may affect results:

    (i) Interrupted time-course: If significant peptide depletion is observed at early time-points but not at following time points, there is either an intrinsic non-linear reaction for the test chemical or an experimental variation. In such cases the run is repeated. If the same pattern is reproducible, a non-linear kinetic is proven and the rate-constant observed at early time points is accepted.

    (ii) Non-linear concentration-response: There are few cases where the concentration-response is not linear, but clear depletion is noted. In such cases no rate constant is calculated by the slope method, as regression coefficient is R^2 < 0.90. Alternatively, rate constants can also be calculated based on individual depletion values according to the formula:

    $$k = [\ln(100/(100 - dp))]/(E \times t)$$

    Where 'dp' is depletion in %, 'E' is the concentration of test chemical and 't' is the incubation (exposure) time. Rate constants according to this formula are calculated at each time point 't' and at each concentration 'E' with depletion values above the threshold of 13.89%. For each time point 't' the average of the values for the different concentrations is taken, and then again the k_{max} for the highest rate at any given time point is reported.

    In such a case a repetition should be performed to check whether this non-linear behaviour is intrinsic to the test chemical, or whether an experimental variation is the cause. If the non-linearity is reproducible, this alternative rate calculation based on the individual depletion values is used for the final rating.

    (iii) Fluorescence interference, namely autofluorescence or fluorescence quenching: Based on the control wells with test chemical only in absence of the test peptide, incidences of autofluorescence and fluorescence quenching by the test chemical can be detected. As the values are corrected for the autofluorescence recorded in the test chemical control wells, this shall not be a problem for low autofluorescence, but with a high autofluorescence, the fluorescence of the peptide-adduct and the autofluorescence may not be fully additive, and subtraction of autofluorescence may lead to apparent depletion, which is not due to loss of peptide signal but to this non-additivity. Thus, one should check whether the observed depletion is time dependent. If this is not the case and autofluorescence is observed, then depletion from autofluorescence is assumed to occur. Fluorescence quenching can also lead to 'pseudo-depletion', but this would happen immediately and resulting depletion would not increase with time. If both conditions are met, it is assumed that depletion from quenching occurs. These cases are rare. If this is not clear from the results a run may be repeated, but if the effect is clear-cut no repetition is needed. In such a case, the test chemical cannot be assessed in the kDPRA (technical limitation)

unless the reaction can be measured with an alternative fluorescent probe not leading to autofluorescence or quenching (see Section II of the Annex 1 to DB-ALM protocol (11)).

(iv) All above cases are detailed in the DB-ALM protocol and automatic alerts appear in the Excel template provided with the DB-ALM protocol when evaluating the data.

Prediction model

28. The kDPRA uses kinetic rates of cysteine peptide depletion for discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) according to UN GHS (3). Results obtained with the test method that do not lead to subcategory 1A categorisation should be interpreted in the context of the limitations stated in paragraph 3 and Annex 1 of this appendix.

Table 1: kDPRA prediction model

Reaction rate	kDPRA Prediction
log k_{max} ≥ -2.0	UN GHS subcategory 1A
Non-reactive or log k_{max} < -2.0	Not categorised as UN GHS subcategory 1A* (non-subcategory 1A)

* Further information is needed to discriminate UN GHS subcategory 1B from UN GHS No Category. Depending on the context (e.g. IATA, DA) this information can be generated prior to or after performing the kDPRA.

29. In cases of a log k_{max} result close to the -2.0 threshold falling in the borderline range calculated for kDPRA (i.e., between -1.93 and -2.06 (12)), no conclusive prediction can be made. In this case, re-testing and/or additional data/information is needed before a conclusive prediction can be made.

30. The kinetic rate constant may be further used in integrated approaches such as IATA or DA to assess the skin sensitisation potency of a test chemical in a continuous scale as needed for risk assessment (3) (10).

Test report

31. The test report should include the following information

Test chemical and Controls (positive control and solvent/vehicle)

For all mono-constituent substance (test and control chemicals)

Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;

Physicochemical properties such as physical state, appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;

Purity, chemical identity of impurities as appropriate and practically feasible, etc;

Treatment prior to testing, if applicable (e.g. warming, grinding);

Concentration(s) tested;

Storage conditions and stability to the extent available.

Additional information for positive control

Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

Additional information for solvent/vehicle control

Solvent/vehicle used and ratio of its constituents, if applicable;

Justification for choice of other solvent than acetonitrile and experimental assessment of the solvent effect on peptide stability.

Peptide

Supplier, lot, purity

Fluorescence analysis

Fluorimeter used (e.g., model and type), including wavelengths settings

Proficiency testing

Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals.

Discussion of the results

Description of any unintended modifications to the test procedure.

Discussion of the results obtained with the kDPRA test method and if it is within the ranges described in paragraph 29.

Description of any relevant observations made, such as appearance of precipitate in the reaction mixture at the end of the incubation time, if precipitate was resolubilised or centrifuged.

Conclusion

LITERATURE FOR APPENDIX III

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APPENDIX III, ANNEX 1

KNOWN LIMITATIONS OF THE KINETIC DIRECT PEPTIDE REACTIVITY ASSAY

The table below provides a summary of the known limitations of the kDPRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Hydroquinones, catechols and aromatic amines	Lag time of oxidation may reduce apparent reaction rate	Results with log k_{max} < - 2.0 can only be accepted if low reactivity can be confirmed after oxidation	Para-phenylenediamine; 106-50-3; Human and LLNA 1A
Thiols or thiol-releasers	Test chemicals with primary SH-groups and those decomposing under the conditions of the assay can react with the detection probe	Test chemical cannot be tested in the kDPRA with derivatisation by thiol reactive probes: other kinetic data with the test peptide e.g. by HPLC may need to be generated (not part of this guideline)	Thioglycerol; 96-27-5; LLNA UN GHS category 1B; Human n/a
Test chemicals having an exclusive lysine-reactivity as observed in DPRA or ADRA	kDPRA only measures reactivity with the cysteine peptide	Results with log k_{max} < - 2.0 for chemicals which specifically deplete NH_2-groups, but not SH-groups in DPRA or ADRA are not conclusive	Some acyl-halides, phenol-esters or aldehydes, Dihydrocoumarin, 119-84-6; LLNA UN GHS category 1B; Human n/a, Glutaric aldehyde; 111-30-8; Human and LLNA UN GHS category 1A
Pro-haptens	Test chemicals for which there is evidence that they strictly require enzymatic bioactivation to exert their skin sensitizing potential	Strict pro-haptens may be underestimated. However chemicals which are i) strict pro-haptens (i.e. test chemicals not also acting as direct haptens or prehaptens, too) and ii) strong allergens were found to be rare	Diethylenetriamine; 111-40-0 (human 1A, LLNA UN GHS category 1)
Fluorescent chemicals with excitation in the range of the fluorescent probe	If fluorescence of test chemicals and of the mBrB-peptide adduct is not additive, pseudo-depletion is observed	Follow the considerations in the DB-ALM Protocol n° 217 to evaluate assay interference	Tetrachlorosalicylanilide; 1154-59-; Human and LLNA UN GHS category 1A
Test chemicals absorbing in the emission range of the probe	If test chemical quenches fluorescence emission of the mBrB-peptide adduct, pseudo-depletion is observed	Follow the considerations in the DB-ALM Protocol n° 217 to evaluate assay interference	Vanillin, 121-33-5; LLNA NC; Human n/a
Mixtures of unknown composition, substances of unknown or variable	no information on applicability of kDPRA is available in the published literature	n/a	UVCBs, chemical emissions, products or formulations with

composition, complex reaction products or biological materials			variable or not fully known composition
Test chemicals which cannot be dissolved in water or acetonitrile or a compatible water-miscible solvent	Not sure if sufficient exposure can be achieved	In such cases, a log k_{max} > -2.0 could still be used to support the identification of the test chemical as a UN GHS subcategory 1A skin sensitiser but no firm conclusion should be drawn in case log k_{max} is < -2.0. Alternative vehicle may be used according to the prescriptions given in paragraph 12.	n/a
Test chemicals which precipitate in reaction solution	Not sure if sufficient exposure can be achieved: If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, one cannot be sure how much test chemical remained in the solution to react with the peptide.	In such a case, a positive result (i.e. log kmax ≥ -2.0) could still be used, but a negative result (i.e., non-reactive or log kmax < -2.0 outcome) should be interpreted with due care (see also provisions in paragraph 4 for the testing of chemicals not soluble up to a concentration of 20 mM in the kDPRA).	Methyl-2-nonynoate[5]; 111-80-8; LLNA NC
Test chemicals promoting cysteine-peptide oxidation		May lead to a potential over estimation of peptide reactivity.	DMSO

[5] Roberts, D.W. and A. Natsch, *High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitization potency of michael acceptor electrophiles.* Chem. Res. Toxicol., 2009. **22**(3): p. 592-603

APPENDIX III, ANNEX 2

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: kinetic Direct Peptide Reactivity Assay (kDPRA)

Prior to routine use of the test method described in this appendix, laboratories should demonstrate technical proficiency by correctly obtaining the expected kDPRA prediction for at least 8 of the 9 proficiency substances recommended in Table 1 and by obtaining cysteine rate constants log k_{max} that fall within the respective reference range for 7 out of the 9 proficiency substances. These proficiency substances were selected to represent the range of responses for skin sensitisation hazard and potency. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the kDPRA are available, and that they were used in the industry-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Table 1: Recommended proficiency substances for demonstrating technical proficiency with the kinetic Direct Peptide Reactivity Assay

Proficiency substances	CASRN	Physical state	*In vivo* prediction[1]	UN GHS Category LLNA	UN GHS Category human	kDPRA prediction [2]	Range of log k_{max} [2]
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	1A	1A	1A	(-0.8) – (-0.4)
Methylisothiazolinone	2682-20-4	Solid	Sensitiser (extreme)	1A	1A	1A	(-0.5) – (-0.1)
Oxazolone	15646-46-5	Solid	Sensitiser (extreme)	1A	No data	1A	(-0.3) – (0.0)
Methyl-2-octynoate	111-12-6	Liquid	Sensitiser (strong)	1A	1A	1A	(-1.6) – (-1.2)
Isoeugenol	97-54-1	Liquid	Sensitiser (moderate)	1A	1A	1A	(-1.4) - (-1.1)
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	1B	No data	non-1A (1B or NC)	(-3.2) – (-2.1)
Ethylene glycol dimethacrylate (EGDMA)	97-90-5	Liquid	Sensitiser (weak)	1B	1B	non-1A (1B or NC)	(-2.8) – (-2.1)
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	No Cat.[3]	No Cat.[3]	non-1A (1B or NC)	Not reactive
Chlorobenzene	108-90-7	Liquid	Non-sensitiser	No Cat.[3]	No Cat.[3]	non-1A (1B or NC)	Not reactive

[1]The *in vivo* hazard and (potency) predictions are based on LLNA data (13). The *in vivo* potency is derived using the criteria proposed by ECETOC (14).

[2] Rounded ranges determined on the basis of at least 14 log k_{max} determinations generated by 7 independent laboratories.

[3] Non sensitisers according to the UN GHS.

Section 4
Health effects

Guideline No. 497

Guideline on Defined Approaches for Skin Sensitisation

14 June 2021

OECD Guidelines for the Testing of Chemicals

OECD
BETTER POLICIES FOR BETTER LIVES

OECD GUIDELINE FOR TESTING OF CHEMICALS

Defined Approaches for Skin Sensitisation

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1. Section 1-Introduction

1.1. General Introduction

1. A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). There is general agreement on the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation initiated by covalent binding to proteins has been summarised as an Adverse Outcome Pathway (AOP) (2) that begins with a molecular initiating event, leading to intermediate key events, and terminating with the adverse effect, allergic contact dermatitis.

2. The skin sensitisation AOP focuses on chemicals that react with amino acid residues (*i.e.* cysteine or lysine) such as organic chemicals. In this instance, the molecular initiating event (*i.e.* the first key event), is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation, and the adverse outcome is presentation of allergic contact dermatitis.

3. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman and the Buehler Test (OECD TG 406) (3) assess both the induction and elicitation phases of skin sensitisation. The murine tests, such as the LLNA (OECD TG 429) (4) and its three non-radioactive modifications — LLNA: DA (OECD TG 442A) (5), LLNA: BrdU-ELISA, and BrdU-FCM (OECD TG 442B) (6) — all assess the induction response exclusively and have gained acceptance, since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitisation.

4. Mechanistically-based *in chemico* and *in vitro* test methods (OECD TG 442C, 442D, 442E) (7, 8, 9) addressing the first three key events (KE) of the skin sensitisation AOP can be used to evaluate the skin sensitisation hazard potential of chemicals. None of these test methods are considered sufficient stand-alone replacements of animal data to conclude on skin sensitisation potential of chemicals or to provide information for potency sub-categorisation according to the UN GHS (sub-categories 1A and 1B). However, data generated with these *in chemico* and *in vitro* methods addressing multiple KEs of the skin sensitisation AOP are proposed to be used together, as well as with information sources such as *in silico* and read-across predictions from chemical analogues, within integrated approaches to testing and assessment (IATA) or defined approaches (DAs). Results from the individual information sources can only be used in DAs if the substances fall within the applicability domains of the methods (see "Initial Considerations, Applicability and Limitations" sections of respective methods (TG 442C, Appendix 1; TG 442D, Appendix 1A; TG 442E Annex 1) (7, 8, 9).

5. Results from multiple information sources can be used together in DAs to achieve an equivalent or better predictive capacity than that of the animal tests to predict responses in humans. A DA consists of a fixed data interpretation procedure (DIP) (*e.g.* a mathematical model, a rule-based approach) applied to data (*e.g. in silico* predictions, *in chemico*, *in vitro* data) generated with a defined set of information sources to derive a prediction without the need for expert judgment. Individual DAs for skin sensitisation and their respective information sources were originally described in Guidance Document 256, Annex I/II (10) and a preliminary assessment was published in Kleinstreuer et al (11). The DAs use method combinations intended to overcome some of the limitations of the individual, stand-alone methods in order to provide increased confidence in the overall result obtained. The ultimate goal of DAs is to provide information that is equivalent to that provided by animal studies, *i.e.* information that can be used for hazard identification and/or potency categorisation.

6. Testing laboratories should consider all relevant available information on the test chemical prior to conducting the studies as directed by a DA. Such information could include, for example, the identity and chemical structure of the test chemical and its physico-chemical properties. Such information should be considered in order to determine whether the individual OECD test guideline methods under a specific DA are applicable for the test chemical.

7. When performing a hazard evaluation and/or potency sub-categorisation based on the output from an *in vivo* (LLNA or any other) test, from an *in chemico* test, from an *in vitro* test, from an *in silico* approach, from a DA, and any combination thereof, the same principles always apply, *i.e.* all available information relevant to the chemical in question should be taken into consideration as well as toxicological data on structurally related test chemicals if available.

8. This Guideline was developed with the input of an OECD Expert Group on Defined Approaches for Skin Sensitisation (EG DASS) comprised of scientific experts from regulatory agencies, validation bodies, non-governmental organisations, and industry.

9. Three rule-based DAs are included in this Guideline, and are described with respect to their intended regulatory purpose: hazard identification, *i.e.* discrimination between skin sensitisers and non-sensitisers (1.4.Part I), or potency sub-categorisation (1.6.Part II). The DAs included in Part II are also suitable for hazard identification. The evaluation and review of the DAs are described in detail in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (12).

10. A comprehensive dataset of 196 chemicals with DA predictions, data on individual information sources, highly curated LLNA and Human Patch Predictive Test (HPPT) data, and physicochemical properties, was compiled and is attached as **Annex 2** to the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (12). Out of the 196 chemicals, 168 chemicals have LLNA classifications and 66 chemicals have HPPT classifications, which were all agreed upon by the EG DASS and used to evaluate the performance of the DAs. Due to the availability of data, this dataset contains mainly cosmetic ingredients but also other types of chemicals that are used across sectors such as preservatives, dyes, or food ingredients. The dataset is chemically diverse as shown by the physicochemical properties covered by these chemicals: it contains small and large molecules (molecular weight ranges from 30 to 512 g/mol), hydrophobic and hydrophilic substances (Log P ranges from -3.9 to 9.4), solids and liquids (melting point ranges from -122 to 253 ºC), volatile and non-volatile substances (boiling point ranges from -19 to 445 ºC). Further details on the chemical space characterization of the reference

database are available in **Section 4** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (12).

11. Other DAs may be included in this Guideline following future review and approval. DAs able to provide a quantitative measure of sensitisation potency, such as a point of departure which can be used for risk assessment, may be included in a new Part II to this Guideline in the future.

1.2. DAs and Use Scenarios included in the Guideline

12. The DAs currently described in this guideline are:

- The "2 out of 3" (2o3) defined approach to skin sensitisation hazard identification based on *in chemico* (KE1) and *in vitro* (KE2/KE3) data (13, 14). See Part I.
- The integrated testing strategy (ITSv1) for UN GHS potency categorisation based on *in chemico* (KE1) and *in vitro* (KE3) data, and *in silico* (Derek Nexus) predictions (14, 15), with a DIP developed with expert group (EG DASS) input. See Part II Potency Categorisation.
- A modification of the integrated testing strategy (ITSv2) for UN GHS potency categorisation based on *in chemico* (KE1) and *in vitro* (KE3) data, and *in silico* (OECD QSAR Toolbox) predictions, with a DIP developed with expert group (EG DASS) input. See Part II Potency Categorisation.

13. The DAs described in this guideline are based on the use of validated OECD test methods (DPRA, KeratinoSens™, h-CLAT), for which transferability, within- and between-laboratory reproducibility have been characterised in the validation phase (7, 8, 9).

14. The ITS DAs (ITSv1 and ITS v2) also make use of an *in silico* information source; Derek Nexus v6.1.0 (ITSv1), or OECD QSAR Toolbox v4.5 (ITSv2). Derek Nexus (referred to as Derek hereafter) is an expert knowledge-based tool which provides predictions of skin sensitisation potential using structural alerts, and OECD QSAR Toolbox (referred to as OECD QSAR TB hereafter) is a computational tool which uses an analogue-based read-across approach or structural alerts for protein binding identified by profilers to predict whether a chemical will be a sensitiser.

15. All DAs described in this guideline can each be used to address countries' requirements for discriminating between sensitisers (*i.e.* UN GHS Category 1) from non-sensitisers, though they do so with different sensitivities and specificities (detailed in the respective descriptions of each DA).

16. The ITS DAs (ITSv1 and ITS v2) can also be used to discriminate chemicals into three UN GHS potency categories (Category 1A = strong sensitisers; Category 1B = other sensitisers, and No Categorization (NC = not classified).

17. The known limitations and applicability domains of the individual information sources were used to design workflows for assigning confidence to each of the predictions produced by the DAs described in this guideline. In order to have a high confidence prediction, the underlying data must meet criteria in the respective test guidelines (see TG 442C, Appendix 1; TG 442D, Appendix 1A; TG 442E Annex 1 (7, 8, 9)), DA predictions with high confidence for hazard identification and/or potency are considered conclusive. DA predictions with low confidence are considered inconclusive for hazard identification and/or potency (see **Sections 2.1.4** and **3.1.4** for further information). These 'inconclusive'

predictions may nevertheless be considered in a weight-of-evidence approach and/or within the context of an IATA together with other information sources (*e.g.* demonstration of exposure to the test system, existing *in vivo* data, clinical data, read-across, other *in vitro* / *in chemico* / *in silico* data, etc.).

18. The performance of the DAs described in this guideline for discriminating between sensitisers and non-sensitisers was evaluated using 168 (135 GHS Skin Sens. Category 1, and 33 no classification) test chemicals for which DPRA, KeratinoSens™, h-CLAT, Derek, OECD QSAR TB predictions and classifications based on LLNA reference data agreed upon by the EG DASS are available (for additional details see **Section 2.1** and **Annex 3** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation*) (12). For the purpose of evaluating the performance of the ITS DAs for predicting UN GHS classifications based on potency categorization (sub-category 1A, 1B, or "not classified" (NC)), 156 test chemicals (38 1A, 85 1B, and 33 NC) were used because for 12 test chemicals it was not possible to assign with sufficient confidence the potency sub-category 1A or 1B on the basis of LLNA data. Mixtures and botanicals with undefined structural composition were excluded from the curated LLNA reference data.

19. The performance of the three DAs (high confidence predictions only) against the LLNA reference data for predicting skin sensitisation hazard showed balanced accuracies (average of sensitivity and specificity; BA) in the range of 80-84%, with sensitivities of 82-93% and specificities of 67-85% (see **Table 1.1**). Note that specificity measures are more uncertain than sensitivities due to lower number of negative reference chemicals. Detailed performance statistics are reported in Part I (2o3 DA) and Part II (ITS DA). The performance of the ITSv1 and ITSv2 DAs for UN GHS classifications based on potency categorization (high confidence predictions only, sub-category 1A, 1B, or NC) when compared to the LLNA reference data yielded overall accuracies of 71%, overall balanced accuracies of 78% (ITSv1) or 77% (ITSv2), and balanced accuracies within a predicted sub-category or NC ranging from 72-81% (ITSv1) or 71-80% (ITSv2). There were no strong sensitisers (1A) that were incorrectly predicted as being a non-sensitiser (NC) or vice versa. Detailed performance statistics are reported in Part II and in **Section 5** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (12).

20. The performance of the DAs described in this guideline for discriminating between sensitisers and non-sensitisers was also evaluated using a set of 66, or 65 for 2o3, due to lack of assay data for one chemical, test chemicals (55 sensitisers and 11 non-sensitisers) for which classifications based on Human Predictive Patch Test (HPPT) data have been agreed upon by the EG DASS (for additional details see **Section 2.2** and **Annex 4** *of the Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation*) (12). For the purpose of evaluating the performance of the ITS DAs for predicting UN GHS classifications based on potency categorization (sub-category 1A, 1B, or NC), 63 test chemicals were used (21 1A, 31 1B, and 11 NC) because for 3 test chemicals it was not possible to assign with sufficient confidence the potency sub-category 1A or 1B on the basis of human reference data. Mixtures and botanicals with undefined structural composition were excluded from the curated human reference data.

21. The performance of the DAs (high confidence predictions only) against the human reference data for predicting skin sensitisation hazard showed balanced accuracies in the range of 69-88%, with sensitivities of 89-94% and specificities of 44-88% (see **Table 1.1**). Note that specificity measures are more uncertain than sensitivities due to lower number of negative reference chemicals. Detailed performance statistics are reported in Part I (2o3

DA) and Part II (ITS DA). The performance of the ITSv1 and ITSv2 DAs for UN GHS skin sensitisation potency classification (high confidence predictions only, sub-category 1A, 1B and NC) when compared to the human reference data yielded overall balanced accuracies of 72% (ITSv1) or 73% (ITSv2), and balanced accuracies within a predicted sub-category or NC in the range of 68-79% (ITSv1) or 69-79% (ITSv2). Detailed performance statistics are reported in Part II and in **Section 5** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (12).

22. The overlap between the LLNA and human reference datasets was 56 chemicals for hazard and 47 chemicals for skin sensitisation potency categorisation, respectively, and the performance of the LLNA against the human reference data was evaluated using these chemicals as a basis for comparison. The performance of the LLNA against the human reference for predicting skin sensitisation hazard showed a balanced accuracy of 58%, with sensitivity of 94% and specificity of 22%. Note that the specificity measure is more uncertain than the sensitivity due to a lower number of negative reference chemicals. The performance of the LLNA for UN GHS potency classification when compared to the human reference data yielded an overall balanced accuracy of 64%, and balanced accuracies within a predicted sub-category or NC in the range of 59-73% There were no strong skin sensitisers (1A) in the human reference data that were incorrectly predicted by the DAs, or by the LLNA as not being a sensitiser (no classification) or vice versa. Detailed performance statistics are reported Part I and Part II

Table 1.1. Summary of the DAs Included in this Guideline

DA/Method	Information Sources	Capability (Hazard and/or Potency)	Hazard Performance vs. LLNA	Hazard Performance vs. Human	Potency Performance vs. LLNA (Accuracy)	Potency Performance vs. Human (Accuracy)
2o3 DA	DPRA, KeratinoSens™, h-CLAT	Hazard	84% BA, 82% Sens, 85% Spec	88% BA, 89% Sens, 88% Spec	-	-
ITSv1 DA	DPRA, h-CLAT, DEREK Nexus v6.1.0	Hazard, Potency	81% BA, 92% Sens, 70% Spec	69% BA, 93% Sens, 44% Spec	70% NC, 71% 1B, 74% 1A	44% NC, 77% 1B, 65% 1A
ITSv2 DA	DPRA, h-CLAT, OECD QSAR Toolbox v4.5	Hazard, Potency	80% BA, 93% Sens, 67% Spec	69% BA, 94% Sens, 44% Spec	67% NC, 72% 1B, 72% 1A	44% NC, 80% 1B, 67% 1A
LLNA (provided for comparison)	*in vivo*	Hazard, Potency	-	58% BA, 94% Sens, 22% Spec	-	25% NC, 74% 1B, 56% 1A

Note: For hazard performance, sensitivity (Sens) is the true positive rate, specificity (Spec) is the true negative rate, and balanced accuracy (BA) is the average of sensitivity and specificity. Due to the imbalanced nature of the reference data, the measures of specificity are more uncertain than the measures of sensitivity. For potency performance, accuracy reflects correct classification rate within each UN GHS sub-category. Due to the imbalanced nature of the reference data, the measures of accuracy are more uncertain for smaller classes, *e.g.* for NC chemicals. Statistics reflect conclusive DA predictions only. This represents the data available at the time of initial guideline adoption.

1.3. Limitations

23. **Table 1.1** provides an overview of the DAs included in this Guideline, their information sources used, whether they provide hazard and/or potency prediction, and

summarises their performance against the LLNA and human reference data. The LLNA (OECD TG 429) is included in **Table 1.1** as a basis for comparison. More details are provided in Part I and Part II of this Guideline, as well as in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (12).

24. The identified limitations of the DAs and their individual components are summarised below.

1.3.1. Limitations of individual in chemico/in vitro *information sources*

25. Users should refer to the limitations of the individual *in chemico*/*in vitro* test methods as specified in their respective Test Guidelines, which are revised as new data become available and should be consulted regularly. The most up-to-date published version of the respective TGs should always be used. For example, some types of chemicals such as metals, inorganic compounds, UVCBs and mixtures, may not be within the applicability domain for certain test methods. Individual assay results within borderline ranges (**Annex 1**) may yield inconclusive DA predictions. The consideration of limitations of individual *in chemico*/*in vitro* test methods in each DA is detailed in **Section 2.1.4** (**Figure 2.1**) and **Section 3.1.4** (**Figure 3.1**).

1.3.2. Limitations of in silico information sources

26. Some DAs include *in silico* tools as an information source. These tools can either perform automated read-across or (Q)SAR predictions. (Q)SARs include both structure-activity relationship (SAR) models (*i.e.* structural alerts, expert systems) and quantitative structure-activity relationship (QSAR) models (*i.e.* statistical tools). (Q)SAR models should fulfil the OECD Principles for the Validation, for Regulatory Purposes, of (Q)SAR Models and be described in a QSAR Model Reporting Format (QMRF) document (15)[1]. One of the OECD QSAR validation principles refers to a defined domain of applicability. The defined domain of applicability reflects limitations beyond which less reliable predictions may be obtained (*e.g.* training set ranges of descriptors included in the model and types of chemical structures included in the training set). A given *in silico* model may be associated with more than one defined applicability domain, each of which is associated with its own reliability measures as established in the validation. Depending on the DIP, chemicals outside the applicability domain may result in DA predictions of low confidence that are considered inconclusive. Where a DA for skin sensitisation includes an *in silico* tool, users should refer to the limitations and applicability domain of the individual *in silico* tool. Two of the DAs covered in this Guideline, the ITSv1 and the ITSv2, rely upon the *in silico* tools Derek and OECD QSAR TB, respectively, and their specified limitations and applicability domains are detailed in **Annex 2** of this Guideline.

1.3.3. Limitations of DAs

27. The limitations of the DAs are based on the limitations of the individual *in chemico/in vitro/in silico* information sources. Details on using the limitations of individual information sources to determine confidence in DA predictions are provided in **Sections**

[1] The QMRF has been slightly adapted for reporting other *in silico* model predictions in the context of DASS. The adapted QPRF can be found on the OECD site for spreadsheets and software associated with OECD Test Guidelines on Health Effects: https://www.oecd.org/env/ehs/testing/section4software.htm.

2.1.4 and **3.1.4** and in the respective test guidelines (TG 442C, Appendix 1; TG 442D, Appendix 1A; TG 442E, Annex 1) (7, 8, 9).

28. During the evaluation of the DAs covered in this Guideline it was observed that, with respect to LLNA data, the DPRA (TG 442C), KeratinoSens™ (TG 442D), h-CLAT (TG 422E), as well as the proposed DAs, have lower sensitivity for test chemicals with Log P > 3.5 **(**for details see **Section 3.1.4** and **Annex 5** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation*) (12). It was also noted that the LLNA test may produce a higher number of false positive results for these test chemicals when compared with human reference data, and supporting mechanistic information was provided **(**for details see **Section 3.2** and **Annex 6** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation*) (12). Overall, the analyses and the number of reference chemicals with Log P > 3.5 are insufficient to draw firm conclusions. However, according to TG 442E, negative h-CLAT results for substances with Log P > 3.5 should not be considered, and this limitation is applied to the DAs as described in **Sections 2.1.4** and **3.1.4.**

29. For the 2o3 DA, borderline ranges (BRs) have been defined for the individual assays addressing the three KE of the DA, in order to define areas where lower confidence may exist (for details see **Section 2.1.4** and **Annex 1** of this Guideline, and **Section 3.3** and **Annex 7** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation*) (12). Positive and/or negative test results falling within these BRs as well as individual assay limitations, *e.g.* negative h-CLAT results obtained for a chemical with Log P > 3.5 (according to TG 442E), have lower confidence and may result in inconclusive 2o3 DA predictions.

30. Inconclusive DA predictions may nevertheless be considered in a weight-of-evidence approach and/or within the context of an IATA together with other information sources (*e.g.* demonstration of exposure to the test system, existing *in vivo* data, clinical data, read-across, other *in vitro* / *in chemico* / *in silico* data, etc.).

1.4. References

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Part I. – Section 2 - Defined Approaches for Skin Sensitisation Hazard Identification

31. Part I of this guideline applies to DAs that are intended solely for hazard identification, *i.e.* distinguishing between sensitisers and non-sensitisers. A summary of the DAs for hazard identification is provided below; additional detailed information can be found in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

2.1. "2 out of 3" Defined Approach

2.1.1. Summary

32. The 2 out of 3 (2o3) DA is intended for the identification of the skin sensitisation hazard of a chemical without the use of animal testing, *i.e.* UN GHS Cat. 1 vs. UN GHS NC. The data interpretation procedure (DIP) is currently not designed to provide information on the potency of a sensitiser.

33. The combination of test methods included in the 2o3 DA covers at least two of the first three KEs of the AOP leading to skin sensitisation as formally described by the OECD: KE1: protein binding (*i.e.* via the direct peptide reactivity assay (DPRA; OECD TG 442C)) (2); KE2: keratinocyte activation (*i.e.* KeratinoSens™; OECD TG 442D) (3); and KE3: dendritic cell activation (*i.e.* via the human cell line activation test (h-CLAT; OECD TG 442E)) (4).

34. The DIP entails that two concordant results obtained from methods addressing at least two of the first three KEs of the AOP determine the final classification. The 2o3 DA was compared to 168 chemicals with curated LLNA reference data agreed upon by the EG DASS and demonstrated an accuracy of 83% and a balanced accuracy of 84% (see **Table 2.1**). The 2o3 DA was also compared to 65 chemicals with curated human reference data agreed upon by the EG DASS and exceeded the accuracy, and balanced accuracy, of the LLNA for hazard identification (see **Tables 2.1-2.2**). It should be noted that due to the imbalanced nature of the reference data (higher numbers of positives than negatives), the measures of balanced accuracy are more uncertain, particularly in the case of the human data comparison.

2.1.2. Data interpretation procedure

35. The data interpretation procedure (DIP) in the 2o3 DA is a transparent, rule-based approach requiring no expert judgment (4, 6, 7). The approach predicts skin sensitisation hazard by sequential testing, in an undefined order, in up to three of the following internationally accepted non-animal assays mapping to KE1-3 (*i.e.* DPRA, KeratinoSens™, h-CLAT). Assays are run for two KEs, and if these assays provide consistent results, then the chemical is predicted accordingly as sensitiser or non-sensitiser. If the first two assays provide discordant results, the assay for the remaining KE is run. The overall result is based on the two concordant findings taking into account the confidence on the obtained predictions as described in **Section 2.1.4**.

36. The performance of the 2o3 DA was found to be impacted by the consideration of borderline ranges for each of the methods, as described below in **Section 2.1.4**, and further

detailed in **Section 3.3** and **Annex 7** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1). A decision tree is provided in **Figure 2.1** of **Section 2.1.4** to derive predictions for the 2o3 DA, with no modification of the 2o3 DA Data Interpretation Procedure.

2.1.3. Description and limitations of the individual information sources

37. The individual information sources in the DA are assays included in OECD KE-based test guidelines for skin sensitisation (OECD TG 442C, 442D, 442E) (2, 3, 4), and the protocols are detailed therein.

38. The following assays from those TGs have been characterised and included in the 2o3 DA.

- Direct Peptide Reactivity Assay (DPRA; OECD TG 442C; KE1) (2): Skin sensitisers are generally electrophilic and react with the nucleophilic moieties of proteins. The DPRA measures depletion of two peptides containing either cysteine or lysine residues due to covalent binding. A test chemical that induces mean peptide depletion of cysteine- and lysine-containing peptide above 6.38% (or in the case of co-elution, cysteine-only depletion above 13.89%) is considered to be positive. In case borderline results are obtained for peptide depletion, additional testing should be conducted, as specified in OECD TG 442C and in **Annex 1**.
- KeratinoSens™ assay (*In vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method; OECD TG 442D; KE2) (3); Keratinocytes harbouring a reporter gene construct react to possible sensitisers via the Nrf2-Keap1 pathway. A test chemical that causes >1.5 fold luciferase induction, at viabilities > 70% when compared to the vehicle control, is considered to be positive. In case borderline results are obtained for luciferase induction, additional testing should be conducted, as specified in **Annex** 1.
- Human cell-line activation test (h-CLAT; OECD TG 442E; KE3) (4): Activation of antigen presenting cells is characterised by the up-regulation of CD86 and/or CD54. The h-CLAT is considered to be positive if CD86 induction exceeds 1.5-fold and/or CD54 exceeds 2-fold at viabilities > 50% when compared to the vehicle control. In case borderline results are obtained for CD54 and/or CD86 induction, additional testing should be conducted, as specified in **Annex 1**.

39. The current limitations of individual *in chemico* and *in vitro* test methods, such as limitations with respect to solubility, are described in the respective test guidelines (TG 442C, Appendix 1; TG 442D, Appendix 1A; TG 442E, Annex 1) and the validation studies cited therein (2, 3, 4).

2.1.4. Confidence in the 2o3 DA predictions

40. The first decision on whether each information element can be used is dictated by the limitations of the *in chemico* and *in vitro* methods (*e.g*. for substances that do not provide conclusive results in the individual methods due to solubility reasons) as found in in the respective test guidelines (TG 442C, Appendix 1; TG 442D, Appendix 1A; TG 442E, Annex 1) (2, 3, 4). Additionally, test results are subject to variation and these variations increase the uncertainty of a test result especially when close to a (classification) cut-off, *i.e.* in the borderline range. In order to define areas where lower confidence in the DA results may exist, borderline ranges (BRs) have been defined for output from the individual assays addressing the three KE of the 2o3 DA, (see **Annex 1** of this document, and **Section**

3.3 and **Annex 7** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation)* (1). The specific borderline ranges for each assay, as derived from their respective validation study data, are:

- DPRA BR: mean peptide depletion: 4.95% – 8.32%, Cys-only depletion (in the case of co-elution with lysine peptide): 10.56% – 18.47%;
- KeratinoSens™ BR: Imax: 1.35-fold – 1.67-fold;
- h-CLAT BR: RFI CD54: 157% – 255%; RFI CD86: 122% – 184%.

41. The incorporation of borderline ranges (BRs) into the prediction models (PM) for each of the individual information sources is are described in **Annex 1** of this guideline.

42. For the data with a single run as reported in the reference database, borderline cases in the DPRA are identified based on the borderline range for the mean peptide depletion or Cys-only depletion as described above. In case repeated runs are conducted, the PM in **Annex 1, Figure 1.1** shall be applied.

43. The prediction model of the KeratinoSens™ assay requires multiple runs. For the assessment of whether the outcome of repeated runs yields a positive, negative or borderline final outcome in KeratinoSens™, the PM in **Annex 1, Figure 1.2** shall be applied (adapted from the PM described in TG 442D to be used within the 2o3 DA to conclude on borderline cases). This prediction model introduces a third outcome (borderline) to be used within the 2o3 DA, based on the same decision cut-offs of the prediction model described in TG 442D. Thus, a negative in the original prediction model can only become negative or borderline, while a positive from the original prediction model can only become positive or borderline.

44. The prediction model of h-CLAT requires multiple runs. For the assessment of whether the outcome of repeated runs yields a positive, negative or borderline final outcome in the h-CLAT, the PM in **Annex 1**, **Figure 1.3** shall be applied (adapted from the PM described in TG 442E to be used within the 2o3 DA to conclude on borderline cases). This prediction model introduces a third outcome (borderline) to be used within the 2o3 DA, based on the same decision cut-offs of the prediction model described in TG 442E. Thus, a negative in the original prediction model can only become negative or borderline, while a positive from the original prediction model can only become positive or borderline.

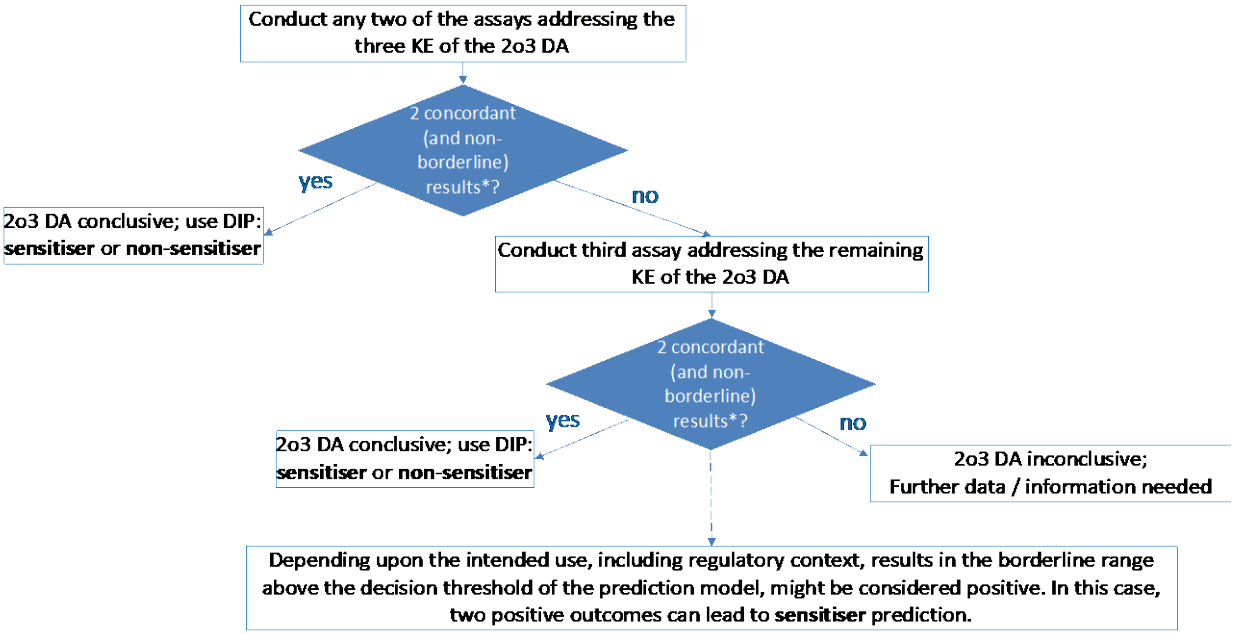
45. Positive and negative test results falling within these BRs as well as inconclusive results due to limitations in the *in chemico/in vitro* test guidelines are of lower confidence. For example, negative h-CLAT results obtained for a chemical with Log P > 3.5 (according to TG 442E (4)) are of lower confidence, and affect the outcome of the 2o3 DA as described below:

- In case the result of one of the 2o3 DA test methods falls into the respective test method's BR, a 2o3 DA prediction can still be made if the outcomes of the other two test methods composing the 2o3 DA are concordant and have high confidence (*i.e.*, results falling outside of the respective BRs).
- Similarly, in case a negative h-CLAT result is obtained for a chemical with Log P > 3.5, a 2o3 DA prediction can still be made if the outcomes of the other two test methods composing the 2o3 DA are concordant and have high confidence (*i.e.*, results falling outside of the respective BRs).

- However, if the result of one of the 2o3 DA test methods falls into the respective test method's BR or a negative h-CLAT result is obtained for a chemical with Log P > 3.5, and the other two methods composing the 2o3 do not provide concordant and high confidence results, the 2o3 DA prediction is considered 'inconclusive'. These inconclusive predictions may nevertheless be considered in a weight-of-evidence approach and/or within the context of an IATA together with other information sources. Depending on the intended use, including regulatory context, results in the borderline range above the decision threshold of the prediction model might still be considered positive; in this case, two positive outcomes can lead to an overall positive (sensitiser) prediction.

46. These borderline considerations and their impact on the confidence of the 2o3 DA predictions are visualized in **Figure 2.1.** DA predictions with high confidence for hazard identification are considered conclusive. DA predictions with low confidence are considered inconclusive for hazard identification. These 'inconclusive' predictions may nevertheless be considered in a weight-of-evidence approach and/or within the context of an IATA together with other information sources.

Figure 2.1. Decision tree to be used for the 2o3 DA, taking into account borderline results



Note: Borderline results are determined based on workflows given in **Annex 1**.
* The use of information elements is dictated by the limitations as found in in the respective test guidelines (TG 442C, Appendix 1; TG 442D, Appendix 1A; TG 442E, Annex 1). For example, in case a negative h-CLAT result is obtained for a chemical with Log P > 3.5 (according to the limitation described in TG 442E (4)), a 2o3 DA prediction can only be made if the outcomes of the other two test methods composing the 2o3 DA are concordant and are non-borderline.

2.1.5. Predictive capacity of the 2o3 DA vs. the LLNA

47. The predictive capacity of the "2o3" DA is reported based on data generated by the LLNA (see **Table 2.1**), curated as agreed upon by the EG DASS (see **Section 2.1** and **Annex 3** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation)*. The borderline range analyses were applied as described above to

assign confidence to the 2o3 DA predictions. Performance statistics are reported for conclusive (high confidence) predictions as compared to LLNA reference data, and inconclusive (low confidence) results are indicated. DA predictions for specific chemicals and further details are available in **Section 5** and **Annex 2** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

Table 2.1. Hazard identification performance of the "2o3" DA in comparison to LLNA reference data

	LLNA	
2o3 DA	Non	Sens
Non	22	19
Sens	4	89
Inconclusive	7	27

DA Performance vs. LLNA Data (N=134)	**2o3**
Accuracy (%)	83%
Sensitivity (%)	82%
Specificity (%)	85%
Balanced Accuracy (%)	84%

Note: Accuracy is the correct classification rate, sensitivity is the true positive rate, specificity is the true negative rate, and balanced accuracy is the average of sensitivity and specificity. Performance is reported based on DPRA, KeratinoSens™, and h-CLAT. Statistics reflect conclusive predictions only; inconclusive predictions are shown in grey. Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation.*

48. The application of the BR analyses and the designation of high/low confidence for the 2o3 DA predictions is applied as described above in **Section 2.1.4** and **Annex 1,** and further detailed in **Section 3.3** and **Annex 7** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

49. Due to the imbalanced nature of the reference data, the measure of specificity (based on 26 LLNA negative chemicals) is more uncertain than the measure of sensitivity (based on 108 LLNA positive chemicals).

2.1.6. Predictive capacity of the 2o3 DA vs. Human Data

50. The predictive capacity of the "2o3" DA is also reported based on Human Predictive Patch Test (HPPT) data (see **Table 2.2**), curated as agreed upon by the EG DASS (see **Section 2.2** and **Annex 4** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1)*)*. The borderline range analyses were applied as described above to assign confidence to the 2o3 DA predictions. Performance statistics are reported for conclusive (high confidence) predictions as compared to human reference data, and inconclusive (low confidence) results are indicated. DA predictions for specific chemicals and further details are available in **Section 5 and Annex 2** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

Table 2.2. Hazard identification performance of the "2o3" DA in comparison to human reference data

	Human	
2 of 3 DA	Non	Sens
Non	7	5
Sens	1	42
Inconclusive	3	7

DA Performance vs. Human Data (N=55)	**2o3**
Accuracy (%)	89%
Sensitivity (%)	89%
Specificity (%)	88%
Balanced Accuracy (%)	88%

Note: Accuracy is the correct classification rate, sensitivity is the true positive rate, specificity is the true negative rate, and balanced accuracy is the average of sensitivity and specificity with respect to HPPT data. Performance is reported based on DPRA, KeratinoSens™, and h-CLAT. Statistics reflect conclusive predictions only; inconclusive predictions are shown in grey. Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

51. The application of the BR analyses and the designation of high/low confidence for the 2o3 DA predictions is applied as described above in **Section 2.1.4** and **Annex 1**, and further detailed in **Section 3.3 and Annex 7** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

52. Due to the imbalanced nature of the reference data, the measure of specificity (based on 8 human negative chemicals) is more uncertain than the measure of sensitivity (based on 47 human positive chemicals).

2.1.7. Predictive capacity of the LLNA vs. Human Data

53. To provide a basis for comparison for the DA performance statistics given above, the predictive capacity of the LLNA is reported based on data from the Human Predictive Patch Test (see **Table 2.3**) curated as agreed upon by the EG DASS. Data for specific chemicals and further details are available in **Section 5** and **Annex 2** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

Table 2.3. Hazard identification performance of the LLNA in comparison to Human reference data

	Human	
LLNA	Non	Sens
Non	2	3
Sens	7	44

LLNA Performance vs. Human Data (N=56)	**LLNA**
Accuracy (%)	82%
Sensitivity (%)	94%
Specificity (%)	22%
Balanced Accuracy (%)	58%

Note: Accuracy is the correct classification rate, sensitivity is the true positive rate, specificity is the true negative rate, and balanced accuracy is the average of sensitivity and specificity with respect to Human HPPT-based data. Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

54. The hazard identification performance of the conclusive 2o3 DA predictions vs. human HPPT data was 89% accuracy, 89% sensitivity, 88% specificity, and 88% balanced accuracy, comparable to and/or exceeding the performance of the LLNA vs human HPPT data in every measure.

55. As previously noted, due to the imbalanced nature of the reference data, the measures of specificity are more uncertain than the measures of sensitivity.

2.1.8. Proficiency chemicals

56. The 2o3 DA relies on a simple, rule-based data interpretation procedure and requires no expert judgment. Proficiency chemicals for the individual information sources (KE1-3) are defined in the respective guidelines (2, 3, 4). Proficiency for the individual information sources demonstrates proficiency for the DA.

2.1.9. Reporting of the DA

57. The reporting of the DA application should follow the template described in OECD GD 255 (8), and should include at a minimum the following elements:

- Test chemical identification (*e.g.* chemical name, structural formula, composition, isomers, impurities including their quantities as available, CAS number, batch and lot number, and other relevant identifiers)
- Individual test reports performed per corresponding guideline (OECD TG 442C, 442D, 442E). Note that the chemical identity for each test report should match that above.
- Application of the individual prediction models adapted to be used within the 2o3 DA to determine borderline outcomes, as described in **Annex 1**
- Outcome of the DA application (hazard identification, *i.e.* skin sensitiser or not skin sensitiser or inconclusive result)
- Any deviation from or adaptation of the 2o3 DA

- Conclusion

2.2. References

1. OECD (2021). Series on Testing and Assessment No. 336: Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation. Organisation for Economic Cooperation and Development, Paris. Available at: [https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm].
2. OECD (2020). OECD Guideline for the Testing of Chemicals No. 442C: *In chemico* Skin Sensitisation: Assays addressing the Adverse Outcome Pathway key even on covalent binding to proteins).*In chemico*. Paris, France: Organisation for Economic Cooperation and Development. Available at: (oecd-ilibrary.org).
3. OECD (2018). OECD Key Event based test Guideline 442D: *In vitro* Skin Sensitisation Assays Addressing AOP Key Event on Keratinocyte Activation. Organisation for Economic Cooperation and Development, Paris. Available at: (oecd-ilibrary.org).
4. OECD (2018). OECD Key event based test Guideline 442E: *In vitro* Skin Sensitisation Assays Addressing the Key Event on Activation of Dendritic Cells on the Adverse Outcome Pathway for Skin Sensitisation. Organisation for Economic Cooperation and Development, Paris. Available at: (oecd-ilibrary.org).
5. OECD (2016). Series on Testing & Assessment No. 256: Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation, Annex 1 and Annex 2.. Organisation for Economic Cooperation and Development, Paris. Available at: [https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm].
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8. OECD (2016). Series on Testing & Assessment No. 255: Guidance Document On The Reporting Of Defined Approaches To Be Used Within Integrated Approaches To Testing And Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm].

Part II. –SECTION 3 - Defined Approaches for Skin Sensitisation Potency Categorisation

58. Part II of the Guideline includes Defined Approaches that allow the allocation of skin sensitizers into UN GHS sub-category 1A, strong sensitizers, or sub-category 1B for other (moderate to weak) skin sensitizers, following the Globally Harmonised System for Classification and Labeling (GHS). These DAs may also be used for hazard identification, *i.e.* to distinguish between sensitisers (UN GHS Category 1) and non-sensitisers (no classification; NC). Currently the ITSv1 DA and ITSv2 DA are included in this section of the Guideline. Additional detailed information can be found in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

3.1. "Integrated Testing Strategy (ITS)" Defined Approach

3.1.1. Summary

59. This defined approach was constructed as an Integrated Testing Strategy (ITS) for prediction of the skin sensitisation hazard potential and potency sub-categorisation according to the UN GHS (sub-categories 1A and 1B) of a chemicals.

60. The ITS DA uses test methods that address key events (KEs) 1 and 3 in the Adverse Outcome Pathway (AOP) and includes an *in silico* prediction of skin sensitisation. Protein binding (KE1) is quantitatively evaluated using the Direct Peptide Reactivity Assay (DPRA; OECD TG 442C) (2). Dendritic cell activation (KE3) is quantitatively evaluated using the human cell line activation test (h-CLAT; OECD TG 442E) (3). The *in silico* prediction of skin sensitisation is provided by either Derek Nexus (ITSv1) or OECD QSAR Toolbox (ITSv2).

61. The ITSv1 DA was evaluated for hazard identification with 167 chemicals and for UN GHS sub-categorisation with 155 chemicals based on LLNA reference data curated as agreed upon by the EG DASS, and achieved accuracies equivalent to the LLNA (see **Tables 3.2-3.3**). The performance of the ITSv1 DA was compared to 64 chemicals with human reference data curated as agreed upon by the EG DASS (see **Tables 3.4-3.5**), and exceeded the accuracy of the LLNA in predicting the same human data for both hazard and potency categorisation.

62. The ITSv2 DA was evaluated for hazard identification for 167 chemicals and for UN GHS sub-categorisation for 153 chemicals based on LLNA reference data curated as agreed upon by the EG DASS, and achieved accuracies equivalent to the LLNA (see **Tables 3.6-3.7**). The performance of the ITSv2 DA was compared to 64 chemicals with human reference data curated as agreed upon by the EG DASS (see **Tables 3.8-3.9**), and exceeded the accuracy of the LLNA in predicting the same human data for both hazard and potency categorisation.

3.1.2. Data interpretation procedure

63. The ITS DIP uses scores assigned to the quantitative results from the h-CLAT (3) and the DPRA (1), and from either Derek Nexus v6.1.0 (2020, Lhasa Limited, https://www.lhasalimited.org/products/derek-nexus.htm) or OECD QSAR TB v4.5 (https://www.oecd.org/chemicalsafety/oecd-qsar-toolbox.htm) to discriminate chemicals

into UN GHS category 1A (strong sensitiser); category 1B (other sensitiser), or Not Classified (non-sensitiser) (**Table 3.1**).

64. The DIP was amended from the original published version of the ITS (4) to change the cut-off for 1A sensitisers from a score of 7 to a score of 6 to optimize the ability of the DA to detect strong sensitisers and to extend the applicability of the ITS to chemicals for which *in silico* predictions cannot be generated. The DIP was also altered from the published version in that it was originally applied to ECETOC categories[2], and is here applied to the UN GHS subcategories.

65. The quantitative results of h-CLAT and DPRA are converted into a score from 0 to 3, as shown in **Table 3.1**. For h-CLAT, the minimum induction threshold (MIT) is converted to a score from 0 to 3 based on the cutoffs of 10 and 150 μg/ml. For DPRA, the mean percent depletion for the cysteine and lysine peptides is converted to a score from 0 to 3, based on the threshold values associated with reactivity classes described in OECD TG 442C (2). In cases where co-elution occurs only with the lysine peptide, the depletion for only cysteine peptides is converted to a score from 0 to 3. For the *in silico* prediction (Derek or OECD QSAR TB), a positive outcome is assigned a score of 1; a negative outcome is assigned a score of 0 (further details on the respective protocols are available in **Annex 2**). When these scores have been assessed, a total battery score ranging from 0 to 7, calculated by summing the individual scores, is used to predict the sensitising potential (hazard identification; UN GHS Cat. 1 vs. UN GHS NC) and potency (UN GHS Cat. 1A, Cat. 1B and NC). The positive criteria for identifying skin sensitisers (UN GHS Cat. 1) are set as a total battery score of 2 or greater. Based on the updated DIP, a total battery score is assigned into three ranks: score of 6-7 is defined as a strong (UN GHS Cat. 1A) sensitiser; score of 2-5 as moderate/weak (UN GHS Cat. 1B) sensitiser; score of 1 or 0, as not classified (*i.e.* a non-sensitiser).

[2] ECETOC Technical Report 087 (2003), Contact Sensitisation: Classification According to Potency. Available at: [https://www.ecetoc.org/publication/tr-087-contact-sensitisation-classification-according-to-potency/]

Table 3.1. Schematic of the ITS defined approach. The DA is a simple score-based system depending on assays from OECD TG 442E and 442C, and an *in silico* structure-based prediction, as shown.

Score	h-CLAT MIT µg/mL	DPRA mean Cysteine and Lysine% depletion	DPRA Cysteine % depletion*	*In silico* (ITSv1: DEREK; ITSv2: OECD TB)
3	≤10	≥42.47	≥98.24	
2	>10, ≤150	≥22.62, <42.47	≥23.09, <98.24	
1	>150, ≤5000	≥6.38, <22.62	≥13.89, <23.09	Positive
0	not calculated	<6.38	<13.89	Negative
	Potency	Total Battery Score		
	UN GHS 1A	6-7		
	UN GHS 1B	2-5		
	Not classified	0-1		

Source: Adapted from Takenouchi (5)

Note: UN GHS 1A correspond to strong sensitisers and UN GHS 1B correspond to other (moderate to weak) sensitisers. Not classified are considered non-sensitisers. *Cysteine-only depletion thresholds are used in the case of co-elution with the lysine peptide.

3.1.3. Description and limitations of the individual information sources

66. The individual *in chemico* and *in vitro* information sources are existing KE-based OECD test guidelines (OECD TG 442C, 442E) (2, 3), and the protocols are detailed therein.

67. The following assays from those TGs have been characterised and included in the ITS DA:

- Human cell-line activation test (h-CLAT; OECD TG 442E; KE3) (3): Activation of antigen presenting cells is characterised by the up-regulation of CD86 and/or CD54. The h-CLAT is considered to be positive if CD86 induction exceeds 1.5-fold and/or CD54 exceeds 2-fold at viabilities > 50% when compared to the vehicle control. From the experimental concentration-response curves, the median concentration(s) inducing 1.5- and/or 2-fold induction of CD86 and/or CD54 are calculated and the lowest of the two values is defined as the minimal induction threshold, MIT:

  $$MIT = \min(EC150\ CD86, EC200\ CD54)$$

  Test chemicals are assigned potency scores based on the MIT thresholds shown in **Table 3.1**.

- Direct Peptide Reactivity Assay (DPRA; OECD TG 442C; KE1) (2): Skin sensitisers are generally electrophilic and react with the nucleophilic moieties of proteins. The DPRA measures depletion of two peptides containing either cysteine or lysine residues due to covalent binding. A test chemical that induces mean peptide depletion of cysteine- and lysine-containing peptide above 6.38% (or in the case of co-elution, cysteine-only depletion above 13.89%) is considered to be positive. In case borderline results are obtained for peptide depletion, additional testing should be conducted, as specified in OECD TG 442C. Test chemicals are assigned potency scores based on the mean peptide depletion thresholds shown in **Table 3.1**.

68. The limitations of the individual *in chemico* and *in vitro* test methods are described in the respective test guidelines and in the respective test guidelines (TG 442C, Appendix 1; TG 442E, Annex 1) (2, 3).

69. The *in silico* information source predictions for ITSv1 are derived from Derek, an expert, knowledge-based software tool comprising alerts on several toxicity endpoints, including skin sensitisation. Derek (Derek Nexus v.6.1.0, 2020, Lhasa Limited) fires alerts based on structural features *i.e.* whether a hapten has potential for electrophilic binding to skin proteins either directly or following metabolism/auto-oxidation. To each alert, a likelihood level is associated. Chemicals firing an alert with a likelihood of certain, probable, plausible, or equivocal are considered to be positive. Chemicals with a negative prediction of 'non-sensitiser with no misclassified or unclassified features' are considered to be negative (https://www.lhasalimited.org/products/skin-sensitisation-assessment-using-derek-nexus.htm#Negative%20Predictions). The approach for characterising the *in silico* applicability domain used in the ITSv1 and the protocol for generating Derek predictions are provided in **Annex 2** of this guideline.

70. The *in silico* information source predictions for ITSv2 are derived from the OECD QSAR TB automated workflow providing skin sensitiser hazard predictions (OECD QSAR TB v4.5). The target compound is profiled for protein binding alerts; auto-oxidation products and skin metabolites are generated and then profiled for protein binding alerts. In case a protein binding alert is identified in the parent or in its (a)biotic metabolites, the same alert is used to identify analogues with experimental skin sensitisation data. If no protein binding alert is identified, then structural profilers are used to identify analogue chemicals and the data gap is filled using read across or directly via profiler outcomes in case no suitable analogues are automatically identified. The approach for characterising the *in silico* applicability domain used in the ITSv2 and the protocol for generating OECD QSAR TB predictions are provided in **Annex 2** of this guideline.

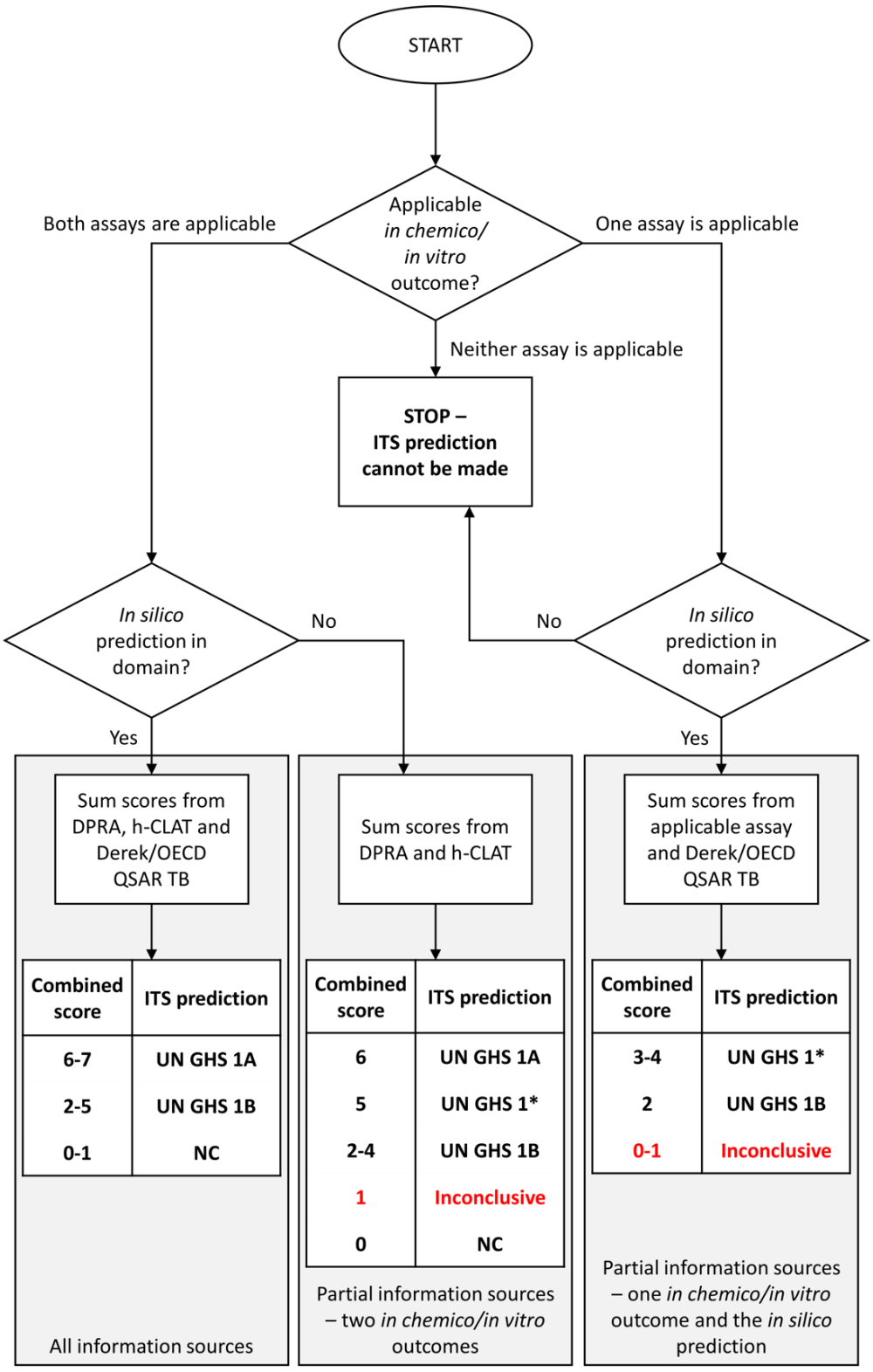
3.1.4. Confidence in the ITS DA predictions

71. The level of confidence of the ITS DA prediction is assigned based on the total DA score and applicability domain of the individual information sources, as shown via the flow chart in **Figure 3.1**. The first decision on whether all information elements can be used is dictated by the limitations of the *in chemico* and *in vitro* methods as found in TG 442C Appendix 1 and TG 442E Annex 1 (3) (*e.g.* for substances that do not provide conclusive results in the individual methods due to limited solubility or negative h-CLAT results for chemicals with Log P > 3.5 which are currently considered unreliable), and by the applicability domain of the *in silico* prediction (**Annex 2**). Partial information sources (*i.e.* two *in chemico/in vitro* outcomes only, or one *in chemico/in vitro* outcome and an *in silico*

prediction) may be used to obtain a DA prediction as shown via the flow chart in **Figure 3.1**.

72. DA predictions with high confidence for hazard identification and potency are considered conclusive. DA predictions with low confidence are considered inconclusive for hazard identification and/or potency. These 'inconclusive' predictions may nevertheless be considered in a weight-of-evidence approach and/or within the context of an IATA together with other information sources. Details including applicability domain and confidence considerations are provided in **Annex 2**.

Figure 3.1. Decision tree for assigning confidence to the ITS DA predictions



***Conclusive for hazard, inconclusive for potency**

3.1.5. Predictive capacity of the ITSv1 DA vs the LLNA

73. The predictive capacity of ITSv1 using Derek is reported based on data from the LLNA (see **Tables 3.2-3.3**), curated as agreed upon by the EG DASS (see **Section 1.1** and **Annex 3** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation)* (1). The workflow shown in **Figure 3.1** was applied to assign confidence to the ITSv1 DA predictions. The designation of conclusive/inconclusive for the ITSv1 DA predictions is further detailed in **Annex 2**. Performance statistics are reported for conclusive predictions as compared to LLNA reference data, and inconclusive results are indicated. DA predictions for specific chemicals and further details are available in **Section 5** and **Annex 2** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

Table3.2. Hazard identification performance of the ITSv1 DA in comparison to LLNA reference data

	LLNA	
ITSv1 DA	Non	Sens
Non	21	11
Sens	9	118
Inconclusive	3	6

DA Performance vs. LLNA Data (N=159)	**ITSv1**
Accuracy (%)	87%
Sensitivity (%)	92%
Specificity (%)	70%
Balanced Accuracy (%)	81%

Note: Accuracy is the correct classification rate, sensitivity is the true positive rate, specificity is the true negative rate, and balanced accuracy is the average of sensitivity and specificity with respect to LLNA data. Statistics reflect high confidence predictions only; inconclusive predictions are shown in grey. Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

74. Due to the imbalanced nature of the reference data, the measure of specificity (based on 30 LLNA negative chemicals) is more uncertain than the measure of sensitivity (based on 129 LLNA positive chemicals).

Table 3.3. Potency categorisation performance of the ITSv1 DA in comparison to LLNA reference data, based on the UN GHS 1A/1B sub-categorisation

	LLNA		
ITSv1 DA	NC	1B	1A
NC	21	11	0
1B	9	55	10
1A	0	12	28
Inconclusive	3	7	0

71% correct classification overall

ITSv1 vs. LLNA reference data: Statistics based on the UN GHS 1A/1B sub-categorisation

Performance (N=146)	NC (N=30)	1B (N=78)	1A (N=38)
Correct classification (%)	70%	71%	74%
Underpredicted (%)	NA	14% (NC)	0% (NC); 26% (1B)
Overpredicted (%)	30% (1B); 0% (1A)	15% (1A)	NA

Note: Statistics reflect high confidence predictions only; inconclusive predictions are shown in grey. For more details on within-class performance (sensitivity, specificity, and balanced accuracy), please see **Section 5** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

75. The designation of high/low confidence for the ITSv1 DA predictions is applied as described above in **Figure 3.1** and further detailed in **Annex 2**.

3.1.6. Predictive capacity of the ITSv2 DA vs the LLNA

76. The predictive capacity of ITSv2 using OECD QSAR TB is reported based on data from the LLNA (see **Tables 3.4-3.5**), curated as agreed upon by the EG DASS (see **Section 2.1** and **Annex 3** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation)* (1). The workflow shown in **Figure 3.1** was applied to assign confidence to the ITSv2 DA predictions. The designation of high/low confidence for the ITSv2 DA predictions is further detailed in **Annex 2**. Performance statistics are reported for high confidence predictions as compared to LLNA reference data, and inconclusive results are indicated. DA predictions for specific chemicals and further details are available in **Section 5** and **Annex 2** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1). **Table 3.4**. Hazard identification performance of the ITSv2 DA in comparison to LLNA reference data.

Table 3.4. Hazard identification performance of the ITSv2 DA in comparison to LLNA reference data.

	LLNA	
ITSv2 DA	Non	Sens
Non	20	9
Sens	10	117
Inconclusive	3	9

DA Performance vs. LLNA Data (N=156)	ITSv2
Accuracy (%)	88%
Sensitivity (%)	93%
Specificity (%)	67%
Balanced Accuracy (%)	80%

Note: Accuracy is the correct classification rate, sensitivity is the true positive rate, specificity is the true negative rate, and balanced accuracy is the average of sensitivity and specificity with respect to LLNA data. Statistics reflect conclusive predictions only; inconclusive predictions are shown in grey. Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

77. Due to the imbalanced nature of the reference data, the measure of specificity (based on 30 LLNA negative chemicals) is more uncertain than the measure of sensitivity (based on 126 LLNA positive chemicals).

Table 3.5. Potency categorisation performance of the ITSv2 DA in comparison to LLNA reference data, based on the UN GHS 1A/1B sub-categorisation

	LLNA		
ITSv2 DA	NC	1B	1A
NC	20	9	0
1B	10	54	10
1A	0	12	26
Inconclusive	3	10	2

71% correct classification overall

ITSv2 vs. LLNA reference data: Statistics based on the UN GHS 1A/1B sub-categorisation

Performance (N=141)	**NC (N=30)**	**1B (N=75)**	**1A (N=36)**
Correct classification (%)	67%	72%	72%
Underpredicted (%)	NA	12% (NC)	0% (NC); 28% (1B)
Overpredicted (%)	33% (1B); 0% (1A)	16% (1A)	NA

Note: Statistics reflect conclusive predictions only; inconclusive predictions are shown in grey. For more details on within-class performance (sensitivity, specificity, and balanced accuracy), please see **Section 5** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

78. The designation of conclusive/inconclusive for the ITSv2 DA predictions is applied as described above in **Figure 3.1** and further detailed in **Annex 2**.

3.1.7. Predictive capacity of the ITSv1 DA vs Human Data

79. The predictive capacity of ITSv1 using Derek is reported based on data from the Human Predictive Patch Test (see **Tables 3.6-3.7**), curated as agreed upon by the EG DASS. The designation of high/low confidence for the ITSv1 DA predictions is further detailed in **Annex 2**. Performance statistics are reported for high confidence predictions as compared to human reference data, and inconclusive results are indicated. DA predictions for specific chemicals and further details are available in **Section 5** and **Annex 2** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

Table 3.6 Hazard identification performance of the ITSv1 DA in comparison to Human reference data

	Human	
ITSv1 DA	Non	Sens
Non	4	4
Sens	5	51
Inconclusive	2	0

DA Performance vs. Human Data (N=64)	**ITSv1**
Accuracy (%)	86%
Sensitivity (%)	93%
Specificity (%)	44%
Balanced Accuracy (%)	69%

Note: Accuracy is the correct classification rate, sensitivity is the true positive rate, specificity is the true negative rate, and balanced accuracy is the average of sensitivity and specificity with respect to Human HPPT-based data. Statistics reflect conclusive predictions only; inconclusive predictions are shown in grey. Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

80. Due to the imbalanced nature of the reference data, the measure of specificity (based on 9 Human negative chemicals) is more uncertain than the measure of sensitivity (based on 55 Human positive chemicals).

Table 3.7 Potency categorisation performance of the ITSv1 DA in comparison to Human reference data, based on the UN GHS 1A/1B sub-categorisation

	Human		
ITSv1 DA	NC	1B	1A
NC	4	4	0
1B	5	24	7
1A	0	3	13
Inconclusive	2	0	1

68% correct classification overall

ITSv1 vs. Human reference data: Statistics based on the UN GHS 1A/1B sub-categorisation

Performance (N=60)	**NC (N=9)**	**1B (N=31)**	**1A (N=20)**
Correct classification (%)	44%	77%	65%
Underpredicted (%)	NA	13% (NC)	0% (NC); 35% (1B)
Overpredicted (%)	56% (1B); 0% (1A)	10% (1A)	NA

Note: Statistics reflect conclusive predictions only; inconclusive predictions are shown in grey. For more details on within-class performance (sensitivity, specificity, and balanced accuracy), please see **Section 5** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

81. The designation of conclusive/inconclusive for the ITSv1 DA predictions is applied as described above in **Figure 3.1** and further detailed in **Annex 2**.

82. Due to the imbalanced nature of the reference data and the small numbers of chemicals, the measures of accuracy are more uncertain for smaller classes, *e.g.* for NC chemicals.

3.1.8. Predictive capacity of the ITSv2 DA vs Human Data

83. The predictive capacity of ITSv2 using OECD QSAR Toolbox is reported based on data from the Human Predictive Patch Test (see **Tables 3.8-3.9**), curated as agreed upon by the EG DASS. The designation of high/low confidence for the ITSv2 DA predictions is further detailed in **Annex 2**. Performance statistics are reported for conclusive predictions as compared to human reference data, and inconclusive results are indicated. DA predictions for specific chemicals and further details are available in **Section 5** and **Annex 2** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

Table 3.8 Hazard identification performance of the ITSv2 DA in comparison to Human reference data

	Human	
ITSv2 DA	Non	Sens
Non	4	3
Sens	5	50
Inconclusive	2	2

DA Performance vs. Human Data (N=62)	**ITSv2**
Accuracy (%)	87%
Sensitivity (%)	94%
Specificity (%)	44%
Balanced Accuracy (%)	69%

Note: Accuracy is the correct classification rate, sensitivity is the true positive rate, specificity is the true negative rate, and balanced accuracy is the average of sensitivity and specificity with respect to Human HPPT-based data. Statistics reflect conclusive predictions only; inconclusive predictions are shown in grey. Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation*(1).

84. Due to the imbalanced nature of the reference data, the measure of specificity (based on 9 Human negative chemicals) is more uncertain than the measure of sensitivity (based on 53 Human positive chemicals).

Table 3.9. Potency categorisation performance of the ITSv2 DA in comparison to Human reference data, based on the UN GHS 1A/1B sub-categorisation

	Human		
ITSv2 DA	NC	1B	1A
NC	4	3	0
1B	5	24	6
1A	0	3	12
Inconclusive	2	1	3

70% correct classification overall

ITSv2 vs. Human reference data: Statistics based on the UN GHS 1A/1B sub-categorisation

Performance (N=57)	NC (N=9)	1B (N=30)	1A (N=18)
Correct classification (%)	44%	80%	67%
Underpredicted (%)	NA	10% (NC)	0% (NC); 33% (1B)
Overpredicted (%)	56% (1B); 0% (1A)	10% (1A)	NA

Note: Statistics reflect conclusive predictions only; inconclusive predictions are shown in grey. For more details on within-class performance (sensitivity, specificity, and balanced accuracy), please see **Section 5** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

85. The designation of conclusive/inconclusive for the ITSv2 DA predictions is applied as described above in **Figure 3.1** and further detailed in **Annex 2**.

86. Due to the imbalanced nature of the reference data and the small numbers of chemicals, the measures of accuracy are more uncertain for smaller classes, *e.g.* for NC chemicals.

3.1.9. Predictive capacity of the LLNA vs. Human Data

87. To provide a basis for comparison for the DA performance, the predictive capacity of the LLNA is reported based on data from the Human Predictive Patch Test (see **Tables 3.10-3.11**) curated as agreed upon by the EG DASS. Data for specific chemicals and further details are available in **Section 5** and **Annex 2** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

Table 3.10 Hazard identification performance of the LLNA in comparison to Human reference data

	Human	
LLNA	Non	Sens
Non	2	3
Sens	7	44

LLNA Performance vs. Human Data (N=56)	LLNA
Accuracy (%)	82%
Sensitivity (%)	94%
Specificity (%)	22%
Balanced Accuracy (%)	58%

Note: Accuracy is the correct classification rate, sensitivity is the true positive rate, specificity is the true negative rate, and balanced accuracy is the average of sensitivity and specificity with respect to Human HPPT-based data. Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

88. The hazard identification performance of the conclusive ITSv1 DA predictions vs. human data was 86% accuracy, 93% sensitivity, 44% specificity, and 69% balanced accuracy, comparable to and/or exceeding the performance of the LLNA in every measure.

89. The hazard identification performance of the conclusive ITSv2 DA predictions vs. human data was 87% accuracy, 94% sensitivity, 44% specificity, and 69% balanced accuracy, comparable to and/or exceeding the performance of the LLNA in every measure.

90. As previously noted, due to the imbalanced nature of the reference data, the measures of specificity are more uncertain than the measures of sensitivity.

Table 3.11 Potency categorisation performance of the LLNA in comparison to Human reference data, based on the UN GHS 1A/1B sub-categorisation

Additional performance characterisation is available in the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

	Human		
LLNA	NC	1B	1A
NC	2	3	0
1B	6	17	7
1A	0	3	9

60% correct classification overall

LLNA vs. Human reference data: Statistics based on the UN GHS 1A/1B sub-categorisation

Performance (N=47)	**NC (N=8)**	**1B (N=23)**	**1A (N=16)**
Correct classification (%)	25%	74%	56%
Underpredicted (%)	NA	13% (NC)	0% (NC); 44% (1B)
Overpredicted (%)	75% (1B); 0% (1A)	13% (1A)	NA

Note: For more details on within-class performance (sensitivity, specificity, and balanced accuracy), please see **Section 5** of the *Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation* (1).

91. The performance of the conclusive ITSv1 DA predictions vs. human data for potency sub-categorisation showed 68% correct classification overall, with accuracies of 44% for NC, 77% for 1B, and 65% for 1A, comparable to and/or exceeding the performance of the LLNA in every measure.

92. The performance of the conclusive ITSv2 DA predictions vs. human data for potency sub-categorisation showed 70% correct classification overall, with accuracies of 44% for NC, 80% for 1B, and 67% for 1A, comparable to and/or exceeding the performance of the LLNA in every measure.

93. As previously noted, due to the imbalanced nature of the reference data and the small numbers of chemicals, the measures of accuracy are more uncertain for smaller classes, *e.g.* for NC chemicals.

3.1.10. Proficiency chemicals

94. The ITS DA relies on a simple, rule-based data interpretation procedure and no expert judgment is required. Proficiency chemicals for the individual *in chemico* and *in vitro* information sources (KE1 and KE3) are defined in the respective guidelines (OECD TG 442C, 442E) (2, 3). The protocol details for the *in silico* information source options, Derek and OECD QSAR Toolbox, are included in **Annex 2** of this guideline. Proficiency has been demonstrated for Derek Nexus v6.1.0 and OECD QSAR Toolbox v4.5, and these

are the software versions that are intended for use in the ITSv1 and ITSv2 DAs, respectively. Proficiency for the individual information sources demonstrates proficiency for the DA.

3.1.11. Reporting of the DA

95. The reporting of the ITS DA should follow the template described in OECD GD 255 (6), and should include at a minimum the following elements:

- Test chemical identification (*e.g.* chemical name, structural formula, composition, isomers, impurities including their quantities as available, CAS number, batch and lot number, and other relevant identifiers)
- Individual test reports for the individual tests performed per corresponding guideline (OECD TG 442C, 442E). Note that the chemical identity for each test report should match that above.
- Description of protocol used for *in silico* prediction **(Annex 2)** and outcome, *e.g.* reported via a QPRF (7).
- Outcome of the DA application (hazard identification and potency categorisation according to UN GHS categories, or inconclusive result)
- Any deviation from the ITS DA
- Conclusion

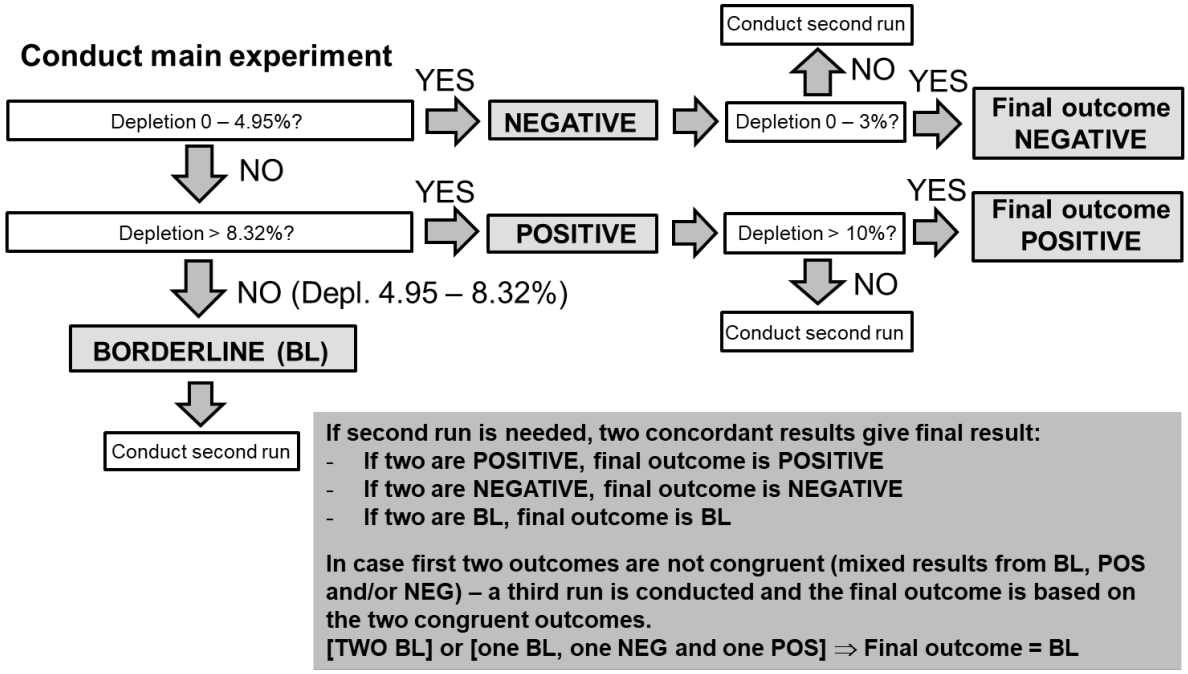
3.2. References

1. OECD (2021). Series on Testing and Assessment No. 336: Supporting document to the Guideline (GL) on Defined Approaches (DAs) for Skin Sensitisation. Organisation for Economic Cooperation and Development, Paris. Available at: [https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm].
2. OECD (2020). OECD Guideline for the Testing of Chemicals No. 442C: *In chemico* Skin Sensitisation: Assays addressing the Adverse Outcome Pathway key even on covalent binding to proteins). *In chemico*. Paris, France: Organisation for Economic Cooperation and Development. Available at:(oecd-ilibrary.org).
3. OECD (2018). OECD Key event based test Guideline 442E: *In vitro* Skin Sensitisation Assays Addressing the Key Event on Activation of Dendritic Cells on the Adverse Outcome Pathway for Skin Sensitisation. Organisation for Economic Cooperation and Development, Paris. Available at: (oecd-ilibrary.org).
4. OECD (2016). Series on Testing & Assessment No. 256: Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation, Annex 1 and Annex 2. Organisation for Economic Cooperation and Development, Paris. Available at: [https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm].
5. Takenouchi O, Fukui S, Okamoto K, Kurotani S, Imai N, Fujishiro M, Kyotani D, Kato Y, Kasahara T, Fujita M, Toyoda A, Sekiya D, Watanabe S, Seto H, Hirota M, Ashikaga T, Miyazawa M. (2015). Test battery with the human cell line activation test, direct peptide reactivity assay and DEREK based on a 139 chemical data set for predicting skin sensitizing potential and potency of chemicals. J Appl Toxicol, 35:1318-32.
6. OECD (2016). Series on Testing & Assessment No. 255: Guidance Document On The Reporting Of Defined Approaches To Be Used Within Integrated Approaches To Testing And Assessment. ENV/JM/HA(2016)28. Organisation for Economic Cooperation and Development, Paris. Available at: [https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm].
7. ECHA (2008). see "CHAPTER R.6 – QSARS AND GROUPING OF CHEMICALS" *in* Guidance on Information Requirements and Chemical Safety Assessment. European Chemicals Agency [Guidance on Information Requirements and Chemical Safety Assessment - ECHA (europa.eu)]

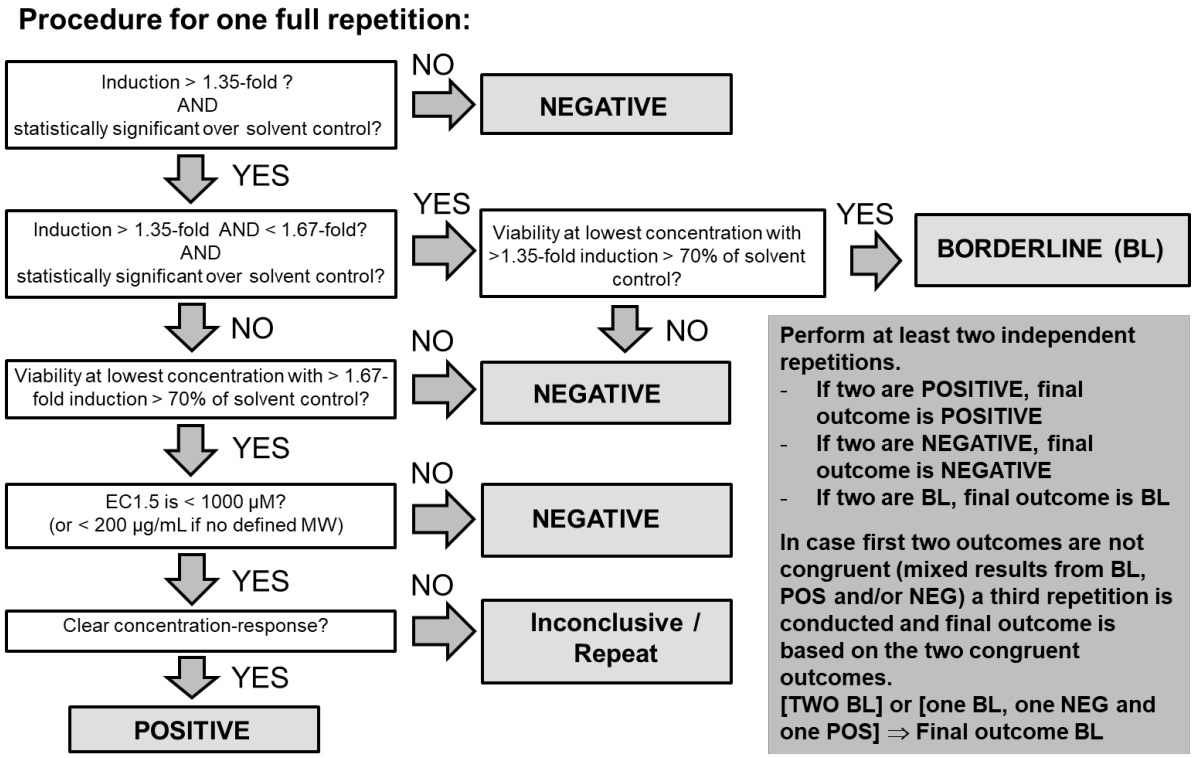
Annex 1: Prediction model for the individual *in chemico*/*in vitro* tests with multiple runs for use in 2o3 DA

96. The individual prediction models of h-CLAT and KeratinoSens™ require multiple runs (independent repetitions). An adaptation of the prediction model was used to determine borderline cases in the individual runs for the purpose of making predictions within the 2o3 DA. These adaptations (Figures 1.2. and 1.3) below should be used in these methods to come to the final conclusion of the individual tests.

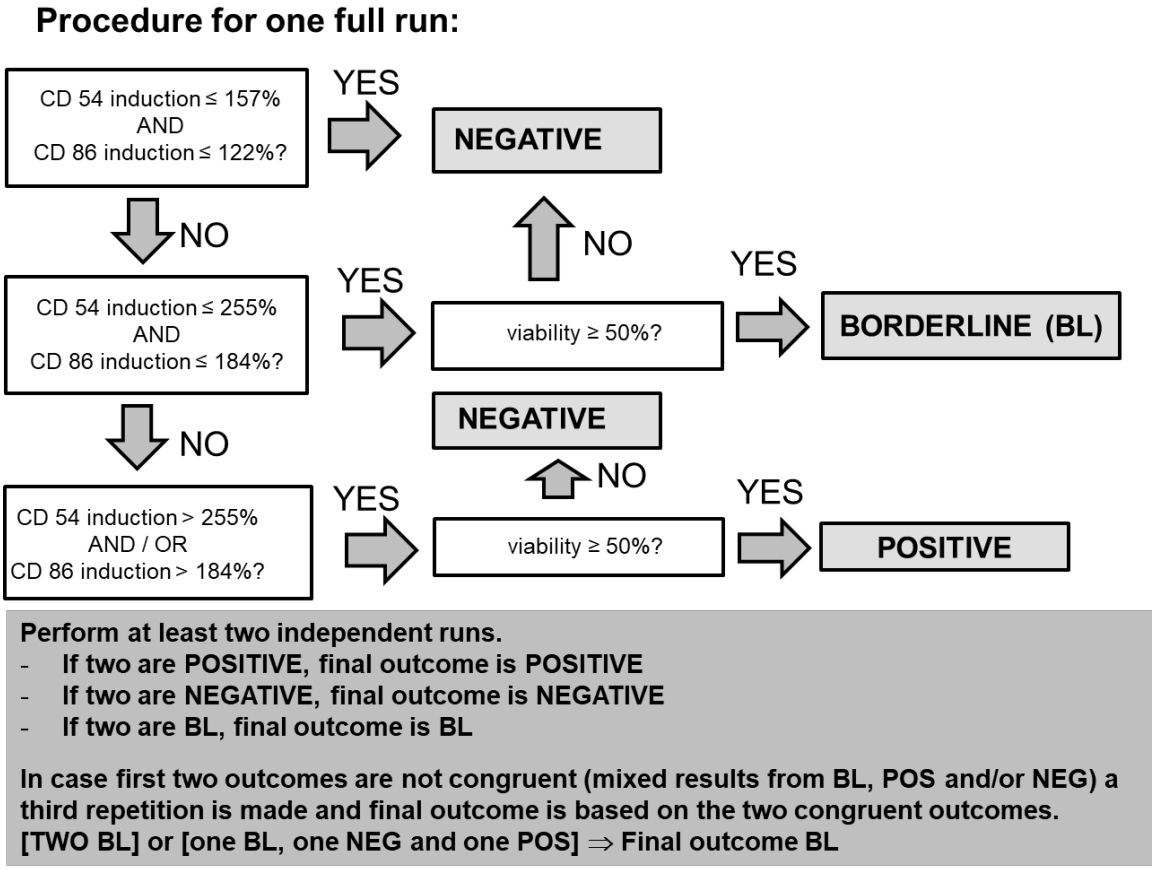
97. For the DPRA, repeated runs are required to be conducted if average depletion is within the range 3 - 10% (9 – 17% in case of Cysteine only depletion model is used). For this adaptation, the flowchart in Figure 1.1 is used to decide on run repetition and borderline assessment within the 2o3 DA.



Annex 1, Figure 1.1. Flow-chart of the DPRA prediction model (mean depletion) taking into borderline ranges and multiple runs conclude on borderline results within the 2o3 DA. The original threshold for a positive classification is 6.38%, and the statistically derived borderline range around this threshold is 4.95% - 8.32%. The same flowchart applies to the cysteine-only prediction model, whereby the following thresholds apply: 9% instead of 3%, >17 % instead of >10%, 10.56 % instead of 4.95% and > 18.47 % instead of >8.32%.



Annex 1, Figure 1.2. Flow-chart of the KeratinoSens™ prediction model taking into account borderline ranges and multiple runs to conclude on borderline results within the 2o3 DA. The original threshold for a positive classification is 1.5-fold induction, and the statistically derived borderline range around this threshold is 1.35 – 1.67-fold. Note: An independent run is referred to as 'repetition' in 442D, while it is called a 'run' in 442C and 442E; these nomenclatures do mean the same thing.



Annex 1, Figure 1.3. Flow-chart of the h-CLAT prediction model taking into account borderline ranges and multiple runs to conclude on borderline results within the 2o3 DA. The original threshold for a positive classification is 150% induction of CD86 with a statistically derived borderline range around this threshold of 122 – 184% and 200% induction of CD54 with a statistically derived borderline range around this threshold of 157 – 255%.

Annex 2: Defining the applicability domain and assessing confidence in DASS ITS predictions and protocols for generating *in silico* predictions

Introduction

98. As described in **Section 3.1** of the ***Guideline for Defined Approaches for Skin Sensitisation*** the ITS defined approaches (DAs) are based on three information sources: two *in chemico/in vitro* assays (DPRA; OECD TG 442C (OECD, 2015) and h-CLAT; OECD TG 442E (OECD, 2018)) and one *in silico* tool (prediction from either Derek Nexus (ITSv1) or OECD QSAR Toolbox (ITSv2) (referred to hereafter as *in silico*)). For each information source a score is given depending on the outcome of the individual assay and/or prediction, that is then summed to obtain the DA prediction.

Applicability domain of the individual information sources

In chemico/in vitro *information source (DPRA and h-CLAT)*

99. A test chemical is considered to be within the *in chemico*/*in vitro* domain (i.e. applicable) of DPRA and/or h-CLAT if it can be tested according to the individual protocols, taking into account the technical and chemical type limitations of each assay (as defined in the respective test guidelines OECD TG 442C and OECD TG 442E (OECD, 2015, 2018)). The *in chemico*/*in vitro* results are considered applicable, in case there are no technical or chemical space specific limitations and no reason why the results obtained from the assay cannot be considered.

In silico *information source*

100. The ITS DAs use *in silico* information sources that are based on chemical structures. These *in silico* sources rely on molecular representation of the chemicals: input usually by drawing the chemical structure, or by entering the Simplified Molecular-Input Line-Entry System (SMILES) or the IUPAC International Chemical Identifier (InChi). As a single chemical can be represented by several CAS or EC numbers (due to differences in composition e.g. stereochemical differences, present as varied salt forms, present as the main component in a mixture), it is important to specify the exact structure if possible. Resources such as the US EPA CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard) or NIH PubChem (https://pubchem.ncbi.nlm.nih.gov/) may be useful in mapping chemical names or structures to SMILES or InChi format. Available guidance can be consulted regarding minimum purity level of substances used in *in silico* predictions based on molecular structure.[34]

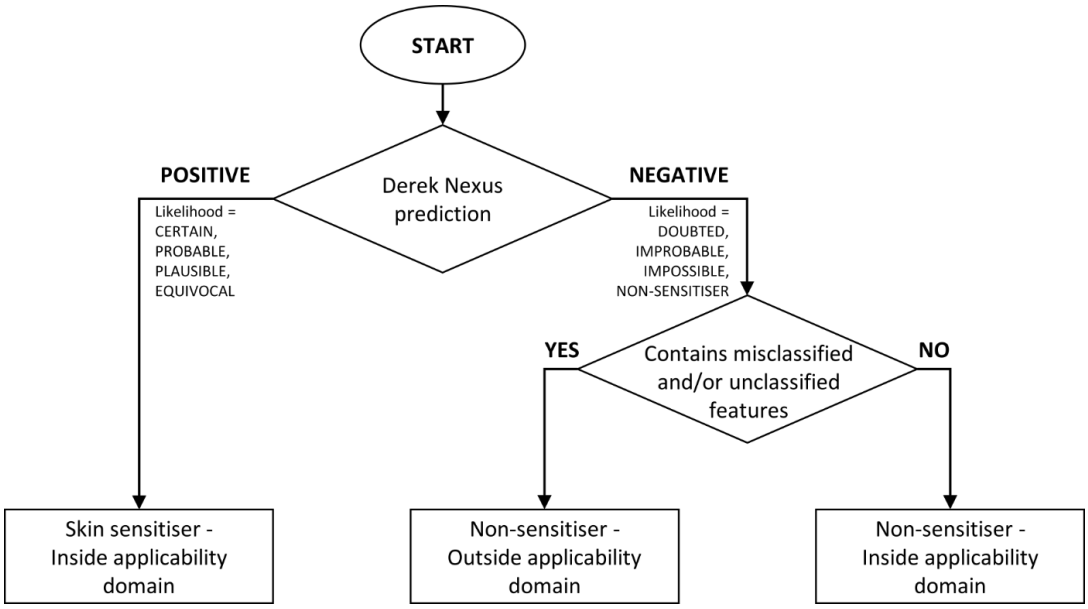
[3] OECD (2017), *Guidance on Grouping of Chemicals, Second Edition*, OECD Series on Testing and Assessment, No. 194, OECD Publishing, Paris, https://doi.org/10.1787/9789264274679-en.

[4] ECHA (2008) CHAPTER R.6 – QSARS AND GROUPING OF CHEMICALS *in* Guidance on Information Requirements and Chemical Safety Assessment. European Chemicals Agency [Guidance on Information Requirements and Chemical Safety Assessment - ECHA (europa.eu)

Derek Nexus (ITSv1)

101. Skin sensitisation predictions from Derek Nexus v6.1.0 are used in ITSv1. The protocol for running Derek Nexus (Derek) predictions is defined in **Appendix 1** of this document. All positive predictions (likelihood = certain, probable, plausible or equivocal) are considered to be inside the applicability domain. Negative predictions (likelihood = doubted, improbable, impossible or non-sensitiser) are also considered to be in the applicability domain unless they contain misclassified and/or unclassified features. A prediction of non-sensitiser with misclassified features indicates the presence of a fragment that has been observed exclusively in known sensitisers which Derek fails to alert for. A prediction of non-sensitiser with unclassified features indicates the presence of a fragment that has not been observed in publicly available data (although Derek may have seen this in proprietary data) (Chilton et al., 2018). Usually expert review is recommended for predictions containing these features but as a fixed data interpretation procedure, required in a DA, does not permit expert review these are best considered as out of domain for use in ITSv1 (**Figure A2.1**).

Figure A2.0.1. Applicability domain for Derek Nexus skin sensitisation predictions used in ITSv1.



QSAR Toolbox (ITSv2)

102. Skin sensitisation predictions from the QSAR Toolbox automated workflow "Skin sensitisation for defined approaches" (Yordanova et al., 2019) are used in ITS v2. The protocol for running QSAR Toolbox predictions is defined in **Appendix 2** of this document.

103. The calculation of the applicability domain of the predictions is automatically provided by Toolbox when running DASS AW predictions and consists of three layers: structural, parametric and mechanistic. The applicability domain layers considered for each individual prediction depend on the type and outcome of the prediction, as summarised in Table A2.1. A detailed description of the three layers and the rationale for their selection is

explained in **Appendix 3** of this document. Toolbox results within applicability domain are considered as applicable in the DA.

Table A2.1. Applicability domain layers for the QSAR Toolbox automated workflow "Skin sensitisation for defined approaches" predictions.

Toolbox DASS AW outcome		Applicability domain layer		
		Structural	Parametric	Mechanistic
Positive	Read-across	Not considered	Not considered	**Considered**
	Profiling	Not considered	Not considered	**Met by definition**
Negative	Read-across	Not considered	Not considered	**Considered**
	Profiling	**Considered**	**Considered**	**Met by definition**

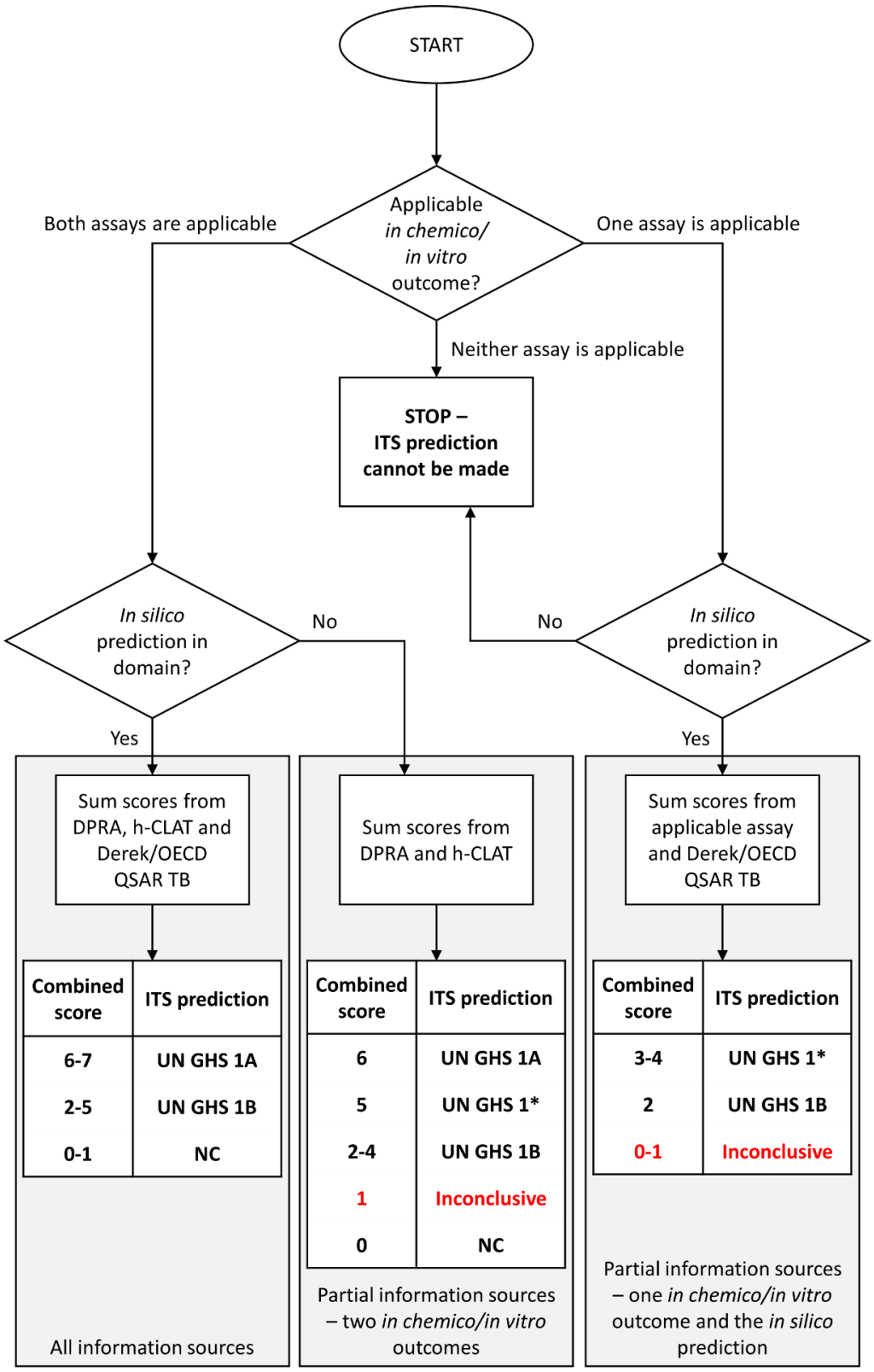
Confidence in ITS predictions

104. The applicability domain of the individual information sources used in the ITS DA are assessed and this determines whether the ITS predictions can be considered conclusive (i.e. high confidence) or inconclusive (i.e. low confidence) for hazard identification and/or potency.

How to apply the data interpretation procedure (DIP) for the ITS

105. The ITS was originally developed to use three information sources (DPRA, h-CLAT, and an *in silico* tool (Derek Nexus or OECD QSAR Toolbox)). Where all three information sources are applicable, a conclusive ITS prediction can be made. In some cases, a conclusive ITS prediction can be made, if there are two information sources with applicable results (**Figure A2.2**).

Figure A2.0.2. Workflow for data interpretation procedure for the ITS.



***Conclusive for hazard, inconclusive for potency**

106. Depending on the applicability of the individual information sources, three different scenarios for the ITS DA are possible (see Figure A2.2 and Table A2.2). In Scenario 1, all three information sources are applicable. In Scenarios 2 and 3, only two information sources are applicable. Details are provided below:

107. Scenario 1: all of the information sources i.e. *in chemico/in vitro* outcomes are applicable and can be considered (as prescribed in each individual assay) and the *in silico* prediction is in domain. The obtained ITS DA prediction is conclusive and of high confidence

108. Scenario 2: *in silico* prediction out of domain, however *in chemico*/*in vitro* methods are in domain and provide conclusive predictions (i.e. *in chemico*/*in vitro* methods are applicable).

- Combined DA score of 0, 2, 3, 4 or 6, *in silico* prediction out of *in silico* domain: DA conclusion is possible based on the two *in chemico/in vitro* outcomes. Conclusive prediction as the *in silico* prediction would not lead to a different DA prediction.
- Combined DA score of 5, *in silico* prediction out of *in silico* domain: DA conclusion possible for hazard identification (conclusive positive DA prediction for hazard identification). DA conclusion not possible for potency (inconclusive DA prediction for potency).
- Combined DA score of 1, *in silico* prediction out of *in silico* domain: DA conclusion not possible. Inconclusive DA prediction for hazard identification and potency.

109. Scenario 3: one *in chemico/in vitro* method out of domain or the result of that method cannot be considered (inapplicable):

- Combined DA score of 2 based on one *in chemico/in vitro* and *in silico* prediction: DA conclusion possible. Conclusive DA prediction as UN GHS 1B, as the outcome of the other *in chemico/in vitro* method would not to a different DA prediction.
- Combined DA score of 3 or 4, based on one *in chemico/in vitro* and *in silico* prediction: DA conclusion possible for hazard identification (conclusive positive DA prediction for hazard identification). DA conclusion not possible for potency (inconclusive DA prediction for potency).
- Combined DA score of 0 or 1, one *in chemico/in vitro* and *in silico* prediction: DA conclusion not possible. Inconclusive prediction for hazard identification and potency.

Table A2.2. Applicability domain and confidence of the ITS.

Scenario	Combined score[5]	ITS prediction	Confidence	DA prediction including confidence considerations
1	**0-1**	NC	High	Conclusive prediction Not Classified (NC).
	2-5	UN GHS 1B	High	Conclusive prediction UN GHS 1B.
	6-7	UN GHS 1A	High	Conclusive prediction UN GHS 1A.
2	**0**	NC	High	Conclusive prediction NC.
	1	Inconclusive	Low	Inconclusive prediction whether positive or negative.
	2-4	UN GHS 1B	High	Conclusive prediction UN GHS 1B.
	5	UN GHS 1	High	Conclusive positive prediction for hazard identification.
			Low	Inconclusive prediction for potency.
	6	UN GHS 1A	High	Conclusive prediction UN GHS 1A.
3	**0-1**	Inconclusive	Low	Inconclusive prediction whether positive or negative.
	2	UN GHS 1B	High	Conclusive prediction UN GHS 1B.
	3-4	UN GHS 1	High	Conclusive positive prediction for hazard identification.
			Low	Inconclusive prediction for potency.

[5]Total scores calculated only from information sources that are applicable/in domain.

References

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Yordanova, D., Schultz, T. W., Kuseva, C., Tankova, K., Ivanova, H., Dermen, I., Pavlov, T., Temelkov, S., Chapkanov, A., Georgiev, M., Gissi, A., Sobanski, T., & Mekenyan, O. G. (2019). Automated and standardized workflows in the OECD QSAR Toolbox. *Computational Toxicology*, *10*, 89–104. https://doi.org/10.1016/j.comtox.2019.01.006

Appendix 1: Protocol for Derek Nexus predictions

110. The following protocol may be used to generate predictions for skin sensitisation hazard using Derek Nexus v.6.1.0 with Derek Knowledge Base (KB) 2020 1.0 to be used as the *in silico* information source for the ITSv1 defined approach.

Protocol for generating predictions for skin sensitisation hazard using Derek Nexus v.6.1.0 with Derek KB 2020 1.0

Single chemical

1. Open Nexus
2. Input structure using one of the following options:
    a. Input structure manually by drawing on the canvas
    b. Go to File>Open Structure(s) to input a single structure from a file (.mol, .sdf, .smi, .csv, .cdx (file list not exhaustive))
    c. Go to File>Type Chemistry to enter or paste SMILES, InChi or MOL file
    d. Go to File>New Structure to input structure by drawing a structure
3. Set up prediction
    a. Go to Prediction>Derek Prediction>Derek Prediction Setup
4. Apply processing constraints
    a. Knowledge Bases
        i. For Nexus v6.1.0, ensure Derek KB 2020 1.0 is selected
        ii. For newer releases, use the default Derek KB supplied
    b. Perception
        i. Ensure 'Perceive tautomers' and Perceive mixtures' are selected
        ii. Ensure 'Match alerts without rules' is unselected
    c. Species
        i. Select 'mammal'
    d. Endpoints
        i. Click 'Deselect all' then expand 'Skin sensitisation (ALL)' to view 'Photoallergenicity' and 'Skin sensitisation'. Select 'Skin sensitisation'
    e. Structure properties
        i. Ensure the 'Overwrite' box(es) for logP, logKp, and average molecular mass are unselected to use the values calculated by Derek Nexus, otherwise, check the 'Overwrite' box(es) to input own values.
5. Generate prediction
    a. Click 'Start Prediction'
    b. If an alert is fired: Knowledge base, endpoint, species, reasoning level, alert fired, EC3 prediction (if applicable), and example matched (if applicable) are shown in the prediction navigator.
        i. Click the likelihood (certain, probable, plausible, equivocal) to view the reasoning rules leading to the likelihood level.

ii. Click the Alert in the prediction navigator to view alert match(es), description image, comments, validation comments, endpoint, references, patterns, and examples associated with the alert.

c. If no alert is fired, a negative prediction is generated: Knowledge base, endpoint, species and negative prediction reasoning (non-sensitiser) and negative prediction overview (absence or presence of misclassified and/or unclassified features) are shown in the prediction navigator.
    i. Click the negative prediction overview ('No misclassified or unclassified features', 'Contains misclassified/unclassified features') to view information about the negative prediction. Similar nearest neighbours are available to view for misclassified features.

d. Use the Derek likelihood to classify each compound as positive or negative (alert fired with certain, probable, plausible, or equivocal is classified as positive, alert fired with doubted, improbable, impossible, or a negative prediction of non-sensitiser with no misclassified or unclassified features is classified as negative).
    i. Negative predictions of non-sensitiser with misclassified and/or unclassified features are of lower confidence and are not used in ITSv1.
    ii. In cases where more than one alert is fired or structures in a mixture generate different likelihoods, the most conservative classification is applied (positive > negative).
    iii. A positive outcome from Derek is scored as 1 in the ITSv1 and a negative outcome is scored as 0.

Multiple chemicals

1. Open Nexus
2. Input structures
    a. Go to File>Open Structure(s) to input a file containing multiple structures (.mol, .sdf, .smi, .csv, .cdx (file list not exhaustive))
    b. Select the fields from the file which will be mapped to structure properties used during the prediction (Name, Average Molecular Mass, LogP, LogKp). If left unchanged then the values set by Derek will be used.
3. Set up batch prediction
    a. Go to Prediction>Derek Prediction>Derek Batch Setup
4. Apply processing constraints
    a. Knowledge Bases
        i. For Nexus v6.1.0, ensure Derek KB 2020 1.0 is selected
        ii. For newer releases, use the default Derek KB supplied
    b. Perception
        i. Ensure 'Perceive tautomers' and Perceive mixtures' are selected
        ii. Ensure 'Match alerts without rules' is unselected
    c. Species
        i. Select 'mammal'
    d. Endpoints

i. Click ‘Deselect all’ then expand ‘Skin sensitisation (ALL)’ to view ‘Photoallergenicity’ and ‘Skin sensitisation’. Select ‘Skin sensitisation’

e. Report configuration
   i. Directory - Leave as default directory or map to preferred location.
   ii. Pick type - Select report for batch (left side icon)
   iii. Pick format - Select desired file type (e.g. Excel)
   iv. Pick design - Select desired design (e.g. Tabular Report)
   v. Filename - input desired filename

f. Report display options
   i. Ensure ‘Show predictions of at least impossible’ is selected
   ii. Select ‘Show Negative Predictions’
   iii. Select ‘Filter All Nearest Neighbours by Misclassified Features’
   iv. Select ‘Show Open Likelihood’
   v. Select ‘Show Rapid Prototypes’

5. Generate batch prediction
   a. Click ‘Start Batch Prediction’
      i. Once the batch prediction is finished, select the ‘Open Report Directory’ when prompted
   b. Use the Derek likelihood to classify each compound as positive or negative (alert fired with certain, probable, plausible, or equivocal is classified as positive, alert fired with doubted, improbable, impossible, or a negative prediction of non-sensitiser with no misclassified or unclassified features is classified as negative).
      i. Negative predictions of non-sensitiser with misclassified and/or unclassified features are of lower confidence and are not used in ITSv1.
      ii. In cases where more than one alert is fired or structures in a mixture generate different likelihoods, the most conservative classification is applied (positive > negative).
   c. A positive outcome from Derek is scored as 1 in the ITSv1 and a negative outcome is scored as 0.

Appendix 2: Protocol for OECD QSAR Toolbox predictions

111. The following protocol may be used to generate predictions for skin sensitisation hazard using OECD QSAR Toolbox v.4.5 with the automated workflow for defined approaches for skin sensitisation (DASS AW) to be used as the in silico information source for the ITSv2 defined approach.

Protocol for generating predictions for skin sensitisation hazard using DASS AW in Toolbox 4.5.

Step 1: Input the chemical in the "Input module". SMILES is the preferred way to input the structure. (If other identifiers such as the CAS number are used as input, the Toolbox will assign the SMILES based on its internal database. In this case, the user needs to make sure that Toolbox identifies and consequently uses for the prediction the correct structure.)

Step 2: Go to the "Data gap filling module" and click on "Automated" button. Select "EC3 from LLNA or Skin sensitization from GPMT assays for defined approaches" and click OK. The scheme with the implemented logic will be shown.

Step 3: Click the Run button -  or press F5 key of the keyboard and confirm with "Yes". The workflow will run automatically.

Step 4: If a substance is predicted "positive" or "negative" as a result of read-across, the prediction will appear on the data matrix with "R" in front of the result (e.g. "R: Negative). If a substance is predicted "positive" or "negative" as a result of profiling, then the result will appear next to the name of the customized profiler "Skin sensitization for DASS".

Step 5: Affiliation of the substance to the domain of the automated workflow for DASS will be automatically determined and presented.

Appendix 3: Information on applicability domain for OECD QSAR Toolbox

Technical aspects

112. The Toolbox prediction used by DA ITS v.2 is calculated using the DASS automated workflow (DASS AW) included in OECD QSAR Toolbox v.4.5. The workflow also includes the automatic calculation of the applicability domain of Derek skin described below.

Calculation of the in silico domain of Toolbox

113. Applicability domain of the QSAR Toolbox Skin sensitisation predictions for use in the ITS defined approach approaches automated workflow (DASS AW) is defined by based on the training set substances of the same automated workflow. The training set (TS) consists of 2268 substances having LLNA and/or GPMT skin sensitisation experimental data[6](the full list of substances can be consulted in the QSAR Toolbox). The TS substances are part of the following OECD QSAR Toolbox databases:

- Skin sensitisation;
- REACH Skin sensitisation (normalized) databases.

114. Based on the correctly predicted training set substances, three layers of applicability domain are automatically calculated by the Toolbox: 1) parametric; 2) structural and 3) mechanistic layers. Depending on the Toolbox prediction approach (read-across or profiling predictions) and prediction outcomes (positive or negative), one or more of these layers are taken into account to establish the overall Toolbox domain of the specific prediction.

115. The applicability domain layers considered for different types of Toolbox predictions are summarised in the table here:

Toolbox DASS AW outcome		Applicability domain layer		
		Structural	Parametric	Mechanistic
Positive	Read-across	Not considered	Not considered	**Considered**
	Profiling	Not considered	Not considered	**Met by definition**
Negative	Read-across	Not considered	Not considered	**Considered**
	Profiling	**Considered**	**Considered**	**Met by definition**

116. Explanation and rationale for the use of different domain layers:

1. Positive predictions (both by read-across and profiling): the presence of an alert (which is the requirement for positive Toolbox prediction to be considered within in the mechanistic domain) is sufficient to consider the prediction to be within the Toolbox domain. Substances triggering an alert are considered as in domain because they contain the toxicophore that has been observed experimentally in skin sensitisers. No further checks are needed in this context to consider the prediction within the Toolbox *in silico* domain.

[6] In case of multiple data points for one substance, the most conservative scenario is taken into account.

2. Negative predictions by read-across: the structural and parametric domains are not taken into account because the Toolbox has already ensured some level of similarity with other substances in its training set that met the requirements to be selected as suitable analogues for read-across (these requirements are explained in detail in the DASS AW description).
3. Negative prediction by profiling predictions: all domain layers are taken into account to ensure the highest possible reliability level for the Toolbox prediction. Stricter requirements are needed mainly for two reasons: 1. lack of alerts is not equal to proof of lack of sensitisation potential and 2. to apply a cautious approach since acceptance of negative predictions may lower the human health protection level risk in case of a false negative predictions.

Calculation of applicability domain layers

1. Parametric layer

Four physico-chemical parameters of the substances are taken into consideration: log Kow, molecular weight, vapour pressure and water solubility[7]. The ranges of variation for the selected parameters are defined based on the training set substances that are correctly predicted by the DASS AW.

A substance is considered within the parametric domain of the DASS AW if its physico-chemical parameter values as calculated by the QSAR Toolbox fall into the ranges of variation given in the table below. It is noted that the ranges include parametric values calculated using EPISuite models implemented in Toolbox that in some cases are wider than that covered by existing test methods.

Physico-chemical parameter	Calculated Parameter range
Log Kow	-9.66 ÷ 18.6
Molecular weight	16 Da ÷ 2290 Da
Vapour pressure*	0 Pa ÷ 3.45 x 10^{7} Pa
Water solubility	2.48 x 10^{-15} mg/L ÷ 1.00 x 10^{6} mg/L

*EPIWIN Vapor Pressure (Antoine method) is used for calculation

2. Structural layer

The structural layer is defined based on the atom centred fragments (ACF) derived from the structural characteristics of the TS substances that are correctly predicted[8] by the DASS AW.

The ACF are defined according to the following Toolbox default values for ACF:

- Any atom distance = 1

[7] QSAR Toolbox is used for the calculation of the physico-chemical properties.

[8] All ACF that are extracted from the correctly predicted TS test chemicals “good space”. The “bad space” is formed from the ACF present in the incorrectly predicted test chemicals. The default QSAR Toolbox settings for ACF are used. Supplementary file with the ACF forming the good and the bad space are available.

- Heteroatom distance = 1
- Extract C (sp^3) fragments = YES
- Include whole aromatic rings = NO

For each substance, the following values are calculated:

- % Correct fragments: percentage of ACF occurring in correctly predicted structures in the training set
- % incorrect fragments: percentage of ACF occurring in incorrectly predicted structures in the training set
- % unknown fragments: percentage of ACF not occurring in the training set.

A substance is considered within the structural domain of the DASS AW if 100% of its ACF belong to the correct fragments.

3. Mechanistic layer

The predicted capability of a substance to interact with the skin proteins without and after (a)biotic activation is taken into consideration. The Toolbox endpoint-specific profiler *Protein binding for skin sensitization by OASIS* and two metabolic simulators – *Autoxidation simulator* and *Skin metabolism simulator* are used to predict such interaction.

A positive prediction is considered within the mechanistic domain if the substance triggers "*Protein binding for skin sensitization by OASIS*" alerts without or after (a)biotic activation.

A negative prediction is considered within the mechanistic domain if the substance does not permit expert review these are best considered as out of domain for use in the ITS "*trigger Protein binding for skin sensitization by OASIS*" without or after (a)biotic activation.

117. Note that predictions obtained by profiling results will meet the mechanistic layer requirements by definition because positive Toolbox predictions by profiler are triggered exactly by the presence of alert. If the test chemical cannot be tested or the outcome/prediction cannot be considered in at least two of the information sources (*in chemico/in vitro and/or in silico)* then the DA cannot be applied.

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

In vivo と相関性のある *in vitro* 毒性評価系による AOP 及び TG の実験データ支援

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研究要旨

本分担研究は、皮膚、肝臓並びに消化管組織の代替法において AOP の成立に寄与する知見を得ることを目的とした。本年度は、皮膚組織における代替法の検討として、3D 皮膚再構成系を用いて folpet 曝露の影響を評価した。肝臓における代替法の検討として正常肝細胞株と肝癌由来細胞株を用いて各種脂肪酸曝露の影響を評価した。さらに腸管上皮における毒性評価として *in vivo* と *in vitro* の試験系からえられる種々の結果を比較した。

その結果、3D 皮膚再構成系を用いた評価において、folpet は、OECD-TG439 による皮膚刺激性試験で陰性であったが、24 時間曝露条件下で濃度が高くなると、細胞生存率の低下と炎症性サイトカインの mRNA 発現の増加を示した。肝細胞株における脂肪毒性に対する評価においては、脂肪酸の種類において細胞毒性が異なることや、本実験で使用した正常と腫瘍細胞株において、特に多価不飽和脂肪酸曝露に対する細胞毒性が異なる可能性が示唆された。腸管に関する検討では、マウス、Caco-2 細胞及びマウス空腸由来オルガノイドで、DSS の曝露によりいずれも TJP-1 (tight junction protein-1)の変動、IL-1b の遺伝子発現減少を認め、AOP の成立に寄与する可能性が示された。

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A. 研究目的

本研究は、皮膚、肝臓並びに消化管組織の代替法において AOP の成立に寄与する知見を得ることを目的として、以下の検討を行った。

皮膚組織における代替法の検討としては、化学物質による皮膚への影響を *in vitro* の実験系において評価することを目的とし、2 次元培養として HaCaT 細胞を用い、3 次元培養としてヒト 3D 皮膚再構成系を用いて folpet 曝露の影響を評価した。

肝臓における代替法の検討としては、化学物質による肝臓への影響を *in vitro* の実験系において評価することを目的とし、ラット正常肝細胞株 Clone 9 とヒト肝癌由来細胞株 HepG2 を用いて各種脂肪酸曝露の影響を評価した。

腸管由来組織における代替法の検討としては、化学物質による腸管への影響を *in vivo*・*in vitro* の各実験系において評価・比

較することを目的とし、マウスに投与すると共に、2 次元培養として Caco-2 細胞を用い、3 次元培養としてマウス空腸由来のオルガノイドを用いてデキストラン硫酸ナトリウム (DSS) 曝露に対する影響を評価した。

B. 研究方法

B.1. 皮膚組織における代替法の検討 : ヒト 3D 皮膚再構成系及びヒト表皮角化細胞単層培養系を用いた folpet に対する経皮毒性

ヒト 3D 皮膚再構成系としては、LabCyte EPI 24 モデル（株式会社ジャパン・ティッシュ・エンジニアリング）を、当該モデルに添付の培養液と共に用いた。単層培養系としては、ヒト表皮角化細胞株 HaCaT 細胞を適宜継代して用いた。

被験物質としては、皮膚刺激性試験で陰性を示すものの 21 日間反復投与毒性を示す folpet（フタルイミド系殺菌剤）を使用した。

ヒト 3D 皮膚再構成系を用いた実験は、OECD TG439 に従った皮膚刺激性試験（15 分曝露・42 時間後培養）と、24 時間曝露の 2 条件で、同じ濃度の folpet（0、100、300、1000、2000 μg/mL）による細胞毒性を（3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide（MTT）アッセイにて細胞傷害率を測定し、比較評価した。また、濃度依存（0、100、300、1000 μg/mL、24 時間）・時間依存（0、3、8、24 時間、300 μg/mL）における各種炎症性サイトカイン関連遺伝子発現を qPCR にて解析した。

HaCaT 細胞を用いた実験は、folpet を 0、1、3、10、30、40、60、80、100、120 μg /mL の濃度で 24 時間曝露し、WST-8 アッセイで細胞毒性を評価した。

B.2. 肝臓における代替法の検討 : 正常または腫瘍肝細胞における脂肪毒性に対する検討

ラット正常肝細胞株 Clone 9 と、ヒト肝癌由来細胞株 HepG2、マウス肝癌由来細胞株 Hepa1-6 を用い、各種脂肪酸（パルミチン酸、オレイン酸、エライジン酸、リノール酸、アラキドン酸、EPA、DHA）を 1-1600 μM の濃度で 24 時間曝露し、WST-8 アッセイで細胞毒性を評価した（表 1）。

B.3. 腸管由来組織における代替法の検討

B.3.1. 腸管上皮由来 Caco-2 細胞を用いた平面培養による検討

DSS (MP Biomedicals)を 6 週齢の雄性 C57BL/6J マウスに 1 週間飲水投与し、小腸及び大腸の病理組織学的解析、遺伝子発現解析を行い、同様に溶媒を投与した対照群のマウスと比較検討した（各群 6 匹）。

B.3.2. 腸管上皮由来 Caco-2 細胞を用いた平面培養による検討

DSS を通常培地(DMEM: Low Glu に 10% 胎仔ウシ血清（FBS）、1% 非必須アミノ酸溶液（NEAA）、1%ペニシリン・ストレプトマイシン添加)に各種の濃度%(w/v)で混じ、Caco-2 に 24 時間曝露した。

B.3.2.1. 遺伝子発現解析

12-well plate の各 well に Caco-2 を 4×10^4 cell/mL で播種して 2 日毎に培地交換を行い、コンフルエントになってから 5 日後に 1、3、5%の濃度、別の 12-well plate で 5、10、15%の濃度の DSS を曝露した。

DSS 10、15%においては RNA 量、cDNA 量共に測定できたにもかかわらず、内部標準遺伝子発現が僅かで、DSS 15%では内部因子を含めその他のターゲット遺伝子すべてに発現が見られなかった。従って DSS

10%以上の曝露は、評価に適さない状況にあるものと考えられた。

B.3.2.2. 蛍光免疫組織学的染色

Cell culture slide (4-well タイプ) の各 well に Caco-2 を 1.3 × 10^5 cell/mL で播種した。培地は 400 µL/well とした。2 日毎に培地交換を行い、約 2 ヶ月後に DSS を 1、5、30%の濃度で曝露した。DSS 処理後に 3%パラホルムアルデヒドで固定し、0.2% Triton-X100 で透過処理を行い、更に 1% FBS でブロッキング後、E-cadherin rabbit polyclonal antibody (ProteinTech) を添加し一晩処理し、donkey anti-Rabbit IgG (H+L) highly cross adsorbed secondary antibody, Alexa Fluor 555 (Thermo Fisher Scientific) を反応させ、4',6-diamidino-2-phenylindole（DAPI）添加剤にて封入した。

B.3.2.3. 生細胞数測定 (WST-8)

96-well plate に Caco-2 を 8,000 cell/well で播種して 2 日毎に培地交換し、コンフルエントになってから 5 日後に 5、10、15、20、25 及び 30%の濃度の DSS を曝露した後、Cell Counting Kit-8 (同人化学:富士フィルム和光純薬株式会社) を添加してインキュベートした。

WST-8 アッセイによる細胞毒性評価においては、WST-8 試薬添加後のインキュベート時間も考慮して、1 時間経過ごとに 4 時間まで、450 µm (マルチモードプレートリーダー:バイオテックジャパン)にて吸光度測定を行った。

B.3.3. マウス空腸由来のオルガノイドを用いた検討

正常 C57BL/6J マウスに DSS 誘発大腸炎マウスの病理組織学的解析や遺伝子発現解析とそのマウスから作製した腸管オルガノイドの形成効率や増殖活性（EdU アッセイ）などの結果を比較した。さらに Caco-2 細胞へ DSS 培地添加した際の影響や、通常の腸管オルガノイドに DSS を培地添加、マイクロインジェクションした際の影響も解析した。

B.3.3.1. 培地添加による曝露

0.5、1、3% DSS (in phosphate-buffered saline (PBS))を調製し、24-well plate に 0.5、1%濃度になるよう培地に添加した。Control 群においては、PBS を 1%になるように添加した。培地容量は、PBS 及び DSS 共に、400 µL/well とした。

B.3.3.2. インジェクションによる曝露

15、30% DSS (in PBS)を調製し、オルガノイドにインジェクションした。オルガノイドの体積とインジェクションする液量の条件から、投与液は 30 倍希釈されることが明らかとなっている。そのため、インジェクション後オルガノイド中の DSS 濃度は、0.5、1%となった。Control 群には、PBS をインジェクションした。

（倫理面への配慮）

腸管組織における検討においては、マウス回腸由来のオルガノイド培養組織を実施するにあたりマウスを使用した。ただし、その使用は最少匹数に留め、東京農業大学動物実験委員会より承認を受けた申請内容に則り実施した。

C. 研究結果

C.1. 皮膚組織における代替法の検討

ヒト 3D 皮膚再構成系を用いた folpet に対する経皮毒性評価において、OECD TG439 に従った皮膚刺激性試験では 2000 µg/mL の最高濃度まで陰性であったのに

対し、24 時間曝露では同条件の対照群と比較して 2000 μg/mL より細胞毒性がみられた（図 1)。24 時間曝露における qPCR では、炎症性サイトカインの mRNA 発現が 1000 μg/mL で有意に増加した（図 2)。また、濃度依存による検討では、3 時間あるいは 8 時間の曝露より炎症性サイトカインの上昇がみられた（図 3)。

HaCaT 細胞を用いた 24 時間曝露では、60 μg/mL 以上で細胞毒性を示した。

C.2. 肝臓における代替法の検討

Clone 9、HepG2、Hepa1-6 において、パルミチン酸やリノール酸に比してオレイン酸やオレイン酸のトランス異性体であるエライジン酸の細胞毒性は弱かった（図 4)。多価不飽和脂肪酸は、Clone 9 と比較して HepG2 と Hepa1-6 において、低濃度曝露で細胞毒性を示した（図 5)。

C.3. 腸管由来組織における代替法の検討

C3.1.1. DSS 曝露によるマウス腸管への影響

DSS 群の小腸では、病理組織学的に明らかな変化がなかったが、細胞接着関連因子(TJP-1)及び炎症関連因子(IL-1b)の遺伝子発現の減少傾向が見られた（図６－１、図７)。DSS 群の大腸では、軽度の炎症性病変が誘発された。

C3.2.1. Caco-2 の遺伝子発現解析

DSS 添加により control と比較し 1%濃度以上で、TJP-1 並びに IL-1b の遺伝子発現が減少傾向を示した（図６－２、図７)。DSS 5%濃度においては、e-cadherin と IL-1β の発現に加え、ZO-1、IL-6、TNF-α についても control と比較し、発現がやや低下する傾向が認められた。DSS 10、15%濃度においては、内部標準遺伝子の発現自体が少なく、PCR の結果が得られなかった。このことから DSS 10%濃度以上では、細胞の死滅が生じているものと考えられた。

C.3.2.2. Caco-2 の蛍光免疫組織学的染色及び遺伝子発現解析

2 週間培養し 24 時間 DSS を曝露したところ、10%では明らかな細胞の脱落・離開が見られた。5%では細胞培養の状態に control と差が見られなかったが、e-cadherin の免疫組織化学では細胞間の陽性部位の減弱が認められた。DSS 1 及び 3%濃度では、明らかな差が見られなかった。

C.3.2.3. Caco-2 の生細胞数測定 (WST-8)

WST-8 試薬添加後のインキュベート 1 時間後の評価において、control に比較し、DSS 5％濃度以上で、細胞が死滅し毒性が生じているものと考えられた（図８)。

C.3.3.1. マウス空腸由来オルガノイドの培地中曝露

添加後 1 時間で、DSS 0.5、1%濃度培地で死滅しているオルガノイドが見られたことから、1 時間足らずでオルガノイドへの影響が確認できなくなり、マトリゲルが破壊され緩くなることから、さらに低濃度による検討を要することが明らかとなった。

C.3.3.2. マウス空腸由来オルガノイドのインジェクションによる曝露

インジェクション後に DSS 1％濃度でもオルガノイドは死滅せず、大型化・変形した。TJP-1 の遺伝子発現は 0.5%濃度以上で一過性の顕著な増加を示したが、24 時間後には、control と差はなく 1%濃度では一転し発現が低下した（図６－３)。さらに IL-1b の遺伝子発現は減少傾向を示した（図７）

D. 考察

D.1. 皮膚組織における代替法の検討

ヒト 3D 皮膚再構成系を用いた評価において、folpet は、OECD TG439 による皮膚刺激性試験で陰性であったが、24 時間暴露条件下で濃度が高くなると細胞生存率が下降傾向を示し、炎症性サイトカインの mRNA 発現が 1000 μg/mL で有意に増加した。したがって、後者のプロトコルは、OECD TG439 による皮膚刺激性試験で陰性の化学物質の細胞傷害性や、in vivo の反復投与経皮毒性に相当する変化を検出できる可能性が示唆された。今後、表皮の毒性に鋭敏な因子の探索や、より簡便なスクリーニングを目指すための HaCaT 細胞を用いた検討を行う予定である。

D.2. 肝臓における代替法の検討

正常または腫瘍肝細胞における脂肪毒性に対する評価において、本実験で使用した細胞株では、特に多価不飽和脂肪酸曝露に対する細胞毒性が異なることが明らかとなった。今後、正常または腫瘍肝細胞の種類を増やし、脂肪酸曝露による細胞毒性の差異を精査する予定である。

D.3. 腸管由来組織における代替法の検討

腸管由来組織における動物実験代替法の確立を目的として、今年度は、マウスへの DSS の投与、腸管由来の株化細胞 Caco-2 を用いた 2 次元培養による影響を検討し、マウス空腸由来のオルガノイドを用いた 3 次元培養による評価について検討した。いずれの実験系も陽性対照物質として DSS を用いた。

DSS を投与したマウスにおいて、回腸に明らかな影響は認められなかった。遺伝子発現解析では、TJP-1 の発現が DSS 群において減少傾向にあった。その他 IL-1b などの炎症促進に関与するサイトカインの遺伝子発現量も低下した。

2 次元培養において、遺伝子発現解析の結果では、DSS 10%濃度以上で細胞が離開し、毒性が生じているものと考えられた。また、WST-8 アッセイの結果、DSS 5%濃度以上で死滅が生じているものと考えられた。今後は、死細胞数測定(lactic acid dehydrogenase (LDH) assay)も行い、実際に細胞死について検討することで、２次元培養条件下における毒性兆候をより明らかにする予定である。

マウス空腸由来のオルガノイドを用いた DSS の評価では、培地中への曝露により DSS 0.5、1%といった低濃度からオルガノイド組織の死滅を示唆する変化が認められた。一方、オルガノイド管腔内にインジェクション曝露した場合には、オルガノイド自体に大型化など形態的変化を認めたものの、組織が死滅に至るような傷害性変化を観察しなかった。このことから、DSS を同濃度に設定して曝露したにもかかわらず、上記のように結果に差が生じたことから、腸管管腔側（インジェクション曝露）よりも基底膜側（培地内曝露）からの DSS 処理による影響が大きいことが明らかとなった。

0.5%濃度以上の DSS のインジェクションにより TJP-1 遺伝子発現の変化や IL-1b 遺伝子発現の低下が見られた。

以上、腸管に関連したいずれの実験系においても DSS の曝露により TJP-1 遺伝子発現への影響や IL-1b 遺伝子発現低下が認められた。これは新たな腸管毒性評価における AOP となり得る可能性を示唆するものと考えた。

E. 結論

本研究は、皮膚組織・肝臓・腸管由来組

織における動物実験代替法の確立に向けた検討を２次元培養ないし３次元培養条件下で実施し、それぞれ、代替法の開発に資する基礎的情報を得た。今後は、実験条件の精査や *in vivo* 試験で得られる結果との比較検討を行うと共に、表現型の背景メカニズムの解析を行い、具体的な代替法開発に取り組む予定である。

F. 研究発表

F.1. 論文発表

なし

F.2. 学会発表

1. 宍戸健太、煙山紀子、美谷島克宏、中江大、他、単層培養系および 3D 再構成系における二酸化チタンナノ粒子のヒト表皮細胞毒性評価、第 48 回日本毒性学会学術年会（2021 年 7 月 7 日，兵庫県神戸市）

G. 知的所有権の取得状況

１．特許取得

なし

２．実用新案登録

なし

３．その他

なし

表１.

脂肪酸曝露濃度

脂肪酸	濃度[μg/mL]
パルミチン酸	0/50/100/200/400/600/800/1000
オレイン酸	0/200/400/800/1000/1200/1400/1600
エライジン酸	0/200/400/800/1000/1200/1400/1600
リノール酸	0/200/400/800/1000/1200/1400/1600
アラキドン酸	0/0.62/12.5/25/50/100/200/400
DHA	0/0.62/12.5/25/50/100/200/400
EPA	0/0.62/12.5/25/50/100/200/400

図１.

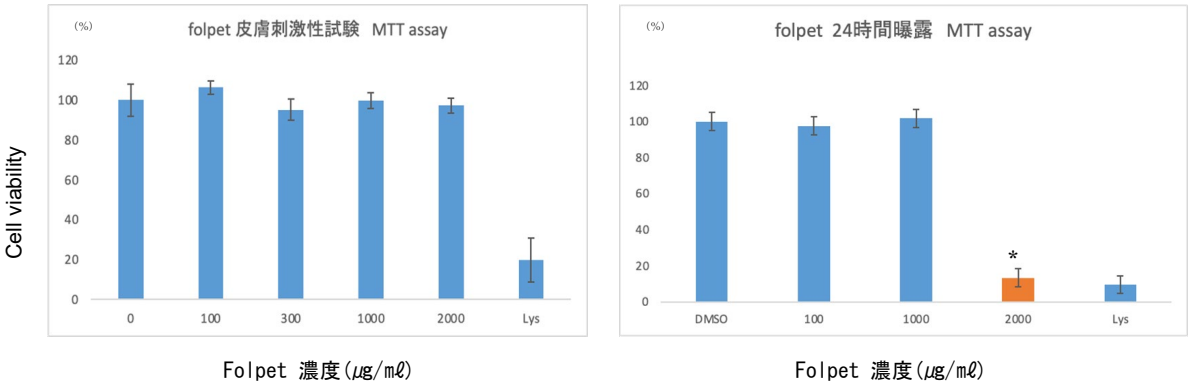


図２.

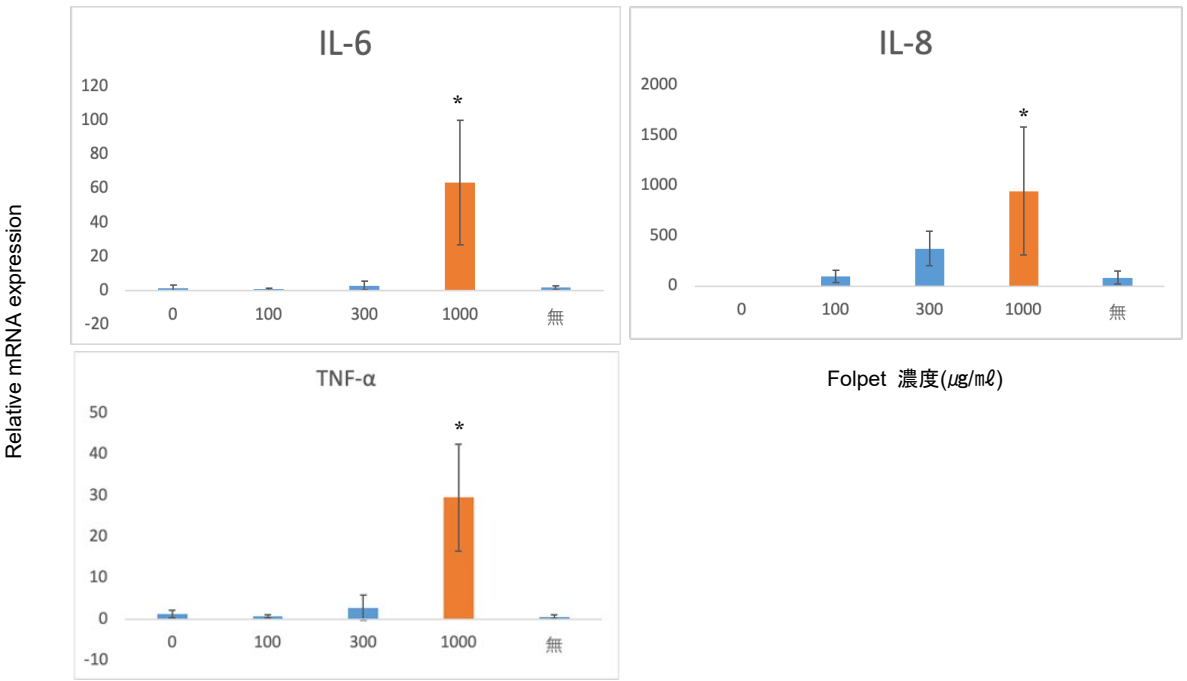


図３.

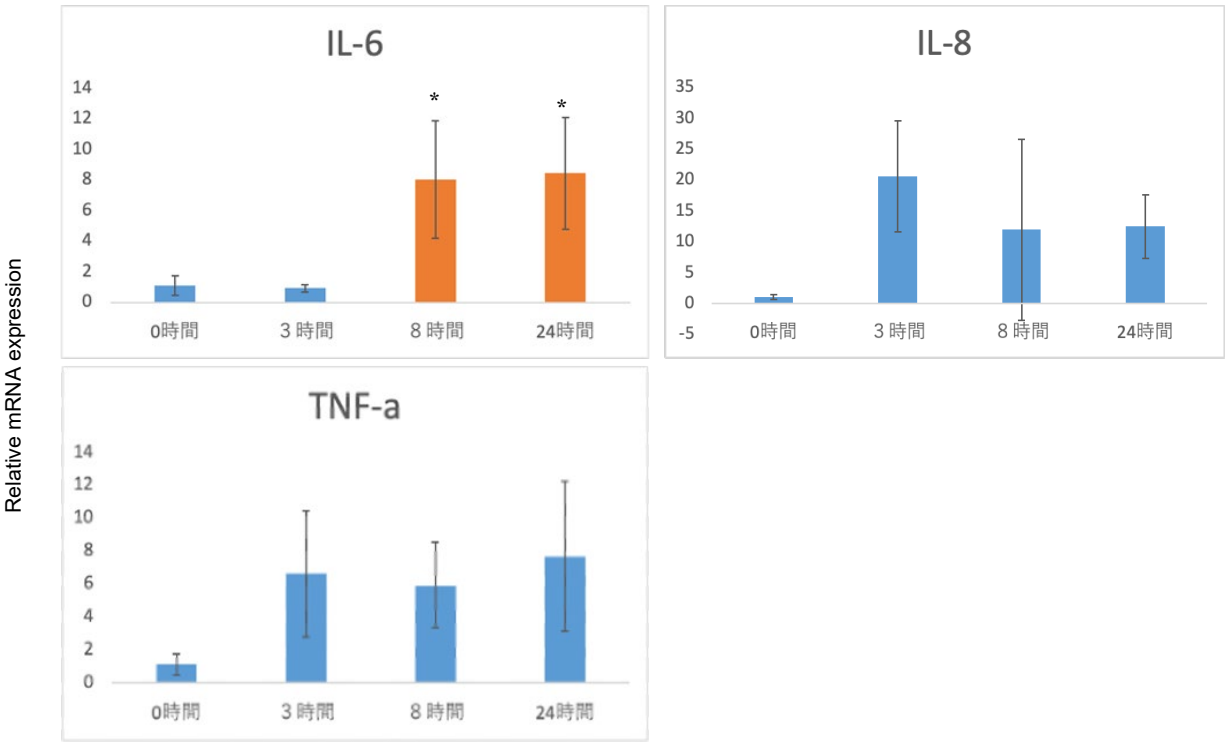

Relative mRNA expression
IL-6
14
12
10
8
6
4
2
0
*
*
0時間
3 時間
8 時間
24時間
IL-8
35
30
25
20
15
10
5
0
-5
0時間
3 時間
8 時間
24時間
TNF-a
14
12
10
8
6
4
2
0
0時間
3 時間
8 時間
24時間

図４.

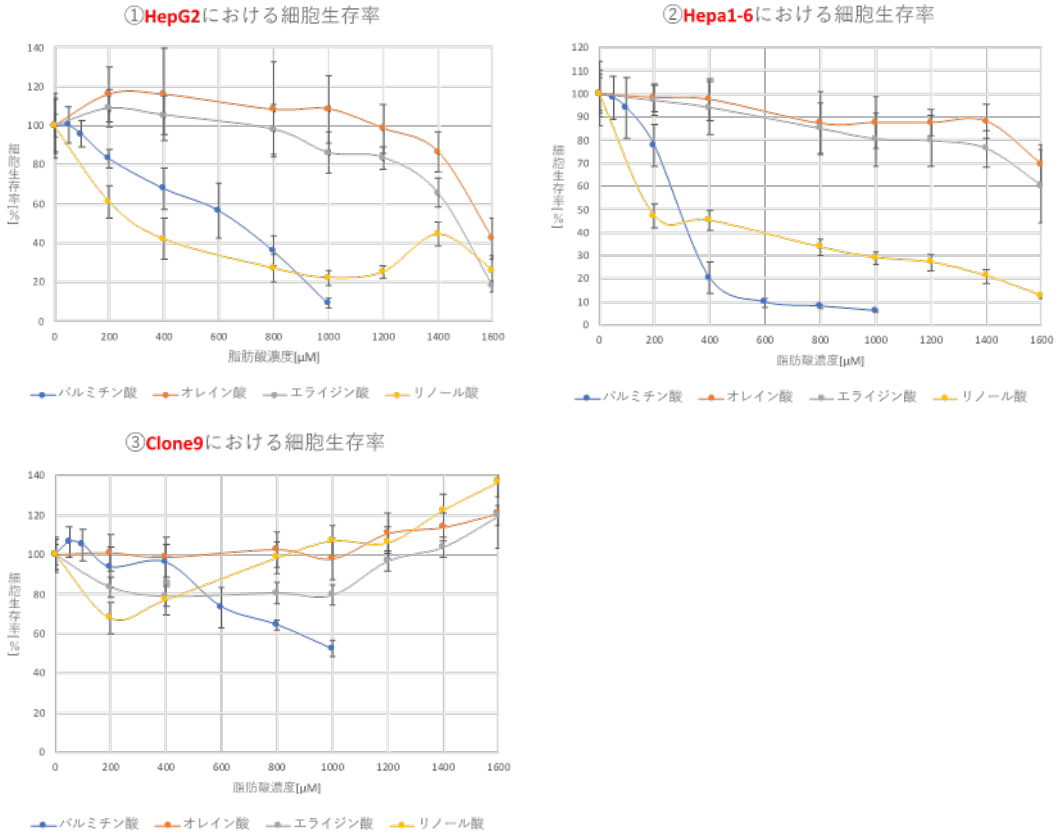

①HepG2における細胞生存率
細胞生存率[%]
脂肪酸濃度[μM]
パルミチン酸
オレイン酸
エライジン酸
リノール酸
②Hepa1-6における細胞生存率
細胞生存率[%]
脂肪酸濃度[μM]
パルミチン酸
オレイン酸
エライジン酸
リノール酸
③Clone9における細胞生存率
細胞生存率[%]
脂肪酸濃度[μM]
パルミチン酸
オレイン酸
エライジン酸
リノール酸

図５.

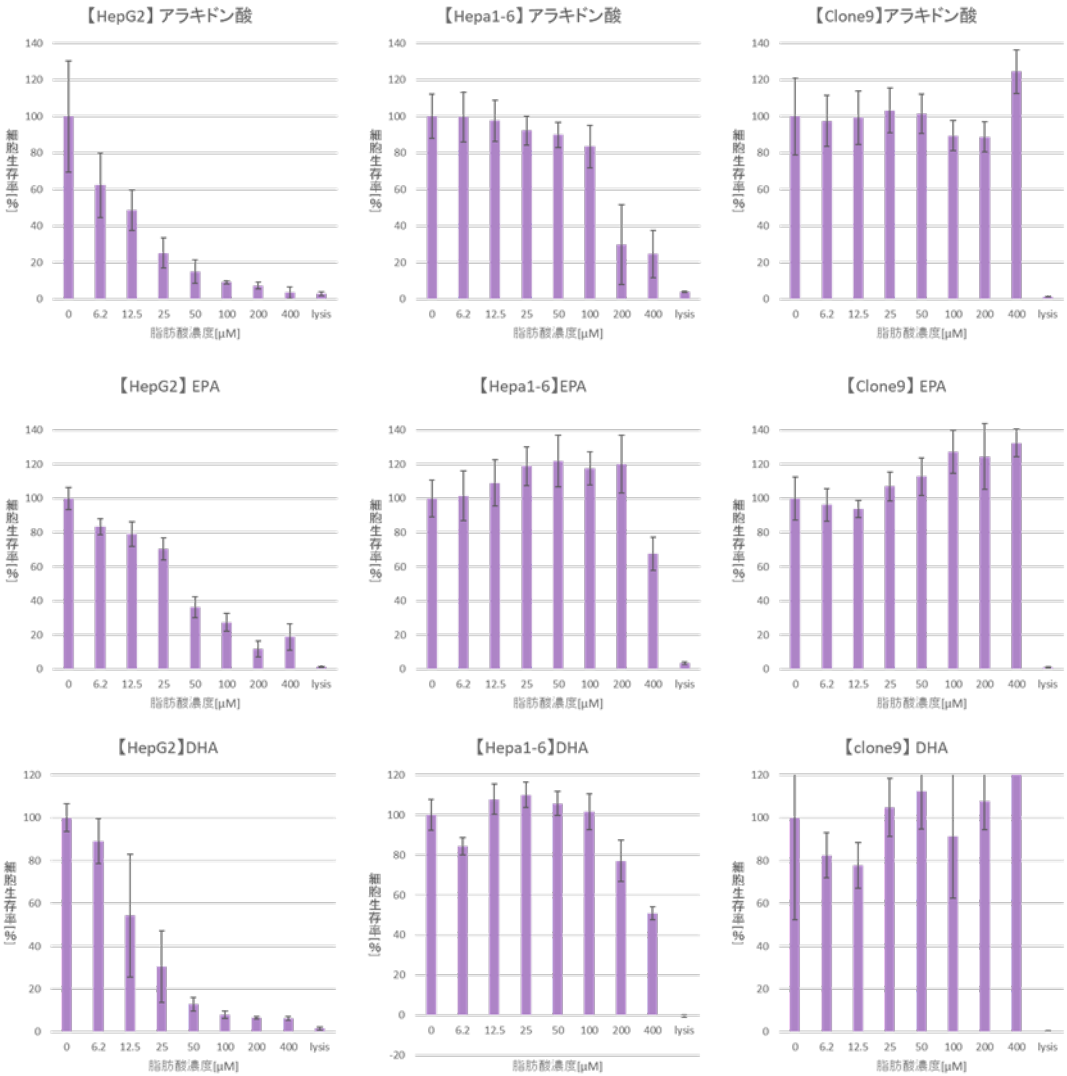

【HepG2】アラキドン酸
【Hepa1-6】アラキドン酸
【Clone9】アラキドン酸
【HepG2】EPA
【Hepa1-6】EPA
【Clone9】EPA
【HepG2】DHA
【Hepa1-6】DHA
【clone9】DHA
細胞生存率[%]
脂肪酸濃度[μM]
0 6.2 12.5 25 50 100 200 400 lysis

図６－１．マウス小腸の遺伝子発現解析（TJP-1）

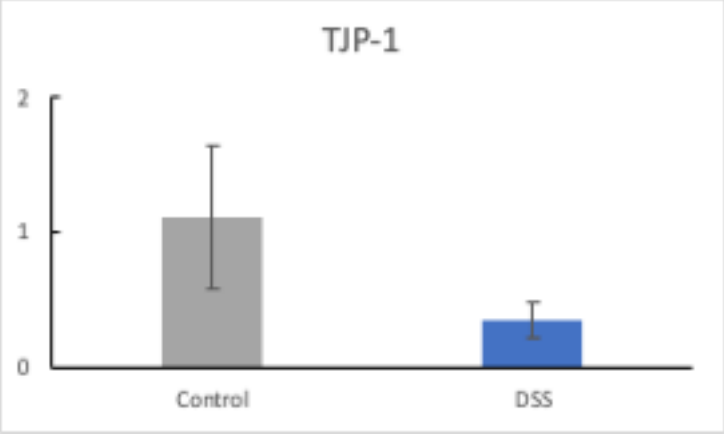


図６－２．Caco-2細胞の遺伝子発現解析（TJP-1）

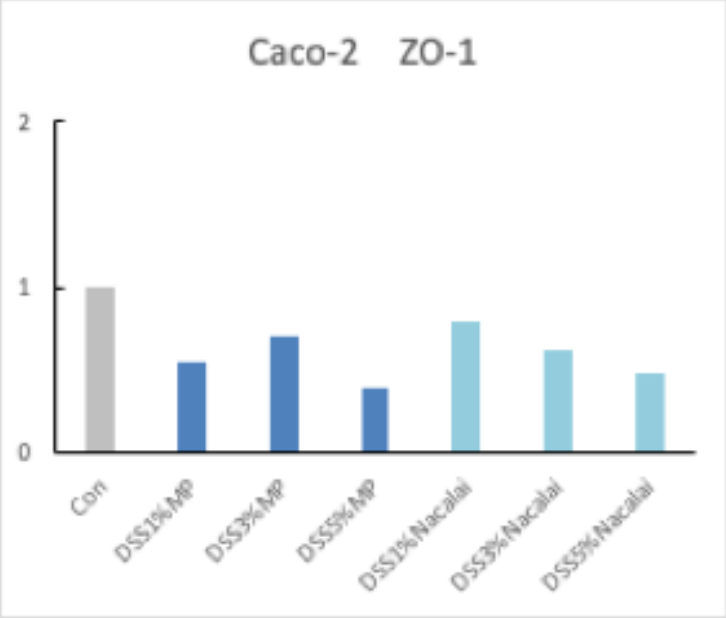
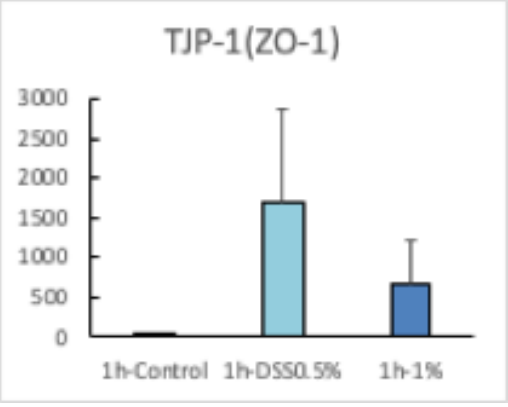


図６－３．マウス由来小腸オルガノイドの遺伝子発現解析（TJP-1）



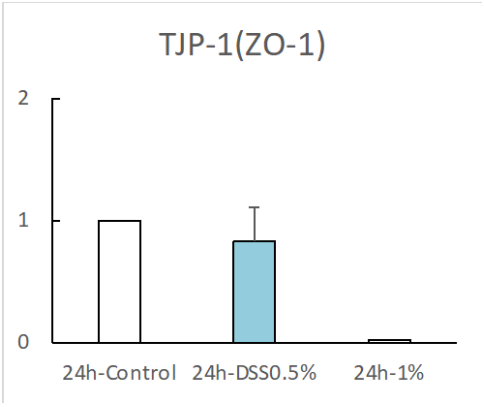


図７．遺伝子発現解析（IL-1β）

マウス小腸　　Caco-2細胞　　マウス由来小腸オルガノイド

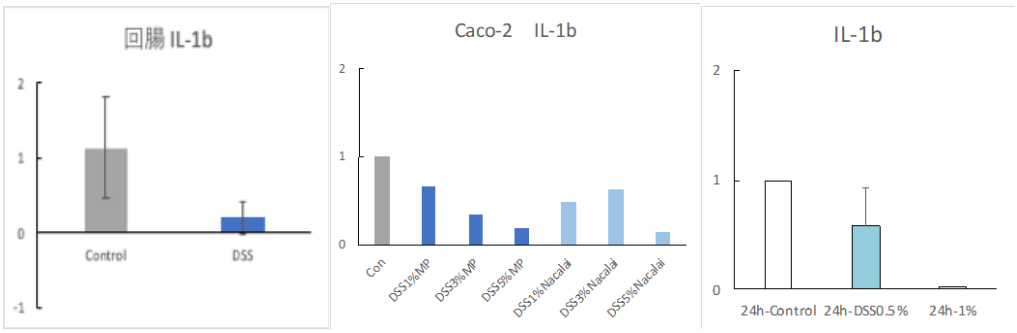
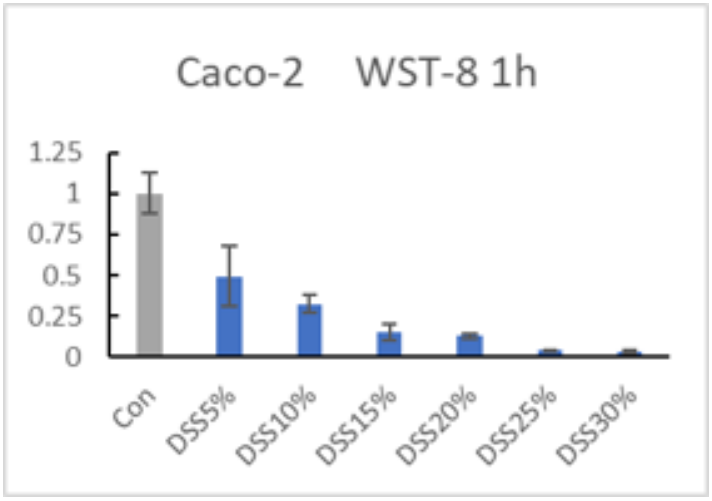


図８．生細胞数測定(WST-8)



厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）
OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

発がん性試験のIATA及びAOP開発に関する研究

研究分担者　小川久美子
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研究要旨

2016 年に経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）において、 非遺伝毒性発がん物質の統合的評価手法の確立を目標とした integrated approach to the testing and assessment (IATA) of non-genotoxic carcinogens (NGTxC) の専門家グループが立ち上げられた。非遺伝毒性発がん性の機序に関連する事象を抽出し、それぞれの事象を主に *in vitro* の試験系で評価する方法とその妥当性について、議論が継続されている。本研究では、当該 IATA 開発に協力すると共に、発がん性の有害転帰経路（Adverse Outcome Pathway: AOP）の開発、並びに生体における発がん機序に関する調査研究を実施し、化学物質のヒト発がん性に関する適切な評価を推進し、以て、日本の厚生労働行政に資することを目的としている。

各種化学物質暴露による鼻腔発がん全般の AOP について論文化に必要な情報を収集し、げっ歯類における化学物質誘発鼻腔発がんを網羅的に解析したところ、各種鼻腔腫瘍の前駆病変は、遺伝毒性の有無に関係なく、腫瘍タイプの化学物質誘発性の細胞毒性と一般的に関連している可能性があり、分子開始イベント後の経路は、遺伝毒性発がん物質と非遺伝毒性発がん物質の間で大きく重複している可能性が示唆された。

また、OECD で進められている非遺伝毒性発がん性の IATA 開発に協力し、cell proliferation、resistance to apoptotic cell death、cell transformation、 indicator of oxidative stress 及び resistance of apoptosis cell death のサブグループに参画し、アッセイブロックの評価を行った。新規評価法としては、米国環境保護庁(Environmental Protection Agency: EPA)の Dr. Chris Corton による eSTAR (Emerging Systems Toxicology for the Assessment of Risk)に関する webinar があり、肝発がんにおいては、AhR, CAR, ER, PPARa の活性及び cytotoxicity が非遺伝毒性発がん性の分子開始イベント(Molecular Initiating Event: MIE)として利用可能との提案があった。さらに、オランダ ライデン大学の Dr. Bob van de Water による weighted gene co-expression network analysis (WGCNA)に関する webinar では、TXG-MAPr webtool を用いた初代ヒト肝細胞のストレス応答遺伝子共発現分析について紹介があり、非遺伝毒性発がん物質と遺伝毒性発がん物質で誘導される遺伝子発現パターンの相違について、これらのデータの利用が提案された。IATA 開発においては、新規アッセイ法の有用性について注視すると共に、アッセイ系の評価が適切になされるよう、引き続き協力を続ける必要があると考えられた。

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A. 研究目的

2016 年に経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）において、非遺伝毒性発がん物質の統合的評価手法の確立を目標とした integrated approach to the testing and assessment (IATA) of non-genotoxic carcinogens (NGTxC) の専門家グループが立ち上げられた。非遺伝毒性発がん性の機序に関連する事象を抽出し、それぞれの事象を主に *in vitro* の試験系で評価する方法とその妥当性について、議論が継続されている。本研究では、当該 IATA 開発に協力すると共に、発がん性の有害転帰経路（Adverse Outcome Pathway: AOP）の開発、並びに生体における発がん機序に関する調査研究を実施し、化学物質のヒト発がん性に関する適切な評価を推進し、以て、日本の厚生労働行政に資することを目的としている。

B. 研究方法

B.1. 発がん性の AOP 開発

研究分担者小川は、研究協力者西川の協力を得て、ホルムアルデヒド誘発鼻腔発がん機序に関する論文に引き続き、各種化学物質暴露による鼻腔発がん全般の AOP について論文化に必要な情報を収集し、それらを解析した。ラット、マウス、ハムスターに鼻腔腫瘍を誘発する化学物質について、PubMed の文献に加えて、NTP、IARC、日本バイオアッセイ研究センターのデータベースを使用して抽出し、誘発された鼻腔腫瘍について、動物種、投与経路、組織型などを分類した。また、関連する非腫瘍性病変及び遺伝毒性のデータについても抽出し、腫瘍発生経路の推定をおこなった。

B-2. 非遺伝毒性発がん性の IATA 開発への協力

非遺伝毒性発がん性IATA開発専門家のweb会議に参加し、開発方針に関する議論及び最新の評価方法に関するwebinarに参加した。全体会合に加えて、当該IATAにおける13のアッセイブロックの内2つまたは3つを分担し、そのサブグループ会議にも参加し、現在あるアッセイの利用に関する考え方など論文化について検討した。

（倫理面への配慮）
該当なし

C. 研究結果

C.1. 発がん性の AOP 開発

網羅的に情報収集した鼻腔発がん物質のうち 40 種の吸入暴露による発がん物質（ラット 38 物質、マウス 11 物質、ハムスター5 物質）及び 38 種の非吸入暴露による発がん物質（ラット 36 物質、マウス 5 物質、ハムスター17 物質）について誘発された鼻腔腫瘍を、国際統一毒性病理用語・診断基準 (International Harmonization of Nomenclature and Diagnostic Criteria: INHAND)に基づいて分類した結果、扁平上乳頭腫、扁平上皮癌、腺腫、腺癌、腺扁平上皮癌、神経上皮癌、未分化癌、非特異的な癌、線維肉腫、血管腫、血管肉腫、粘表皮腫、横紋筋腫、横紋筋肉腫が報告されていた。最も高頻度の鼻腔腫瘍は扁平上皮癌であり、投与経路に関係なく認められ、その前駆病変として、扁平上皮化生及び/または扁平上皮乳頭腫と呼吸上皮過形成が示唆された。 2 番目に多いのは腺癌であり、その前駆病変として主に嗅上皮過形成が示唆されたが、腺腫の前駆病変は

呼吸上皮病変と考えられた。これらの経路はげっ歯類間で共通していると考えられるが、マウスまたはハムスターのデータは限定的であった。

C.2. 非遺伝毒性発がん性の IATA 作成への協力

OECD で進められている非遺伝毒性発がん性の IATA 開発に協力した。平成 30 年 6 月の会議において、非遺伝毒性発がん性に係る試験・検査のパラメータを優先順位に関して 4 つのカテゴリーに分けることになった。また、候補となるアッセイを 13 のブロックに分けて、分担してレヴューしている。

IATA 開発について、小川研究分担者は cell proliferation 及び resistance to apoptotic cell death のサブグループに、西川研究協力者は cell transformation, indicator of oxidative stress 及び resistance of apoptosis cell death のサブグループに参画し、アッセイブロックの評価を行った。cell proliferation においては、細胞増殖の評価に関する *in vitro*/*ex-vivo*/short term *in vivo* assay の非遺伝毒性発がん性のキーイベントとしての網羅的文献検索、オミックス解析の利用の可能性、規制への応用の可能性などを内容とする論文作成について議論された。

新規評価法としては、米国環境保護庁 (Environmental Protection Agency: EPA) の Dr. Chris Corton による eSTAR (Emerging Systems Toxicology for the Assessment of Risk)に関する webinar があり、肝発がんにおいては、AhR, CAR, ER, PPARa の活性及び cytotoxicity が非遺伝毒性発がん性の分子開始イベント(Molecular Initiating Event: MIE)として利用可能との紹介があった (Corton JC,et al., Toxicol Sci,177; 11-26, 2020)。また、オランダ　ライデン大学の Dr. Bob van de Water による weighted gene co-expression network analysis (WGCNA)に関する webinar があり、TXG-MAPr webtool (available at https://txgmapr.eu/WGCNA_PHH/TGGATEs_PHH/) を用いた初代ヒト肝細胞のストレス応答遺伝子共発現分析について紹介があった (Callegro G et al., Archive Tox, 95; 3745-75, 2021)。非遺伝毒性発がん物質と遺伝毒性発がん物質で誘導される遺伝子発現パターンの相違について、これらのデータを比較することも有用と考えられた。

D. 考察

D.1. 発がん性の AOP 開発

げっ歯類における化学物質誘発鼻腔発がんの網羅的解析結果は、各種鼻腔腫瘍の前駆病変は、遺伝毒性の有無に関係なく、腫瘍タイプの化学物質誘発性の細胞毒性と一般的に関連している可能性があり、分子開始イベント後の経路は、遺伝毒性発がん物質と非遺伝毒性発がん物質の間で大きく重複している可能性が示唆された。

D.2. 非遺伝毒性発がん性の IATA 開発への協力

OECDの非遺伝毒性発がん性のIATA開発においては、新規アッセイ法の有用性について注視すると共に、アッセイ系の評価が適切になされるよう、引き続き協力を続ける必要があると考えられた。

E. 結論

E.1. 発がん性の AOP 開発

げっ歯類における化学物質誘発鼻腔発がんの網羅的解析は、化学物質によって誘発される鼻腔腫瘍の病因の包括的な理解に貢献し、細胞毒性から鼻腔腫瘍発生に至る

AOPの解明、及び非遺伝毒性発がん物質のIATA開発などのOECDの活動に資するものと考えられた。

E.2. 非遺伝毒性発がん性の IATA 開発への協力

OECD の非遺伝毒性発がん性の IATA 開発に引き続き協力していくことが必要と考えられた。

F. 研究発表

F.1. 論文発表

1. Nishikawa A, Nagano K, Kojima H, Ogawa K. A comprehensive review of mechanistic insights into formaldehyde-induced nasal cavity carcinogenicity. Regul Toxicol Pharmacol. 2021; 123: 104937. doi: 10.1016/j.yrtph.2021.104937.
2. Nishikawa A. Perspectives on the elimination of animal assays in the assessment of carcinogenicity. Regul Toxicol Pharmacol. 2021; 126:105031. doi: 10.1016/j.yrtph.2021.105031.

F.2. 学会発表

該当なし

G. 知的財産権の出願・登録状況

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）
OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

発がん性試験におけるAOP及びTGの実験データ支援

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研究要旨

我々はこれまでに、DNA 損傷マーカーであるγ-H2AX が、化学物質の膀胱発がん性早期検出に有用な指標となることを示してきた。本研究では、腎発がん物質検出におけるγ-H2AX の感度・特異度の検証を目的とする。令和 3 年度は、腎発がん物質 5 種を用いたラット 28 日間反復経口投与試験を実施し、腎臓における病理組織学的検索およびγ-H2AX 形成の免疫組織化学的解析を行った。その結果、いずれの投与群でも、尿細管上皮細胞におけるγ-H2AX 形成が有意に増加することが明らかとなった。以上より、γ-H2AX 免疫染色は腎発がん物質の早期検出に有用である可能性が示唆された。

A. 研究目的

近年、化学物質の安全性評価の効率化・迅速化及び実験動物福祉（3R）の観点から、長期がん原性試験をより短期間の試験で代替する手法の開発が求められている。我々はこれまでに、DNA 損傷マーカーであるγ-H2AX が、化学物質の膀胱発がん性早期検出に有用な指標となり得ることを報告してきた。一方で、主要な毒性標的臓器である肝臓及び腎臓への応用を検討する必要があると考えられる。腎臓を対象として、これまでに 16 物質について検討した結果、γ-H2AX 免疫染色は膀胱と同様、腎発がん物質の早期検出にも有用である可能性が示唆された。

そこで本研究では、腎発がん物質早期検出におけるγ-H2AX 形成の感度及び特異度を検証することを目的とする。具体的には、新規の被験物質を用いた 28 日間反復経口投与試験を実施し、腎臓におけるγ-H2AX 形成の定量解析を行う。また、過去に実施した 28 日間反復投与試験で得られた腎臓についても同様に検討し、γ-H2AX 形成を指標とした腎発がん物質検出法の妥当性を評価する。

B. 研究方法

令和 3 年度の新規被験物質として、腎発がん物質 5 種 : Hexachlorobutadiene (HCBD)、1-Amino-2,4-dibromoanthraquinone (ADBAQ)、Dimethylnitrosamine (DMN)、*N*-Ethyl-*N*-hydroxyethylnitrosamine (EHEN) 及び Azoxymethane (AOM)を、6 週齢の雄 F344 ラットに 28 日間混餌（HCBD・ADBAQ・DMN）または飲水（EHEN・AOM）投与した（各群 5 匹）。各物質の投与濃度は短期試験における最大耐量として、300 ppm HCBD, 10000 ppm ADBAQ, 500 ppm DMN, 1000 ppm EHEN 及び 40 ppm AOM に設定した。ただし EHEN 投与群については、顕著な体重増加抑制が認められたため、3 週目以降投与濃度を 500 ppm に変更した。

投与期間終了時に解剖し、腎臓及び肝臓の重量を測定した。腎臓の病理組織学的検索を実施するとともに、免疫組織化学的手法によるγ-H2AX 形成の定量解析を実施した。右腎横断面から、皮質及び髄質外層外帯の特定部位を顕微鏡下（x400）でそれぞれ 4 か所撮影し、尿細管上皮細胞の総数ならびにγ-H2AX 陽性細胞数を元に、陽性細胞率を計測した。

（倫理面への配慮）

動物の数は最小限にとどめ、実験は国立医薬品食品衛生研究所の実験動物取扱い規定に基づき、動物の苦痛を最小限とするよう配慮して行った。

C. 研究結果

対照群及び各被験物質投与群の、体重・摂餌量・飲水量・被験物質摂取量を表 1 に示す。投与期間終了時点で、HCBD・DMN・EHEN・AOM 投与群において、対照群と比較して有意な体重増加抑制が認められた。摂餌量は HCBD・DMN・EHEN・AOM 投与群、飲水量は EHEN・AOM 投与群で低値の傾向を示した。

各群における臓器重量を表 2 に示す。HCBD・ADBAQ 投与群では腎絶対・相対重量の有意な増加が、DMN・EHEN・AOM 投与群では腎絶対重量の低下及び相対重量の増加が認められた。また、ADBAQ 投与群で肝絶対・相対重量の増加、HCBD 投与群で肝相対重量の増加、DMN・EHEN・AOM 投与群では肝絶対重量の低下が観察された。

腎臓の病理組織学的検索の結果を表 3 に示す。HCBD・ADBAQ 投与群では再生尿細管及び尿細管上皮への好酸性顆粒沈着増加等が、DMN・EHEN・AOM 投与群では間質における炎症性細胞浸潤、尿細管上皮細胞の変性/壊死及び核の大型化等が種々の程度で認められた。

腎尿細管上皮細胞におけるγ-H2AX 形成を免疫組織化学的に検討した結果、対照群では陽性細胞は稀であったのに対し、被験物質を投与した各群ではいずれもγ-H2AX 陽性細胞の有意な増加が認められた（図 1）。

D. 考察

令和 3 年度は、新規被験物質として腎発がん物質 5 種について、短期試験での最大耐量を用いたラット 28 日間反復経口投与試験を実施し、腎臓における病理組織学的検索及びγ-H2AX 形成の免疫組織化学的解析を行った。その結果、被験物質投与群ではいずれも、腎臓における病理組織学的所見及び尿細管上皮細胞でのγ-H2AX 形成が誘発されることが明らかとなった。これまでの検討結果を総合すると、腎発がん物質 14 物質中 13 種がγ-H2AX 陽性率の増加を引き起こした一方、非腎発がん物質 7 種ではいずれも対照群と同じレベルにとどまった。以上の結果から、γ-H2AX 免疫染色によって化学物質の腎発がん性を、短期間かつ高い感度及び特異度で検出可能であることが示唆された。

E. 結論

今年度の新規被験物質（腎発がん物質 5 種）はいずれも短期間の投与により、ラット腎尿細管上皮細胞におけるγ-H2AX 形成を有意に増加させることが明らかとなり、γ-H2AX 免疫染色は腎発がん物質の早期検出に有用である可能性が示唆された。

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G. 知的財産権の出願・登録状況

1. 特許取得
該当なし
2. 実用新案登録
該当なし
3. その他
該当なし

表 1. 各群における体重・摂餌量・飲水量・被験物質摂取量。

Table 1. Body weight and chemical intake data for male F344 rats

Treatment	Body weight (g)		Food consumption (g/rat/day)	Water intake (g/rat/day)	Chemical intake (mg/rat/day)
	Initial	Day 28			
Control	116.5 ± 3.4	225.2 ± 5.4	14.7	22.1	-
HCBD	116.7 ± 3.3	198.6 ± 8.8**	11.6	20.6	20.6
ADBAQ	116.7 ± 3.6	221.5 ± 5.8	13.7	21.9	742.5
DMN	116.4 ± 8.1	170.5 ± 12.1**	10.1	17.2	33.1
EHEN	116.7 ± 4.5	119.3 ± 28.4**	7.0	8.4	60.8
AOM	116.5 ± 4.0	171.6 ± 5.9**	11.8	14.0	3.8

**; $P < 0.01$ *vs.* Control by *t*-test.

HCBD, hexachlorobutadiene; ADBAQ, 1-amino-2,4-dibromoanthraquinone; DMN, dimethylnitrosamine; EHEN, *N*-ethyl-*N*-hydroxyethylnitrosamine; AOM, azoxymethane.

表 2. 各群における腎および肝重量。

Table 2. Liver and kidney weight data for male F344 rats

Treatment	Kidney weight		Liver weight	
	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
Control	1.53 ± 0.04	0.68 ± 0.02	8.0 ± 0.28	3.55 ± 0.08
HCBD	1.66 ± 0.08*	0.84 ± 0.02**	7.6 ± 0.31	3.81 ± 0.15**
ADBAQ	1.69 ± 0.02**	0.76 ± 0.01**	10.0 ± 0.34**	4.51 ± 0.24**
DMN	1.30 ± 0.04**	0.76 ± 0.05**	6.3 ± 0.69**	3.67 ± 0.20
EHEN	1.03 ± 0.15**	0.88 ± 0.08**	3.8 ± 1.20**	3.18 ± 0.55
AOM	1.29 ± 0.03**	0.75 ± 0.02**	6.4 ± 0.17**	3.71 ± 0.19

*, **; $P < 0.05$ and 0.01 *vs.* Control by *t*-test, respectively.

HCBD, hexachlorobutadiene; ADBAQ, 1-amino-2,4-dibromoanthraquinone; DMN, dimethylnitrosamine; EHEN, *N*-ethyl-*N*-hydroxyethylnitrosamine; AOM, azoxymethane.

表3. 各群における腎臓の病理組織学的所見。

Table 3. Histopathological evaluation in the kidney of male F344 rats treated with renal carcinogens for 28 days

	Control	HCBD	ADBAQ	DMN	EHEN	AOM
Findings						
Regenerative tuble (±, +)	0	5** (5, 0)	5** (3, 2)	4* (4, 0)	1 (1, 0)	1 (1, 0)
Inflammation, interstitial (±)	0	2	2	5**	0	4*
Degeneration/necrosis (±)	0	5**	3	1	5**	4*
Eosinophilic granule (±)	0	5**	5**	0	0	0
Karyomegaly (±)	0	0	0	0	3	2

* and **: significantly different from control at $P < 0.05$ and 0.01, respectively.

± and +: slight and mild, respectively.

HCBD, hexachlorobutadiene; ADBAQ, 1-amino-2,4-dibromoanthraquinone; DMN, dimethylnitrosamine; EHEN, *N*-ethyl-*N*-hydroxyethylnitrosamine; AOM, azoxymethane.

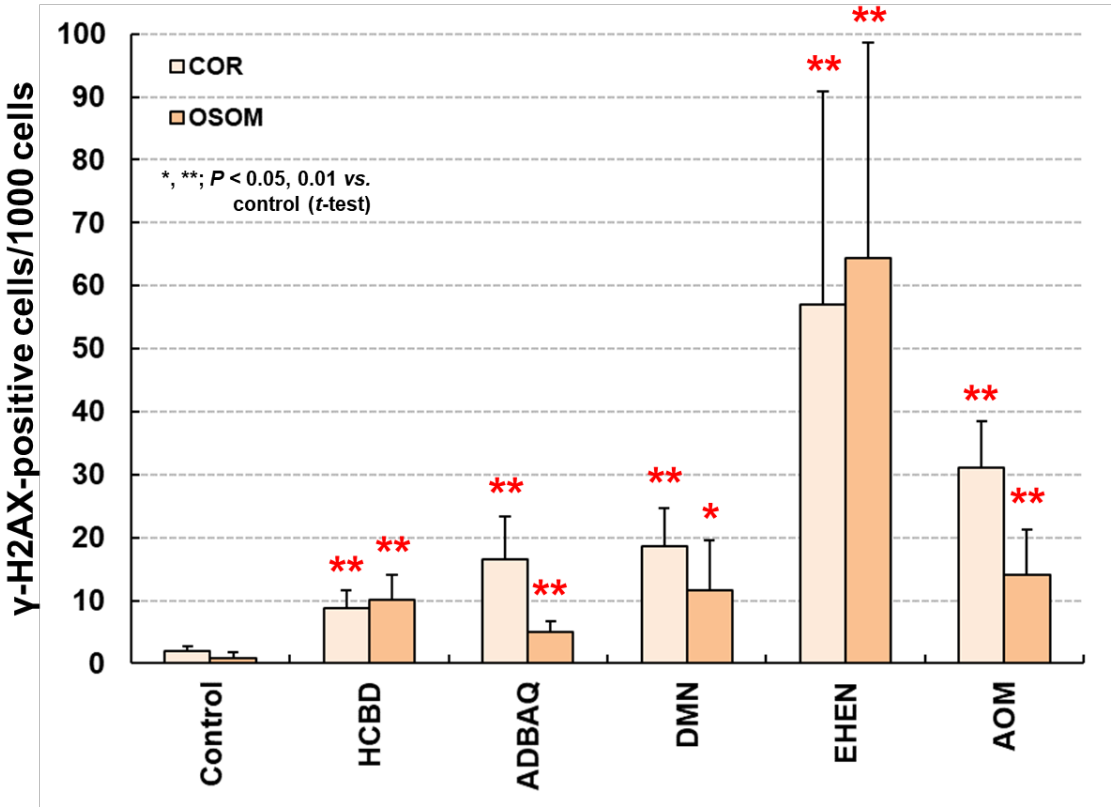

図 1. 腎尿細管上皮細胞における γ-H2AX 陽性率。COR：皮質、OSOM：髄質外層外帯。

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）
OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

遺伝毒性のAOP開発

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研究要旨

発がん性（遺伝毒性）の AOP への組み込みを想定し、遺伝毒性初期応答反応の早期検出システムの構築を試みた。遺伝毒性初期応答反応の検出にはクロマチン免疫沈降法（Chromatin immunoprecipitation; ChIP）を応用し、定量的 PCR を用いた DNA 損傷応答の分子生物学的解析を実施した。特に、数百コピーから成るクラスターを形成し、転写のメカニズムについての知見も豊富である rDNA を DNA 損傷応答解析の標的領域とした。RPA194、γH2AX、または Ku80 を ChIP の標的タンパク質とした解析結果から、本手法が DNA 上で直接的に生じている DNA 損傷応答を定量・定性的かつ早期に検出できることが示された。また、ChIP に用いる抗体をアレンジすることで解析の標的タンパク質を自在に設定することができるため、汎用性が高く、この試験法の発がん性・遺伝毒性 AOP 開発に対する高い有効性を示すことができると考えられる。

A. 研究目的

化学物質の生体曝露から遺伝毒性発現（遺伝毒性 AOP）に至る流れを全体的に捉えると、図 1 で示す経路で示すことができる。すなわち、化学物質に曝露後、場合によっては代謝を経たのちに DNA 上に付加体や DNA 損傷が形成される。それに対し、DNA 修復や DNA 複製などの損傷応答を経由することで、最終的に突然変異、DNA 鎖切断または染色体異常といったエンドポイントとしての遺伝毒性が発現する。したがって、遺伝毒性の発現結果そのものを検出する従来の一般的な遺伝毒性試験法では、結果を得るためにこれらの遺伝毒性発現サイクルが完了するまでの時間を要する。言い換えれば、バクテリアを用いる試験では遺伝毒性発現サイクルが短いために短期間で試験が終了するが、トランスジェニック変異試験のような試験ではより長い期間を要する。

その一方で、これらの遺伝毒性発現の基本的な概念は、バクテリア、培養細胞、高等生物で普遍的であり、また一般的に曝露から損傷応答反応が生じる期間は遺伝毒性発現期間と比べて非常に短時間である。すなわち、この流れをタイミングよく効率的に捉えることで化学物質の遺伝毒性を短期間で検出することができる。そのためには特に、DNA 付加体や DNA 損傷そのものはそれ自体が最終的に遺伝毒性に結びつかないことがあるため、遺伝毒性に直接結びつく細胞内の応答反応を直接捉える必要がある。以上を踏まえ、本研究では、発がん性の AOP への組み込みを想定し、遺伝毒性初期応答反応の早期検出システムを構築することを研究目的とする。

B. 研究方法

遺伝毒性初期応答反応の早期検出システムを構築するため、クロマチン免疫沈降法（Chromatin immunoprecipitation; ChIP）及び定量的 PCR を用いた DNA 損傷応答の分子生物学的解析を実施した。なお、一般的なコーディング DNA 領域と比べて、リボソーム DNA（ribosomal DNA; rDNA）は 1 細胞あたりヒトでは 200～700 コピーから成るクラスターを形成しており、また、DNA 代謝反応である転写のメカニズムについての知見も豊富であることから、rDNA を ChIP 及び定量的 PCR を利用した DNA 損傷応答解析の標的領域とした。

DNA 損傷の定量化が可能な紫外線照射による DNA 損傷を誘導した Flp-In 293 細胞を用いて、rDNA 上の転写装置である RNA polymerase I （RNAPI）の構成サブユニット RPA194、DNA 損傷応答マーカーであるヒストンバリアント γH2AX、または DNA 損傷応答タンパク質の一つとして知られる Ku80 を標的とした ChIP を実施し、RNAPI、γH2AX または Ku80 が局在する DNA 画分をそれぞれ調製した。これらの DNA 画分を鋳型 DNA とし、rDNA unit を転写領域及び非転写領域を含む領域に分けてそれらを標的とした 9 つのプライマーセット（H1～H42.9、図 2A）を用いた定量的 PCR により、DNA 損傷誘導時における RNAPI、γH2AX または Ku80 の rDNA 上での位置的相対量変化を解析することで、DNA 上で直接的に生じている DNA 損傷応答の定量・定性的検出を試みた。

（倫理面への配慮）
該当なし

C. 研究結果

遺伝毒性初期応答反応の早期検出システム構築に用いた rDNA unit 上のプライマーセット H1～H42.9 の内、H1～H13 は転写領域、H18～H42.9 は非転写領域を検出することができる。DNA 損傷を誘発しない条件において、RNAPI 共沈 DNA 中には H1～H13 の転写領域の DNA のみが均一に存在する一方で、紫外線 DNA 損傷誘発時の RNAPI 共沈 DNA 中には、転写領域の中でも H1 と H4 の DNA 領域が特に多く存在することが示された（図 2B）。一方で、DNA 損傷を誘発しない条件において、γH2AX 及び Ku80 共沈 DNA 中には rDNA unit 全領域の H1～H42.9 がわずかに、かつ、均一に存在しているが、紫外線 DNA 損傷誘発時の γH2AX 及び Ku80 共沈 DNA 中には H1～H42 がさらに多く存在し、その中でも特に H18 及び H27 の DNA 領域が多く存在することが示された（図 2C 及び D）。

D. 考察

発がん性の AOP への組み込みを想定した遺伝毒性初期応答反応の早期検出システム構築の研究では、RNAPI、γH2AX または Ku80 を標的とした ChIP 及び rDNA unit 領域の定量的 PCR の結果から、通常時の RNAPI は rDNA unit 中の転写領域のみに満遍なく存在している一方で、紫外線 DNA 損傷誘導時には転写領域の上流に局在することが明らかになった。これらのことから、通常時の RNAPI は滞りなく rDNA を転写しているが、紫外線 DNA 損傷誘導時には DNA 損傷上で転写が阻害され、RNAPI が転写開始領域近辺に蓄積していることが示唆される。他方、γH2AX 及び Ku80 については、通常時には rDNA 領域にはわずかにしか存在せず、DNA 損傷誘導時に強く rDNA 領域に局在することが明らかになった。特に、これらの局在は rDNA の非転写領域の上流 H18 及び H27 に偏っ

ており、この DNA 領域においてγH2AX 及び Ku80 が修復に関わるような DNA 損傷である DNA 鎖切断が生じていることを示唆する。

本手法は ChIP に用いる抗体をアレンジすることで解析の標的タンパク質を自在に設定することができる。今後は化学物質による DNA 損傷誘導時、そして、RPA194、γH2AX、または Ku80 以外の DNA 損傷応答タンパク質や DNA 修復・複製タンパク質等を標的とした同様の解析により、この試験法の発がん性・遺伝毒性 AOP 開発に対する高い有効性を示すことができる。

E. 結論

発がん性の AOP への組み込みを想定した遺伝毒性初期応答反応の早期検出システム構築の研究では、RPA194、γH2AX、または Ku80 を標的とした ChIP 及び rDNA 領域の定量的 PCR を利用した DNA 損傷応答の解析結果から、本手法が DNA 上で直接的に生じている DNA 損傷応答を定量・定性的かつ早期に検出できることが示された。また、ChIP に用いる抗体をアレンジすることで解析の標的タンパク質を自在に設定することができるため、汎用性が高く、この試験法の発がん性・遺伝毒性 AOP 開発に対する高い有効性を示すことができると考えられる。

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G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

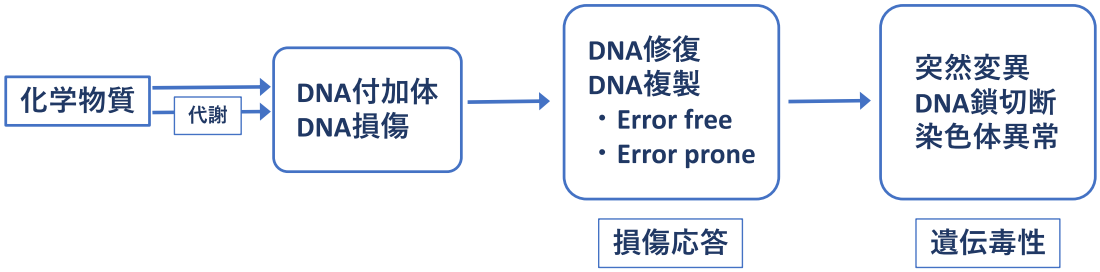

図 1. 化学物質の曝露から遺伝毒性発現まで

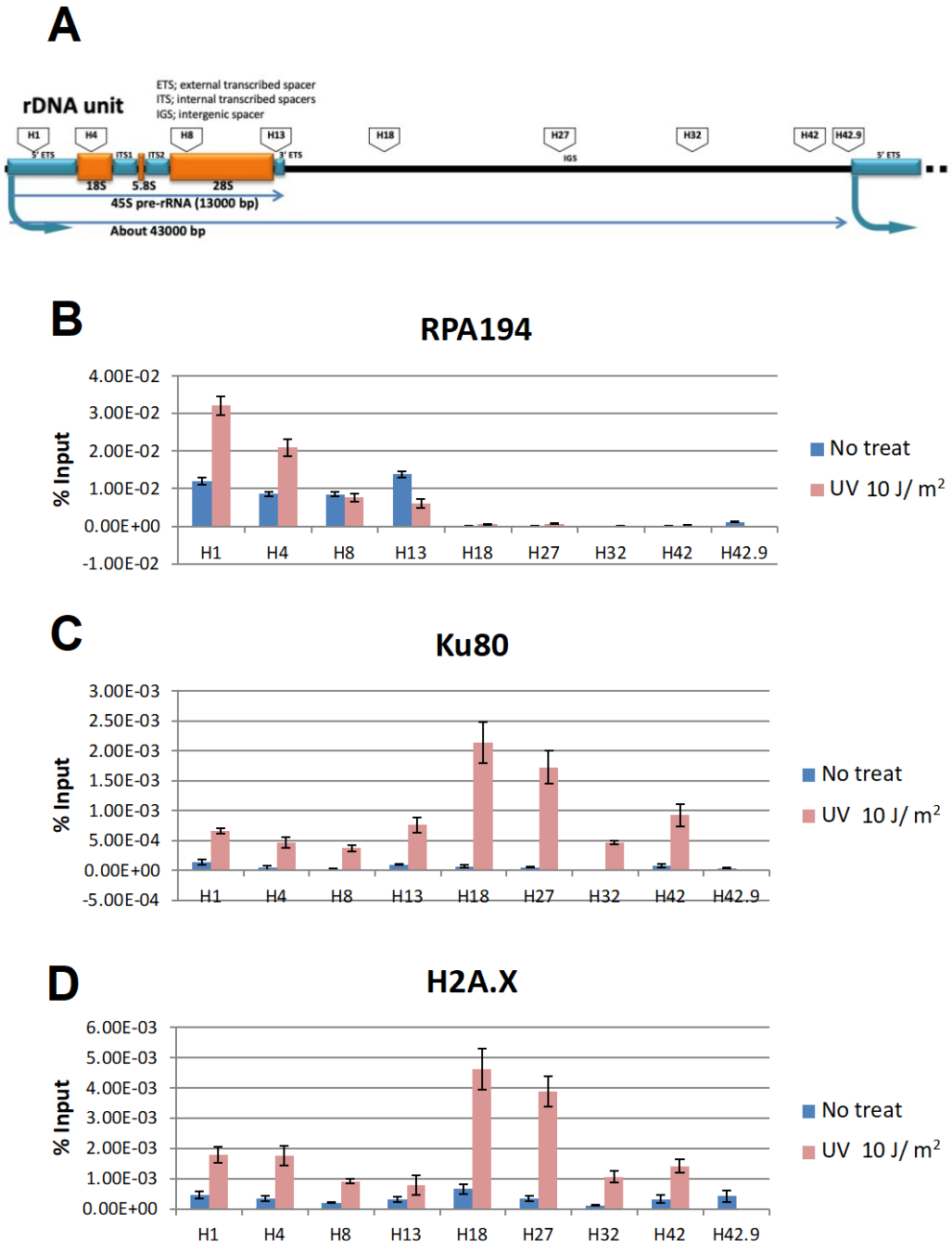

図 2.クロマチン免疫沈降法及び定量的 PCR を用いた DNA 損傷応答解析

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

免疫毒性のAOP及びTG開発

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研究要旨

日本免疫毒性学会 AOP 検討小委員会とともに、免疫毒性に関する 5 種の有害性発現経路（AOP: Adverse Outcome Pathway）の開発を行った。

昨年度外部レビューに進んだ「Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response (AOP154)」については、全ての評価プロセスが終了し、OECD Library において公開された。

AOP313, 314 及び 315 については、コーチ制による内部レビューを受けており、そのうち AOP315 については内部レビューが終了し、今後専門家による外部レビューに進む予定である。

本年度より新たに取り組むこととなった AOP277 については、external review に相当する scientific review に進んでいる。

A. 研究目的

免疫毒性は化学物質の安全性を評価するうえで重要な項目であり、その複雑さから IATA (Integrated Approaches to Testing and Assessment）による総合的な評価が必要と考えられている。IATA 確立のためには、免疫毒性に関する複数の AOP (Adverse Outcome Pathway)を開発し、ネットワーク化する必要がある。免疫毒性に関する各種 AOP を日本主導で開発することにより、将来の IATA 開発に大きく貢献するだけでなく、当該分野における日本の活動を世界にアピールすることを目的とする。

B. 研究方法

日本免疫毒性学会会員をメンバーとする同学会試験法委員会 AOP 検討小委員会に免疫毒性 AOP の開発を委託している（研究分担者も本委員会のメンバー）。文献調査の結果に基づいて、MIE (Molecular Initiating Event)、AO (Adverse Outcome)及びその間に介在する KE (Key Event)を定めて、OECD に指定された外部（または scientific）レビュアー及びコーチの指摘事項に対応することで開発を進めた。

（倫理面への配慮）
該当なし

C. 研究結果

「カルシニューリン阻害による T 細胞依存的抗体産生抑制 : AOP154」については、WNT/WPHA に提出したのち、ドイツからコメントがあり、AOP Wiki の改訂作業を行った。ドイツからの主な指摘は、TDAR (T cell Dependent Antibody Response)アッセイ

の方法をガイドラインごとに詳細に記載すること、本 AOP のみで免疫毒性試験が免除されることはなく、本 AOP は IATA 開発に利用されるべきであること、本 AOP の EU 地域での規制上の重要性についても記載することなどであった。指摘事項に対応し、OECD 事務局に改訂完了の連絡と著者回答ファイルを提出したところ、OECD 事務局から、本 AOP が WNT/WPHA で承認されたという通知があった。その後 OECD 事務局に著作権譲渡に関する著者全員の署名書類を提出し、2021 年 10 月 15 日に OECD iLibrary において公開された。

「TLR (Toll-like receptor) 7/8 への結合による乾癬様皮膚疾患の増悪 : AOP313」については、コーチとの web ミーティング時の指摘事項（測定結果だけでなく測定対象及び方法を記載すること、Taxonomic Applicability は AOP が環境毒性からきているためにある項目であり、サポートする evidence を KE や KER に記載すること、IL-23 について構造だけでなく機能も記載すること、IL-17 及び Th17 に focus するのであればそのことを明記することなど）に対応中である。

「免疫細胞に存在する ER (Estrogen Receptor)の活性化による全身性リテマトーデス(SLE)の増悪 : AOP314」については、AO 及び KE の置き換えなど大幅な見直しを検討していることをコーチに連絡した。

「JAK3 の阻害による T 細胞依存的抗体産生抑制 : AOP315」については、コーチに改訂完了の連絡と著者回答ファイルを提出し、その後コーチコメント受領した。コーチコメントに対する AOP Wiki 改訂作業を行い、再びコーチに改訂版を提出した。コーチからの主な指摘は、本 AOP の AO が AOP154 の AO と類似しているので同じイベントの場合は AOP154 の AO（KE984）にリンクさせ、別のイベントを作成したい場合は全体を修正すること、KER3 の定量的考察はデキサメタゾンがどのように STAT5DNA 結合を阻害するかをより詳細に説明すること、biological plausibility of KERs, essentiality of KEs 及び empirical support for KERs についてハンドブックに従ってサマリーテーブルを加えることなどであった。OECD より、外部レビューに入れる AOP があればチェックリストを提出して欲しいとの連絡を受けたコーチが、2021 年 11 月 8 日にチェックリストを OECD に提出したことから、今後外部レビューに進むことが想定される。AOP154 の場合外部レビュアーが決まってから OECD に承認されるまで約 1 年半かかっており、AOP315 も同程度の期間内の承認を目指す。

「IL-1 receptor 結合阻害 : AOP277」については、今年度より本分担研究に追加されたものであり、AOP 開発の引継ぎに関する web 会議を実施し、本 AOP の開発者である東北大学の相場節也先生、木村裕先生から説明を受けた。さらに Scientific レビュアーとの web 会議が行われ、review 結果について説明を受けた。その後 OECD から scientific review report を受領した。Review report における主な推奨事項は、IL-1R シグナルを阻害するストレッサーに特異抗体だけでなく化合物/医薬品を加えること、AP-1 など NF-kB が関与しない経路も考慮すること、T cell のタイプを明確にすること、増加する感染のタイプを明確にすることなどであった。現在対応案を作成中である。

D. 考察

開発中の AOP については、コーチ及び scientific reviewer のコメントに基づいて修正を行っている。IL-23 の機能の説明のよ

うに既存の情報を収集することで対応できるものもあるが、医薬品だけでなく化学物質のストレッサーを示すことなど情報がないものについては調査したことを示して納得いただく必要がある。また、AO が疾患の憎悪である AOP については、行政活用の観点からその汎用性に疑問が投げかけられており、すでに承認された AOP154 のように AO を TDAR (T cell Dependent Antibody Response)に変更するなど対応を検討する。今回 OECD に承認された AOP154 の KE の一つは IL-2 産生抑制であり、本年度日本から別途 OECD に提案した免疫毒性スクリーニング試験 IL-2 Luc assay のテストガイドライン化の理論的基盤となるため、その意義は大きいと考える。

E. 結論

AOP154 については全ての評価プロセスが終了し、OECD iLibrary において公開された。AOP313, 314 及び 315 については、コーチ制による internal review を受けており、そのうち AOP315 については今後外部レビューに進む予定である。本年度より新たに担当することとなった AOP277 については、external review に相当する scientific review に進んでいる。

F. 添付資料

1. OECD Series on Adverse Outcome Pathways No. 18
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4. AOP314-2022-01-12
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年会(2022.3.27, On line)

H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

OECD Series on Adverse Outcome Pathways No. 18

Adverse Outcome Pathway on inhibition of calcineurin activity leading to impaired T-cell dependent antibody response

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https://dx.doi.org/10.1787/3c988dde-en

Adverse Outcome Pathway on Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response

Series on Adverse Outcome Pathways No. 18

AOP No. 154 in the AOP Wiki platform

Foreword

This Adverse Outcome Pathway (AOP) on Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response, has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Party of the National Coordinators for the Test Guidelines Programme (WNT). The AOP has been reviewed internally by the EAGMST, externally by experts nominated by the WNT, and has been endorsed by the WNT and the Working Party on Hazard Assessment (WPHA) on 22 July 2021.

Through endorsement of this AOP, the WNT and the WPHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The OECD's Chemicals and Biotechnology Committee agreed to declassification of this AOP on 7 October 2021.

This document is being published under the responsibility of the OECD's Chemicals and Biotechnology Committee.

The outcome of the internal and external reviews are publicly available respectively in the AOP Wiki and the eAOP Portal of the AOP Knowledge Base at the following links: [internal review] [external review].

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Abstract

Calcineurin (CN), a protein phosphatase, is known to impair immune function when its phosphatase activation is inhibited. The relationship between CN and immune functions is well understood, and immunosuppressants that work by inhibiting CN have been developed.

CN inhibitors (CNIs) inhibit CN phosphatase activity to suppress many kinds of immune functions and have been used to prevent hyper immune reactions such as rejection and graft versus host disease (GVHD), and treat autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CNIs are reported to induce immunosuppression-derived adverse effects such as increased frequency and/or severity of infections and increased tumor incidences. CNIs might affect several T-cell derived immune functions to induce compromised host. Among the affected immune functions, T-cell dependent antibody response (TDAR) is an important factor to resist infections and thought to be the useful endpoint on evaluating immunotoxicity of chemicals; therefore, this AOP describes the linkage between the inhibition of CN activity and impairment of TDAR.

CN activity is inhibited when stressors bind to CN with their respective immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes with activator protein-1 (AP-1) that bind at the site of IL-2, IL-4 and other T cell -derived cytokine promoters is reduced, thereby suppressing production of these cytokines. Among the affected cytokines from each of the helper T cell subsets, reduced production of IL-2 and IL-4 affects the proliferation and differentiation of B-cells to suppress TDAR.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for inhibition of CN activity leading to impaired TDAR.

Since CN is produced in a vast variety of species, this AOP might be applicable to many mammal species, including humans and rodents.

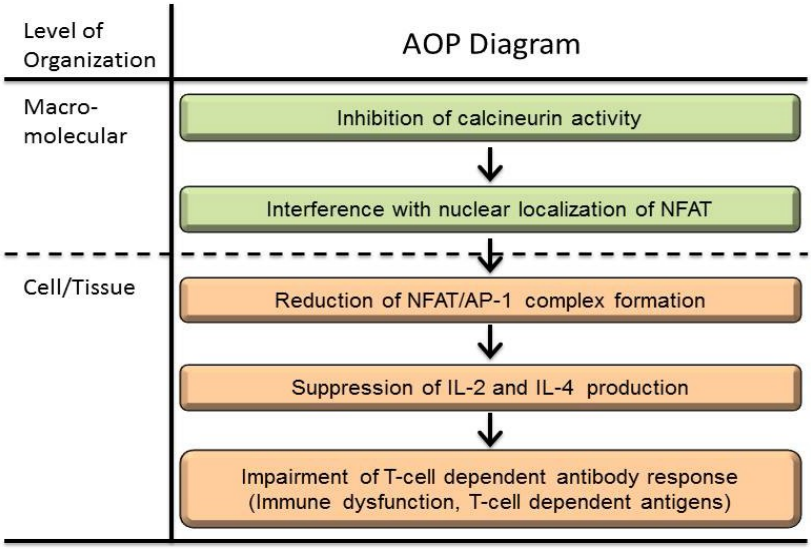
Contents

Background

Although there are other stressors that inhibit CN activity, this AOP is mainly based on an understanding of immunosuppression caused by the complex of FK506 and FKBP12 and cyclophilin and CsA, on which a significant body of scientific literature has been published.

We look forward to future amendments to this AOP with up-to-date information on other stressors, which will more clarify the linkage between inhibition of CN activity and impairment of TDAR.

Graphical Representation



Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	EventID	Title	Short name
1	MIE	980	Inhibition, Calcineurin Activity (https://aopwiki.org/events/980)	Inhibition, Calcineurin Activity
2	KE	979	Interference, nuclear localization of NFAT (https://aopwiki.org/events/979)	Interference, nuclear localization of NFAT
3	KE	981	Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/events/981)	Reduction, NFAT/AP-1 complexformation
4	KE	1202	Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)	Suppression, IL-2 and IL-4production
5	AO	984	Impairment, T-cell dependent antibody response (https://aopwiki.org/events/984)	Impairment, T-cell dependent antibody response

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Calcineurin Activity (https://aopwiki.org/relationships/1508)	adjacent	Interference, nuclear localization of NFAT	Moderate	Moderate
Interference, nuclear localization of NFAT (https://aopwiki.org/relationships/1017)	adjacent	Reduction, NFAT/AP-1 complex formation	High	High
Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/relationships/1509)	adjacent	Suppression, IL-2 and IL-4production	High	High
Suppression, IL-2 and IL-4 production (https://aopwiki.org/relationships/1510)	adjacent	Impairment, T-cell dependentantibody response	High	High

Stressors

Name	Evidence
Tacrolimus (also FK506)	High
Cyclosporin	High
Pimecrolimus	High
Gossypol	Moderate
Kaempferol	Moderate
Dodecylbenzene sulfonate	Moderate
Dibefurin	Moderate
Ascomycin	Moderate
1,5-dibenzoyloxymethyl-norcantharidin	Moderate

Overall Assessment of the AOP

Inhibition of CN might induce suppression of cytokines production from all the T helper cell subsets as well as other immune functions of other immune cells. Suppression of cell-mediated immunity is involved in the pharmacology of preventing hyper immune reactions such as rejection and GVHD, and treatment of autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CN inhibition might induce immunosuppression-derived adverse outcomes. One of the effects is increased frequency and/or severity of infections. Compromised host might be related with impairment of multiple immune functions; however, impaired TDAR deems to be usually related. Moreover, TDAR is the frequently used measurable endpoint in immunotoxicity testing according the ICH S8 or US EPA OPPTS 870.7800 immunotoxicity testing guideline. Therefore, the present AOP focus on CN inhibition-induced impairment of TDAR.

When stressors bind to calcineurin-A (CnA) with immunophilin, CN phosphatase activity is inhibited. Immunophilins are composed of a family of highly conserved proteins that have the ability to bind immunosuppressive drugs. This interfere with the nuclear localization of NFAT, the substrate for CN. As a result, the formation of functional NFAT/ AP-1 complexes that bind at the site of IL-2, IL-4 and other cytokine promoters in each of the T helper cell subsets is reduced, thereby suppressing production of these cytokines. Among the affected cytokines TDAR is impaired mainly by the suppression of production of IL-2 and IL-4, which affect the proliferation and differentiation of B-cells to lower TDAR. We have identified a number of key events (KEs) along this pathway, and based on these key event relationships (KERs), created an AOP for inhibition of CN activity leading to impaired TDAR.

Since each KE involving MIE and AO is quantifiable, and shows similar dose responses with the CNIs in vitro, this AOP is useful for understanding immunosuppression due to inhibition of CN activity. In addition, each KER is based on sufficient scientific evidence and exhibits no contradiction with dose responses of adjacent KEs.

Since CN/NFAT system is conserved among vast variety of species and the function in immune system is common in at least human and mice, this AOP might be applicable to many mammalian species, including humans and rodents.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Macaca fascicularis	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Unspecific	High

The proposed AOP regarding inhibition of CN activity leading to impaired TDAR is not dependent on life stage, sex, or age. Since tacrolimus (FK506) ointment (Protopic) is approved for pediatric atopic dermatitis, the MOA for immunosuppression appears to be applicable to all life stages. The applicable state is considered supported by the draft FDA guidance for immunotoxicology that was recently issued (2020) indicating that “example of immunotoxicology testing could included TDAR assay” to address the concern of immunotoxicity in offspring in juvenile animal studies.

Since FK506 or CsA-induced outcomes in humans are mimicked by similar responses in a variety of animal models including non-human primates and rodents, immunosuppression induced by inhibition of CN activity is considered to occur across a variety of mammalian species.

In addition to the drugs, it is known that CN activity is suppressed by alkeylbenzene sulfonate (dodecylbenzene sulfonate) extracted from an acrylonitrile butadiene rubber (Ito et al. 2013), suggesting that the proposed AOP would be applicable to non-pharmacological agents.

For the chemicals such as pesticide, the TDAR assay is also recommended in the US EPA OPPTS 870.7800 immunotoxicity testing guideline.

Essentiality of the Key Events

Essentiality is supported by several knockout animals as follows.

Stage	Essentiality	Evidence	Supported by literatures
MIE and later	CnA-KO mice	Strong	The CN molecule consists of two regions, CnA and CnB, of which CnA exhibits phosphatase activity. In CnA-KO mice, T-cell proliferation in response to ovalbumin stimulation is lower than that for wild-type mice and is not complemented by normal antibody producing cells. In addition, when stimulated with ovalbumin, CnA-KO mice produce less IFN-γ, IL 2, and IL 4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to TNP-ovalbumin, which means that CnA deficiency affects only T cell-dependent antibody response (TDAR) (Zhang et al. 1996).
KE1 and later	NFAT-KO mice	Strong	The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly, moderately enhanced B- and T-cell responses, with bias towards Th2-cell response, decreased IFN-γ production in response to T-cell receptor (TCR) ligation, reduced proliferative responses by T cells, impaired repopulation of the thymus and lymphoid organs, impaired Th2- cell responses and IL-4 production, grossly impaired T-cell effector functions, profound defects in cytokine production and cytolytic activity, B-cell hyperactivity, impaired development of CD4 and CD8 single-positive cells, increased apoptosis of double-positive thymocytes, and mild hyperactivation of peripheral T cells. Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by CN. Suppression of T-cell-derived cytokines is noted both in CnA-knockout and NFAT-knockout mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system (Macian, 2005).
Stressor	FKBP12-KO mice	Moderate	FK506 induces suppression of immune responses; however, there is no literature showing a relationship of a relationship between FKBP12 knockout and the immune system in the FKBP12-KO mouse model. Steric structure of FKBP12/FK506 complex is considered the key factor for inhibition of CN phosphatase activity, but not for the enzymatic activities of FKBP12.

Weight of Evidence Summary

Biological Plausibility

T-cell functions are mainly regulated by the CN-NFAT system and suppression of CN activity in T cells is known to induce multiple types of immunosuppression, including T cell-dependent antibody response (TDAR).

Experiments with T cells indicate that TCR stimulation brings about increases in intracellular concentrations of Ca2+ that trigger CN activity, thereby inducing nuclear localization of substrate NFAT per dephosphorylation. The localized NFAT forms complexes with activator protein 1 (AP-1) at the promoter sites of the T cell cytokine genes and induces production of the cytokines.

CN phosphatase activity is known to be inhibited by the formation of immunophilin-CN inhibitor (CNI) complexes, such as CsA/cyclophilin complexes or FK506/FK506-binding protein (FKBP) 12 complexes. Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity, but there is no similarity between amino-acid sequences of the two classes of immunophilins. The three-dimensional structures of immunophilin complexes are essential to the inhibition of CN phosphatase activity, even though their enzymatic activities are not.

It is also known that one of the effects on immune function when CNI forms complexes with its respective immunophilin and inhibits CN activity is the suppression of IL-2 and other T-cell derived cytokine production. It is further known that inhibition of CN leads to suppression of TDAR because IL 2 and IL 4 mainly promote the proliferation, class switching, differentiation, and maturation of B-cells.

Furthermore, CN-NFAT also exists in B cells and it has been reported that CNIs do suppress production of certain cytokines from them. At the time of our review of the literature, however, we did not find any reports of a direct effect of CN inhibition on B cells, such as changes in proliferation, class switching, differentiation, or maturation of B cells.

Also, although CN-NFAT is known to exist in dendritic cells, natural killer T (NKT) cells, and other types of cells in which it regulates the expression of IL-2 receptors, there are no reports of effects on the production of T cell-dependent antibodies due to CNI-induced alteration in expression of IL-2 receptors in these cells.

CN-NFAT system-mediated immunosuppression is well understood based on the pharmacology of some CNI drugs (mostly FK506 and Cs A); therefore, AOP of CN inhibition-induced suppression of TDAR is useful for prediction of CN-mediated immunotoxicity.

KER	KEup-KEdown	Evidence	Rationales supported by literatures
KER 1	CN inhibitionto interference, NFAT nuclear translocation	Moderate	CN phosphatase activation through TCR stimulation dephosphorylates NFAT, thereby promoting nuclear localization of NFAT. CN phosphatase activity in T cells could be inhibited by CNI/immunophilin complexes, thus interfering with dephosphorylation and nuclear localization of NFAT. The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPlase activity, but modification of these functions is unrelated to inhibition of CN activity and thus thought to arise in the molecular structure of the complexes (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).
KER 2	Interference, nuclear localization NFAT to reduction, NFAT/AP-1 complex formation	Strong	CN activity dephosphorylates NFAT, thereby promoting its nuclear translocation. Nuclear-located NFAT binds with AP-1 at the promoter regions of the cytokine genes to promote T-cell cytokine production. Inhibition of dephosphorylation of NFAT by CNIs prevents nuclear export of NFAT and resultant binding with AP-1 at the promoter region of the T cell cytokine genes. NFAT has NLS and NES domains among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS domain is exposed whereas the NES domain is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000). CNIs interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).
KER 3	Reduction, NFAT/AP-1 complex formation to suppression of IL-2 and IL-4 production	Strong	NFAT/AP-1 complexes bind to the promoter regions of the cytokine genes, which promotes production of cytokines in T cells. Of these cytokines, IL-2 and IL-4 have a major role in promoting proliferation, maturation and class-switching of B cells, and development of TDAR. Reduction of NFAT/AP-1 complex formation in the nucleus due to inhibition CN activity by CNIs suppresses production of T-cell derived cytokines, including IL-2 and IL-4. T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015). Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001). Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).

KER 4	Suppression of IL-2 and IL-4 production to impaired TDAR	Strong	T cell-derived cytokines play important roles in TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation. Inhibition of CN activity by CNIs is known to suppress production of multiple cytokine species from T cells. Of these cytokines and receptors, suppression of IL-2 and IL-4 production mainly leads to impairment of TDAR. Suppressed production of other cytokines due to inhibition of CN activity exhibits only minor effects, if any, on TDAR. CsA is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in reduced productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015). Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production. Th2 cells produce cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 in Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells. IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one of the IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Empirical Support

KER	KEup-KEdown	Evidence	Empirical support of KERs
			CN phosphatase activity is inhibited with IC50 values of 0.5 nM (FK506) and 5nM (CsA) after 1 hours treatment (Fruman et al.1992).
KER 1	Inhibition, calcineurin activity leadsto interference, nuclear localization of NFAT	Moderate	Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at concentrations from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 μM (1000 nM) under the conditions of 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991).
			CN phosphatase activity and nuclear translocation of NFAT seems to be suppressed by CNIs at the similar ranges of doses and reaction times of 1 to 2 hours.

KER 2	Interference, nuclear localization of NFAT leads to reduction, NFAT/AP-1 complex formation	Strong	Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 μM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013) Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991). Gel mobility shift assays using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1μM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to bind IL-2 promoters (Flanagan et al. 1991). NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997). Quantitative data on NFAT/AP-1 complex formation in the nucleus is insufficient; however, inhibition of nuclear localization of NFAT and following NFAT/AP-1 complex formation in the nucleus are simultaneously detected by gel mobility shift assays at the concentration of FK506 within the range for inhibition of nuclear translocation of NFAT using imaging flowcytometry after 2 hours culture of T cells.
		Moderate	In NFATp- and NFAT4-deficient mice, cultured splenocytes boundanti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).
			In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 μg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 μg/mL (Yoshida et al. 2015).
			In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 μM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 μM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM for 4 h (Checker et al. 2012).
			Gel mobility shift assay revealed that treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders NFAT nuclear translocation and following formation of NFAT/AP-1 complexes in the nucleus (Flanagan et al. 1991).
KER	Reduction, NFAT/AP-1		Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993). In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at 10 nM (Dumont et al. 1998).

3	complex formation leads to suppression, IL-2 and IL-4 production		Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).
			In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).
			Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).
			In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003).
			Therefore, concentration of CNI needed for inhibition of NFAT/AP-1 complex formation in the nucleus is higher than that for inhibition of IL-2 and IL-4 production. A time lag is found between the two KEs; 2 hours for KE2 and 22 to 48 hours for KE3.

KER 4	Suppression ,IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response	Strong	Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015). In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4 weeks (Sanofi K.K. 2018). In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines such as IL-4 and IL-5 on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013). 1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010). In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH (keyhole limpet hemocyanine) and decrease in anti-KLH IgG(Alessandro, B. et al. 2003). FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a). In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998). Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004). Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b). After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2010). After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001). In vitro suppression of T-cell–derived cytokines and T-cell–dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two events, which showed earlier onset of cytokine production and delayed onset of antibody production.

Based on these findings of empirical support, each KE involving MIE and AO except for KE2 shows similar dose responses to the CNIs in vitro; however, culture time lag is noted, in that, 1 hour for MIE, 2 hours for KE1 and KE2, 22 to 24 hours for KE3 and more than days for AO.

Quantitative Understanding

KER1

No literature is available showing a clear quantitative relationship between the inhibition of CN phosphatase activity and nuclear translocation of NFAT; however, the dose responses of CN phosphatase activity and nuclear translocation of NFAT to CNI deem to be the same.

KER2:

Gel mobility shift assay of activated T cells showed that NFAT/AP-1 complexes are only found in nuclear extract, which indicates a strong relationship between the nuclear translocation of NFAT and simultaneous complex formation with AP-1 in the nucleus. CNI treatment clearly suppresses the complex formation of nuclear located NFAT and AP-1 in the nucleus, which also shows the solid relationship between these adjacent two KEs although quantitative data on suppressed NFAT/AP-1 complex formation is insufficient (Flaganan W.M. et al. 1991).

KER3:

The quantitative relationship between the decreased formation of NFAT/AP-1 complexes and the production of IL2/IL-4 formation induced by CNIs has not been reported.

However, as mentioned in the empirical support, nuclear localization of NFAT is strongly related to NFAT/AP-1 complex formation in the nucleus based on the fact that these two events are detected simultaneously by gel mobility shift assay, and the dose responses of IL2/IL-4 production and nuclear translocation of NFAT inhibited by CNI are similar; therefore, dose ranges of CNI in the inhibitions of IL2/IL-4 production and NFAT/AP-1 complex formation in the nucleus might also be the same.

In addition, T-5224 and ursolic acid inhibit AP-1 DNA binding activity or production of NF-κB, NFAT and AP-1, respectively, and both suppress the IL-2 and/or IL-4 production with dose dependent manner including the doses of inhibiting NFAT-AP-1 system (Yoshida et al. 2015, Checker et al. 2012).

KER4:

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).

Inhibition of IL-4 production in mice treated with oral administration of suplatast tosilate suppresses antigen-specific IgE production in a dose-dependent manner (Taiho Pharmaceutical 2013). In the inhibition of IL-4 production in human cell culture by suplatast tosilate at the concentration of 10 μg/mL for 10 days, antigen specific IgE production was suppressed from 56 to 72% and IL-4 production was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the levels of T-cell cytokines including IL-2 and IL-4 and inhibited IgM and IgG productions with a dose-dependent manner (Heidt S. 2010).

These results show the quantitative relationships between the inhibition of IL-4 or IL-2 by specific antibodies or CNI and suppression of antibody production.

Considerations for Potential Applications of the AOP (optional)

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

In AOP154, it was difficult to define susceptibility to infection as an AO for the AOP154, so TDAR, which is recommended as an indicator of immunosuppresoin in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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Appendix 1 - MIE, KEs and AO

List of MIEs in this AOP

Event: 980: Inhibition, Calcineurin Activity (https://aopwiki.org/events/980)

Short Name: Inhibition, Calcineurin Activity

Key Event Component

Process	Object	Action
binding	FK506-binding protein 15	increased
binding	FKBP12 (Arabidopsis thaliana)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	MolecularInitiatingEvent

Stressors

Name
Tacrorimus
Cyclosporin
Pimecrolimus
Dodecylbenzene sulfonate
Dibefurin
Gossypol
Ascomycin
Kaempferol
1,5-dibenzoyloxymethyl-norcantharidin
Tacrolimus (also FK506)

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

CN inhibitory activities (IC50) are shown in follows.

Tacrorimus: 0.4nM

Cyclosporin: 7nM

Pimecrolimus: 0.4 nM

Dodecylbenzene sulfonate 9.3 uM

Dibefurin: 44 uM

Gossypol: 17 uM

Ascomycin: 0.7 nM

1,5-dibenzoyloxymethyl-norcantharidin: 7 uM

Kaempferol: 51.3 uM

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus rattus	Rattus rattus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10117)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN is broadly distributed in T-cells, B cells, and throughout the body. The structure of CnA and CnB is highly conserved from yeasts to humans. Also highly conserved are the amino acid sequences of the catalytic and regulatory domains of CnA isoforms from different organisms (Kincaid. 1996).

As for immunophilins, of which complexes inhibit the CN activity, FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989a). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all living beings have PPIase activity (Wang P et al. 2005).

However, inhibition of CN phosphatase activity through immunophilin-CNI complex has been reported at least in rodents and humans.

Key Event Description

Calcineurin (CN) is a heterodimer that comprises a catalytic subunit (CnA), which handles phosphatase activity as well as calmodulin binding, and a Ca-binding regulatory subunit (CnB), which regulates intracellular calcium as well as CnA (Klee et al. 1988, Zhang et al. 1996). CnA, a 59kDa protein, has a serine-threonine phosphatase domain.

Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity (Barik. 2006) and an immunophilin-CN inhibitor (CNI) complex such as FKBP12- FK506 and cyclophilin-CsA binds directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which inhibits the phosphatase activity of CN without any contribution of PPIase activity (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

How it is Measured or Detected

Phosphatase activity can be measured using a phosphatase assay. CN, calmodulin, FK506, and FKBP are incubated together, and the phosphatase activity is measured at various concentrations of FKBP. Kinetic analysis of FKBP12 concentration-dependent phosphatase activity and calculation of the CN inhibition constant Ki by the FKBP12-FK506 complex are conducted. (Bram et al. 1993). Phosphatase activity of CN in the presence of cyclosporin A (CsA) , gossypol or dibefurin can also be determined in a similar manner (Sieber et al. 2009).

Immunophilin-CNI complexes directly inhibit phosphatase activity of CN, therefore, as a surrogate measurement of the CN activity, the binding of CsA with cyclophilin can be detected using an ELISA kit. Microtiter plates precoated with BSA and conjugated to cyclosporin are incubated with cyclophilin. Bound cyclophilin is then revealed by incubation with anti-cyclophilin rabbit antiserum followed by incubation with anti-rabbit globulin goat IgG coupled to alkaline phosphatase (Quesniaux et al. 1987).

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List of Key Events in the AOP

Event: 979: Interference, nuclear localization of NFAT (https://aopwiki.org/events/979)

Short Name: Interference, nuclear localization of NFAT

Key Event Component

Process	Object	Action
genetic interference	NFAT protein	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientifi cTerm	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

Key Event Description

The nuclear factor of activated T cells (NFAT) is a substrate of calcineurin (CN) (Rao et al. 1997). A NFAT has an N-terminal with a plurality of SP motifs rich in serine and proline, which are controlled by means of phosphorylation and dephosphorylation. There is a nuclear localization signal (NLS) held between these SP regions as well as a nuclear export signal (NES) in the N-terminal adjacent to the SP motifs (Beals et al. 1997, Zhu and McKeon 1999, Serfling et al. 2000). SP motifs ordinarily are phosphorylated, which covers the NLS and leaves the NES exposed, so that NFAT localizes in cytoplasm. When SP motifs are dephosphorylated by activated CN, the NLS is exposed and the NES is covered, thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999). When T-cell activation takes place, T-cell–receptor- mediated stimulus increases the intracellular concentration of calcium and activates a regulatory subunit (CnB), which subsequently induces a catalytic subunit (CnA) phosphatase activation, leading to dephosphorylation of NFAT thereby promoting nuclear localization of NFAT. CNI-immunophilin complexes inhibit CN phosphatase activation, thereby interfering with NFAT nuclear localization (Bhattacharyya et al.2011).

Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at concentrations from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 μM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013).

How it is Measured or Detected

Nuclear translocation of NFAT can be tested by imaging flowcytometer, in which lymphocytes are treated with fluorescence-labeled anti-NFAT antibody and DAPI (nuclear stain) and intracellular distribution of NFAT is analyzed by imaging flowcytometry with image analysis (Maguire O et al. 2013).

Interference with translocation of NFAT to the nucleus can be detected using gel mobility shift assays of nuclear or cytoplasmic extracts electrophoresed with end-labeled NFAT-binding site from human IL-2 enhancer (Flanagan et al. 1991).

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Event: 981: Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/events/981)

Short Name: Reduction, NFAT/AP-1 complex formation

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	NFAT activation molecule 1	decreased
cell activation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientifi cTerm	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Description

Activated nuclear factor of activated T cells (NFAT) that has localized to the nucleus binds cooperatively at the site of the Interleukin-2 (IL-2) promoter with activator protein-1 (AP-1), which is a heterodimer comprising a Fos and a Jun protein (Schreiber and Crabtree 1992, Jain et al. 1992), thereby inducing transcription of IL-2 (Jain et al. 1993). Interfered nuclear localization of NFAT, induced by FK506, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991).

Besides IL-2, NFAT is known to bind cooperatively at the promoters of other T-cell cytokines, such as Interleukin-4 (IL-4) (Macian et al. 2005).

Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991).

How it is Measured or Detected

Reductions in NFAT/AP-1 complex formation can be detected using a gel shift assay to test nuclear extracts from either stimulated or unstimulated Ar-5 T cells with radio-labelled NFAT binding oligonucleotide from murine IL-2 promoter. Anti-Fos and anti-Jun antibodies are used to examine NFAT/AP-1 complex formation (Jain et al. 1992).

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Event: 1202: Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)

Short Name: Suppression, IL-2 and IL-4 production

Key Event Component

Process	Object	Action
interleukin-2 production	interleukin-2	decreased
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin
Dexamethasone
Azathioprine
Methotrexate
Benzo(a)pyrene
Urethane
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
cynomolgu smonkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNIs suppress production of IL-2, IL-3, IL-4, IL-5, IFN-γ, Granulocyte Macrophage colony-stimulating Factor (GM-CSF), and other cytokines, as induced by CD2/CD3 or CD3/CD26 stimulation, in human peripheral blood mononuclear cells (PBMC) (Sakuma et al. 2001a). Also, CNIs (FK506 and CsA) suppress production of IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, Tumor Necrosis Factor-α, IFN-γ, and GM-CSF, as induced by CD3/PMA stimulation, in human PBMC (Dumont et al. 1998).

CNIs (FK506 and CsA) exhibit suppression of IL-2 production induced from mixed lymphocyte reactions in mice and humans (Kino, T et al. 1987a).

Treatment with CsA or 2C1.1 resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

These facts indicate that Calcineurin-NFAT system-mediated suppression of cytokines is commonly found in humans, monkey and mice.

Key Event Description

Production of T cell cytokines including Interleukin (IL)-2 and IL-4 is regulated by nuclear factor of activated T cells (NFAT)/ activator protein-1 (AP-1) complexes. Activated NFAT/AP-1 complex that bind at the site of the IL-2 and IL-4 promoters, thereby induces transcription of IL-2 and IL-4 (Jain et al. 1993). For IL-2, NFAT proteins are necessary for IL-2 gene expression and cooperation of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab

reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016).

Calcineurin inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) hinder the formation of the functional NFAT/AP-1 complexes by interfering with NFAT nuclear localization (Flanagan et al. 1991). Reduced binding of NFAT/AP-1 complexes at the promoter site of the IL-2 gene lowers the transcription of the mRNA of IL-2 and the following cytokine production.

Transcription of IL-4 is also inhibited by CNIs in the same manner as IL-2 (Dumont et al. 1998).

In CD3/ phorbol 12-myristate-13-acetate (PMA)-activated human T cells, FK506 suppressed production of IL-2, IL-4, and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at 10 nM (Dumont et al. 1998).

Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (KLH) (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).

Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

CNIs is considered to increase carcinogenicity through the suppression of IL-2 and IL-4 production.

- Renal transplant patients on immunosuppressive therapy were found to develop cancer within 10 years after surgery (Luster, M.I. et al. 1993).

In experimental animal studies, carcinogenicity of FK506 was reported as follows.

- In mice subjected to topical application testing, in which 100 μL of FK506 ointment was applied once daily for two years to roughly 40% of the total body area, an increased incidence of lymphoma was found in mice of the 0.1% ointment group showing high blood concentrations of the drug (Maruho Co., Ltd 2014).
- In hairless albino mice, virtually all of which developed skin tumors after a 40-week exposure to ultraviolet light, application of a 1% FK506 ointment reduced the time to outbreak of the skin tumors. (Maruho Co., Ltd 2014).

How it is Measured or Detected

Quantitation of cytokine content was done on appropriately diluted samples, run in duplicate, using Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) kits to test matched Antibody pairs with biotin-horseradish peroxidase-streptavidin detection and 3,3',5,5'-tetramethylbenzidine substrate. ELISA plates were scanned in a Molecular Devices UVmax plate reader (Menlo Park, CA), using SOFT max software (Molecular Devices) (Dumont et al. 1998).

Ex vivo whole blood stimulated cytokine (IL-2, IL-4, IL-5, and IL-17) production assay in the supernatants were determined using an electrochemiluminescent immunoassay from Meso Scale Discovery (MSD; Gaithersburg, MD) (Kevin, G. et al. 2014).

Total RNA was extracted using RNeasy mini kit (Qiagen, Chatsworth, CA) and quantitated by absorbance at 260 nm. Cytokine mRNAs were detected using a RiboQuant MultiProbe RPA system (PharMingen, San Diego, CA). Riboprobes were 32P-labeled and hybridized overnight with 10 to 30 mg of the RNA samples. The hybridized RNA was treated with RNase and purified according to the RiboQuant protocol. The samples were then electrophoresed in 6% polyacrylamide-Tris-borate-EDTA-urea gels using the Seqi -Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Hercules, CA), or minigels (Novex, San Diego, CA). The gels were dried, exposed and quantitated in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software (Dumont et al. 1998).

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List of Adverse Outcomes in this AOP

Event: 984: Impairment, T-cell dependent antibody response (https://aopwiki.org/events/984)

Short Name: Impairment, T-cell dependent antibody response

Key Event Component

Process	Object	Action
Immunosuppression		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	Adverse Outcome

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
cynomolgus monkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence

All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNIs induced impairment of TDAR is demonstrated with rodent studies. That is, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino et al. 1987). Likewise, oral administration of FK506 to rats over a four-week period reduced production of both anti-KLH-IgG and IgM antibodies after subcutaneous immunization with KLH (Ulrich et al. 2004). Moreover, Treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum SRBC-specific IgM and IgG (Kevin, G. et al. 2014). As for humans, in vitro experiments showed that treatment with FK506 or CsA of peripheral blood mononuclear cells from blood-bank donors suppressed the production of IgM and IgG antibodies specific to T-cell–dependent antigens. (Heidt et al, 2009) Also, in SKW6.4 cells (IL-6–dependent, IgM-secreting, human B-cell line) cultures, FK506 or CsA suppressed the production of IgM antibodies in the presence of T-cell activation. (Sakuma et al. 2001b) Considering that FKF506 and CsA reduce T cell-derived cytokines including IL-2 and IL-4, these findings strongly suggest that impairment of TDAR following reduced production of such cytokines occurs at least in common among humans monkey and rodents.

Key Event Description

Antibody production to T-cell–dependent antigens is established through the coordination of B cells, antigen-presenting cells as well as T-cell–derived cytokines, which stimulate B cells to proliferate and differentiate. T-cell–dependent antibody response (TDAR) might be altered if any of these cell populations is affected.

Interleukin (IL)-2 stimulates B cells to proliferate through surface IL-2 receptors. IL-4 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Suppressing the production of these B-cell–related cytokines appears to impaire TDAR, as seen in the result of FK506 treatment (Heidt et al, 2009).

IL-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production. IL-2 promotes differentiation of B cells through IL-2 stimulates differentiation of the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs TDAR (Alberts et al. 2008).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) decrease in anti- keyhole limpet hemocyanine (KLH) immunoglobulin (Ig)G. (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene (DBA) exposure reduced total IgG antibody in spleen cell culture supernatants after in vitro stimulation with lipopolysaccharide (LPS) (Donna, C. et al. 2010).

Treatment with cyclosporin A (CsA) at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum sheep red blood cells (SRBC)-specific IgM and IgG (Kevin, G. et al. 2014).

After a 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41.6 and 83.2 nM) of CsA (Heidt et al, 2009).

After a 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated peripheral blood mononuclear cells (PBMC) culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.012 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.083 to 83.2 nM) of CsA (Sakuma et al. 2001b).

Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG was reduced at the dose level of 3 mg/kg/day (Ulrich et al. 2004).

Mice were treated with FK506 or CsA for 4 days, and immunized with SRBC, after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).

As immunosuppression-derived adverse outcomes by calcineurin inhibition, FK506 and CsA increase the frequency and/or severity of infections and allergic reactions impaired TDAR deems to be one of the causative factors for these side effects . Some clinical trials of FK506 and CsA revealed these adverse effects as follows.

- In clinical trials of renal transplantation using FK506 or CsA, opportunistic infections such as candida, cytomegalovirus and herpes simplex virus were reported (Ekberg et al. 2007).
- In recipients of liver transplants treated with FK506 or CsA, opportunistic infections such as cytomegalovirus, hepatitis C virus, hepatitis B and herpes simplex virus were reported (Fung et al. 1991).
- Cardiac transplant patients treated with cyclosporin developed pulmonary infections within the first year after surgery (Luster, M.I. et al. 1993).
- In patients of X-linked autoimmune enteropathy treated with CsA or FK506, serum levels of IgE developed extremely high during the immunosuppressive therapy (Kawamura et al. 1997).
- Renal transplant recipients treated with belatacepy/mycophenolate (MMF)/predonisone or FK506/MMF/prednisone showed significantly lower the geometric mean hemagglutination inhibition titer against influenza vaccine, hemagglutination-specific IgG and isotype IgG1 antibodies, and IgG-antibody secreting cells response (Gangappa et al. 2019).

How it is Measured or Detected

TDAR could be examined in vivo and in vitro.
In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with Enzyme-Linked ImmunoSorbent Assay (ELISA) or with a plaque-forming cell (PFC) assay.

- Rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH, after which the serum was examined for T-cell–dependent, antigen-specific, IgM and IgG levels using a Sandwich ELISA kit (Ulrich et al. 2004).
- Mice were repeatedly administered calcineurin inhibitors (CNIs) including FK506 and CsA orally for 4 days and immunized with SRBC, after which spleen cells were examined using a PFC assay (Kino et al. 1987).
- Cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBC, after which the serum was examined for Anti-SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Kevin, G. et al. 2014).
- Mice were exposed a single pharyngeal aspiration of DBA, after which supernatants of splenocytes cultured for 24 h in the presence of LPS and assayed using a mouse IgM or IgG matched pairs antibody kit (Bethyl Laboratories, Montgomery, TX) (Donna, C. et al. 2010).

For in vitro studies, total IgM and IgG levels in culture supernatant are often measured after polyclonal T-cell activation rather than measuring antigen stimulation in immune cell cultures.

- T cells and B cells isolated from human peripheral blood mononuclear cells (PBMC) were co-cultured with a CNIs for nine days in the presence of polyclonal–T-cell stimulation, after which supernatants were tested for immunoglobulin IgM and IgG levels using a Sandwich ELISA kit. Treatment with FK506 or CsA reduced the levels of IgM and IgG at the concentrations of 0.3 and 1.0 ng/mL or 50 and 100 ng/mL (Heidt et al, 2009).
  SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) were cultured with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. After culturing for four days, IgM produced in the culture

- supernatants was measured using an ELISA kit. FK506 or CsA reduced the levels of IgM at the concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma et al. 2001b).
- In order to examine class switching, T cells derived from human PBMCs were cultured with CNIs, and cytokine mRNA levels of Interferon-gamma, IL-2, IL-4, IL-5, IL-10, IL-13, and other B-cell–stimulatory cytokines produced in T cells were measured by quantitative PCR (Dumont et al. 1998).

Regulatory Significance of the AO

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

In AOP154, it was difficult to define susceptibility to infection as an AO for the AOP154, so TDAR, which is recommended as an indicator of immunosuppresoin in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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Appendix 2: List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1508: Inhibition, Calcineurin Activity leads to Interference, nuclear localization of NFAT (https://aopwiki.org/relationships/1508)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell DependentAntibody Response (https://aopwiki.org/aops/154)	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	ScientifiCTerm	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculoides	Mus musculoides	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=60742)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN is broadly distributed throughout the body, and the structure of CnA and CnB is highly conserved from yeasts to humans (Kincaid. 1993).

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells, and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all have PPIase activity (Wang P et al. 2005). They form binary complexes with their ligand cyclosporine A.

These facts indicate that CN and immunophilins are conserved among animals and plants although they show multiple physiological functions.

In addition, CNI/immunophilin complex-induced inhibition of CN phosphatase activity resulting in suppression of immune responses is found in humans and mice.

Key Event Relationship Description

The phosphatase activity of calcineurin (CN) is known to be inhibited by CN inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) through the formation of complexes with immunophilins.

Immunophilins of FK506-binding protein (FKBP) and cyclophilin bind with CNIs FK506 and CsA to form complexes, which inhibit CN activity (Barik. 2006).

While FKBP12, FKBP12.6, FKBP13, and FKBP52 are all part of the FK506-binding FKBP family, FKBP12 has a significant involvement in the mechanism of action for FK506-induced immunosuppression (Siekierka et al. 1989, Kang et al. 2008).

FKBP12 is a 12-kDa protein localized in cytoplasm and has been isolated from Jurkat T-cells as a receptor that binds to FK506 (Bram et al. 1993). FKBP12 has an FK506-binding domain (FKBD) that comprises 108 amino acids, and is expressed in T cells, B cells, Langerhans cells, and mast cells (Siekierka et al. 1990, Panhans-Gross et al. 2001, Hultsch et al. 1991).

Cyclophilin and FKBP both exhibit peptidyl propyl isomerase (PPIase) activity, but inhibition of PPIase activity is not related to CN regulation.

CN is a heterodimer that comprises a catalytic subunit (CnA) and a Ca-binding regulatory subunit (CnB). CnA handles phosphatase activity as well as calmodulin binding, and CnB regulates intracellular calcium and CnA (Klee et al. 1988, Zhang et al. 1996). CnA is a 59kDa protein with a serine-threonine phosphatase domain.

CNI-immunophilin complexes such as FK506/FKBP complexes and cyclophilin/CsA complexes bind directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which in turn inhibits phosphatase activity of CN (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

The nuclear factor of activated T cells (NFAT) is a substrate of CN (Rao et al. 1997).

When T-cell activation takes place, T-cell–receptor-mediated stimulus increases the intracellular concentration of calcium and activates CnB, which subsequently induces CnA phosphatase activation, leading to dephosphorylation of NFAT. In that process, dephosphorylated SP motifs expose the nuclear localization signal (NLS) and cover nuclear export signal (NES), thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

When CN activity is inhibited by the binding of immunophilin complexes, dephosphorylation does not occur in NFAT, thereby resulting in nuclear export.

Evidence Supporting this KER

Biological Plausibility

The molecular structures and functions of CN and NFAT are based on sufficient scientific evidence as mentioned above The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPlase activity, but the isomerase activity per se is not relevant for CN activity indicating that the latter is affected by the molecular structure of the complex (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

As mentioned above, inhibition of CN phosphatase activity interferes with the dephosphorylation of NFAT, which leads to the suppression of its nuclear localization.

Empirical Evidence

Much experimental data is available that supports the inhibition of CN activity induced by CNI/immunophilin complexes, which subsequently suppress nuclear localization of NFAT. In addition, CN phosphatase activity is inhibited by 24 hours treatment with CNI of FK506 and CsA with IC50 values of 0.5 and 5 nM, respectively (Fruman et al.1992).

Also, concentration-dependent reduction of in vitro nuclear localization of NFAT was evident using imaging flowcytometry at the maximum concentration of 1 μM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM (T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991).

These findings show that dose responses and temporality of MIE and KE1 seem to be the same.

Uncertainties and Inconsistencies

CN and NFAT are expressed in T cells and other immune cells including B cells, DC, and NKT cells and related to cytokine productions from these immune cells. Also, expression of IL-2 receptors (IL-2R) in DCs are lowered due to the inhibition of CN phosphatase activity by CNI treatment. Of these, reduced production of IL-2 and IL-4 from T cells plays a major role in suppression of TDAR due to lower proliferation, differentiation, and class switching of B cells. There have been no reports of CNI-induced reduction of cytokines other than IL-2 and IL-4 or reduced expression of IL-2R resulting in TDAR suppression.

FKBP12, a specific immunophilin that binds with FK506, is also an accessory molecule that binds to IP3 and Ryanodine receptors, both of which occur in Ca channels located on the membrane of the endoplasmic reticulum and participate in the regulation of intracellular Ca concentration. When binding with FK506, FKBP12 leaves these receptors to increase the influx of Ca2+ from the endoplasmic reticulum to cytoplasm, which should increase CN activity. Treatment with FK506, however, suppresses NFAT nuclear localization. In addition, FKBP12-knock out mice show no changes in immune function, including T-cell function. These facts suggest that the inhibition of CN-NFAT systems induced by FK506 treatment results from direct inhibition of CN phosphatase activity by FK506/FKBP12 complexes and not by affecting Ryanodine and IP3 receptors associated with FKBP12.

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

Dose-response analysis of the effects of FK506 on CN phosphatase activity in mast cell-derived KiSVMC4W cells transfected with human FKBP12 cDNA showed that increased expression of FKBP12 resulted in a greater than ten-fold increase in sensitivity to FK506-mediated inhibition, as indicated by an IC50 value of roughly 2 nM with linear inverse dose-response curve after 1 hour incuvation (Fruman et al.1995). Another phosphatase assay showed that FK506 inhibition of CN activity was concentration-dependent reverse sigmoidal and that IC50 values for CN inhibition were approximately 0.5 nM for FK 506 and 5 nM for CsA after 1 hour culture (Fruman et al.1992).

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing CNI concentrations from 0.1 nM (Jurkat human T cells) up to 1 μM (1000 nM) using imaging flowcytometry. Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again at maximal concentrations of 1 μM with minimum concentration of 10nM (Maguire et al. 2013).

So far, there is no evidence available that the dose response of inhibition of CN phosphatase activity is correlated with nuclear translocation of NFAT; however, the concentration ranges of CNIs for inhibition of CN phosphatase activity and nuclear translocation of NFAT seem to be the same range.

Time-scale

Inhibition of CN phosphatase activity was examined after 1 hour culture of T cells (Fruman et al.1995, Fruman et al.1992), and inhibition of nuclear translocation of NFAT was measured by imaging flowcytometry after 2 hour culture of T cells with CNI (Maguire et al. 2013).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1017: Interference, nuclear localization of NFAT leads to Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/relationships/1017)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientifi cTerm	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents, and other mammalian species (Rao et al. 1997).
CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Relationship Description

Activated (dephosphorylated) nuclear factor of activated T cells (NFAT) is translocated into the nucleus through the molecular changes of exposing nuclear localization signal (NLS) and concomitant masking of nuclear export signal (NES) due to dephosphorylation of the SP motifs of NFAT. (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

In the nucleus NFAT binds with AP 1 at the IL-2 promoter region, (Schreiber and Crabtree 1992; Jain et al. 1992) and induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

Once CN phosphatase activity is inhibited, dephosphorylation of NFAT and subsequent nuclear localization of NFAT decreases, which results in a decrease of NFAT/AP-1 complex formation at the cytokine promoter sites (Rao et al. 1997).

Evidence Supporting this KER

Biological Plausibility

As has been mentioned, NFAT has NLS and NES domains among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS domain is exposed whereas the NES doman is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000).

It is well known from the experiments using CN inhibitors (CNIs) that interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).

In contrast to T cells, B-cell receptor-mediated increases in intracellular concentration of calcium in B cells leads to NFAT nuclear localization, thereby producing some classes of cytokines in the same manner as T-cells (Bhattacharyya et al.2011). However, there has been no report of any evidence that CNI acts directly on B cells to effect antibody production.

Expression of IL-2 receptors in dendritic cells and NKT cells is also reported to be regulated by this CN-NFAT system (Panhans-Gross A et al. 2001; Kim et al. 2010), but there is no report showing that CNIs suppress TDAR through the changes in IL-2R expression in these cells.

Empirical Evidence

The relationship of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

Imaging flowcytometry revealed that concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the maximum concentration of 1 μM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM (CD4+T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013).

Gel mobility shift assays using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1μM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991) NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997).

Quantitative understanding of NFAT/AP-1 complex formation in the nucleus is insufficient although nuclear NFAT/AP-1 complex formation was shown to be inhibited by FK506 at concentrations within the range of FK506 for the inhibition of nuclear translocation of NFAT.

Uncertainties and Inconsistencies

Nothing especially

Quantitative Understanding of the Linkage

Response-response relationship

The relationship of the interference of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing FK506 concentrations from 0.01nM (Jarkat T cells) up to 1 μM (1000 nM). Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again from 10nM to maximal concentrations of 1 μM (Maguire et al. 2013). Both parameters were measured after 2 hour culture of T cells with FK506.

KE2:

Reduction in generation of NFAT/AP-1 complexes can be detected using a gel shift assay (Rao et al. 1997, Jain et al. 1992, Jain et al. 1993).

Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991). As mentioned above, gel mobility shift assays also showed that NFAT/AP-1 complexes were formed only in the nucleus after T cell activation with unchanged preexisting NFAT in the cytoplasm and that treatment of T cells with 1μM FK506 led to decease the levels of NFAT/AP-1 complex (Jain et al. 1992).

These findings suggest that nuclear translocation of NFAT after T cell stimulation is strongly related to the complex formation with AP-1 in the nucleus, and FK506 was shown to inhibit NFAT/AP-1 complex formation in

the nucleus at the concentrations within the concentration range of FK506 for suppressing nuclear translocation of NFAT (Maguire et al. 2013).

Time-scale

Nuclear translocation of NFAT was shown to be inhibited in vitro using imaging flowcytometry after 2 hours culture of T cells with FK506 (Maguire et al. 2013), and gel mobility shift assays revealed the inhibition of nuclear translocation of NFAT and following complex formation with AP-1 within the nucleus after 2 hours culture of T cells with FK506 (Jain et al. 1992, Flanagan et al. 1991).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1509: Reduction, NFAT/AP-1 complex formation leads to Suppression, IL-2 and IL-4 production(https://aopwiki.org/relationships/1509)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell DependentAntibody Response (https://aopwiki.org/aops/154)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1, IL-2 production and CD25 (IL-2R) up-regulation (Yoshida et al. 2015).
In splenic lymphocytes and/or CD4+ T cells, ursolic acid suppressed products of NF-kB, NFAT and AP-1, and inhibits secretion of IL-2 and IL-4, mRNA level of IL-2 and CD25 expression (Checker et al. 2012).
NFATp- and NFAT4-deficient mice indicate decreased production of IL-2 (Ranger et al. 1998).
NFAT/AP-1 complex formation in the nucleus was shown using murine and human T cells lines (Jain J et al. 1992). In addition to data on suppression of cytokine production by CNI in rodents, FK506 is reported to inhibit expression of both IL-2 and mRNA in human anti-CD3/PMA-activated cells (Dumont et al. 1998).

Key Event Relationship Description

Localized nuclear factor of activated T cells (NFAT) in the nucleus of T cells forms complexes with activator protein-1 (AP-1) at the Interleukin (IL)-2 promoter region (Schreiber and Crabtree 1992; Jain et al. 1992), which induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

For IL-2, NFAT proteins are necessary for IL-2 gene expression and interaction of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

Lowered nuclear localization of NFAT by calcineurin inhibitor (CNI) results in decreased formation of NFAT/AP-1 complex at the promoter region of IL-2 genes in the nucleus of T cells thereby reducing the transcription of IL-2 (Dumont et al. 1998). Production in T cells of IL-4 and other classes of cytokines is also suppressed in the same manner as IL-2 (Dumont et al. 1998).

Evidence Supporting this KER

Biological Plausibility

T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015).

Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001).

Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).

NFATp- and NFAT4-deficient mice indicate decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is generally accepted that NFAT, translocated to the nucleus after T-cell stimulation, binds with AP-1 to the promoter regions of the cytokine genes to mount transcription, which follows production of these T-cell–derived cytokines. Of these cytokines, IL-2 and IL-4 promote proliferation, maturation, and class-switching of B cells to enhance TDAR.

There is also sufficient evidence to support the hypothesis that CNI-induced decreases in T-cell–derived cytokine production is mediated through suppressed nuclear localization of NFAT, with a resultant decrease in the amount of NFAT/AP-1 complex binding to the promoter regions of T-cell-derived cytokines.

When stimulated with ovalbumin, calcineurin A (CnA)-knockout (KO) mice produce less Interferon (IFN)- γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to trinitrophenol-ovalbumin (Zhang et al. 1996).

The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly; moderately enhanced B- and T-cell responses, with bias towards Th2- cell responses; decreased IFN-γ production in response to TCR ligation; reduced proliferative responses by T cells; impaired repopulation of the thymus and lymphoid organs; impaired Th2-cell responses and IL-4 production; grossly impaired T-cell effector functions, with profound defects in cytokine production and cytolytic activity; B-cell hyperactivity; impaired development of CD4 and CD8 single-positive cells, with increased apoptosis of double-positive thymocytes; and mild hyperactivation of peripheral T cells (Macian, 2005).

Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by calcineurin (CN). Suppression of T-cell-derived cytokines is noted both in CnA-KO and NFAT-KO mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system.

FK506-FKBP12 complex decreased CN phosphatase activity, which inhibits. Because NF-ATp is an essential transcription factor regulating the IL-2 gene, FK506 ultimately blocks the T-cell response by inhibiting IL-2 transcription (Panhans-Gross A et al. 2001). FK506 inhibited IL-2 mRNA expression in anti-CD3/phorbol 12-myristate-13-acetate (PMA)-activated cells (Dumont et al. 1998).

These facts indicate that although NFAT is widely involved in the function of T cells, the effect of CNIs is to suppress production of some classes of T cell–derived cytokines through reducing the formation of NFAT/AP-1 complexes induced by inhibition of CN phosphatase activity.

Empirical Evidence

Empirical support of Reduction, NFAT/AP-1 complex formation leading to Suppression, IL-2 and IL-4 production is strong.

Rationale:

- In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 μg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 μg/mL (Yoshida et al. 2015).
- In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 μM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 μM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM for 4 h (Checker et al. 2012).
- In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is well established that inhibition of NFAT/AP-1 complex formation at the promoter sites reduces the production of T-cell–derived cytokines including IL-2 and IL-4, which are mainly involved in T-cell–dependent antibody response.

- NFAT/AP-1 complex formation is inhibited by CNI shown by gel shit mobility assay using human T cell line or CD4+ T cells from heathy donners after 2 hours treatment with cyclosporin A (CsA) at 1μM.. Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993).
- In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA in a dose-dependent (10 nM) manner after 3 day culture (Dumont et al. 1998).
- Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).
- In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).
- Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003).

Reduced nuclear translocation of NFAT followed by NFAT/AP-1 complex formation and suppression of IL-2/IL-4 productions are shown to occur under similar dose ranges and treatment duration.

Uncertainties and Inconsistencies

CNIs are reported to suppress IL-17 release from Th17 cells and development of Th17 cells from naïve T cells (Tsuda et al, 2012). On the other hand, Yadav reported that Th17 cells increased and Treg cells decreased in number and that the levels of RORC mRNA increased and those of FOXP3 decreased in renal transplanted patients with chronic calcineurin inhibitor toxicity (Yadav, 2015). From these findings, CNIs suppress the functions of Th17 and Treg cells, which enhance Th17 cells to develop chronic CNI toxicity.

FK506 suppresses expression of IL-2 receptor (IL-2R: CD25) and costimulatory molecules CD80 (B7.1)/CD40 in Langerhans cells (Panhans-Gross A et al. 2001).

In human NK cells, FK506 suppresses IL-2 responsive proliferation and cytokine production as well as lowers cytotoxicity directed toward K562 tumor cells (Kim et al. 2010). FK506 suppresses IL-2 production of NKT cell line DN32.D3 induced by stimulus from PMA/calcium -ionophore (van Dieren et al. 2010).

The relationship between these FK506-induced mechanisms and NFAT and contribution of those to TDAR are unclear.

In addition to NFAT/AP-1 complexes, NFAT forms complexes at the site of IL-3 and IL-4 enhancers with avian musculoaponeurotic fibrosarcoma oncogene homolog, early growth response 1, early growth response 4, interferon-regulatory factor 4, octamer-binding transcription factor, and other transcriptional partners to induce transcription of a variety of cytokines (Macian 2005). The production of cytokine induced by these transcriptional partners also suppressed by CNI; however, contribution of these additional transcription factors to TDAR is also unclear.

Quantitative Understanding of the Linkage

Response-response relationship

In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 at 80 μg/mL. On the other hand, T-5224 inhibits IL-2 production in a dose-dependent manner from 40, 60 and 80 μg/mL after 48 hours culture. T-5224 also inhibits CD25 (IL-2R) up-regulation at 80 μg/mL (Yoshida et al. 2015).

In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibits secretion of IL-2 and IL-4 at 0.5, 1 and 5 μM. In lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid also inhibits secretion of IL-2 and IL-4 at 5 μM. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM (Checker et al. 2012).

These findings showed that T-5244 and ursolic acid treated for 24 hours inhibit NFAT/AP-1 complex formation at a single concentration each and that these compounds suppress IL-2 and IL-4 production with dose dependent manner including the doses for inhibition of NFAT/AP-1 complex formation.

FK506 suppressed proliferation in human T cells induced by anti-CD3 mAb in the presence of adherent autologous peripheral blood mononuclear cells (mean IC50 = 0.06 nM). FK506 suppressed, in a dose-dependent (1.2 to 12.5 nM) manner after 22-24 hours culture, production of IL-2, IL-4, and IFN-γ by human T cells stimulated with anti-CD3 mAb in the presence of PMA, as well as inhibited, also in a dose-dependent (10 nM) manner, expression of IL-2, IL-4, and IFN-γ mRNA in anti-CD3/PMA- activated cells (Dumont et al. 1998). On the other hand, the quantitative data for the decreased formation of NFAT/AP-1 complexes by CNI is insufficient, although the formation was suppressed by FK506 at the concentration within the range needed for suppressed production of IL2/IL-4 by FK506 after 2 hours culture.

Time-scale

Inhibition of NFAT/AP-1 complex is detected by gel mobility shit assay after 2 hours culture with CNI; however, suppression of IL2/IL-4 could be measured after 22-48 hours in vitro culture.

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1510: Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response(https://aopwiki.org/relationships/1510)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell DependentAntibody Response (https://aopwiki.org/aops/154)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
cynomolgu smonkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In cynomolgus monkeys, the effects of CsA on production of IL-2 and IL-4, and antigen-specific IgM and IgG in TDAR were demonstrated (Gaida K. 2015).

Suppressed IgE and antigen specific IgG1 productions by the blocking of IL-4 receptor were reported in mice using dupilumab (anti-IL-4/13R antibody) (Sanofi K.K. 2018).

Suppressed antigen specific IgE production by the inhibition of IL-4 production was reported in mice using suplatast tosilate (Taiho Pharmaceutical 2013).

Suppressed antigen specific IgE and IL-4 productions by the inhibition of IL-4 production were reported in human cell culture using suplatast tosilate(Taiho Pharmaceutical 2013).

The effects of FK506 on serum concentration of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over four weeks and immunized with KLH (Ulrich et al. 2004). The effects of FK506 and CsA on antigen-specific plaque-forming splenocytes have been demonstrated in mice treated with FK506 or CsA for 4 days and immunized with SRBC (Kino et al. 1987b).

The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt et al, 2009, Sakuma et al, 2001).

The effects of FK506 and CsA on production of IL-2 and IL-4 have been demonstrated using mice and human cells (Kino et al. 1987a, Dumont et al. 1998).

These facts suggest that there are no species differences between humans, monkeys and rodents in inhibitions of IL-2 and IL-4 production and TDAR induction.

Key Event Relationship Description

Interleukin (IL)-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of T-cell dependent antibody response (TDAR), both of which induces/enhances T cell dependent antibody production. IL-4 affects maturation and class switching of B cells as well as proliferation, IL-2 promotes differentiation of B cells through IL-2 receptors and stimulates the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs T cell dependent antibody production (Alberts et al. 2008).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR

T cell-derived cytokines play important roles in the development of TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production.

Thus, suppressing the production of IL-2, IL-4, and other cytokines in T cells reduces stimulation of B cells including proliferation, activation, and class switching, and leading to impairment of TDAR. Therefore, suppressing the production of these B-cell-related cytokines appears to be the main factor in impairment of TDAR by inhibitors of T-cell–dependent-antibody production.

Evidence Supporting this KER

Biological Plausibility

Cyclosporin A (CsA) is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in the reduction of the productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015).

It is established that IL-2 stimulates B cells to proliferate through the surface IL-2 receptors and that IL-4 stimulates B cells to proliferate, to induce class switch, and to differentiate into plasma and memory cells.

Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production.

Th2 cell produces cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 from Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells.

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). They suggest that the blocking of IL-2 signaling by anti-IL-2R antibody results in decreased rejection through the inhibition of the activation of antigen specific T cell with reduced antibody production.

FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Several in vivo studies in rodents showed decreased TDAR by the treatment of FK506 (Kino et al. 1987b, Ulrich et al. 2004). In in vitro tests examining antibody production in blood samples obtained from blood-bank donors, peripheral blood mononuclear cells (PBMC) treated with FK506 and CsA suppressed the production of IgM and IgG antibodies to T-cell dependent antigens (Heidt et al, 2009).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

However, as for the suppression of humoral immunity induced by the inhibition of calcineurin (CN) phosphatase activity, calcineurin inhibitors (CNIs) do not affect B cells directly but rather indirectly through T cells. That is, FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B cell response by FK506 and CsA appears due solely to inhibition of T helper cells (Heidt et al, 2010).

Therefore, it is concluded that decreased amounts of IL-2 and IL-4 secreted from helper T cells is the main factor for suppression of TDAR induced by CN phosphatase inhibition.

Empirical Evidence

Empirical support of the suppression, IL-2 and IL-4 production leads to impairment, T-cell dependent antibody response is strong.

Rationale:

- Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).
- In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018).
- In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013).
- In the clinical study of renal transplantation, basiliximab decreased incidence of acute rejection at 20 mg/kg (Novartis Pharma 2016). In human T cell culture immunized with PPD, basiliximab reduced activation of antigen specific T cell at the concentration of 300 ng/mL (Novartis Pharma 2016).
- In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998).
- FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a).
- After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2009).
- After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture

supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001).

- Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004).
- Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).
- 1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH and decrease in anti-KLH IgG(Alessandro, B. et al. 2003).

In vitro suppression of T-cell–derived cytokines and T-cell-dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two KEs, which showed earlier onset of cytokine production and delayed onset of antibody production.

Uncertainties and Inconsistencies

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Therefore, reduced production of both IL-2 and IL-4 might certainly induce suppression of TDAR; however, there remains some possibility of additional suppression of other immune functions.

Quantitative Understanding of the Linkage

Response-response relationship

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In the blocking of IL-4 receptor in mice by dupilumab (anti-IL-4/13R antibody) at 25 mg/kg of twice weekly subcutaneous administration for 4weeks, IgE production was suppressed to about 1/100 and antigen specific IgG1 production was suppressed to about 1/200 (Sanofi K.K. 2018).

In the inhibition of IL-4 production in mice by suplatast tosilate at 10, 20, 50 and 100 mg/kg of oral administration for 5 days, antigen specific IgE production was suppressed from about 1/10 to 1/100 (Taiho Pharmaceutical 2013). In human T cell culture by suplatast tosilate at the concentration of 10 μg/mL, antigen specific IgE production after 10 days was suppressed from 56 to 72% and IL-4 production after 3 days was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. That is, murine small resting B cells, cultured with irradiated hapten-specific TH1 clone, were induced to enter cell cycle at 2 days and to secret antibody at 5 days. An anti-IL-2 and anti-IL-2R antibodies completely inhibited this T-cell dependent antibody production (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the m-RNA levels of T-cell cytokines at 8h post-stimulation including IL-2 and IL-4 at 1.0ng/mL (1.24nM) FK506 or 100ng/mL (90.7nM) CsA and inhibited IgM and IgG productions after 9 days at 0.3 and 1.0ng/mL FK506 and 50 and 100ng/mL CsA (Heidt S. 2010).

Time-scale

In CsA-treatment for 24 days at 50 mg/kg BID, cynomolgus monkeys showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In human T cell culture, suplatast tosilate inhibits IL-4 production after 3 days and antigen specific IgE production after 10 days (Taiho Pharmaceutical 2013).

In the human T-B cell co-culture, CNIs of FK506 and CsA lowered the m-RNA levels of IL-2 and IL-4 at 8h post-stimulation and inhibited IgM and IgG productions after 9 days (Heidt S. 2010).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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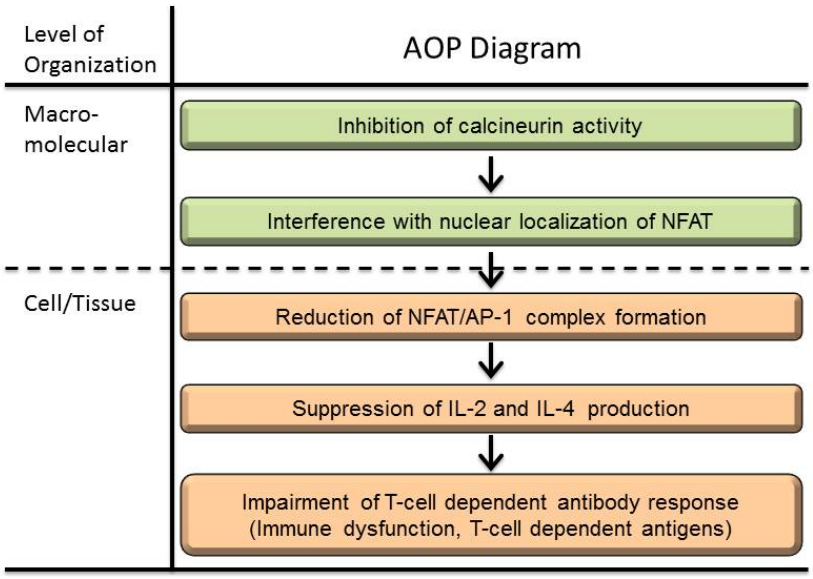
AOP ID and Title:

AOP 154: Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response
Short Title: Immunosuppression

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for comment. Do not cite	EAGMST Under Review	1.38	Included in OECD Work Plan

Abstract

Calcineurin (CN), a protein phosphatase, is known to impair immune function when its phosphatase activation is inhibited. The relationship between CN and immune functions is well understood, and immunosuppressants that work by inhibiting CN have been developed.

CN inhibitors (CNIs) inhibit CN phosphatase activity to suppress many kinds of immune functions and have been used to prevent hyper immune reactions such as rejection and graft versus host disease (GVHD), and treat autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CNIs are reported to induce immunosuppression-derived adverse effects such as increased frequency and/or severity of infections and increased tumor incidences. CNIs might affect several T-cell derived immune functions to induce compromised host. Among the affected immune functions, T-cell dependent antibody response (TDAR) is an important factor to resist infections and thought to be the useful endpoint on evaluating immunotoxicity of chemicals; therefore, this AOP describes the linkage between the inhibition of CN activity and impairment of TDAR.

CN activity is inhibited when stressors bind to CN with their respective immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes with activator protein-1 (AP-1) that bind at the site of IL-2, IL-4 and other T cell -derived cytokine promoters is reduced, thereby

suppressing production of these cytokines. Among the affected cytokines from each of the helper T cell subsets, reduced production of IL-2 and IL-4 affects the proliferation and differentiation of B-cells to suppress TDAR.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for inhibition of CN activity leading to impaired TDAR.

Since CN is produced in a vast variety of species, this AOP might be applicable to many mammal species, including humans and rodents.

Background

Although there are other stressors that inhibit CN activity, this AOP is mainly based on an understanding of immunosuppression caused by the complex of FK506 and FKBP12 and cyclophilin and CsA, on which a significant body of scientific literature has been published.

We look forward to future amendments to this AOP with up-to-date information on other stressors, which will more clarify the linkage between inhibition of CN activity and impairment of TDAR.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	980	Inhibition, Calcineurin Activity	Inhibition, Calcineurin Activity
2	KE	979	Interference, nuclear localization of NFAT	Interference, nuclear localization of NFAT
3	KE	981	Reduction, NFAT/AP-1 complex formation	Reduction, NFAT/AP-1 complex formation
4	KE	1202	Suppression, IL-2 and IL-4 production	Suppression, IL-2 and IL-4 production
5	AO	984	Impairment, T-cell dependent antibody response	Impairment, T-cell dependent antibody response

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Calcineurin Activity	adjacent	Interference, nuclear localization of NFAT	Moderate	Moderate
Interference, nuclear localization of NFAT	adjacent	Reduction, NFAT/AP-1 complex formation	High	High
Reduction, NFAT/AP-1 complex formation	adjacent	Suppression, IL-2 and IL-4 production	High	High
Suppression, IL-2 and IL-4 production	adjacent	Impairment, T-cell dependent antibody response	High	High

Stressors

Name	Evidence
Tacrolimus (also FK506)	High
Cyclosporin	High
Pimecrolimus	High
Gossypol	Moderate
Kaempferol	Moderate
Dodecylbenzene sulfonate	Moderate

Name	Evidence
Dibefurin	Moderate
Ascomycin	Moderate
1,5-dibenzoyloxymethyl-norcantharidin	Moderate

Tacrolimus (also FK506)

also known as FK506

Overall Assessment of the AOP

Inhibition of CN might induce suppression of cytokines production from all the T helper cell subsets as well as other immune functions of other immune cells. Suppression of cell-mediated immunity is involved in the pharmacology of preventing hyper immune reactions such as rejection and GVHD, and treatment of autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CN inhibition might induce immunosuppression-derived adverse outcomes. One of the effects is increased frequency and/or severity of infections. Compromised host might be related with impairment of multiple immune functions; however, impaired TDAR deems to be usually related. Moreover, TDAR is the frequently used measurable endpoint in immunotoxicity testing according the ICH S8 or US EPA OPPTS 870.7800 immunotoxicity testing guideline. Therefore, the present AOP focus on CN inhibition-induced impairment of TDAR.

When stressors bind to calcineurin-A (CnA) with immunophilin, CN phosphatase activity is inhibited. Immunophilins are composed of a family of highly conserved proteins that have the ability to bind immunosuppressive drugs. This interfere with the nuclear localization of NFAT, the substrate for CN. As a result, the formation of functional NFAT/ AP-1 complexes that bind at the site of IL-2 , IL-4 and other cytokine promoters in each of the T helper cell subsets is reduced, thereby suppressing production of these cytokines. Among the affected cytokines TDAR is impaired mainly by the suppression of production of IL-2 and IL-4, which affect the proliferation and differentiation of B-cells to lower TDAR. We have identified a number of key events (KEs) along this pathway, and based on these key event relationships (KERs), created an AOP for inhibition of CN activity leading to impaired TDAR.

Since each KE involving MIE and AO is quantifiable, and shows similar dose responses with the CNIs in vitro, this AOP is useful for understanding immunosuppression due to inhibition of CN activity. In addition, each KER is based on sufficient scientific evidence and exhibits no contradiction with dose responses of adjacent KEs.

Since CN/NFAT system is conserved among vast variety of species and the function in immune system is common in at least human and mice, this AOP might be applicable to many mammalian species, including humans and rodents.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Macaca fascicularis	Macaca fascicularis	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

The proposed AOP regarding inhibition of CN activity leading to impaired TDAR is not dependent on life stage, sex, or age. Since tacrolimus (FK506) ointment (Protopic) is approved for pediatric atopic dermatitis, the MOA for immunosuppression appears to be applicable to all life stages. The applicable state is considered supported by the draft FDA guidance for immunotoxicology that was recently issued (2020) indicating that “example of immunotoxicology testing could included TDAR assay” to address the concern of immunotoxicity in offspring in juvenile animal studies.

Since FK506 or CsA-induced outcomes in humans are mimicked by similar responses in a variety of animal models including non-human primates and rodents, immunosuppression induced by inhibition of CN activity is considered to occur across a variety of mammalian species.

In addition to the drugs, it is known that CN activity is suppressed by alkeylbenzene sulfonate (dodecylbenzene sulfonate) extracted from an acrylonitrile butadiene rubber (Ito et al. 2013)., suggesting that the proposed AOP would be applicable to non-

pharmacological agents.

For the chemicals such as pesticide, the TDAR assay is also recommended in the US EPA OPPTS 870.7800 immunotoxicity testing guideline.

Essentiality of the Key Events

Essentiality is supported by several knockout animals as follows.

Stage	Essentiality	Evidence	Supported by literatures
MIE and later	CnA-KO mice	Strong	The CN molecule consists of two regions, CnA and CnB, of which CnA exhibits phosphatase activity. In CnA-KO mice, T-cell proliferation in response to ovalbumin stimulation is lower than that for wild-type mice and is not complemented by normal antibody producing cells. In addition, when stimulated with ovalbumin, CnA-KO mice produce less IFN-γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to TNP-ovalbumin, which means that CnA deficiency affects only T cell-dependent antibody response (TDAR) (Zhang et al. 1996).
KE1 and later	NFAT-KO mice	Strong	The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly, moderately enhanced B- and T-cell responses, with bias towards Th2-cell response, decreased IFN-γ production in response to T-cell receptor (TCR) ligation, reduced proliferative responses by T cells, impaired repopulation of the thymus and lymphoid organs, impaired Th2- cell responses and IL-4 production, grossly impaired T-cell effector functions, profound defects in cytokine production and cytolytic activity, B-cell hyperactivity, impaired development of CD4 and CD8 single-positive cells, increased apoptosis of double-positive thymocytes, and mild hyperactivation of peripheral T cells. Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by CN. Suppression of T-cell-derived cytokines is noted both in CnA-knockout and NFAT-knockout mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system (Macian, 2005).
Stressor	FKBP12-KO mice	Moderate	FK506 induces suppression of immune responses; however, there is no literature showing a relationship of a relationship between FKBP12 knockout and the immune system in the FKBP12-KO mouse model. Steric structure of FKBP12/FK506 complex is considered the key factor for inhibition of CN phosphatase activity, but not for the enzymatic activities of FKBP12.

Weight of Evidence Summary

Biological Plausibility

T-cell functions are mainly regulated by the CN-NFAT system and suppression of CN activity in T cells is known to induce multiple types of immunosuppression, including T cell-dependent antibody response (TDAR).

Experiments with T cells indicate that TCR stimulation brings about increases in intracellular concentrations of Ca2+ that trigger CN activity, thereby inducing nuclear localization of substrate NFAT per dephosphorylation. The localized NFAT forms complexes with activator protein 1 (AP-1) at the promoter sites of the T-cell cytokine genes and induces production of the cytokines.

CN phosphatase activity is known to be inhibited by the formation of immunophilin-CN inhibitor (CNI) complexes, such as CsA/cyclophilin complexes or FK506/FK506-binding protein (FKBP) 12 complexes. Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity, but there is no similarity between amino-acid sequences of the two classes

of immunophilins. The three-dimensional structures of immunophilin complexes are essential to the inhibition of CN phosphatase activity, even though their enzymatic activities are not.

It is also known that one of the effects on immune function when CNI forms complexes with its respective immunophilin and inhibits CN activity is the suppression of IL-2 and other T-cell derived cytokine production. It is further known that inhibition of CN leads to suppression of TDAR because IL-2 and IL-4 mainly promote the proliferation, class switching, differentiation, and maturation of B-cells.

Furthermore, CN-NFAT also exists in B-cells and it has been reported that CNIs do suppress production of certain cytokines from them. At the time of our review of the literature, however, we did not find any reports of a direct effect of CN inhibition on B-cells, such as changes in proliferation, class switching, differentiation, or maturation of B-cells.

Also, although CN-NFAT is known to exist in dendritic cells, natural killer T (NKT) cells, and other types of cells in which it regulates the expression of IL-2 receptors, there are no reports of effects on the production of T cell-dependent antibodies due to CNI-induced alteration in expression of IL-2 receptors in these cells.

CN-NFAT system-mediated immunosuppression is well understood based on the pharmacology of some CNI drugs (mostly FK506 and Cs A); therefore, AOP of CN inhibition-induced suppression of TDAR is useful for prediction of CN-mediated immunotoxicity.

KER	KE_{up}-KE_{down}	Evidence	Rationales supported by literatures
KER1	CN inhibition to interference, NFAT nuclear translocation	Moderate	CN phosphatase activation through TCR stimulation dephosphorylates NFAT, thereby promoting nuclear localization of NFAT. CN phosphatase activity in T cells could be inhibited by CNI/immunophilin complexes, thus interfering with dephosphorylation and nuclear localization of NFAT. The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPIase activity, but modification of these functions is unrelated to inhibition of CN activity and thus thought to arise in the molecular structure of the complexes (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).
KER2	Interference, nuclear localization NFAT to reduction, NFAT/AP-1 complex formation	Strong	CN activity dephosphorylates NFAT, thereby promoting its nuclear translocation. Nuclear-located NFAT binds with AP-1 at the promoter regions of the cytokine genes to promote T-cell cytokine production. Inhibition of dephosphorylation of NFAT by CNIs prevents nuclear export of NFAT and resultant binding with AP-1 at the promoter region of the T cell cytokine genes. NFAT has NLS and NES domains among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS domain is exposed whereas the NES domain is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000). CNIs interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).
	Reduction,		NFAT/AP-1 complexes bind to the promoter regions of the cytokine genes, which promotes production of cytokines in T cells. Of these cytokines, IL-2 and IL-4 have a major role in promoting proliferation, maturation and class-switching of B cells, and development of TDAR. Reduction of NFAT/AP-1 complex formation in the nucleus due to inhibition CN activity by CNIs suppresses production of T-cell derived cytokines, including IL-2 and IL-4. T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding

KER3	NFAT/AP-1 complex formation to suppression of IL-2 and IL-4 production	Strong	activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015). Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001). Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).
KER4	Suppression of IL-2 and IL-4 production to impaired TDAR	Strong	T cell-derived cytokines play important roles in TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation. Inhibition of CN activity by CNIs is known to suppress production of multiple cytokine species from T cells. Of these cytokines and receptors, suppression of IL-2 and IL-4 production mainly leads to impairment of TDAR. Suppressed production of other cytokines due to inhibition of CN activity exhibits only minor effects, if any, on TDAR. CsA is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in reduced productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015). Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production. Th2 cells produce cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 in Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells. IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one the of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Empirical Support

KER	KE_{up}-KE_{down}	Evidence	Empirical support of KERs
			CN phosphatase activity is inhibited with IC50 values of 0.5 nM (FK506) and 5nM (CsA) after 1 hours treatment (Fruman et

KER1	Inhibition, calcineurin activity leads to interference, nuclear localization of NFAT	Moderate	al.1992). Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at concentrations from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991). CN phosphatase activity and nuclear translocation of NFAT seems to be suppressed by CNIs at the similar ranges of doses and reaction times of 1 to 2 hours.
KER2	Interference, nuclear localization of NFAT leads to reduction, NFAT/AP-1 complex formation	Strong	Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013). Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991). Gel mobility shift assays using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1µM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to bind IL-2 promoters (Flanagan et al. 1991). NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997). Quantitative data on NFAT/AP-1 complex formation in the nucleus is insufficient; however, inhibition of nuclear localization of NFAT and following NFAT/AP-1 complex formation in the nucleus are simultaneously detected by gel mobility shift assays at the concentration of FK506 within the range for inhibition of nuclear translocation of NFAT using imaging flowcytometry after 2 hours culture of T cells.
			In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998). In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 µg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 µg/mL (Yoshida et al. 2015). In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 µM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 µM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 µM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 µM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 µM for 4 h (Checker et al. 2012).

KER3	Reduction, NFAT/AP-1 complex formation leads to suppression, IL-2 and IL-4 production	Moderate	Gel mobility shift assay revealed that treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders NFAT nuclear translocation and following formation of NFAT/AP-1 complexes in the nucleus (Flanagan et al. 1991). Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993). In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at 10 nM (Dumont et al. 1998). Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018). In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010). Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014). In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003). Therefore, concentration of CNI needed for inhibition of NFAT/AP-1 complex formation in the nucleus is higher than that for inhibition of IL-2 and IL-4 production. A time lag is found between the two KEs; 2 hours for KE2 and 22 to 48 hours for KE3.
			Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015). In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018). In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines such as IL-4 and IL-5 on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013).

KER4	Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response	Strong	1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010). In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH (keyhole limpet hemocyanine) and decrease in anti-KLH IgG(Alessandro, B. et al. 2003). FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a). In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998). Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004). Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b). After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2010). After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001). In vitro suppression of T-cell–derived cytokines and T-cell–dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two events, which showed earlier onset of cytokine production and delayed onset of antibody production.

Based on these findings of empirical support, each KE involving MIE and AO except for KE2 shows similar dose responses to the CNIs in vitro; however, culture time lag is noted, in that, 1 hour for MIE, 2 hours for KE1 and KE2, 22 to 24 hours for KE3 and more than days for AO.

Quantitative Consideration

KER1

No literature is available showing a clear quantitative relationship between the inhibition of CN phosphatase activity and nuclear translocation of NFAT; however, the dose responses of CN phosphatase activity and nuclear translocation of NFAT to CNI deem to be the same.

KER2:

Gel mobility shift assay of activated T cells showed that NFAT/AP-1 complexes are only found in nuclear extract, which indicates a

strong relationship between the nuclear translocation of NFAT and simultaneous complex formation with AP-1 in the nucleus. CNI treatment clearly suppresses the complex formation of nuclear located NFAT and AP-1 in the nucleus, which also shows the solid relationship between these adjacent two KEs although quantitative data on suppressed NFAT/AP-1 complex formation is insufficient (Flaganan W.M. et al. 1991).

KER3:

The quantitative relationship between the decreased formation of NFAT/AP-1 complexes and the production of IL2/IL-4 formation induced by CNIs has not been reported.

However, as mentioned in the empirical support, nuclear localization of NFAT is strongly related to NFAT/AP-1 complex formation in the nucleus based on the fact that these two events are detected simultaneously by gel mobility shift assay, and the dose responses of IL2/IL-4 production and nuclear translocation of NFAT inhibited by CNI are similar; therefore, dose ranges of CNI in the inhibitions of IL2/IL-4 production and NFAT/AP-1 complex formation in the nucleus might also be the same.

In addition, T-5224 and ursolic acid inhibit AP-1 DNA binding activity or production of NF-κB, NFAT and AP-1, respectively, and both suppress the IL-2 and/or IL-4 production with dose dependent manner including the doses of inhibiting NFAT-AP-1 system (Yoshida et al. 2015, Checker et al. 2012).

KER4:

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).

Inhibition of IL-4 production in mice treated with oral administration of suplatast tosilate suppresses antigen-specific IgE production in a dose-dependent manner (Taiho Pharmaceutical 2013). In the inhibition of IL-4 production in human cell culture by suplatast tosilate at the concentration of 10 µg/mL for 10 days, antigen specific IgE production was suppressed from 56 to 72% and IL-4 production was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. T (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the levels of T-cell cytokines including IL-2 and IL-4 and inhibited IgM and IgG productions with a dose-dependent manner (Heidt S. 2010).

These results show the quantitative relationships between the inhibition of IL-4 or IL-2 by specific antibodies or CNI and suppression of antibody production.

Considerations for Potential Applications of the AOP (optional)

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and

immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

In AOP154, it was difficult to define susceptibility to infection as an AO for the AOP154, so TDAR, which is recommended as an indicator of immunosuppresoin in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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Appendix 1

List of MIEs in this AOP

Event: 980: Inhibition, Calcineurin Activity

Short Name: Inhibition, Calcineurin Activity

Key Event Component

Process	Object	Action
binding	FK506-binding protein 15	increased
binding	FKBP12 (Arabidopsis thaliana)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	MolecularInitiatingEvent

Stressors

Name
Tacrorimus
Cyclosporin
Pimecrolimus
Dodecylbenzene sulfonate
Dibefurin
Gossypol
Ascomycin
Kaempferol
1,5-dibenzoyloxymethyl-norcantharidin
Tacrolimus (also FK506)

Biological Context

Level of Biological Organization

Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

CN inhibitory activities (IC50) are shown in follows.

Tacrorimus: 0.4nM

Cyclosporin: 7nM

Pimecrolimus: 0.4 nM

Dodecylbenzene sulfonate 9.3 uM

Dibefurin: 44 uM

Gossypol: 17 uM

Ascomycin: 0.7 nM

1,5-dibenzoyloxymethyl-norcantharidin: 7 uM

Kaempferol: 51.3 uM

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus rattus	Rattus rattus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN is broadly distributed in T-cells, B-cells, and throughout the body. The structure of CnA and CnB is highly conserved from yeasts to humans. Also highly conserved are the amino acid sequences of the catalytic and regulatory domains of CnA isoforms from different organisms (Kincaid. 1996).

As for immunophilins, of which complexes inhibit the CN activity, FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989a). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all living beings have PPIase activity (Wang P et al. 2005).

However, inhibition of CN phosphatase activity through immunophilin-CNI complex has been reported at least in rodents and humans.

Key Event Description

Calcineurin (CN) is a heterodimer that comprises a catalytic subunit (CnA), which handles phosphatase activity as well as calmodulin binding, and a Ca-binding regulatory subunit (CnB), which regulates intracellular calcium as well as CnA (Klee et al. 1988, Zhang et al. 1996). CnA, a 59kDa protein, has a serine-threonine phosphatase domain.

Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity (Barik. 2006) and an

immunophilin-CN inhibitor (CNI) complex such as FKBP12- FK506 and cyclophilin-CsA binds directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which inhibits the phosphatase activity of CN without any contribution of PPIase activity (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

How it is Measured or Detected

Phosphatase activity can be measured using a phosphatase assay. CN, calmodulin, FK506, and FKBP are incubated together, and the phosphatase activity is measured at various concentrations of FKBP. Kinetic analysis of FKBP12 concentration-dependent phosphatase activity and calculation of the CN inhibition constant Ki by the FKBP12-FK506 complex are conducted. (Bram et al. 1993). Phosphatase activity of CN in the presence of cyclosporin A (CsA) , gossypol or dibefurin can also be determined in a similar manner (Sieber et al. 2009).

Immunophilin-CNI complexes directly inhibit phosphatase activity of CN, therefore, as a surrogate measurement of the CN activity, the binding of CsA with cyclophilin can be detected using an ELISA kit. Microtiter plates precoated with BSA and conjugated to cyclosporin are incubated with cyclophilin. Bound cyclophilin is then revealed by incubation with anti-cyclophilin rabbit antiserum followed by incubation with anti-rabbit globulin goat IgG coupled to alkaline phosphatase (Quesniaux et al. 1987).

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List of Key Events in the AOP

Event: 979: Interference, nuclear localization of NFAT

Short Name: Interference, nuclear localization of NFAT

Key Event Component

Process	Object	Action
genetic interference	NFAT protein	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

Key Event Description

The nuclear factor of activated T cells (NFAT) is a substrate of calcineurin (CN) (Rao et al. 1997). A NFAT has an N-terminal with a plurality of SP motifs rich in serine and proline, which are controlled by means of phosphorylation and dephosphorylation. There is a nuclear localization signal (NLS) held between these SP regions as well as a nuclear export signal (NES) in the N-terminal adjacent to the SP motifs (Beals et al. 1997, Zhu and McKeon 1999, Serfling et al. 2000). SP motifs ordinarily are phosphorylated, which covers the NLS and leaves the NES exposed, so that NFAT localizes in cytoplasm. When SP motifs are dephosphorylated by activated CN, the NLS is exposed and the NES is covered, thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999). When T-cell activation takes place, T-cell–receptor- mediated stimulus increases the intracellular concentration of calcium and activates a regulatory subunit (CnB), which subsequently induces a catalytic subunit (CnA) phosphatase activation, leading to dephosphorylation of NFAT thereby promoting nuclear localization of NFAT. CNI-immunophilin

complexes inhibit CN phosphatase activation, thereby interfering with NFAT nuclear localization (Bhattacharyya et al.2011).

Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at concentrations from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013).

How it is Measured or Detected

Nuclear translocation of NFAT can be tested by imaging flowcytometer, in which lymphocytes are treated with fluorescence-labeled anti-NFAT antibody and DAPI (nuclear stain) and intracellular distribution of NFAT is analyzed by imaging flowcytometry with image analysis (Maguire O et al. 2013).

Interference with translocation of NFAT to the nucleus can be detected using gel mobility shift assays of nuclear or cytoplasmic extracts electrophoresed with end-labeled NFAT-binding site from human IL-2 enhancer (Flanagan et al. 1991).

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Event: 981: Reduction, NFAT/AP-1 complex formation

Short Name: Reduction, NFAT/AP-1 complex formation

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	NFAT activation molecule 1	decreased
cell activation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

T cell

Organ term

Organ term

immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Description

Activated nuclear factor of activated T cells (NFAT) that has localized to the nucleus binds cooperatively at the site of the Interleukin-2 (IL-2) promoter with activator protein-1 (AP-1), which is a heterodimer comprising a Fos and a Jun protein (Schreiber and Crabtree 1992, Jain et al. 1992), thereby inducing transcription of IL-2 (Jain et al. 1993). Interfered nuclear localization of NFAT, induced by FK506, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991).

Besides IL-2, NFAT is known to bind cooperatively at the promoters of other T-cell cytokines, such as Interleukin-4 (IL-4) (Macian et al. 2005).

Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991).

How it is Measured or Detected

Reductions in NFAT/AP-1 complex formation can be detected using a gel shift assay to test nuclear extracts from either stimulated or unstimulated Ar-5 T cells with radio-labelled NFAT binding oligonucleotide from murine IL-2 promoter. Anti-Fos and anti-Jun antibodies are used to examine NFAT/AP-1 complex formation (Jain et al. 1992).

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Event: 1202: Suppression, IL-2 and IL-4 production

Short Name: Suppression, IL-2 and IL-4 production

Key Event Component

Process	Object	Action
interleukin-2 production	interleukin-2	decreased
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin
Dexamethasone
Azathioprine
Methotrexate
Benzo(a)pyrene
Urethane
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
cynomolgus monkey	Macaca fascicularis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNIs suppress production of IL-2, IL-3, IL-4, IL-5, IFN-γ, Granulocyte Macrophage colony-stimulating Factor (GM-CSF), and other cytokines, as induced by CD2/CD3 or CD3/CD26 stimulation, in human peripheral blood mononuclear cells (PBMC) (Sakuma et al. 2001a). Also, CNIs (FK506 and CsA) suppress production of IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, Tumor Necrosis Factor-α, IFN-γ, and GM-CSF, as induced by CD3/PMA stimulation, in human PBMC (Dumont et al. 1998).

CNIs (FK506 and CsA) exhibit suppression of IL-2 production induced from mixed lymphocyte reactions in mice and humans (Kino, T et al. 1987a).

Treatment with CsA or 2C1.1 resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

These facts indicate that Calcineurin-NFAT system-mediated suppression of cytokines is commonly found in humans, monkey and mice.

Key Event Description

Production of T cell cytokines including Interleukin (IL)-2 and IL-4 is regulated by nuclear factor of activated T cells (NFAT)/ activator protein-1 (AP-1) complexes. Activated NFAT/AP-1 complex that bind at the site of the IL-2 and IL-4 promoters, thereby induces transcription of IL-2 and IL-4 (Jain et al. 1993). For IL-2, NFAT proteins are necessary for IL-2 gene expression and cooperation of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016).

Calcineurin inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) hinder the formation of the functional NFAT/AP-1 complexes by interfering with NFAT nuclear localization (Flanagan et al. 1991). Reduced binding of NFAT/AP-1 complexes at the promoter site of the IL-2 gene lowers the transcription of the mRNA of IL-2 and the following cytokine production.

Transcription of IL-4 is also inhibited by CNIs in the same manner as IL-2 (Dumont et al. 1998).

In CD3/ phorbol 12-myristate-13-acetate (PMA)-activated human T cells, FK506 suppressed production of IL-2, IL-4, and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at 10 nM (Dumont et al. 1998).

Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC_{25} and IC_{50} for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC_{25} and IC_{50} for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (KLH) (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).

Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

CNIs is considered to increase carcinogenicity through the suppression of IL-2 and IL-4 production.

- Renal transplant patients on immunosuppressive therapy were found to develop cancer within 10 years after surgery (Luster, M.I. et al. 1993).

In experimental animal studies, carcinogenicity of FK506 was reported as follows.

- In mice subjected to topical application testing, in which 100 µL of FK506 ointment was applied once daily for two years to roughly 40% of the total body area, an increased incidence of lymphoma was found in mice of the 0.1% ointment group showing high blood concentrations of the drug (Maruho Co., Ltd 2014).
- In hairless albino mice, virtually all of which developed skin tumors after a 40-week exposure to ultraviolet light, application of a 1% FK506 ointment reduced the time to outbreak of the skin tumors. (Maruho Co., Ltd 2014).

How it is Measured or Detected

Quantitation of cytokine content was done on appropriately diluted samples, run in duplicate, using Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) kits to test matched Antibody pairs with biotin-horseradish peroxidase-streptavidin detection and 3,3',5,5'-tetramethylbenzidine substrate. ELISA plates were scanned in a Molecular Devices UVmax plate reader (Menlo Park, CA), using SOFT max software (Molecular Devices) (Dumont et al. 1998).

Ex vivo whole blood stimulated cytokine (IL-2, IL-4, IL-5, and IL-17) production assay in the supernatants were determined using an electrochemiluminescent immunoassay from Meso Scale Discovery (MSD; Gaithersburg, MD) (Kevin, G. et al. 2014).

Total RNA was extracted using RNeasy mini kit (Qiagen, Chatsworth, CA) and quantitated by absorbance at 260 nm. Cytokine mRNAs were detected using a RiboQuant MultiProbe RPA system (PharMingen, San Diego, CA). Riboprobes were 32P-labeled and hybridized overnight with 10 to 30 mg of the RNA samples. The hybridized RNA was treated with RNase and purified according to the RiboQuant protocol. The samples were then electrophoresed in 6% polyacrylamide-Tris-borate-EDTA-urea gels using the Seqi - Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Hercules, CA), or minigels (Novex, San Diego, CA). The gels were dried, exposed and quantitated in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software (Dumont et al. 1998).

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List of Adverse Outcomes in this AOP

Event: 984: Impairment, T-cell dependent antibody response

Short Name: Impairment, T-cell dependent antibody response

Key Event Component

Process	Object	Action
Immunosuppression		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	AdverseOutcome

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
cynomolgus monkey	Macaca fascicularis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNIs induced impairment of TDAR is demonstrated with rodent studies. That is, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino et al. 1987). Likewise, oral administration of FK506 to rats over a four-week period reduced production of both anti-KLH-IgG and IgM antibodies after subcutaneous immunization with KLH (Ulrich et al. 2004). Moreover, Treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum SRBC-specific IgM and IgG (Kevin, G. et al. 2014). As for humans, in vitro experiments showed that treatment with FK506 or CsA of peripheral blood mononuclear cells from blood-bank donors suppressed the production of IgM and IgG antibodies specific to T-cell–dependent antigens. (Heidt et al, 2009) Also, in SKW6.4 cells (IL-6–dependent, IgM-secreting, human B-cell line) cultures, FK506 or CsA suppressed the production of IgM antibodies in the presence of T-cell activation. (Sakuma et al. 2001b) Considering that FKF506 and CsA reduce T cell-derived cytokines including IL-2 and IL-4, these findings strongly suggest that impairment of TDAR following reduced production of such cytokines occurs at least in common among humans monkey and rodents.

Key Event Description

Antibody production to T-cell–dependent antigens is established through the coordination of B cells, antigen-presenting cells as well as T-cell–derived cytokines, which stimulate B cells to proliferate and differentiate. T-cell–dependent antibody response (TDAR) might be altered if any of these cell populations is affected.

Interleukin (IL)-2 stimulates B cells to proliferate through surface IL-2 receptors. IL-4 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Suppressing the production of these B-cell–related cytokines appears to impaire TDAR, as seen in the result of FK506 treatment (Heidt et al, 2009).

IL-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production. IL-2 promotes differentiation of B cells through IL-2 stimulates differentiation of the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs TDAR (Alberts et al. 2008).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) decrease in anti- keyhole limpet hemocyanine (KLH) immunoglobulin (Ig)G. (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene (DBA) exposure reduced total IgG antibody in spleen cell culture supernatants after in vitro stimulation with lipopolysaccharide (LPS) (Donna, C. et al. 2010).

Treatment with cyclosporin A (CsA) at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum sheep red blood cells (SRBC)-specific IgM and IgG (Kevin, G. et al. 2014).

After a 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41.6 and 83.2 nM) of CsA (Heidt et al, 2009).

After a 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated peripheral blood mononuclear cells (PBMC) culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.012 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.083 to 83.2 nM) of CsA (Sakuma et al. 2001b).

Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG was reduced at the dose level of 3 mg/kg/day (Ulrich et al. 2004).

Mice were treated with FK506 or CsA for 4 days, and immunized with SRBC, after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).

As immunosuppression-derived adverse outcomes by calcineurin inhibition, FK506 and CsA increase the frequency and/or severity of infections and allergic reactions impaired TDAR deems to be one of the causative factors for these side effects . Some clinical trials of FK506 and CsA revealed these adverse effects as follows.

- In clinical trials of renal transplantation using FK506 or CsA, opportunistic infections such as candida, cytomegalovirus and herpes simplex virus were reported (Ekberg et al. 2007).
- In recipients of liver transplants treated with FK506 or CsA, opportunistic infections such as cytomegalovirus, hepatitis C virus, hepatitis B and herpes simplex virus were reported (Fung et al. 1991).
- Cardiac transplant patients treated with cyclosporin developed pulmonary infections within the first year after surgery (Luster, M.I. et al. 1993).
- In patients of X-linked autoimmune enteropathy treated with CsA or FK506, serum levels of IgE developed extremely high during the immunosuppressive therapy (Kawamura et al. 1997).
- Renal transplant recipients treated with belatacepy/mycophenolate (MMF)/predonisone or FK506/MMF/prednisone showed significantly lower the geometric mean hemagglutination inhibition titer against influenza vaccine, hemagglutination-specific IgG and isotype IgG1 antibodies, and IgG-antibody secreting cells response (Gangappa et al. 2019).

How it is Measured or Detected

TDAR could be examined in vivo and in vitro.

In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with Enzyme-Linked ImmunoSorbent Assay (ELISA) or with a plaque-forming cell (PFC) assay.

- Rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH, after which the serum was examined for T-cell–dependent, antigen-specific, IgM and IgG levels using a Sandwich ELISA kit (Ulrich et al. 2004).
- Mice were repeatedly administered calcineurin inhibitors (CNIs) including FK506 and CsA orally for 4 days and immunized with SRBC, after which spleen cells were examined using a PFC assay (Kino et al. 1987).
- Cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBC, after which the serum was examined for Anti-SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Kevin, G. et al. 2014).
- Mice were exposed a single pharyngeal aspiration of DBA, after which supernatants of splenocytes cultured for 24 h in the

presence of LPS and assayed using a mouse IgM or IgG matched pairs antibody kit (Bethyl Laboratories, Montgomery, TX) (Donna, C. et al. 2010).

For in vitro studies, total IgM and IgG levels in culture supernatant are often measured after polyclonal T-cell activation rather than measuring antigen stimulation in immune cell cultures.

- T cells and B cells isolated from human peripheral blood mononuclear cells (PBMC) were co-cultured with a CNIs for nine days in the presence of polyclonal–T-cell stimulation, after which supernatants were tested for immunoglobulin IgM and IgG levels using a Sandwich ELISA kit. Treatment with FK506 or CsA reduced the levels of IgM and IgG at the concentrations of 0.3 and 1.0 ng/mL or 50 and 100 ng/mL (Heidt et al, 2009).
- SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) were cultured with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. After culturing for four days, IgM produced in the culture supernatants was measured using an ELISA kit. FK506 or CsA reduced the levels of IgM at the concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma et al. 2001b).
- In order to examine class switching, T cells derived from human PBMCs were cultured with CNIs, and cytokine mRNA levels of Interferon-gamma, IL-2, IL-4, IL-5, IL-10, IL-13, and other B-cell–stimulatory cytokines produced in T cells were measured by quantitative PCR (Dumont et al. 1998).

Regulatory Significance of the AO

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

In AOP154, it was difficult to define susceptibility to infection as an AO for the AOP154, so TDAR, which is recommended as an indicator of immunosuppresoin in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1508: Inhibition, Calcineurin Activity leads to Interference, nuclear localization of NFAT

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Mus musculoides	Mus musculoides	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Sex **Evidence**

CN is broadly distributed throughout the body, and the structure of CnA and CnB is highly conserved from yeasts to humans (Kincaid. 1993).

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells, and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all have PPIase activity (Wang P et al. 2005). They form binary complexes with their ligand cyclosporine A.

These facts indicate that CN and immunophilins are conserved among animals and plants although they show multiple physiological functions.

In addition, CNI/immunophilin complex-induced inhibition of CN phosphatase activity resulting in suppression of immune responses is found in humans and mice.

Key Event Relationship Description

The phosphatase activity of calcineurin (CN) is known to be inhibited by CN inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) through the formation of complexes with immunophilins.

Immunophilins of FK506-binding protein (FKBP) and cyclophilin bind with CNIs FK506 and CsA to form complexes, which inhibit CN activity (Barik. 2006).

While FKBP12, FKBP12.6, FKBP13, and FKBP52 are all part of the FK506-binding FKBP family, FKBP12 has a significant involvement in the mechanism of action for FK506-induced immunosuppression (Siekierka et al. 1989, Kang et al. 2008).

FKBP12 is a 12-kDa protein localized in cytoplasm and has been isolated from Jurkat T-cells as a receptor that binds to FK506 (Bram et al. 1993). FKBP12 has an FK506-binding domain (FKBD) that comprises 108 amino acids, and is expressed in T cells, B cells, Langerhans cells, and mast cells (Siekierka et al. 1990, Panhans-Gross et al. 2001, Hultsch et al. 1991).

Cyclophilin and FKBP both exhibit peptidyl propyl isomerase (PPIase) activity, but inhibition of PPIase activity is not related to CN regulation.

CN is a heterodimer that comprises a catalytic subunit (CnA) and a Ca-binding regulatory subunit (CnB). CnA handles phosphatase activity as well as calmodulin binding, and CnB regulates intracellular calcium and CnA (Klee et al. 1988, Zhang et al. 1996). CnA is a 59kDa protein with a serine-threonine phosphatase domain.

CNI-immunophilin complexes such as FK506/FKBP complexes and cyclophilin/CsA complexes bind directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which in turn inhibits phosphatase activity of CN (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

The nuclear factor of activated T cells (NFAT) is a substrate of CN (Rao et al. 1997).

When T-cell activation takes place, T-cell–receptor-mediated stimulus increases the intracellular concentration of calcium and activates CnB, which subsequently induces CnA phosphatase activation, leading to dephosphorylation of NFAT. In that process, dephosphorylated SP motifs expose the nuclear localization signal (NLS) and cover nuclear export signal (NES), thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

When CN activity is inhibited by the binding of immunophilin complexes, dephosphorylation does not occur in NFAT, thereby resulting in nuclear export.

Evidence Supporting this KER

Biological Plausibility

The molecular structures and functions of CN and NFAT are based on sufficient scientific evidence as mentioned above The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPIase activity, but the isomerase activity per se is not relevant for CN activity indicating that the latter is affected by the molecular structure of the complex (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

As mentioned above, inhibition of CN phosphatase activity interferes with the dephosphorylation of NFAT, which leads to the suppression of its nuclear localization.

Empirical Evidence

Much experimental data is available that supports the inhibition of CN activity induced by CNI/immunophilin complexes, which subsequently suppress nuclear localization of NFAT. In addition, CN phosphatase activity is inhibited by 24 hours treatment with CNI of FK506 and CsA with IC50 values of 0.5 and 5 nM, respectively (Fruman et al.1992).

Also, concentration-dependent reduction of in vitro nuclear localization of NFAT was evident using imaging flowcytometry at the maximum concentration of 1 µM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM (T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991).

These findings show that dose responses and temporality of MIE and KE1 seem to be the same.

Uncertainties and Inconsistencies

CN and NFAT are expressed in T cells and other immune cells including B cells, DC, and NKT cells and related to cytokine productions from these immune cells. Also, expression of IL-2 receptors (IL-2R) in DCs are lowered due to the inhibition of CN phosphatase activity by CNI treatment. Of these, reduced production of IL-2 and IL-4 from T cells plays a major role in suppression of TDAR due to lower proliferation, differentiation, and class switching of B cells. There have been no reports of CNI-induced reduction of cytokines other than IL-2 and IL-4 or reduced expression of IL-2R resulting in TDAR suppression.

FKBP12, a specific immunophilin that binds with FK506, is also an accessory molecule that binds to IP3 and Ryanodine receptors, both of which occur in Ca channels located on the membrane of the endoplasmic reticulum and participate in the regulation of intracellular Ca concentration. When binding with FK506, FKBP12 leaves these receptors to increase the influx of Ca2+ from the endoplasmic reticulum to cytoplasm, which should increase CN activity. Treatment with FK506, however, suppresses NFAT nuclear localization. In addition, FKBP12-knock out mice show no changes in immune function, including T-cell function. These facts suggest that the inhibition of CN-NFAT systems induced by FK506 treatment results from direct inhibition of CN phosphatase activity by FK506/FKBP12 complexes and not by affecting Ryanodine and IP3 receptors associated with FKBP12.

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

Dose-response analysis of the effects of FK506 on CN phosphatase activity in mast cell-derived KiSVMC4W cells transfected with human FKBP12 cDNA showed that increased expression of FKBP12 resulted in a greater than ten-fold increase in sensitivity to FK506-mediated inhibition, as indicated by an IC50 value of roughly 2 nM with linear inverse dose-response curve after 1 hour incuvation (Fruman et al.1995). Another phosphatase assay showed that FK506 inhibition of CN activity was concentration-dependent reverse sigmoidal and that IC50 values for CN inhibition were approximately 0.5 nM for FK 506 and 5 nM for CsA after 1 hour culture (Fruman et al.1992).

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing CNI concentrations from 0.1 nM (Jurkat human T cells) up to 1 µM (1000 nM) using imaging flowcytometry. Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again at maximal concentrations of 1 µM with minimum concentration of 10nM (Maguire et al. 2013).

So far, there is no evidence available that the dose response of inhibition of CN phosphatase activity is correlated with nuclear translocation of NFAT; however, the concentration ranges of CNIs for inhibition of CN phosphatase activity and nuclear translocation of NFAT seem to be the same range.

Time-scale

Inhibition of CN phosphatase activity was examined after 1 hour culture of T cells (Fruman et al.1995, Fruman et al.1992), and inhibition of nuclear translocation of NFAT was measured by imaging flowcytometry after 2 hour culture of T cells with CNI (Maguire et al. 2013).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1017: Interference, nuclear localization of NFAT leads to Reduction, NFAT/AP-1 complex formation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents, and other mammalian species (Rao et al. 1997).

CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Relationship Description

Activated (dephosphorylated) nuclear factor of activated T cells (NFAT) is translocated into the nucleus through the molecular changes of exposing nuclear localization signal (NLS) and concomitant masking of nuclear export signal (NES) due to dephosphorylation of the SP motifs of NFAT. (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

In the nucleus NFAT binds with AP 1 at the IL-2 promoter region, (Schreiber and Crabtree 1992; Jain et al. 1992) and induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

Once CN phosphatase activity is inhibited, dephosphorylation of NFAT and subsequent nuclear localization of NFAT decreases, which results in a decrease of NFAT/AP-1 complex formation at the cytokine promoter sites (Rao et al. 1997).

Evidence Supporting this KER

Biological Plausibility

As has been mentioned, NFAT has NLS and NES domains among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS domain is exposed whereas the NES doman is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000).

It is well known from the experiments using CN inhibitors (CNIs) that interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).

In contrast to T cells, B-cell receptor-mediated increases in intracellular concentration of calcium in B cells leads to NFAT nuclear localization, thereby producing some classes of cytokines in the same manner as T-cells (Bhattacharyya et al.2011). However, there has been no report of any evidence that CNI acts directly on B cells to effect antibody production.

Expression of IL-2 receptors in dendritic cells and NKT cells is also reported to be regulated by this CN-NFAT system (Panhans-Gross A et al. 2001; Kim et al. 2010), but there is no report showing that CNIs suppress TDAR through the changes in IL-2R expression in these cells.

Empirical Evidence

The relationship of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

Imaging flowcytometry revealed that concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the maximum concentration of 1 μM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM ($CD4^+$T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013).

Gel mobility shift assays using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1μM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991) NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997).

Quantitative understanding of NFAT/AP-1 complex formation in the nucleus is insufficient although nuclear NFAT/AP-1 complex formation was shown to be inhibited by FK506 at concentrations within the range of FK506 for the inhibition of nuclear translocation

of NFAT.

Uncertainties and Inconsistencies

Nothing especially

Quantitative Understanding of the Linkage

Response-response relationship

The relationship of the interference of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing FK506 concentrations from 0.01nM (Jarkat T cells) up to 1 µM (1000 nM). Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again from 10nM to maximal concentrations of 1 µM (Maguire et al. 2013). Both parameters were measured after 2 hour culture of T cells with FK506.

KE2:

Reduction in generation of NFAT/AP-1 complexes can be detected using a gel shift assay (Rao et al. 1997, Jain et al. 1992, Jain et al. 1993).

Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991). As mentioned above, gel mobility shift assays also showed that NFAT/AP-1 complexes were formed only in the nucleus after T cell activation with unchanged preexisting NFAT in the cytoplasm and that treatment of T cells with 1µM FK506 led to decease the levels of NFAT/AP-1 complex (Jain et al. 1992).

These findings suggest that nuclear translocation of NFAT after T cell stimulation is strongly related to the complex formation with AP-1 in the nucleus, and FK506 was shown to inhibit NFAT/AP-1 complex formation in the nucleus at the concentrations within the concentration range of FK506 for suppressing nuclear translocation of NFAT (Maguire et al. 2013).

Time-scale

Nuclear translocation of NFAT was shown to be inhibited in vitro using imaging flowcytometry after 2 hours culture of T cells with FK506 (Maguire et al. 2013), and gel mobility shift assays revealed the inhibition of nuclear translocation of NFAT and following complex formation with AP-1 within the nucleus after 2 hours culture of T cells with FK506 (Jain et al. 1992, Flanagan et al. 1991).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1509: Reduction, NFAT/AP-1 complex formation leads to Suppression, IL-2 and IL-4 production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1, IL-2 production and CD25 (IL-2R) up-regulation (Yoshida et al. 2015).

In splenic lymphocytes and/or CD4+ T cells, ursolic acid suppressed products of NF-kB, NFAT and AP-1, and inhibits secretion of IL-2 and IL-4, mRNA level of IL-2 and CD25 expression (Checker et al. 2012).

NFATp- and NFAT4-deficient mice indicate decreased production of IL-2 (Ranger et al. 1998).

NFAT/AP-1 complex formation in the nucleus was shown using murine and human T cells lines (Jain J et al. 1992). In addition to data on suppression of cytokine production by CNI in rodents, FK506 is reported to inhibit expression of both IL-2 and mRNA in human anti-CD3/PMA-activated cells (Dumont et al. 1998).

Key Event Relationship Description

Localized nuclear factor of activated T cells (NFAT) in the nucleus of T cells forms complexes with activator protein-1 (AP-1) at the Interleukin (IL)-2 promoter region (Schreiber and Crabtree 1992; Jain et al. 1992), which induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

For IL-2, NFAT proteins are necessary for IL-2 gene expression and interaction of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

Lowered nuclear localization of NFAT by calcineurin inhibitor (CNI) results in decreased formation of NFAT/AP-1 complex at the promoter region of IL-2 genes in the nucleus of T cells thereby reducing the transcription of IL-2 (Dumont et al. 1998). Production in T cells of IL-4 and other classes of cytokines is also suppressed in the same manner as IL-2 (Dumont et al. 1998).

Evidence Supporting this KER

Biological Plausibility

T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015).

Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent

glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001).

Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).

NFATp- and NFAT4-deficient mice indicate decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is generally accepted that NFAT, translocated to the nucleus after T-cell stimulation, binds with AP-1 to the promoter regions of the cytokine genes to mount transcription, which follows production of these T-cell–derived cytokines. Of these cytokines, IL-2 and IL-4 promote proliferation, maturation, and class-switching of B cells to enhance TDAR.

There is also sufficient evidence to support the hypothesis that CNI-induced decreases in T-cell–derived cytokine production is mediated through suppressed nuclear localization of NFAT, with a resultant decrease in the amount of NFAT/AP-1 complex binding to the promoter regions of T-cell-derived cytokines.

When stimulated with ovalbumin, calcineurin A (CnA)-knockout (KO) mice produce less Interferon (IFN)- γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to trinitrophenol-ovalbumin (Zhang et al. 1996).

The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly; moderately enhanced B- and T-cell responses, with bias towards Th2- cell responses; decreased IFN-γ production in response to TCR ligation; reduced proliferative responses by T cells; impaired repopulation of the thymus and lymphoid organs; impaired Th2-cell responses and IL-4 production; grossly impaired T-cell effector functions, with profound defects in cytokine production and cytolytic activity; B-cell hyperactivity; impaired development of CD4 and CD8 single-positive cells, with increased apoptosis of double-positive thymocytes; and mild hyperactivation of peripheral T cells (Macian, 2005).

Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by calcineurin (CN). Suppression of T-cell-derived cytokines is noted both in CnA-KO and NFAT-KO mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system.

FK506-FKBP12 complex decreased CN phosphatase activity, which inhibits. Because NF-ATp is an essential transcription factor regulating the IL-2 gene, FK506 ultimately blocks the T-cell response by inhibiting IL-2 transcription (Panhans-Gross A et al. 2001). FK506 inhibited IL-2 mRNA expression in anti-CD3/phorbol 12-myristate-13-acetate (PMA)-activated cells (Dumont et al. 1998).

These facts indicate that although NFAT is widely involved in the function of T cells, the effect of CNIs is to suppress production of some classes of T-cell–derived cytokines through reducing the formation of NFAT/AP-1 complexes induced by inhibition of CN phosphatase activity.

Empirical Evidence

Empirical support of Reduction, NFAT/AP-1 complex formation leading to Suppression, IL-2 and IL-4 production is strong.

Rationale

- In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 μg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 μg/mL (Yoshida et al. 2015).
- In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 μM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 μM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM for 4 h (Checker et al. 2012).
- In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is well established that inhibition of NFAT/AP-1 complex formation at the promoter sites reduces the production of T-cell–derived cytokines including IL-2 and IL-4, which are mainly involved in T-cell–dependent antibody response.

- NFAT/AP-1 complex formation is inhibited by CNI shown by gel shit mobility assay using human T cell line or CD4+ T cells from heathy donners after 2 hours treatment with cyclosporin A (CsA) at 1μM.. Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993).
- In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA in a dose-dependent (10 nM) manner after 3 day culture (Dumont et al. 1998).

- Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).
- In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).
- Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003).

Reduced nuclear translocation of NFAT followed by NFAT/AP-1 complex formation and suppression of IL-2/IL-4 productions are shown to occur under similar dose ranges and treatment duration.

Uncertainties and Inconsistencies

CNIs are reported to suppress IL-17 release from Th17 cells and development of Th17 cells from naïve T cells (Tsuda et al, 2012). On the other hand, Yadav reported that Th17 cells increased and Treg cells decreased in number and that the levels of RORC mRNA increased and those of FOXP3 decreased in renal transplanted patients with chronic calcineurin inhibitor toxicity (Yadav, 2015). From these findings, CNIs suppress the functions of Th17 and Treg cells which enhance Th17 cells to develop chronic CNI toxicity.

FK506 suppresses expression of IL-2 receptor (IL-2R: CD25) and costimulatory molecules CD80 (B7.1)/CD40 in Langerhans cells (Panhans-Gross A et al. 2001).

In human NK cells, FK506 suppresses IL-2 responsive proliferation and cytokine production as well as lowers cytotoxicity directed toward K562 tumor cells (Kim et al. 2010). FK506 suppresses IL-2 production of NKT cell line DN32.D3 induced by stimulus from PMA/calcium -ionophore (van Dieren et al. 2010).

The relationship between these FK506-induced mechanisms and NFAT and contribution of those to TDAR are unclear.

In addition to NFAT/AP-1 complexes, NFAT forms complexes at the site of IL-3 and IL-4 enhancers with avian musculoaponeurotic fibrosarcoma oncogene homolog, early growth response 1, early growth response 4, interferon-regulatory factor 4, octamer-binding transcription factor, and other transcriptional partners to induce transcription of a variety of cytokines (Macian 2005). The production of cytokine induced by these transcriptional partners also suppressed by CNI; however, contribution of these additional transcription factors to TDAR is also unclear.

Quantitative Understanding of the Linkage

Response-response relationship

In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 at 80 µg/mL. On the other hand, T-5224 inhibits IL-2 production in a dose-dependent manner from 40, 60 and 80 µg/mL after 48 hours culture. T-5224 also inhibits CD25 (IL-2R) up-regulation at 80 µg/mL (Yoshida et al. 2015).

In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 µM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibits secretion of IL-2 and IL-4 at 0.5, 1 and 5 µM. In lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid also inhibits secretion of IL-2 and IL-4 at 5 µM. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 µM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 µM (Checker et al. 2012).

These findings showed that T-5244 and ursolic acid treated for 24 hours inhibit NFAT/AP-1 complex formation at a single concentration each and that these compounds suppress IL-2 and IL-4 production with dose dependent manner including the doses for inhibition of NFAT/AP-1 complex formation.

FK506 suppressed proliferation in human T cells induced by anti-CD3 mAb in the presence of adherent autologous peripheral blood mononuclear cells (mean IC50 = 0.06 nM). FK506 suppressed, in a dose-dependent (1.2 to 12.5 nM) manner after 22-24 hours culture, production of IL-2, IL-4, and IFN-γ by human T cells stimulated with anti-CD3 mAb in the presence of PMA, as well as inhibited, also in a dose-dependent (10 nM) manner, expression of IL-2, IL-4, and IFN-γ mRNA in anti-CD3/PMA- activated cells (Dumont et al. 1998). On the other hand, the quantitative data for the decreased formation of NFAT/AP-1 complexes by CNI is insufficient, although the formation was suppressed by FK506 at the concentration within the range needed for suppressed production of IL2/IL-4 by FK506 after 2 hours culture.

Time-scale

Inhibition of NFAT/AP-1 complex is detected by gel mobility shit assay after 2 hours culture with CNI; however, suppression of IL2/IL-4 could be measured after 22-48 hours in vitro culture.

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1510: Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
cynomolgus monkey	Macaca fascicularis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In cynomolgus monkeys, the effects of CsA on production of IL-2 and IL-4, and antigen-specific IgM and IgG in TDAR were demonstrated (Gaida K. 2015).

Suppressed IgE and antigen specific IgG1 productions by the blocking of IL-4 receptor were reported in mice using dupilumab (anti-IL-4/13R antibody) (Sanofi K.K. 2018).

Suppressed antigen specific IgE production by the inhibition of IL-4 production was reported in mice using suplatast tosilate (Taiho Pharmaceutical 2013).

Suppressed antigen specific IgE and IL-4 productions by the inhibition of IL-4 production were reported in human cell culture using suplatast tosilate(Taiho Pharmaceutical 2013).

The effects of FK506 on serum concentration of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over four weeks and immunized with KLH (Ulrich et al. 2004). The effects of FK506 and CsA on antigen-specific plaque-forming splenocytes have been demonstrated in mice treated with FK506 or CsA for 4 days and immunized with SRBC (Kino et al. 1987b).

The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt et al, 2009, Sakuma et al, 2001).

The effects of FK506 and CsA on production of IL-2 and IL-4 have been demonstrated using mice and human cells (Kino et al. 1987a, Dumont et al. 1998).

These facts suggest that there are no species differences between humans, monkeys and rodents in inhibitions of IL-2 and IL-4 production and TDAR induction.

Key Event Relationship Description

Interleukin (IL)-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of T-cell dependent antibody response (TDAR), both of which induces/enhances T cell dependent antibody production. IL-4 affects maturation and class switching of B cells as well as proliferation, IL-2 promotes differentiation of B cells through IL-2 receptors and stimulates the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs T cell dependent antibody production (Alberts et al. 2008).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR

T cell-derived cytokines play important roles in the development of TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production.

Thus, suppressing the production of IL-2, IL-4, and other cytokines in T cells reduces stimulation of B cells including proliferation, activation, and class switching, and leading to impairment of TDAR. Therefore, suppressing the production of these B-cell-related cytokines appears to be the main factor in impairment of TDAR by inhibitors of T-cell–dependent-antibody production.

Evidence Supporting this KER

Biological Plausibility

Cyclosporin A (CsA) is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in the reduction of the productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015).

It is established that IL-2 stimulates B cells to proliferate through the surface IL-2 receptors and that IL-4 stimulates B cells to proliferate, to induce class switch, and to differentiate into plasma and memory cells.

Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody

results in the decrease in T cell dependent antibody production.

Th2 cell produces cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 from Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells.

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). They suggest that the blocking of IL-2 signaling by anti-IL-2R antibody results in decreased rejection through the inhibition of the activation of antigen specific T cell with reduced antibody production.

FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Several in vivo studies in rodents showed decreased TDAR by the treatment of FK506 (Kino et al. 1987b, Ulrich et al. 2004). In in vitro tests examining antibody production in blood samples obtained from blood-bank donors, peripheral blood mononuclear cells (PBMC) treated with FK506 and CsA suppressed the production of IgM and IgG antibodies to T-cell dependent antigens (Heidt et al, 2009).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

However, as for the suppression of humoral immunity induced by the inhibition of calcineurin (CN) phosphatase activity, calcineurin inhibitors (CNIs) do not affect B cells directly but rather indirectly through T cells. That is, FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B cell response by FK506 and CsA appears due solely to inhibition of T helper cells (Heidt et al, 2010).

Therefore, it is concluded that decreased amounts of IL-2 and IL-4 secreted from helper T cells is the main factor for suppression of TDAR induced by CN phosphatase inhibition.

Empirical Evidence

Empirical support of the suppression, IL-2 and IL-4 production leads to impairment, T-cell dependent antibody response is strong.

Rationale

- Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).
- In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018).
- In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013).
- In the clinical study of renal transplantation, basiliximab decreased incidence of acute rejection at 20 mg/kg (Novartis Pharma 2016). In human T cell culture immunized with PPD, basiliximab reduced activation of antigen specific T cell at the concentration of 300 ng/mL (Novartis Pharma 2016).
- In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998).
- FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a).
- After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2009).
- After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001).
- Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004).
- Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).
- 1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH and decrease in anti-KLH IgG(Alessandro, B. et al. 2003).

In vitro suppression of T-cell–derived cytokines and T-cell-dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two KEs, which showed earlier onset of cytokine production and delayed onset of antibody production.

Uncertainties and Inconsistencies

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Therefore, reduced production of both IL-2 and IL-4 might certainly induce suppression of TDAR; however, there remains some possibility of additional suppression of other immune functions.

Quantitative Understanding of the Linkage

Response-response relationship

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In the blocking of IL-4 receptor in mice by dupilumab (anti-IL-4/13R antibody) at 25 mg/kg of twice weekly subcutaneous administration for 4weeks, IgE production was suppressed to about 1/100 and antigen specific IgG1 production was suppressed to about 1/200 (Sanofi K.K. 2018).

In the inhibition of IL-4 production in mice by suplatast tosilate at 10, 20, 50 and 100 mg/kg of oral administration for 5 days, antigen specific IgE production was suppressed from about 1/10 to 1/100 (Taiho Pharmaceutical 2013). In human T cell culture by suplatast tosilate at the concentration of 10 µg/mL, antigen specific IgE production after 10 days was suppressed from 56 to 72% and IL-4 production after 3 days was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. That is, murine small resting B cells, cultured with irradiated hapten-specific TH1 clone, were induced to enter cell cycle at 2 days and to secret antibody at 5 days. An anti-IL-2 and anti-IL-2R antibodies completely inhibited this T-cell dependent antibody production (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the m-RNA levels of T-cell cytokines at 8h post-stimulation including IL-2 and IL-4 at 1.0ng/mL (1.24nM) FK506 or 100ng/mL (90.7nM) CsA and inhibited IgM and IgG productions after 9 days at 0.3 and 1.0ng/mL FK506 and 50 and 100ng/mL CsA (Heidt S. 2010).

Time-scale

In CsA-treatment for 24 days at 50 mg/kg BID, cynomolgus monkeys showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In human T cell culture, suplatast tosilate inhibits IL-4 production after 3 days and antigen specific IgE production after 10 days (Taiho Pharmaceutical 2013).

In the human T-B cell co-culture, CNIs of FK506 and CsA lowered the m-RNA levels of IL-2 and IL-4 at 8h post-stimulation and inhibited IgM and IgG productions after 9 days (Heidt S. 2010).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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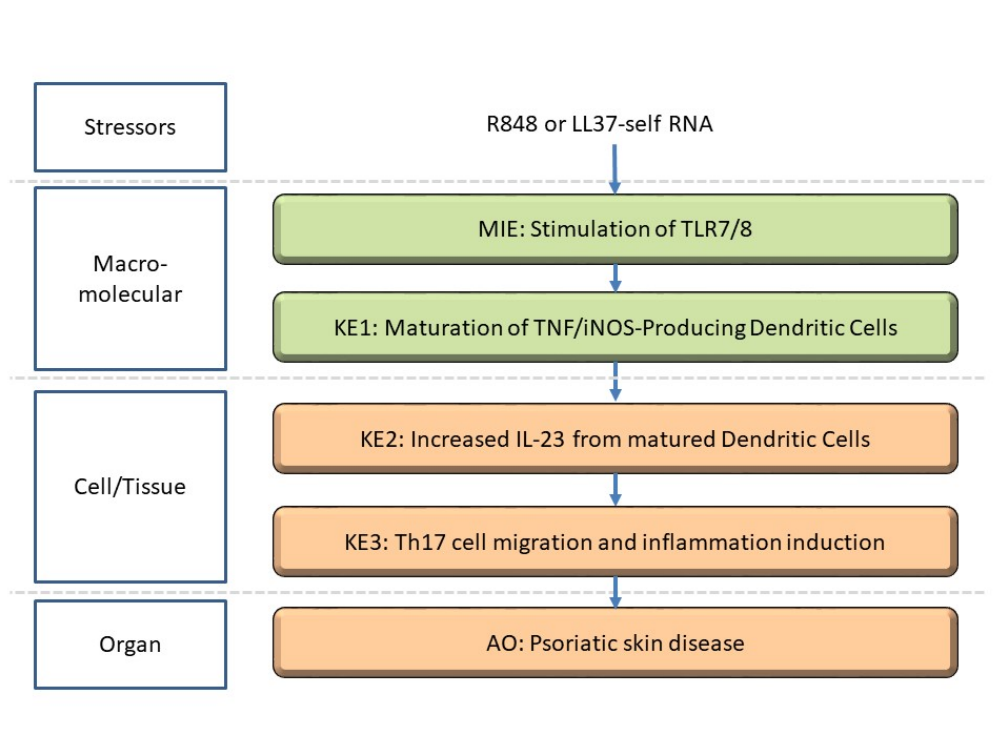
14. Donna C. S, Matthew J. S and Kimber L. W Jr. (2010) Systemic immunosuppression following a single pharyngeal aspiration of 1,2:5,6-dibenzanthracene in female B6C3F1 mice, Journal of Immunotoxicology, 7:3, 219-231

AOP ID and Title:

AOP 313: Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease
Short Title: Skin disease by stimulation of TLR7/8

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.75	Included in OECD Work Plan

Abstract

Toll-like receptor (TLR) 7 and TLR8 are pattern recognition receptors that are known to activate antiviral reaction of immune system, hyperactivation of which can lead to psoriatic skin disease when hyperactivation of them occurred. The relationship between TLR7/8 and immune functions is well understood, and antiviral compound that work by stimulating TLR7/8 have been developed. TLR7/8 agonists such as imidazoquinolin compounds stimulate these TLRs through the formation of homodimer. This signal activates the IL-23/IL-17 axis, which leads to psoriasis and other related skin diseases.

Activation of the IL-23 / IL-17 axis and causes abnormal proliferation and inflammation of the epidermis, which is a pathological condition of psoriasis. This AOP shows an association between TLR7 / 8 stimulation and psoriatic skin disease.

TLR7-mediated signaling in plasmacytoid dendric cells (pDC) is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response, secreting vast amounts of IFN type 1. Similarly, upon engagement of ligands in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation. IFN-α and TNF-α cooperatively mature myeloid dendritic cells. TLR7/8 agonist stimulates a specific population of inflammatory dermal dendritic cells referred as TNF and inducible nitric oxide synthase–expressing DCs (Tip-DCs) to produce IL-23 after maturation by enhanced transcriptional activity.

IL-23R is mainly expressed in Th17 cells. In chronic psoriasis, the cytokines IL-12 and IL-23 produced by resident DC are the main causes. Not only does the expression of IL-23 increases in the skin tissue of the lesion, Th17 cells also increase.

Mature Th17 cells are activated by IL-23 stimulation. Signaling through IL-23 produces cytokines IL-17 and IL-22 that mediate the psoriasis response and promote neutrophil migration into the epidermis, epidermal cell proliferation, and similar responses, which lead to the development of a psoriasis rash. In mice, psoriasis-like hyperplasia is induced by the application of IL-23 but does not occur in IL-17A and IL-22 KO mice, so IL-17A and IL-22 play an important role downstream of IL-23.

IL-17 receptor form heterodimers, and IL-17RA / IL-17RC appears in a variety of cells, including fibroblasts and epidermal cells. IL-17RE / IL-17RA expressed in epidermal cells and IL-17C binding are also important in the pathology of psoriasis. Immunohistochemically, IL-17A is expressed only in cells of the dermal papilla layer, while IL-17C is widely expressed in cells such as hyperproliferative overexpressed keratinocytes, leukocytes, and vascular endothelial cells. IL-17C produces keratinocytes by bacterial stimulation and further stimulates keratinocytes to induce the production of various cytokines and chemokines. Keratinocytes are known to be self-activated by IL-17C.

IL-17 and IL-22 secreted from Th17 act on keratinocytes, causing abnormalities in keratinocytes through the secretion of inflammatory cytokines, chemokines, growth factors, and antimicrobial peptides, and thereby exacerbating the skin symptoms of psoriasis.

The creation of this AOP began with an examination of important event relationships brought about by TLR7 / 8 activity due to environmental or genetic factors and resulting in abnormal differentiation of keratinocytes, which leads to thickening of the epidermis and its resultant autoimmune skin disease, psoriasis

Background

Psoriasis is an chronic autoimmune disease characterized by chronic epithelial inflammatory disease induced by environmental factors such as infection, stress, smoking or alcohol consumption as well as by genetic factors. The onset of psoriasis has been reported to be triggered by drugs and chemical substances use, including beta-blockers, chloroquine, lithium, ACE inhibitors, indomethacin, terbinafine, and interferon alpha. Diagnosis is based on the type and distribution of the lesions.

Psoriasis occurs when abnormal differentiation (keratosis) of keratinocytes leads to thickening of the epidermis. Patients often exhibit an erythema with a clear border and epidermal hyperplasia, stratum corneum hyperplasia, heterocytosis in the stratum corneum, mixed skin moist cells of neutrophilic granulocytes and T cells in the epidermis. Dendritic cells (DC) and macrophages are associated with silver-white plaque. Neutrophilic effusion (Munro microabscesses) are observed in the epidermis, and CD8+ T cells (Tc17) increase the expression of angiogenesis related genes.

The main therapeutic agents are mild topical treatments such as emollients, salicylic acid, coal tar preparations, anthralin, corticosteroids, vitamin D3 derivatives, retinoids, calcineurin inhibitors or tazarotene. UV therapy is also used for moderate or severe psoriasis. Widespread psoriasis is treated with systemic therapies such as immunomodulators methotrexate, cyclosporin, retinoids and other immunosuppressants used alone or in combination.

Although there are stressors that are well known to induce psoriasis-like skin inflammation in mice, this AOP is based primarily on an understanding of stimulation caused by imiquimod, resiquimod or LL37-selfRNA complexes, for which a significant body of scientific literature has been published.

As a test model for psoriasis, an Autoimmune skin disease, mouse tests that induce skin inflammation like psoriasis are frequently conducted using the imidazoquinoline derivative imiquimod. This AOP is primarily based on an understanding of stimuli caused by imiquimod, resiquimod, or LL37-selfRNA complexes.

Imiquimod is derived from imidazoquinoline and is often used to create mouse models. It is our hope that this AOP will contribute to greater knowledge about the development of psoriatic skin diseases that start from stimulation of TLR as well as the development of new treatment targets for psoriasis.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1706	Stimulation, TLR7/8	Stimulation of TLR7/8
2	KE	1822	Maturation of TNF/iNOS-Producing Dendritic Cells	Maturation, TNF/iNOS-Producing Dendritic Cells
3	KE	1707	Increase, IL-23 from matured dendritic cells	Increase of IL-23

Sequence	Type	Event ID	Title	Short name
4	KE	1708	Th17 cell migration and inflammation induction	Th17 cell migration and inflammation induction
5	AO	1709	Psoriatic skin disease	Skin disease

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Stimulation, TLR7/8	adjacent	Increase, IL-23 from matured dendritic cells	High	High
Increase, IL-23 from matured dendritic cells	adjacent	Th17 cell migration and inflammation induction	High	High
Th17 cell migration and inflammation induction	adjacent	Psoriatic skin disease	High	High

Stressors

Name	Evidence
Imiquimod	High
Resiquimod	High

Overall Assessment of the AOP

TLR7/8 is stimulated when imidazoquinolin compounds or stimilar agonists from homodimers TLR7-mediated signaling in plasmacytoid dendritic cells (pDC) is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response,thereby secreting large amounts of IFN-α. Similarly, the engagement of ligands in endosomescauses TLR8 initiate the MyD88-dependent pathway, culminating in synthesis and release of TNF-a and other proinflammatory mediators, via NF-κB activation.

IFN-α and TNF-α cooperatively mature myeloid dendritic cells. TLR7/8 agonist stimulates a specific population of inflammatory dermal dendritic cells referred as Tip-DCs to produce IL-23 after maturation by enhanced transcriptional activity.

Naive T cells differentiate into Naive Th17 by both IL-6 and TGF-β cells that express the transcription factors ROR-γt, ROR-α, and STAT3. These naive Th17 cells are self-activated by IL-21 in an autocrine manner and mature into Th17 cells which express IL-23 receptor on cell surface. Mature Th17 cells are activated by IL-23 stimulation. IL-23-mediated signal transduction produces cytokines IL-17.

IL-17 mediates the psoriasis response, promoting such activities as neutrophil migration to the epidermis,and proliferation of epidermal cells, which leads to the outbreak of psoriasis rash. Thus, psoriatic skin is induced mainly by overproduction of IL-17, which leads to a variety of adverse effects. We have identified a number of key events (KEs) along this pathway and created an AOP for stimulation of TLR7/8 that leads to psoriatic skin disease based on these key event relationships(KERs).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Mixed	High

The proposed AOP for psoriasis-like skin thickening resulting from abnormal differentiation of keratinocytes, starting with Toll-like receptor (TLR) 7/8 activity, is independent of life stage, gender, or age (Lowes et al. 2007). The pathogenesis of psoriasis, an

autoimmune disease, is genetically predisposed (3), but the autoantigen that causes psoriasis has not been identified (Zaba et al. 2008). Other causes of psoriasis are caused by external and internal triggers such as mild trauma, sunburn, infection, systemic drugs, and stress (Hansel et al. 2011). Stimulation of TLR7 / 8 releases INF-α and TNF-α in large amounts to produce IL-23, and Th17 cells mature by the stimulation to produce IL-17 and IL-22. In psoriasis skin formation, cytokines such as TNF-α, IL-23, and IL-17 work continuously. Since TNF-α inhibitors significantly suppressed IL-17A and IL-23p19 expression in psoriatic eruptions (Leonardi et al. 2012), by suppressing self-activation of Tip-DC by TNF-α, It can be seen that IL-23 and IL-17A production was suppressed. Anti-IL-17 and anti-IL-17RA antibodies suppress IL-17A and IL-17C, which are highly expressed in psoriatic eruptions. In particular, anti-IL-17RA antibody has been shown to normalize the expression of keratinocyte-related genes and IL-17C production two weeks after administration, followed by normalization of IL-17A production from leukocytes.

In mice, subcutaneous administration of IL-23 induced psoriatic eruption and IL-17A expression (K. A. et al. 2013), and IL-17C transgenic mice overexpressing IL-17C in keratinocytes showed psoriatic eruption. As shown in (8), the reaction of psoriasis-like eruption occurs in mice due to the chain of stimulation to T cells and epidermal cells starting from TLR.

Essentiality of the Key Events

Stressor, MIE and later events:MyD88 knock out(KO) mice

TLR7 (TLR7 / 8 in human) recognizes the imidazoquinoline derivative, binds to the adapter molecule MyD88, activates IRAKs (IL-1 receptor associated kinases), interacts with TRAF6 (TNF receptor associated factor 6) and IKK (Activates the IκB kinase complex). It phosphorylates IκB, induces its degradation, and transfers the transcription factor NF-κB to the nucleus. This pathway is called MyD88-dependent pathway and is essential for the production of inflammatory cytokines such as TNF-α (Akira S, Takeda K .: Nat Rev Immunol. Jul; 4: 499-511, 2004). When pDC is stimulated with a TLR7 / 8 ligand, the transcription factor IRF7 constitutively expressing pDC and MyD88 associate directly. IRF7 activity does not occur when pDCs of MyD88 KO mice are stimulated with TLR7 / 8 ligand. IRF7 is also activated by binding to TRAF6, leading to IFN-α production, which requires the Myd88 / TRAF6 / IRF7 complex. (Satoshi U, Shizuo A: Virus 54; 2: 145-152,2004)

Imiquimod 5% cream was applied to the left flank of female SKH-1 hairless mice (25 g body weight). The IFN-α and TNF-α concentrations in the skin after 1 and 2 hours of application increased these concentrations compared to the untreated skin.

In C57BL / 6 mice (8-12 weeks old) sensitized with 0.5% dinitrofluorobenzene (DNFB) as an antigen, imiquimod 5% cream was applied to the auricle once a day for 3 days. The application of imiquimod 5% cream promoted edema of the ears of mice (promoted DTH) compared to the base cream group. Imiquimod activates antigen-specific T cells by topical application to the skin. (Beserna Cream Interview Form Mochida Pharmaceutical Co., Ltd.)

KE-1 and later event:IL-17, IL-22 KO mice

In mice, psoriasis-like hyperplasia is induced by the application of IL-23, but this effect does not occur in IL-17A and IL-22 KO mice. IL-17A deficient mice show little epidermal hyperplasia after intradermal administration of IL-23. WT mice treated with anti-IL-17A Ab did not show IL-23-induced epidermal hyperplasia. IL-17 KO mice treated with IL-23 do not induce TNF-α mRNA and do not cause epidermal thickening. IL-22 did not increase in IL-17-/-mice after IL-23 administration, and IL-17 clearly increased in IL-22-/-mice. In IL-17-/-, IL-22-/-and WT mice treated with IL-23, immunohistochemically CD3 + T cells, CD11c (dendritic cells), F4 / 80 (macrophages), Gr-1 (Neutrophils) were analyzed. There was no difference in F4 / 80 and Gr-1 + cells in IL-17A-/-compared to WT mice, and CD3 + T cells decreased, but there was no obvious difference in IL-22-/-mice .

These data suggest that cytokines alone are not sufficient to mediate IL-23-induced epidermal changes, and that IL-17 and IL-22 are downstream mediators of mouse skin IL-23-induced changes. Therefore, Th17 cytokines are required for the generation of IL-23-mediated skin lesions.

KE-2 and later events: Mouse psoriasis-like dermatitis model

When TPA (12-O-tetradecanoy1phorbol-13-acetate) on the dorsal skin of K14 / mIL-1F6 gene-modified mice overexpress mouse IL-1F6 (IL-36a) selectively under the keratin 14 promoter was applied, skin pathological findings specific to psoriasis were observed, such as epidermal hyperplasia, epidermal exfoliation and micro-abscess formation, and wet inflammatory cells in the dermis. Quantitative RT-PCR measures mRNA expression levels of inflammatory chemokines and cytokines in skin tissues, and includes inflammatory chemokines: CCL3, CCL4, CXCL10, CXCL1, and cytokines: IL-23, IL-12, IL-1β, etc. These expressions were elevated. (Kyowa Hakko Kirin Co., Ltd.)

References

Appendix 1

List of MIEs in this AOP

Event: 1706: Stimulation, TLR7/8

Short Name: Stimulation of TLR7/8

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

TLR7 and TLR8 are conserved among humans and mice (Gupta et al. 2016). In addition, these molecules are also conserved among humans and rhesus monkeys.

Alignment of amino acid residues between human toll-like receptor 7 (NP_057646.1) and murine toll-like receptor 7 (NP_573474.1, NP_001277684.1, NP_001277685.1, NP_001277686.1, NP_001277687.1, XP_006528776.1, XP_011246087.1 and XP_011246088.1) was 80.74-80.76% identification. Murine TLR7 proteins have 1050 or 1053 amino acids. Human and rhesus toll-like receptor 7 (NP_001123898.1) was 98.00% identification. Rhesus TLR7 protein has 1049 amino acid residues.

In addition, alignment of amino acid residues between human toll-like receptor 8 (NP_619542.1) and murine toll-like receptor 8 (NP_001300689.1) was 70.97% identification. Likewise, human and rhesus toll-like receptor 8 (NP_001123899.1) was 96.73% identification. Murine and rhesus TLR8 referred here have 1029 and 1039 amino acids residues, respectively.

Studies of DC subsets isolated from humans and mice have revealed that TLRs have distinct expression patterns. TLR7 is expressed in freshly isolated human pDCs, whereas TLR8 is expressed in $CD11c^+$ human myeloid DCs (mDCs). In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas other reports showed that TLR7 was exclusively expressed in pDCs (Iwasaki and Medzhitov. 2004).

In mice, $CD4^+$ dendritic cells (DC), CD4/CD8 double negative DC and pDC express TLR7. All splenic DC subsets express TLR8. Moreover, mouse $CD8\alpha^+$ DCs lack TLR7 expression and fail to respond to TLR7 agonists. (Iwasaki and Medzhitov. 2004).

Key Event Description

Toll-like receptors (TLRs) are members of interleukin-1 (IL-1) receptor/TLR superfamily, as they share the intracellularToll-IL-1 receptor (TIR) domain with the IL-1 receptor.

Toll-like receptor (TLR) 7 and TLR8 is known to mediate the recognition of guanosine- and uridine-rich single-stranded RNA (ssRNA) derived from ssRNA viruses and synthetic antiviral imidazoquinoline components specifically that lead to activation of sequential signalling pathway (Akira et al. 2006, Blasius and Beutler. 2010). They also mediate the recognition of self RNA that is released from dead or dying cellsand activation of Myeloid differentiation primary response 88 (MyD88)-dependent signals can occur that leads to inflammation process as well as ssRNA derived from viruses.

TLR7 are exclusively expressed in plasmacytoid DCs (pDCs), which have the capacity to secrete vast amounts of type I interferon (IFN) in rapid response to viral infection (Gilliet et al. 2008, Reizis et al. 2011). TLR8 is express in various tissues, with its highest expression in monocytes. Myeloid DCs (mDCs) also express TLR8 in human (Iwasaki and Medzhitov. 2004). Thus, TLR8 ligands can directly activate mDCs via TLR8. TLR8 signalling activates mDCs to secrete TNF-α and IL-6 (Ganguly et al. 2009). TLR7 and TLR8 are localize in the endoplasmic reticulum of expressing cells (Lai et al. 2017).

Human TLR7 (hTLR7) and human TLR8 (hTLR8) contain 1049 and 1041 amino acid residues, respectively with molecular weight of 120.9 kDa and 119.8 kDa, respectively (Chuang and Ulvitch. 2000). The full-length hTLR7 protein includes a signal peptide of 26 amino acids (1–26 aa). The mature hTLR7 protein ectodomain, trans-membrane, and TIR domain are composite structure of 27–839, 840–860, and 889–1,036 amino acids, respectively (Gupta et al. 2016).

hTLR7 and hTLR8 form a subfamily of proteins that each contain an extracellular domain of >800 residues and share functional and structural features. hTLR7 and hTLR8 contains 27 and 26 leucine-rich repeats (LRRs), which is the largest number of LRRs among TLRs whose structures have been reported (Tanji et al. 2013).

As mentioned above, TLR7 and TLR8 are localize in the endoplasmic reticulum of expressing cells. They are deliver to the endosomes by interacting UNC93B1, which is a 12 membrane-spanning protein (Kawai and Akira. 2011, Itoh et al. 2011). After the trafficking, they initiate cellular responses upon their activation by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Lai et al. 2017).

Structural characterization was conducte with recombinant TLR7 from monkey (*Macaca mulatta*; 96.8% sequence identity with human TLR7) expressed in *Drosophila* S2 cells (Zhang et al. 2016). Rhesus TLR7 exists as a monomer in the absence of ligands. This TLR7 is activate by dimerization triggered by guanosine and uridine-containing ssRNA, which are degradation products of ssRNA, synergistically. Specifically, this TLR7 molecule has two ligand-binding sites. The first site conserved in TLR7 and TLR8 is use for small ligand-binding essential for its activation. The second site spatially distinct from that of TLR8 is use for ssRNA-binding that enhances the affinity of the first-site ligands. The first site preferentially recognize guanosine and the second site specifically bound to non-terminal uridine in ssRNA which have more than 3 bases. Rhesus TLR7 is also activate by dimerization induced by resiquimod alone, which is bound to only the first site (Zhang et al. 2016).

In contrast, hTLR8 exists as preformed dimer before ligand recognition. hTLR8 molecule has two ligand-binding sites as well as TLR7. The first site preferentially recognize uridine and second site recognize short-oligonucleotide. hTLR8 transforms into activated form upon binding of these two degradation products of ssRNA. hTLR8 is also activated by transformation induced by resiquimod alone, which is bound to only the first site (Tanji et al. 2015).

The key residues involved in TLR7 dimerization are LYS410, ASN503, SER504, GLY526, ASN527, SER530, THR532, ARG553, and TYR579 (Gupta et al. 2016).

Cellular signalling initiated by TLR7 activation with ssRNA in pDC is mediated in a Myeloid differentiation primary response 88 **(**MyD88)-dependent fashion, and activates NF-κB and IRF7, which results in induction of inflammatory cytokines and type I interferon, respectively (Kawai and Akira. 2011).

MyD88-dependent IRF7 activation in pDCs is mediate by activation of IRAK1, TRAF6, TRAF3, and IKKα and is facilitate by IFN-inducible Viperin expressed in the lipid body (Kawai and Akira. 2011).

IRF7, which is constitutively expresse by pDCs, binds MyD88 and forms a multiprotein signalling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (Kawai and Akira. 2008). In this complex, IRF7 becomes phosphorylate by IRAK1 and/or IKKα, dissociates from the complex and translocates into the nucleus to induce transcription of type I IFN by binding to its promoter proximal region (Kawai and Akira. 2008, Génin et al. 2009).

Signalling initiated by TLR8 engagement with ssRNAs in endosomes is also mediated the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

How it is Measured or Detected

In general, quantification of TLR7/8 activation can be done by:

- Reporter gene assay
- ELISA

Measurement of transcriptional activation of human TLR and NF-κB-luciferase co-transfected cells

HEK293 cells were transiently co-transfected with human TLR7 and a NF-κB-luciferase reporter. The cells were incubated for 48 hours following transfection and then stimulated with various concentrations of resiquimod or imiquimod. Luciferase activity was measured 48h post-stimulation and the results are reported as fold-increase in luciferase production relative to medium control (Gibson et al. 2002). Likewise, resiquimod (0.001-10 µg/mL) induced NF-κB activation in HEK293 cells transfected with human TLR7 or human TLR8 and a NF-κB-luciferase reporter is detected in the same manner (Jurk et al. 2002).

Measuring of cytokine levels in supernatants

IFN-α in cell-free supernatants collected after stimulation of human PBMC and/or pDC-enriched cells by imidazoquinoline derivatives is detected by ELISA (Gibson et al. 2002).

TNF-α and IL-6 in cell-free supernatants collected after stimulation of mDCs by RNA-LL37 are measured by ELISA (Ganguly et al. 2009).

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List of Key Events in the AOP

Event: 1822: Maturation of TNF/iNOS-Producing Dendritic Cells

Short Name: Maturation, TNF/iNOS-Producing Dendritic Cells

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Tip-DCs are also observed in mice. Murine Tip-DCs are defined as splenic $CD11c^+$, $CD11b^+$, $MHC\text{-}II^+$, $CD40^+$, and $CD86^+$ cells producing iNOS and TNF. CD11b expression is observed in murine Tip-DC, however it is lacking on human cells (Lowes et al. 2005).

Key Event Description

Monocytes are formed in the bone marrow and continuously enter the blood circulation, where they constitute 10% of the total

leukocyte population in humans (Sprangers et al. 2016). They are recruited to inflammatory sites and differentiate into immature dendritic cells in situ (Tang-Huau and Segura. 2019). These immature dendritic cells, known as monocyte-derived dendritic cells (mo-DC) are distinguished from conventional or classical DCs which arise from a common DC precursor (Guilliams et al. 2014). They possess typical DC functions of antigen-presenting cells, including the ability to efficiently stimulate naive T cells and the capacity to express CCR7, and potentially enabling their migration to lymph nodes (Tang-Huau and Segura. 2019).

Mo-DC are HLA-DR^{+}CD11c^{+}CD14^{int}CD206^{+}CD1c^{+} cells. By contrast, they lack the macrophage markers CD16 and CD163. They also display a typical DC morphology: they are small size, possess dendrites and lack large cytoplasmic vacuoles (Tang-Huau and Segura. 2019). Human mo-DC are present in lungs, intestine and peritoneum in the steady-state. Peritoneal mo-DC secrete IL-6, TNF-α, IL-1β and IL-12p70 upon ex vivo re-stimulation. Mo-DC from bronchoalveolar lavage also secrete TNF-α upon re-stimulation (Tang-Huau and Segura. 2019).

Pathogen-derived components, such as Toll-like receptor ligands as well as inflammatory mediators induce maturation of mo-DC. These stimulants include LPS, ssRNA, IFN-α, TNF-α, IFN-γ or CD40L (León et al. 2005, Farkas and Kemény. 2011). TNF-α and inducible nitric oxide synthase (iNOS)-producing DCs (Tip-DCs) are abundant in inflamed tissue such as skin in patients of chronic inflammatory skin disease, and not present in the steady-state or normal skin tissue. These cells are derived from monocyte infiltrated during inflammation and contribute to innate immune response to pathogens including bacteria and parasites (Guilliams et al. 2014).

From the above, monocyte is considered to infiltrate into inflammatory site and differentiate to mo-DC and Tip-DC, sequentially in chronically inflamed tissue. This maturation process is induced and/or promoted by IFN-α, TNF-α and GM-CSF (Farkas and Kemény. 2011).

Tip-DCs express HLA-DR, CD40, CD86, as well as maturation markers DC-Lamp and CD83 but lack the CD207/Langerin and CD14 markers of Langerhans cells and monocytes. In addition, Tip-DCs found in psoriasis produce the inflammatory mediators IL-8, IL-1, STAT1, CCL 20, IL-20, IL-23p19, and IL-12/IL-23p40, which mediate Th1 and Th17 responses (Wilsmann-Theis et al. 2013).

How it is Measured or Detected

Detection of Tip-DC is considered to be done by:

- Flowcytometry
- RT-qPCR

Analysis of maturation marker expression on cell surface

Maturation markers such as CD80, CD86, CD40 and CD83 can be analyzed by flowcytometry (Wilsmann-Theis et al. 2013).

Quantification of mRNA expression of TNF-α and iNOS

Expression of TNF-α, iNOS, IL-12p35 and IL-23p19 mRNA in in vitro generated Tip-DC are quantified by RT-qPCR. (Wilsmann-Theis et al. 2013).

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Event: 1707: Increase, IL-23 from matured dendritic cells

Short Name: Increase of IL-23

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
dendritic cell

Organ term

Organ term
immune system

Domain of Applicability

Tip-DCs are also observed in mice. Murine Tip-DCs are defined as splenic CD11c$^+$, CD11b$^+$, MHC-II$^+$, CD40$^+$, and CD86$^+$ cells producing iNOS and TNF. CD11b expression is observed in murine Tip-DC, however it is lacking on human cells (Lowes et al. 2005).

Key Event Description

Increased IL-23 synthesis from TNF-α and inducible nitric oxide synthase (iNOS)-producing DCs (Tip-DCs) is a result of maturation from monocyte-derived dendritic cells (mo-DC) to Tip-DC which is HLA-DR$^+$CD40$^+$CD86$^+$DC-Lamp$^+$ and CD83$^+$ (Farkas and Kemény. 2011, Wilsmann-Theis et al. 2013).

Tip-DCs are abundant in inflamed tissue such as skin in patients of chronic inflammatory skin disease, and not present in the steady-state or normal skin tissue. These cells are derived from monocyte infiltrated during inflammation and contribute to innate immune response to pathogens including bacteria and parasites (Guilliams et al. 2014).

Increased production of cytokines including IL-12/IL-23p40 in Tip-DC stimulated with R848, an agonist of Toll-like receptor (TLR) 7/8 was reported (Hänsel et al. 2011). In addition, maturation-dependent cytokine production including IL-23 from 6 hours in-vitro matured Tip-DC were observed when stimulated with CD40L and TLR ligands, such as LPS, PGN, Pam3Cys and R848 (Hänsel et al. 2011).

IL-23 is a heterodimer, sharing a p40 subunit with IL-12 but having a distinct p19 subunit. IL-23 binds to IL-12Rβ1 but not IL-12Rβ2. The receptor for this cytokine is heterodimeric and uses a novel second subunit, IL-23R, which is a member of the hematopoietin receptor family (Lee et al. 2004).

How it is Measured or Detected

Measurement of IL23 protein

IL-23 in cell-free supernatants collected after R848 stimulation to moDCs is detected by ELISA (Schwarz et al. 2013).

Measurement of IL23 RNA levels

Expression of IL-23 mRNA in R848-stimulated moDCs is measured by qRT-PCR (Schwarz et al. 2013).

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Event: 1708: Th17 cell migration and inflammation induction

Short Name: Th17 cell migration and inflammation induction

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

dendritic cell

Organ term

Organ term

immune system

Domain of Applicability

Ras homolog gene family H (RhoH) is a membrane-bound adapter protein involved in proximal T cell receptor signaling, and spontaneously develops chronic dermatitis that closely resembles human psoriasis in RhoH gene-deficient mice. Ubiquitin protein ligase E3 component N recognition 5 (Ubr5) and nuclear receptor subfamily 2 group F member 6 (Nr2f6) expression levels are decreased at the lesion site, and protein levels and DNA binding activity of retinoic acid-related orphan receptors are increased is doing. As a result, T cells differentiated into Th17 cells due to increased production of IL-17 and IL-22. These results indicate that RhoH suppresses the differentiation of naive T cells into effector Th17 cells. RhoH is a gene expressed in blood cells, and when RhoH expression decreases in T cells, Th17 cells increase, IL-22 is produced in large quantities, and the epidermis thickens, leading to the formation of psoriasis pathology. Humans with low RhoH expression may become more severe if they suffer from psoriasis. Journal of Allergy and Clinical Immunology

The effect of the unique gut flora in psoriasis on the development and reactivity of inflammatory cells on the IL-23 / Th17 axis was analyzed in imiquimod-induced psoriasis model mice. Th17, γδ TCR-bearing lymphocytes in the spleen were measured from sterile (GF) mice, broad-spectrum antibiotic mixture-administered (ATB) mice, and conventional (CV) mice. GF mice and ATB-treated mice had fewer Th17 cells and γδTCR + cells than CV mice. This is thought to be due to the symbiotic bacteria that lack microbiota or changes due to ATB treatment reduce pro-inflammatory T cell response and regulate T cell development. In other words, it is proof that the interaction between the microorganisms of Clostridiales and Elysiperotricales and the host affects the reactivity of Th17 cells and is involved in the etiology of imiquimod-induced skin inflammation. The positive effect of antibiotic regulation of the gut flora on skin severity suggests the involvement of the gut and skin axes and is part of the management of psoriasis patients. (Zizana Z et al. 2016) * Wide-area antibiotic mixture (ATB): A mixture of metronidazole, colistin, streptomycin, and vancomycin.

Key Event Description

Psoriasis is known to play a major role in the etiology of T cell dysfunction, especially in over activation of the Th17 pathway, which Th17 cells were associated with Th1 and Th2 (Lisa C. et al. 2007) Th17 cell was identified as a cell population that produces different IL17. Abnormal activation of Toll-like receptors (TLR7, 8 and 9) is also involved in the initiation and maintenance of psoriasis. IMO-3100 (an antagonist of TLR7 and 9) and IMO-8400 (an antagonist of TLR7, 8 and 9) has been shown to reduce psoriasis-like skin lesions induced by intradermal administration of IL-23 on the back of mice (Mayte S-F et al. 2013). Immune cell infiltration in psoriasis lesions is composed of CD3 + Th1cell, Th17 cells and CD11c + dendritic cells (DC) (Chamian F et al 2005).

Cytokines such as TNF-α, IFN-γ, IL-17, IL-22, IL-23, IL-12, and IL-1β produced from these cells cause an inflammatory cascade. In particular, the IL-23 / Th17 axis plays an important role, and IL-23h is produce in DC, promotes the differentiation of naive CD4 + T cell progenitor cells into the Th17 phenotype, and stimulates the survival and expansion of the Th17 population (Harrington LE et al. 2005) (Veldhoen M et al. 2006). IL-17 produced from Th17 cells regulates the expression of defensin, S100 family protein and LL-37. These are innate immune responses in the skin and show higher expression of IL-23 in keratinocytes and dermal tissues of psoriatic lesions than in non-lesions (Liang SC et al. 2006).

Overproduction of Th1 and TH17 cytokines is a major cause of psoriasis, and glucocorticoid (GC) regulates epidermal differentiation and acts as a potent anti-inflammatory compound to suppress the pathology of psoriasis. Synthetic glucocorticoids are uses to suppress inflammatory disease including psoriasis, and induce the glucocorticoid-induced leucine zipper (GILZ), a protein that inhibits major immune cell signaling pathways. CILZ is deficient in lesioned skin of psoriasis patients and shows a negative correlation with the expression of pro-inflammatory cytokines IL-1, IL-23, IL-22, and STAT3. *Lisa et al.* was identified a T cell-specific role of CILZ that limits Th17 cell formation in vitro in response to the Th17-promoting cytokines IL-1β and IL-23 (Lisa M et al.2019). CILZ has the clinical significance of psoriasis as well as the non-redundant function of controlling pathogenic Th17 responses (Lisa M et al.2019).

One of the causes of psoriasis is an increase in pathogenic Th17 cells in people with a genetic predisposition stimulated by the production of Th17 polarized cytokines by bone marrow cells. The antibacterial peptide LL37, which forms a complex with nucleic acids released from cells, is an autoantigen that promotes the activation of cutaneous plasmacytoid dendritic cells and myeloid DCs, and Th17 cells are effector cytokines such as IL-17A. It activates keratinocytes directly through release. Activated keratinocytes proliferate abnormally and release inflammatory mediators and chemokines to amplify the inflammatory response (Boehncke WH et al.2015).

How it is Measured or Detected

IL17 + cell count measurement

Flow cytometric analysis of psoriasis skin biopsy showed increased IL-17 + and IL-22 + CD4 + T cells,

Measurement of IL17 protein levels (in skin and serum)

Increased frequency of IL-17 +, CCR6 +, and CCR4 + T cells. IL-22-producing cells (Th-22 cells) that do not produce IL-17 or IFNγ also increased (Benham et al. 2013).

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List of Adverse Outcomes in this AOP

Event: 1709: Psoriatic skin disease

Short Name: Skin disease

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	AdverseOutcome

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Mouse psoriasis-like dermatitis model: K14 / mIL-1F6 gene-modified mice overexpress mouse IL-1F6 (IL-36a) selectively under the keratin 14 promoter, and TPA: 12-O- tetradecanoy1phorbol-13-acetate(TPA) was applied, skin pathological featuresfindings specific to psoriasis-such as epidermal hyperplasia, epidemal exfoliation and micro-abscess formation, and wet inflammatory cells in the dermis-were observed. Quantitative RT-PCR. Measures mRNA expression levels of Inflammatory chemokines and cytokines in skin tissues, and includes inflammatory chemokines: CCL3, CCL4, CXCL10, CXCL1 and cytokines: IL-23, IL-12, IL-1β etc. Expression was observed. (Kyowa Hakko Kirin Co., Ltd.)

Epidermal keratinocyte expression genes that were elevated in psoriatic lesions of patients with psoriasis with stage-type skin eruption: mRNA expression level of keratin6a and 16, s100A7A, S100A12, DEFB4, IL-1F6, CCL20, IL-17C, etc. was rapidly reduced by 700 single intravenous dose of brodalumab and decreased to non-lesional skin level two weeks after administration.On the other hand, leukocyte expression genes with increased expression in psoriatic lesion skin: IL-17A, IL-17F, IL-23F, IL-12B, IL-22, IFN-γ and other mRNA expression levels decreased with brodalumab administration However, at 2 weeks after administration, the level did not decrease to the level of the non-lesional skin. Since the expression of pathophysiology-related genes is reduced prior to the decrease in the expression of leukocyte expression genes and the decrease in the PASI score, brodalumab expresses pathophysiology-related genes by blocking IL-17 signaling in the epidermal keratinocytes of psoriatic lesions It is possible to improve the skin eruption promptly. (Kyowa Hakko Kirin Co., Ltd. In-house materials)

The monoclonal antibody-mediated IL-17A (secukinumab / ixekizumab) and the receptor subunit IL-17RA (brodalumab) are effective approaches in the treatment of psoriasis. Blocking IL-17RA results in inhibition of the IL-17 family, including IL-17A, IL-17F, IL-17C, and IL-17E. Other drugs are under clinical development that target bimekizumab targeting IL-17A and IL-17F, IL-23 upstream of the IL-17 pathway, or signal transduction substances downstream. (Conrad C et al. 2018)

Key Event Description

Psoriasis is a complex inflammatory disease caused by activation of Th1 and TH17 cells. The epidermis is composed of keratinocytes that differentiate to form a permeable barrier. Abnormal balance between proliferation and differentiation of keratinocytes affects barrier function and causes inflammatory skin lesions. Psoriasis is a complex combination of genetic and environmental risk factors, and dysregulation of Th1 and Th17 lines is due to overproduction of cytokines including IFN-α, TNF-α, IL-23, IL-17, and IL-22. Causes overgrowth and skin immunity. (Harrington LE et al. 2005)

Histopathological features of psoriasis lesions include epidermal thickening, epidermal differentiation, and epidermal protrusions (reticular ridges), with intraepithelial neutrophil moistening (manlo-like abscess) and marked immune moistening consisting of T cells and dendritic cells. See an increase in. (Boehncke WH et al. 2018) Commonly used psoriasis model mice were produced by topical application of the TLR7 agonist imiquimod, which induces the IL-23-Th17 cell axis and histopathology of human psoriasis pathology. Strictly reproduce the target and molecular features. (Wagner EF et al.2010)

Psoriasis vulgaris shows overexpression of the S100 protein family soriacin (sorazine), cobunericin (kebuneridine), and epidermal antibacterial peptide (AMP). AMP is induced by IL-17 and itself functions as a chemotactic factor and cytokine. Mobilize CD4 + T cells and neutrophils that exacerbate inflammation. (Kanagawa Psoriasis Treatment Study Group)

Serum IL-17 levels in psoriasis patients are significantly higher than in healthy individuals, and brodalumab, a neutralizing antibody against the IL-17A receptor, has been shown to be effective in the treatment of psoriasis (Gilliet et al. 2004). In addition, antibody preparations against IL-17 ixekizumab (John K. et al. 2002) and Szeimies et al. 2004 were used to treat psoriasis, and with positive results, Th17-mediated pathways are important for the etiology of psoriasis. It is believed to play a role. Immunohistological examination of biopsies of skin areas with psoriatic plaques, including surrounding normal skin, shows an increase in the number of activated dendritic cells, especially CD1a-positive Langerhans cells in the epidermis of psoriatic lesions. In CD83-positive CD1a-negative Langerhans, -negative CD11c-positive dermal dendritic cells increased in the epidermis at the border of psoriatic plaques. In normal skin, there were significantly fewer CD3-positive T lymphocytes than lesions.

CD4 and CD8 T cells infiltrate both the epidermis and dermis and show increased expression of IL17A, IL22, and IFNG in epidermal CD4 and CD8 T cells near keratinocytes, but with lower dermal T cell upregulation. IL-22, produced primarily by lesion epidermal CD4 T cells, is associated with keratinocyte activation and the formation of epidermal thickening, a prominent morphological feature of psoriasis. Lesion epithelial CD8 T cells mainly produce IL-17A and promote the production of inflammatory cytokines and chemokines by keratinocytes. IL-17A is an important mediator of psoriatic inflammation through skin recruitment and activation of leukocytes. (Cheuk et al. 2014)

In patients with psoriasis, inflammatory keratin K6 and K16-positive keratinocytes were found, even in areas of normal-appearing skin that were not affected by psoriasis lesions. In addition, the transcription factor C / EBPβ, which is normally expressed only in the stratum granulosum of the healthy epidermis, was expressed throughout the epidermis, including the epidermis of the lesion. This suggests that early inflammatory changes have already occurred in areas that have not yet shown obvious skin lesions, and that these changes are caused by dendritic cells rather than lymphocytes. (Komine et al. 2007)

How it is Measured or Detected

Biopsy of the skin area and surrounding normal skin of patients with psoriasis vulgaris

The dendritic cell surface marker and lymphocyte surface marker of the section were used as the primary antibody.

In the vicinity of psoriasis lesions, an increased number of activated dendritic cells was observed, with CD1a-positive Langerhans cells in the epidermis and CD83-positive CD1a-negative Langerin-negative CD11c-positive dermal dendritic cells at the epidermal border.

There were significantly fewer CD3-positive T lymphocytes than lesions in normal skin.

Inflammatory keratin K6 and K16 positive keratinocytes were found in the normal part.

The transcription factor C / EBPβ, which is normally expressed only in the granular layer of the normal epidermis, was expressed in the entire epidermis in the same manner as the lesion. (Komine et al. 2007)

Serum amyloid A: SAA measurement ... measured in 35 psoriasis patients and healthy humans

DNA microarray analysis in lesions of psoriasis patients ... SAA levels are about 5 times higher than in normal skin. The average SAA of psoriasis patients was 19.1 ug / ml, and the average SAA after treatment was 6.9 ug / ml.

There is a correlation between SAA and psoriasis severity score (PASI).

Amyloid A deposition was observed in the skin-stained area of the psoriasis lesion skin area (Tanizaki H et al. 2013)

Regulatory Significance of the AO

Psoriasis model mice have been developed as a tool for understanding the etiology of this disease and as a preclinical model. Five representative models are based on K14-amphiregulin, K5-Stat3C, K5-Tie2, K5-TGF-β1, and imiquimod. There were statistically significant similarities between the gene expression patterns associated with epidermal development and keratinization in these

models and the gene expression patterns in human psoriasis. Direct high-level activation of keratinocytes via autoclean growth factor (amphiregulin) has the ability to induce cytokine-related genetic circuits that closely resemble human psoriasis. Through transgenes, inward mutations (CARD14), injury, and exposure to specific T cell-producing cytokines, activated keratinocytes induce and lead to a chronic inflammatory response that is highly consistent with psoriasis. However, there were frequent differences in the expression of immune-related genes between the models. (Cook PW et al. 1997)

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2017: Stimulation of TLR7/8 leads to Increase of IL-23

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Thirteen mammalian TLR members (10 in humans and 13 in mice) have been identified so far, of which TLR1, 2, 4, 5, and 6 are membrane bound and catalytic site for pathogenic structural components, whereas TLR3, 7, 8, and 9 expressed within the

endosomal compartment

are dedicated to nucleic acids. TLRs 1–9 are conserved among humans and mice, yet TLR10 is present only in humans and TLR11 strictly restricted to rodents (Gupta et al. 2016).

Mouse TLR10 is not functional because of a retrovirus insertion, and TLR11, TLR12 and TLR13 have been lost from the human genome (Kawai and Akira. 2010).

In addition, alignment of amino acid residues between human toll-like receptor 7 (AAF60188.1) and murine toll-like receptor 7 (AGX25544.1) was 80.74% identification. Both proteins have 1049 amino acid residues.

Structural characterization was conducted with recombinant TLR7 from monkey (*Macaca mulatta*; 96.8% sequence identify with human TLR7) expressed in *Drosophila* S2 cells (Zhang et al. 2016).

Studies of DC subsets isolated from humans and mice have revealed that TLRs have distinct expression patterns. Freshly isolated human pDCs express TLR7 and TLR9, whereas $CD11c^+$ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR8. In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas others found TLR7 was exclusively expressed in pDCs (Iwasaki and Medzhitov. 2004).

In mice, all splenic DC subsets express TLRs 1, 2, 4, 6, 8 and 9. However, mouse pDCs do not express TLR3. Moreover, mouse $CD8\alpha^+$ DCs lack TLR5 and TLR7 expression and fail to respond to TLR7 agonists. In short, $CD4^+$ DC, CD4CD8DN DC and pDC express TLR7 in mice (Iwasaki and Medzhitov. 2004).

Although unpublished, it has been reported that human slanDCs (Tip-DCs) lack the DNA-binding structureTLR9 but can express the endosomal RNA-binding receptorsTLR8 (slanDCs andCD1c$^+$ DCs) and TLR7 (slanDCs but not CD1c$^+$ DCs; Hänsel et al, unpublished data, June 2010) (Hänsel et al. 2011). There are not any other reports which mentioned TLR7 expression in Tip-DCs, so whether or not TLR7 exists in human Tip-DCs is still unknown.

IFN-α, but not TNF-α and IL-6 production by human pDCs after stimulation with self-RNA-LL37 complex was detected (Ganguly et al. 2009). However, in mice, IFN-α production from splenic pDCs was induced by IMQ treatment. The production of TNF-α and IL-23 was also induced by IMQ treatment. Although 4–8% of mPDCA-1$^-$ CD11c$^+$ DCs were contaminated in prepared mPDCA-1$^+$ pDC fraction, it was confirmed that splenic mPDCA-1$^-$ CD11c$^+$ DCs enriched to approximately 80% purity could not produce TNF-α and IL-23 by IMQ stimulation. In $Tlr7^{-/-}$ splenic pDCs, these cytokines (IFN-α, TNF-α and IL-23) were not induced by IMQ treatment, although stimulation by CpG, a TLR9 ligand, resulted in induction of these cytokines at the same level as was produced by wild-type splenic pDCs. These data indicate that, in mice, IMQ application can induce the production via TLR7 of IFN-α, TNF-α and IL-23 from pDCs existing in the skin in vivo (Ueyama et al. 2014).

When BMDCs were generated by 10-day culture with GM-CSF and IL-4 and characterized their phenotypes, CD11c+ BMDCs showed MHC II^{high}, CD11b^{high}, B220$^-$, CD86^{high}, Mac-3$^+$, and had the ability to produce high levels of TNF-α and NO/iNOS in response to LPS stimulation, which represents a similar phenotype to Tip-DCs (Xu et al. 2007, Ueyama et al. 2014).

In these BMDCs which represents a similar phenotype to Tip-DCs, IMQ weakly but significantly induced the production of IL-23. In addition, although IFN-α had no effect alone, co-stimulation with IFN-α and IMQ resulted in marked induction of IL-23 production. However, using BMDCs derived from $Tlr7^{-/-}$ mice, these effects of IMQ and IFN-α was not observed, verifying that it is also mediated via TLR7 (Ueyama et al. 2014).

In mice, purified bone marrow dendritic cells (BMDCs) derived from wild-type mice stimulated with IFN-α showed increase in Tlr7 mRNA expression (Ueyama et al. 2014). In addition, TLR7 expression was also observed in the inflamed skin of IMQ-treated mice (Ueyama et al. 2014). These data suggest that the synergistic effect of IMQ and IFN-α on BMDCs was caused by induction of TLR7 expression by IFN-α (Ueyama et al. 2014).

Taken together, in mice, IFN-α produced by IMQ-primed pDCs may enhance the effects of IMQ to activate Tip-DC, resulting in the release of a large amount of IL-23 in IMQ-induced psoriasis-like skin lesion (Ueyama et al. 2014).

Key Event Relationship Description

Toll-like receptors (TLRs) are members of interleukin-1 (IL-1) receptor/TLR superfamily, as they share the intracellularToll-IL-1 receptor (TIR) domain with the IL-1 receptor.

Toll-like receptor (TLR) 7 and TLR8 is known to mediate the recognition of guanosine- and uridine-rich single-stranded RNA (ssRNA) derived from ssRNA viruses and synthetic antiviral imidazoquinoline components (Akira et al. 2006; Blasius and Beutler. 2010). They also mediate the recognition of self RNA that is released from dead or dying cells.

Human TLR7 (hTLR7) and human TLR8 (hTLR8) contains 1049, 1041 amino acid residues with a calculated molecular weight of 120.9 kDa and 119.8 kDa respectively (Chuang and Ulvitch. 2000).

The full-length hTLR7 protein includes a signal peptide of 26 amino acids (1–26 aa). The mature hTLR7 protein ectodomain, trans-membrane, and TIR domain are composite structure of 27–839, 840–860, and 889–1,036 amino acids, respectively (Gupta et al. 2016).

hTLR7 and hTLR8 form a subfamily of proteins that each contain an extracellular domain of >800 residues and share functional and structural features. TLR8 contains 26 leucine-rich repeats (LRRs), which is the largest number of LRRs among TLRs whose structures have been reported (Tanji et al. 2013).

Monkey TLR7 exists as a monomer in the absence of ligands, and TLR7 dimerization is induced by R848 alone, but not by poly U or guanosine alone, although these two ligands synergistically triggered TLR7 dimerization (Zhang et al. 2016). In contrast, hTLR8 exists as preformed dimer before ligand recognition. TLR8 is activated by R848 alone, or both uridine and ssRNA synergistically (Tanji et al. 2013).

The key residues interacting two TLR7 molecules into dimer confirmation are LYS410, ASN503, SER504, GLY526, ASN527, SER530, THR532, ARG553, and TYR579 (Gupta et al. 2016).

TLR3, TLR7, TLR8, and TLR9 localize to the endoplasmic reticulum and are trafficked to the endosomal compartment where they initiate cellular responses upon their activation by PAMPs and DAMPs (Lai et al. 2017).

TLR7 are exclusively expressed in plasmacytoid DCs (pDCs), which have the capacity to secrete vast amounts of type I IFN rapidly in response to viral infection (Gilliet et al. 2008, Reizis et al. 2011).

TLR8 is expressed in various tissues, with its highest expression in monocytes. Myeloid DCs (mDCs) also express TLR8 in human (Iwasaki and Medzhitov. 2004). Thus, TLR8 ligands can directly activate mDCs via TLR8.

TLR7-mediated signaling in pDC is mediated in a MyD88-dependent fashion, which initiates an IRF7-mediated response, secreting vast amounts of IFN type 1 (Kawai and Akira. 2011).

MyD88-dependent IRF7 activation in pDCs is mediated by activation of IRAK1, TRAF6, TRAF3, and IKKα and is facilitated by IFN-inducible Viperin expressed in the lipid body (Kawai and Akira. 2011).

IRF7, which is constitutively expressed by pDCs, binds MyD88 and forms a multiprotein signaling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (Kawai and Akira. 2008). In this complex, IRF7 becomes phosphorylated by IRAK1 and/or IKKα, dissociates from the complex and translocates into the nucleus.

The interferons (IFNs) are a primary defense against pathogens because of the strong antiviral activities they induce. Three types of IFNs, types I, II and III, have been classified based on of their genetic, structural, and functional characteristics and their cell-surface receptors (Zhou et al. 2014). IFN-α belongs to the type I IFNs, the largest group which includes IFN-β, IFN-ε, IFN-ω, IFN-κ, IFN-δ, IFN-τ and IFN-ζ.

The IFN-α of type I IFN family in humans is composed of 12 subtypes encoded by 14 nonallelic genes including one pseudogene and two genes that encode the same protein. The various IFN-α subtypes have many common points. For example, all are clustered on chromosome 9 (Diaz et al. 1993). IFN-αs, which are composed of 165 to 166 aa, have 80% amino acid sequence identities (Li et al. 2018).

Upon engagement of ssRNAs in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

A distinct population of human blood DCs that are defined by the selective expression of the 6-sulfo LacNAc residue on the P-selectin glycoprotein ligand 1 membrane molecule was described previously. 6-Sulfo LacNAc DCs (slanDCs) stand out by a marked production of TNF-α, and they were recognized as the major source of IL-12p70 among blood leukocytes when stimulated with CD40 ligand or LPS of gramnegative bacteria (Hänsel et al. 2011).

According to the current concept, these inflammatory DCs are $CD1c^{-}$, $CD11c^{+}$ cells locally expressing TNF-α and iNOS. They were also referred to as TNF and inducible nitric oxide synthase–expressing DCs (Tip-DCs) (Lowes et al. 2005) or inflammatory dermal DCs (Zaba et al. 2009). In contrast, resident dermal DCs express CD1c and CD11c and were shown to lack inflammatory markers. The phenotype of slanDCs ($CD11c^{+}$ and $CD1c^{-}$) and their local production of IL-23p19, TNF-α, and iNOS identify slanDCs as being a population of inflammatory dermal DCs and so-called Tip-DCs in psoriasis (Hänsel et al. 2011).

Stimulation of blood DCs with self-RNA–LL37 complexes induced a robust TNF-α response (Hänsel et al. 2011). TNF-α affects Tip-DCs in an autocrine and/or paracrine manner (Zaba et al. 2007).

DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

R848 induces IL-23 production from activated human monocyte-derived DCs (moDCs) by enhanced transcriptional activity (Schwarz et al. 2013).

IL-23 is a heterodimer, sharing a p40 subunit with IL-12 but having a distinct p19 subunit. IL-23 binds to IL-12Rβ1 but not IL-12Rβ2. The receptor for this cytokine is heterodimeric and uses a novel second subunit, IL-23R, which is a member of the hematopoietin receptor family (Lee et al. 2004).

Evidence Supporting this KER

Biological Plausibility

The molecular structure and function of TLR7 and TLR8 are evident based on sufficient scientific findings as mentioned above. The known mechanisms for stimulation of TLR7/8 by each ligand are initiated by the formation of homodimer. TLR7-mediated signaling in pDC is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response, secreting vast amounts of IFN type 1 (Kawai and Akira. 2011).

Similarly, upon engagement of ligands in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

R848 induces IL-23 production from activated human monocyte-derived DCs (moDCs) by enhanced transcriptional activity (Schwarz et al. 2013).

TNF and inducible nitric oxide synthase–expressing DCs also known as Tip-DCs or inflammatory dermal DCs differentiates from moDCs by inflammation (Hänsel et al. 2011).

As mentioned above, stimulation of TLR7 in pDCs, and TLR8 in moDCs and Tip-DCs leads to activation of Tip-DCs, which leads to the overproduction of IL-23 from matured Tip-DCs.

Empirical Evidence

Much experimental data is available that supports the stimulation of TLR7 in pDC induced by TLR7 agonist, which subsequently promote secretion of IFN-α in MyD88-dependent fashion. For example, three populations of cells were evaluated for type I IFN production following imidazoquinoline stimulation: human PBMC, pDC-depleted PBMC, and pDC-enriched cells. Human PBMC produce IFN-α following imiquimod (0.3–30 μM) or resiquimod (0.03–30 μM) treatment. Peak levels of IFN-α were reached with imiquimod and resiquimod at 3 μM. PBMC, depleted of pDC, did not produce detectable levels of IFN-α in response to imiquimod or resiquimod treatment.

The imidazoquinoline-treated pDC-enriched cultures produced 2–20 times more IFN-α than similarly treated PBMC as measured over the entire dose range. Peak levels of Resiquimod- and imiquimod-induced IFN-α production were reached with 0.3 μM and 30 μM, respectively (Gibson et al. 2002).

In addition, pDCs were stimulated with LL37 premixed with total human RNA extracted from U937 cells to confirm that LL37 can interact with self-RNA and convert it into a trigger of IFN-α production. U937-derived self-RNA induced dose-dependent IFN-α production when mixed with LL37, but not when given alone or mixed with the scrambled peptide GL37. Similar to self-DNA (Lande et al., 2007), pDCs activated by self-RNA mixed with LL37 produced high levels of IFN-α, but did not produce TNF-α or IL-6 or undergo maturation as assessed by measuring the expression of costimulatory molecules CD80 and CD86 (Ganguly et al. 2009).

Importantly, self-RNA isolated from a variety of cell types and tissue samples from various types of skin pathologies induced similar levels of IFN-α when mixed with LL37, indicating that cellular- or disease-dependent variations in RNA composition do not play a role in the responses to self-RNA. These data demonstrate that LL37 can convert otherwise nonstimulatory self-RNA into a trigger of pDC activation to produce IFN-α, and thus enable the RNA released during cell death to induce innate immune activation (Ganguly et al. 2009).

IFN-α induced in pDCs by self-RNA–LL37 complexes was inhibited in a dose-dependent manner by bafilomycin, which blocks endosomal acidification and TLR signaling. To specifically inhibit TLR7, we used the short oligonucleotide C661, which selectively blocks TLR7 (Barrat et al. 2005), as shown by the inhibition of IFN-α induction by the synthetic TLR7 agonist R837 but not the TLR9 agonist CpG2006. Pretreatment of pDCs with C661 specifically blocked the IFN-α induction by self-RNA–LL37 complexes, indicating that pDC activation by self-RNA–LL37 complexes occurs through TLR7 (Ganguly et al. 2009).

Self-RNA–LL37 complexes but not self-RNA alone activated mDCs to produce the proinflammatory cytokines TNF-α and IL-6, but not IFN-α (Ganguly et al. 2009). Self-RNA–LL37 complexes also activated mDCs to undergo maturation as shown by the up-regulation of CD80 and CD86 expression (Ganguly et al. 2009) . mDC activation by self-RNA–LL37 complexes was entirely dependent on self-RNA, given that these responses were abrogated by decreasing the amount of self-RNA in the complexes (unpublished data). In contrast to self-RNA–LL37 complexes, self-DNA–LL37 complexes were unable to activate mDCs (Ganguly et al. 2009) . In accordance with these findings, stimulation of mDCs with supernatants of apoptotic cells combined with LL37 induced the secretion of proinflammatory cytokines, and this secretion was entirely dependent on self-RNA because activity was abolished by depletion of self-RNA but not self-DNA (Ganguly et al. 2009).

Compared with stimulation with either supernatant of activated pDCs or self-RNA–LL37 alone, the combination of both significantly enhanced the activation of mDCs to secrete IL-6 and TNF-α and enhanced their differentiation into mature CD83+ DCs (Ganguly et al. 2009). This activity was completely blocked by antibodies against IFN-α, IFN-β and IFN-αβR (Ganguly et al. 2009). Thus, self-RNA–LL37 complexes can trigger mDC activation and maturation, and this process is enhanced by the concomitant activation of pDCs to produce IFN-α.

Self-RNA was also internalized by mDCs when complexed with LL37 but not when given alone. The cytokine production such as TNF-α and IL-6 of mDCs induced by self-RNA–LL37 complexes but not by the TLR4 agonist LPS was completely inhibited by bafilomycin in a dose-dependent manner, demonstrating that mDC activation by self-RNA–LL37 complexes involved endosomal TLR activation. Using 293T cells transfected with TLR8 and TLR3 expression vectors along with a NF-κB luciferase reporter plasmid, it was confirmed that self-RNA–LL37 complexes activated TLR8 but not TLR3. In support of this finding, synthetic short ssRNA sequences that activate TLR8 in human mDCs (Diebold et al. 2004, Heil et al. 2004) also activated mDCs when complexed

with LL37 but not when given alone (Ganguly et al. 2009).

Dose-dependent DC maturation was observed with increasing concentrations from 10 IU/ml up to 1000 IU/ml of IFN-α2a or IFN-α8 added to cultures containing GM-CSF, IL-4, and TNF-α. Both of the IFNs had a similar capacity to up-regulate HLA-A, B, C, CD80, and CD86 and to down-regulate CD1a and CD11b expression on the cell population (Luft et al. 1998).

DC cultured in GM-CSF, TNF-α, and IL-4-containing medium until day 14, and type I IFNs were added daily between days 14 and 17. Proportions of positive cells for each markers were analyzed by FACS on day 17 (Luft et al. 1998).

When GM-CSF, TNF-α, and IL-4-containing cultures were washed on day 14 and continued until day 17 in serum-free medium containing GM-CSF and IL-4, without or with TNF-α (20 ng/ml, standard conditions), IFN-a (1000 IU/ml), or both, IFN-α alone did not enhance DC maturation as compared with standard conditions. When both of TNF-α and IFN-α exist, optimal maturation was observed than either TNF-α or IFN-α alone. Thus, the enhancement of DC activation by IFN-α under serum-free conditions required the presence of TNF-α (Luft et al. 1998).

LL37 is highly expressed in the inflamed skin of psoriasis but is undetectable in inflamed skin of atopic dermatitis or in healthy skin (Lande et al. 2007). To determine whether extracellular self-RNA–LL37 complexes form in vivo, Staining skin sections with Ribogreen and DAPI revealed that numerous extracellular Ribogreen^{+}/ DAPI^{-} complexes in the dermal compartment of psoriatic skin lesions, but not in skin of atopic dermatitis or healthy skin (Ganguly et al. 2009). These tissue RNA complexes presented several features of self-RNA–LL37 complexes generated in vitro, including the size and bead-like branched structures resulting from the aggregation of smaller particles (Ganguly et al. 2009).

Skin sections of psoriatic tissues were stained with an anti-LL37 antibody and Ribogreen to determine whether the self-RNA complexes in the tissues contained LL37 and it was found that the majority of these complexes contained LL37 (Ganguly et al. 2009). Importantly, psoriatic skin also contained substantial numbers of particulate self-DNA–LL37 complexes.

Serial sections of lesional psoriatic skin were stained for RNA complexes and DC-LAMP, a lysosomal marker specific for mature mDCs to determine whether the presence of tissue self-RNA complexes is associated with the presence of activated DCs in psoriatic skin. Consistent with previous reports (Lowes et al. 2005), it was found that large clusters of DC-LAMP–positive mature mDCs (Ganguly et al. 2009). We also found tissue self-RNA–LL37 complexes within these clusters, and, occasionally, even inside the DCs as shown by the colocalization with endolysosomal compartments stained with DC-LAMP (Ganguly et al. 2009). The number of tissue self-RNA complexes significantly correlated with the numbers of DC-LAMP–positive mDCs in psoriatic skin (Ganguly et al. 2009). Together, these findings strongly support *in vitro* data that self-RNA complexes can activate mDCs and suggest that this pathway is operational in psoriasis.

When mRNA expression normalized to HARP for IL-23 subunits, such as p19 and p40 were quantified by RT-PCR in monocyte-derived DCs (moDCs) matured without and with etanercept, a dimeric human tumor necrosis factor recepter p75-Fc fusion protein made of 2 extra-cellular domains of the human 75kD TNFR linked by the constant Fc portion of human IgG1 (Haraoui and Bykerk. 2007), significant decrease in expression of IL-23 subunits p19 and p40 by etanercept were observed (Zaba et al. 2007). MoDCs cultured with etanercept decreased CD86 expression threefold and HLA-DR expression fivefold. In addition, moDCs cultured with etanercept were also an average of two to threefold less stimulatory than control DCs in a mixed leukocyte reaction. Gene array on control moDCs compared with those cultured with etanercept revealed that CD163, a macrophage scavenger receptor, was up-regulated 6.5-fold (Zaba et al. 2007).

In psoriatic dermis, mRNA expression normalized to HARP for multiple inflammatory products of Tip-DCs, including iNOS, TNF-α and IL23 p40 subunit, are reduced within 1–2 weeks after beginning etanercept, whereas the number of CD11c^{+} DCs in the tissue is minimally affected during this time, suggesting an initial blockade of cytokine production by these cells rather than cell reduction (Zaba et al. 2007). These facts suggest that TNF-α is an autocrine or paracrine inducer of IL-23 from Tip-DC (Zaba et al. 2007).

R848-treatment to moDCs, which were generated from monocytes isolated from buffy coats of healthy donors, resulted in concentration-dependent expression of IL-23. 2×10^{5} moDCs/ml were plated in DC medium and stimulated with 0-5 µg/ml R848. After 24 h of TLR stimulation, supernatants were harvested and cytokine expression was measured by ELISA. In addition, the combination of NOD1 and NOD2 agonists with R848 stimulated high levels of IL-23 secretion (Schwarz et al. 2013).

qRT-PCR for moDCs stimulated with TLR agonists in the absence or presence of NOD1 and NOD2 ligands at 8 h and 24 h post induction revealed that synergistic cytokine expression observed in NOD1/NOD2- and R848-stimulated cells is largely mediated by enhanced transcriptional activity (Schwarz et al. 2013).

In time kinetic studies, moDCs were stimulated with R848 in the absence or presence of MDP and iE-DAP which are ligands of NOD1/2, for 30 min, 2 h, 8 h or 24 h and mRNA levels of IL-23 as well as the cumulative cytokine release were measured by qRT-PCR and sandwich-ELISA, respectively. At the mRNA level, synergistic effects of both NOD ligands with R848 are already detectable after 8 h of stimulation. In agreement with IL-23 mRNA expression, synergistic effects are detectable by ELISA after 8 h; nevertheless, these effects are even more pronounced after 24 h of stimulation (Schwarz et al. 2013).

These findings show that dose responses and temporality of MIE and KE1 seem to be in sequence.

Uncertainties and Inconsistencies

Although unpublished, it has been reported that human slanDCs (Tip-DCs) lack the DNA-binding structureTLR9 but can express the endosomal RNA-binding receptorsTLR8 (slanDCs andCD1c^{+} DCs) and TLR7 (slanDCs but not CD1c^{+} DCs; Hänsel et al, unpublished data, June 2010) (Hänsel et al. 2011). There are not any other reports which mentioned TLR7 expression in Tip-DCs,

so whether or not TLR7 exists in human Tip-DCs is still unknown.

In addition, freshly isolated human pDCs have been reported to express TLR7 and TLR9, whereas $CD11c^+$ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR8. In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas others found TLR7 was exclusively expressed in pDCs. Therefore, it is still unknown that whether or not TLR7 exists in human mDCs, and how much it does contribute recognition of R848 or LL37-RNA in these cells (Iwasaki and Medzhitov. 2004).

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

Much experimental data is available that supports the stimulation of TLR7 in pDC induced by TLR7 agonist, which subsequently promote secretion of IFN-α in MyD88-dependent fashion. For example, HEK293 cells were transiently co-transfected with human TLR7 and NF-κB-luciferase reporter. After 48 hours, the cells were stimulated with various concentrations of resiquimod or imiquimod. Luciferase activity was measured 48h post-stimulation and the results are reported as fold-increase relative to medium control. As a result, dose-dependent increase in NF-κB-dependent luciferase activity in HEK293 transfected with hTLR7 was observed with increasing concentrations from 0.01 μM up to 10 μM of resiquimod, and 0.1 μM up to 15 μM of imiquimod. Maximal NF-κB activation with resiquimod is achieved with 10-30 μM, which yields an 18-fold increase in luciferase production. Maximal NF-κB activation with imiquimod requires 10-15 μM compound and induces a 5-6-fold increase in luciferase production (Gibson et al. 2002).

In addition, three populations of cells were evaluated for type I IFN production following imidazoquinoline stimulation: human PBMC, pDC-depleted PBMC, and pDC-enriched cells. Human PBMC produce IFN-α following imiquimod (0.3–30 μM) or resiquimod (0.03–30 μM) treatment. Peak levels of IFN-α were reached with imiquimod and resiquimod at 3 μM. PBMC, depleted of pDC, did not produce detectable levels of IFN-α in response to imiquimod or resiquimod treatment.

The imidazoquinoline-treated pDC-enriched cultures produced 2–20 times more IFN-α than similarly treated PBMC as measured over the entire dose range. Peak levels of Resiquimod- and imiquimod-induced IFN-α production were reached with 0.3 μM and 30 μM, respectively (Gibson et al. 2002).

In different experiments, pDCs were stimulated with LL37 premixed with total human RNA extracted from U937 cells to confirm that LL37 can interact with self-RNA and convert it into a trigger of IFN-α production. U937-derived self-RNA induced dose-dependent IFN-α production when mixed with LL37, but not when given alone or mixed with the scrambled peptide GL37 (Ganguly et al. 2009).

R848 (0.001-10 μg/mL) induced NF-κB activation in HEK293 cells transfected with human TLR8 in a dose-dependent manner (Jurk et al. 2002). In addition, the production of TNF-α and IL-6, and the maturation

of mDCs induced by self-RNA–LL37 complexes but not by the TLR4 agonist LPS was completely inhibited by bafilomycin in a dose-dependent manner, demonstrating that mDC activation by self-RNA–LL37 complexes involved endosomal TLR activation (Ganguly et al. 2009).

Dose-dependent DC maturation was observed with increasing concentrations from 10 IU/ml up to 1000 IU/ml of IFN-α2a or IFN-α8 added to cultures containing GM-CSF, IL-4, and TNF-α. Both of the IFNs had a similar capacity to up-regulate HLA-A, B, C, CD80, and CD86 and to down-regulate CD1a and CD11b expression on the cell population (Luft et al. 1998).

DC cultured in GM-CSF, TNF-α, and IL-4-containing medium until day 14, and type I IFNs were added daily between days 14 and 17. Proportions of positive cells for each markers were analyzed by FACS on day 17 (Luft et al. 1998).

When GM-CSF, TNF-α, and IL-4-containing cultures were washed on day 14 and continued until day 17 in serum-free medium containing GM-CSF and IL-4, without or with TNF-α (20 ng/ml, standard conditions), IFN-a (1000 IU/ml), or both, IFN-α alone did not enhance DC maturation as compared with standard conditions. When both of TNF-α and IFN-α exist, optimal maturation was observed than either TNF-α or IFN-α alone. Thus, the enhancement of DC activation by IFN-α under serum-free conditions required the presence of TNF-α (Luft et al. 1998).

In accordance with these findings, compared with stimulation with either supernatant of activated pDCs or self-RNA–LL37 alone, the combination of both significantly enhanced the activation of mDCs to secrete IL-6 and TNF-α and enhanced their differentiation into mature CD83+ DCs (Ganguly et al. 2009). This activity was completely blocked by antibodies against IFN-α, IFN-β and IFN-αβR (Ganguly et al. 2009). Thus, self-RNA–LL37 complexes can trigger mDC activation and maturation, and this process is enhanced by the concomitant activation of pDCs to produce IFN-α.

KE 1

R848-treatment to moDCs, which were generated from monocytes isolated from buffy coats of healthy donors, resulted in concentration-dependent expression of IL-23. 2×10^5 moDCs/ml were plated in DC medium and stimulated with 0-5 μg/ml R848. After 24 h of TLR stimulation, supernatants were harvested and cytokine expression was measured by ELISA. In addition, the

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qRT-PCR for moDCs stimulated with TLR agonists in the absence or presence of NOD1 and NOD2 ligands at 8 h and 24 h post induction revealed that synergistic cytokine expression observed in NOD1/NOD2- and R848-stimulated cells is largely mediated by enhanced transcriptional activity (Schwarz et al. 2013).

Time-scale

Human PBMC, pDC-deficient PBMC, and pDC -enriched from human PBMC (pDC-enriched) were cultured with various concentrations of resiquimod or imiquimod. After 24 h in culture, cell-free supernatants were collected and IFN-α was analyzed by ELISA (Gibson et al. 2002).

Suspensions containing RNA-LL37 or supernatants of dying cells were added to pDC and mDC cultures. After overnight culture, supernatants of pDCs and mDCs were collected and IFN-α, TNF-α and IL-6 were measured by ELISA (Ganguly et al. 2009). pDCs and mDCs were also stained with fluorochrome-labeled anti-CD80, anti-CD86, and anti-CD83 antibodies and analyzed by flow cytometry. mDCs were also cultured with supernatants of pDCs stimulated for 24 h with self-DNF-LL37 (Ganguly et al. 2009).

In time kinetic studies, moDCs were stimulated with R848 in the absence or presence of MDP and iE-DAP which are ligands of NOD1/2, for 30 min, 2 h, 8 h or 24 h and mRNA levels of IL-23 as well as the cumulative cytokine release were measured by qRT-PCR and sandwich-ELISA, respectively. At the mRNA level, synergistic effects of both NOD ligands with R848 are already detectable after 8 h of stimulation. In agreement with IL-23 mRNA expression, synergistic effects are detectable by ELISA after 8 h; nevertheless, these effects are even more pronounced after 24 h of stimulation (Schwarz et al. 2013).

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Relationship: 2018: Increase of IL-23 leads to Th17 cell migration and inflammation induction

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	adjacent	High	High

Evidence Supporting Applicability of this Relationship

In mice, application of IL-23 causes psoriatic-like epidermal hyperplasia, but this effect does not occur in IL-17A and IL-22KO mice. Therefore, it is thought that IL-17A and IL-22 play an important role downstream of IL-23 Rizzo HL. Et al. 2011 .

Recombinant mIL-23 (rmIL-23) injected into the ear of WT mice induced IL-17A and IL-22 expression, and showed ear swelling and epidermal hyperplasia. When rmIL-23 was injected into IL-22 KO mice, IL-22 was induced, but ear swelling and epidermal hyperplasia were less than in WT mice. When rmIL-23 was injected into IL-17A KO mice, IL-22 was induced, but ear swelling and epidermal hyperplasia hardly occurred. WT mice after administration of IL-22 or IL-17A inhibitor completely inhibited IL-23-induced epidermal hyperplasia. These results indicate that two cytokines, IL-22 and IL-17A, are downstream mediators of IL-23-induced changes in mouse skin and are required for the generation of IL-23-mediated skin lesions. (Hansel et al. 2011)

Key Event Relationship Description

IL-23 is important for differentiation and proliferation of Th17 cells. As a major source of IL-23, Tip-DC is present in the skin lesions of psoriatic patients and works to activate the Th17 pathway (Hansel et al. 2011).

Signaling through the heterodimeric IL-23 receptor (subunits of p19 and p40) of Th17 cells stimulates the production of proinflammatory keratinocyte cytokines that mediate the psoriatic response and induces the production of IL-17. Th17 cells are increased in the peripheral blood and lesion skin of psoriatic patients, and IL-17 and IL-22 produced from Th17 act on epidermal keratinocytes to cause inflammatory chemokines and hyperproliferation (Michelle A. et al. 2005).

IL-17A, which is highly expressed by Th17 cells, has a direct effect on the regulation of genes expressed by keratinocytes that are

involved in innate immune defense, including defensins,8, 9 S100 family proteins, lipocalin, and LL37/cathelicidin, as well as a range of CXCL chemokines that regulate neutrophil trafficking (Gilliet et al. 2004). IL-22, which is expressed by Th22 and Th17 cells, and related IL-20 family members promote keratinocyte hyperproliferation and abnormal differentiation (Krueger et al. 2012).

Evidence Supporting this KER

Biological Plausibility

IL-17A, which is highly expressed by TH17 cells, has a direct effect on the regulation of genes expressed by keratinocytes that are involved in innate immune defense, thorough expressions of defensins,8, 9, S100 family proteins, lipocalin and LL37/cathelicidin, as well as a range of CXCL chemokines that regulate neutrophil trafficking. IL-22, which is expressed by TH22 and TH17 cells, and related IL-20 family members promote keratinocyte hyperproliferation and abnormal differentiation Gilliet et al.2012 .

In vitro Reconstituted Human Epidermis (RHE) model stimulated for 48 hours with medium containing IL-17, IL-22 and TNFα mix (concentration 3 ng / mL) as psoriasis-specific cytokines. Controls were cultured in normal medium. After fixing RHE and embedding in paraffin, 4 µm sections were stained with hematoxylin-eosin or immunolabeled with anti-filaggrin, anti-S100A7, anti-hBD-2 mAb.

RHE stimulated with cytokine mix showed dramatic expression of these protein. In the RHE with normal medium, antibacterial peptide S100A7 was expressed locally, but BD-2 protein was not detected. This is due to the synergistic effect of IL-17 added to the IL-22 / TNFα combination. Filaggrin, S100A7 and BD-2 mRNA expression by RT-qPCR analysis increased 20-fold (S100A7) or -50-fold (BD-2) compared to controls. This is a downstream event that can be modeled using keratinocytes and cytokines and relies on upstream mechanisms of recruitment and activation of other innate adaptive immune cells. Bernard et al. 2012. .

Quantitative Understanding of the Linkage

Response-response relationship

KE1:

IL-23, which maintains Th17 cells, is released from TNF-a and inducible nitric oxide synthase (iNOS) -producing dendritic cells (TIP-DC). TIP-DC activates IL-17, IL-22, IL-23, and TNF-a mRNA expression in psoriatic skin. Cytokine staining analysis of peripheral blood mononuclear cell (PBMC) in patients with psoriasis showed a three-fold increase in Th17 cells compared to normal PBMC. Thl7 cells produce IL-22 and stimulate keratinocyte proliferation. IL-22 activates STAT3 and induces the production of cytokine (such as IL-8), chemokines and the synthesis of antimicrobial peptides (Zaba et al. 2005).

KE 2

The epidermis of psoriasis patients did not have many T cells, but the analysis was similar to peripheral blood and dermis. The proportion of Th17 cells in the dermis was significantly higher than that in normal skin, and TNF and IFN-g were produced from Th17 cells. Skin and peripheral blood contained a subset of Th17 cells producing IFN-g / TNF.

Keratin 16, IL-17, IFN-g, and IL-22 mRNA expression increased in psoriatic skin, but cyclosporine therapy returned these mRNA to normal levels. The average expression of IL-17 / human acidic ribosomal protein (hARP) in non-lesional skin was 0.4 compared to 10.8 in lesional skin, and cyclosporine administration returned to non-lesional levels. That IL-17 mRNA return to baseline, effective treatment supports that Th17 in psoriasis is a central pathogenic.(Lowes et al.2008)

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Relationship: 2019: Th17 cell migration and inflammation induction leads to Skin disease

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Initiation of plaque formation in the Aldara psoriasis mouse model is dependent on RORγt +, skin infiltrating γδ T cells, and innate lymphocyte cells (ILC). Vγ4 + γδT cells and innate lymphoid cells (ILC) are the dominant and important sources of IL-17A, IL-17F, and IL-22 in the formation of acute psoriatic lesions, rather than Th cells (Pantelyushin et al. 2012).

Amyloid A: SAA, an inflammatory marker, is high in the serum of patients with psoriasis. When C57B6 mice were given SAA protein subcutaneously on the back, epidermal thickening and inflammatory cell wetting were frequent on days 5-7. Skin inflammation was significantly suppressed when IL-12 / IL-23p40 protein, a target molecule of psoriasis biologics, was administered in advance. By SAA administration, a similar reaction to psoriatic eruption was formed in the immunological reaction, and a psoriatic eruption model mouse was established. (J Dermatolog Trest. 2013; 24 (6): 477-80)

Key Event Relationship Description

Th17 cells produce the cytokines IL-17 and IL-22. IL-17 is inflammatory, promotes the migration of neutrophils to psoriatic lesions, contributes to the formation of Munro's micro-abscess, and through DCL and memory T cells, including Th17 cells and CCR6, via CCL20 Incorporate into the affected area. IL-22 causes abnormal keratinocyte proliferation by down-regulating genes that control terminal differentiation, leading to altered differentiation and keratinization. Both IL-17 and IL-22 induce keratinocyte expression of the antibacterial S100A7 (psoriacin). Nograles et al. 2008

STAT3 is important for Th17 differentiation. Cytokine signaling SOCS3-deficient mice show increased IL-17 expression by increasing STAT3 activity in response to IL-23 binding to IL-17. Associated with increased activity of STAT3 in response to IL-23 capable of binding to IL-17 and IL-17F promoters. STAT3 overexpression promotes Th17 differentiation, whereas STAT3 deficiency inhibits Th17 differentiation. STAT3 signaling from IL-6, IL-21, IL-23 regulates the expression of lineage specific master transcription factors RORγt22, 63, 66 and RORα67. It has been found that patients with psoriasis with mutations in STAT3 cannot generate a Th17 response. Martinez et al. 2008

Evidence Supporting this KER

Biological Plausibility

The biological activity of the combination of cytokines was investigated. The combination of IL-17A and IFN-γ or IL-17A and TNF-α has a synergistic effect on CXCL8 production by keratinocytes. IL-17A and IL-22 exert a synergistic effect in upregulation of β-defensin 2: BD-2 and S100A9 production] IL-1α, IL-17, IL-22, Oncostatin M: OSM, and TNFα binding are associated with increased expression of inflammatory molecules such as soriacin / S100A7 or BD-2, IL-8 in vitro by NHEK Although very potent synergistic, removal of IL-22 from the cytokine mixture reduces CXCL8 and BD-2 expression by 30% and removal of IL-17 reduces it by 70%.

Ex vivo studies on human skin explants showed upregulation of BD-2, S100A7, and CXCL8 expression in response to the same combination of cytokines, and intradermal injection of cytokines into mice resulted in neutrophil infiltration and early epidermis CXCL1, CXCL2, CXCL3, S100A9, and BD-3 expression related to epidermal thickening was increased. (Bernard et aaal. 2012)

Empirical Evidence

Resident memory tissue T cells (TRM cells) confer both resistance and immunity depending on the local microenvironment, and CD8 TRM can be tracked by phenotypic markers CD49a and CD103. Circulating effector T cells infiltrate the site of skin inflammation and turn into long-lived epidermal TRM cells when the skin inflammation is resolved. Epidermal TRM cells are thought to form pathological site-specific disease memory at the site of recurrent psoriasis.(Cheuk et al. 2014)

Single cell suspensions of epidermis and dermis were analyzed by flow cytometry within 30 hours of sampling. In active psoriasis, CD8 T cells increased about 100-fold in the epidermis compared to normal skin, whereas CD4 T cells increased 10-fold in the dermis. In healthy skin, 20-30% of epidermal CD8 T cells co-expressed integrin CD103 and CD49a, which are phenotypic markers

of TRM cells. In active psoriasis, approximately half of epidermal CD8 T cells co-expressed these TRM phenotypic markers, a 100-fold increase compared to healthy skin. Cheuk et al. 2014

Uncertainties and Inconsistencies

Cytokines cannot be specified for genes associated with abnormalities in psoriatic skin. Many genes that are up-regulated in psoriatic lesions can be attributed to IFN-γ, including IL-17 and IL-22. Cytokines synthesized by Th1 / Th17 cells regulate different gene expression pathways in epidermal keratinocytes and other skin resident cells. The psoriatic transcriptome may result from activation of multiple independent pathways. Nograles et al. 2008

After daily topical application of Aldara containing imiquimod (IMQ) to humans, significant skin thickening, redness and scaling were observed 3 days later (doi: 10.1172 / JCI61862DS1). The clinical course of plaque formation on the ear and back skin and histopathology were similar. Aldara treatment resulted in impaired keratinocyte hyperproliferation and epidermal differentiation, as indicated by epidermal thickening and hyperkeratosis. There was a terminal neutrophil accumulation in the stratum corneum reminiscent of a Munro micro-abscess in psoriasis. Extensive leukocyte infiltration was observed in the dermis.(Pantelyushin et al. 2012)

Quantitative Understanding of the Linkage

Response-response relationship

KE2

High levels of Th17 cytokines were observed in psoriatic skin induced by CD4 + T cells. IL-23 p40 subunit or IL-22 significantly prevented the development of skin lesions.

IL-22-induced acantosis and inflammation were reduced in IL-22-deficient mice compared to WT mice. Blocking IL-22 increases IL-1α, IL-1β, IL-6, IL-17, IL-17F, and TNF-α. (K. A. et al. 2013)

AO

Anti-IL-17 antibody administration results in decreased keratinocyte proliferation and differentiation, leukocyte infiltration, and keratinocyte release of inflammatory cytokines. In psoriatic lesioned keratinocytes, changes in mRNA and protein expression of IL-17 regulatory products occurred. Within 2 weeks of antibody administration, the expression of LL37 (cathelicidin), β-defensin 2, S100A7, and S100A8 proteins was markedly decreased in keratinocytes, and the expression reached normal levels after 6 weeks. (Krueger et al. 2012)

Time-scale

Epidermal keratinocyte expression genes that were elevated in psoriatic lesions of patients with psoriasis with stage-type skin eruption: mRNA expression level of keratin6a and 16, s100A7A, S100A12, DEFB4, IL-1F6, CCL20, IL-17C, etc. was rapidly reduced by 700 single intravenous dose of brodalumab and decreased to non-lesional skin level 2 weeks after administration. On the other hand, leukocyte expression genes with increased expression in psoriatic lesion skin: IL-17A, IL-17F, IL-23F, IL-12B, IL-22, IFN-γ and other mRNA expression levels decreased with brodalumab administration. However, at 2 weeks after administration, the level did not decrease to the level of the non-lesional skin. Since the expression of pathophysiology-related genes is reduced prior to the decrease in the expression of leukocyte expression genes is reduced prior to the decrease in the expression of leukocyte expression genes and the decrease in the PASI score, Brodalumab is reduced expression of pathophysiology-related genes by blocking IL-17 signaling in the epidermal keratinocytes of psoriatic lesions It is possible to improve the skin eruption promptly. (Kyowa Hakko Kirin Co., Ltd.)

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AOP ID and Title:

AOP 314: Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)
Short Title: Binding to ER-α leading to exacerbation of SLE

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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.73	Included in OECD Work Plan

Abstract

This AOP describes the linkage between the binding to estrogen receptor (ER) α in immune cells with the exacerbation of the autoimmune disease systemic lupus erythematosus (SLE).

Estrogen receptors (ERs), ERα and ERβ, are a group of proteins that are activated by the steroid hormone estrogen and are widely expressed in most tissue types, including most immune cells. ERα can be activated with exogenous and endogenous estrogens. Also, there are numerous xenoestrogens that exist in the environment and imitate estrogen. Bisphenol A (BPA) is an example of a xenoestrogen that is considered an endocrine disrupting (ED) compound. SLE is an autoimmune disease characterized by overproduction of a variety of anti-cell nuclear and other pathogenic autoantibodies. It is characterized by B-cell hyperactivity, polyclonal hypergammaglobulinemia, and immune complex deposition.

Binding to ERα in immune cells by a xenoestrogen or endogenous estrogen marks the molecular initiating event (MIE), which results in induction of GATA3 expression (KE1). One theory of immune regulation involves homeostasis between T-helper 1 (Th1) and T-helper2 (Th2) activity, however GATA3 expression induce increase of Th2 cells producing cytokine interleukin-4 (IL-4) (KE2), which results in increase of anti-DNA antibody from autoreactive B cell (KE3). This sequence of pathway means that the immune system skew from a Th1 to a Th2 profile, which results in the adverse outcome (AO) of exacerbated SLE.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for binding to ERα in immune cells leading to exacerbated SLE.

Background

It is well recognized that allergic diseases and autoimmune diseases are markedly increased the last several decades. About the same time, increasing scientific and social attention had been paid to environmentally dispersed chemicals that can enter the body by ingestion or adsorption and that mimic the actions of estrogens. These chemicals are termed endocrine disruptors (EDs) or environmental estrogens and are found in plastics (bisphenol-A, phthalates), pesticides (DDT, hexachlorobenzene, and dieldrin) and the like. Some of these estrogenic chemicals have also been shown to influence the immune system. Endocrine disruptors mimic hormones, block or alter hormone binding to receptors, or alter the metabolism of natural estrogens. It has been widely noted that females have stronger immune capabilities than males, as evidenced by their better immune responses to a variety of self-antigens and non-self-antigens, or vaccination. Paradoxically, the stronger immune response comes at a steep price, which is the high incidence of autoimmune diseases in females. This phenomenon of gender-based immune capability is largely attributed to the effects of sex hormones. Estrogens regulate the level of serum and uterine IgM, IgA, and IgG, and they augment antibody production to several nonself- antigens and self-antigens. It is possible that endocrine disruptors that mimic estrogenic activity may be involved in the increased incidence of autoimmune diseases such as SLE (Yurino H. 2004, Vaishali RM. 2018).

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1710	Binding to estrogen receptor (ER)-α in immune cells	Binding to estrogen receptor (ER)-α

Sequence	Type	Event ID	Title	Short name
	KE	1711	Induction of GATA3 expression	Induction of GATA3 expression
	KE	1712	Increase of Th2 cells producing IL-4	Increase of Th2 cells producing IL-4
	KE	1713	Increase of anti-DNA antibody from autoreactive B cell	Increase of autoantibody production
	AO	1714	Exacerbation of systemic lupus erythematosus (SLE)	Exacerbation of SLE

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells	adjacent	Induction of GATA3 expression	Moderate	Moderate
Induction of GATA3 expression	adjacent	Increase of Th2 cells producing IL-4	Moderate	Moderate
Increase of Th2 cells producing IL-4	adjacent	Increase of anti-DNA antibody from autoreactive B cell	Moderate	Moderate
Increase of anti-DNA antibody from autoreactive B cell	adjacent	Exacerbation of systemic lupus erythematosus (SLE)	Moderate	Moderate

Stressors

Name	Evidence
Bisphenol A	Moderate
17beta-Estradiol	High

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI

Sex Applicability

Sex	Evidence
Mixed	High

It has long been appreciated that most autoimmune disorders are characterized by increased prevalence in females, suggesting a potential role for sex hormones (estrogen) in the etiology of autoimmunity. Females generally exhibit a stronger response to a variety of antigens including ERα ligands than males, which is perhaps one reason that they are more prone to develop autoimmune and allergic diseases such as SLE in greater severity than males. Therefore, this AOP is applicable to females and is dependent on the levels of estrogen, which means it varies with life stage, and age.

SLE frequently develop and progress in setting in which sympathoadrenomedullary and gonadal hormone levels are changing, e.g., during pregnancy, the postpartum period, or estrogen administration in menopause (Wilder RL. 1999). Women using oral contraceptives that contain estrogen or undergoing hormone replacement therapy are susceptible to major flare ups and exacerbation of the disease (Whitelaw DA. 2007).

The mechanisms described in this AOP are applicable to rodents and humans, and then the findings of this AOP are not found in any other species. However, Th2 dominant conditions induced by binding to ERα is considered likely to occur in a variety of mammalian species since ERα are expressed in all vertebrates (Eick GN. 2011).

Essentiality of the Key Events

Stressor , MIE and later events:

The NZB/W F1 mouse is the oldest classical model of lupus generated by the F1 hybrid between the NZB and NZW strains. The administration of the estrogen antagonist tamoxifen diminishes immune complex deposition in the kidneys and increases survival in NZB/W F1 strain. Renal disease was evaluated by the development of albuminuria and histological changes in the kidney (Wu WM. 2000). In females of the NZB/NZW F1 strain, disruption of ERα attenuated glomerulonephritis and increased survival and reduced anti-dsDNA antibodies (Bynote KK. 2008, Isenberg DA. 2007) and ovariectomy of NZB/W F1 mice not only delayed onset of the disease but also decreased autoantibody titer Meanwhile, restoration of estradiol in ovariectomized NZB/W F1 mice reestablished high numbers of autoantibody-producing (DNA-specific) B cells, and thereby suggests a pathogenic role of estrogen in lupus (Daniel P. 2011). Both NZB and NZW display limited autoimmunity, while NZB/W F1 hybrids develop severe lupus-like phenotypes comparable to that of lupus patients. In NZM female mice, ERα inactivation markedly prolonged life-span, lowered proteinuria, and ameliorated glomerulonephritis but resulted in higher serum anti-dsDNA antibody levels (Svenson JL. 2008).

KE1 and later events:

GATA3 mRNA expression has potential to induced IL-4 production in CD4+T cell (Lambert KC. 2005). The differentiation of activated CD4+T cells into the T helper type 1 (Th1) or Th2 fate is regulated by cytokines and the transcription factors T-bet and GATA-3. Early GATA-3 expression, required for Th2 differentiation, was induced by T cell factor 1 (TCF-1) and its cofactor β-catenin, mainly from the proximal Gata3 promoter upstream of exon 1b. TCF-1 blocked Th1 fate by negatively regulating interferon-γ (IFN-γ) expression independently of β-catenin. Thus, TCF-1 initiates Th2 differentiation of activated CD4+T cells by promoting GATA-3 expression and suppressing IFN-γ expression. Higher GATA-3 expression promotes IL-4 production and initiates Th2 differentiation (Qing Y. 2009). GATA-3 mRNA expression also increased in patients with SLE, compared with the healthy control groups (Zheng H. 2015, Sonia GR. 2012).

KE2 and later events:

Administration of mAb against IL-4 before the onset of lupus was effective in preventing the onset of lupus nephritis (Nakajima A. 1997).

KE3 and later events:

In a study to investigate a novel subpopulation of B-1 cells and its roles in murine lupus, anti-double-stranded DNA (anti-dsDNA) autoantibodies were preferentially secreted by a subpopulation of CD5+ B-1 cells that expressed programmed death ligand 2 (L2pB1 cells) (Xuemei Z. 2009). A substantial proportion of hybridoma clones generated from L2pB1 cells reacted to dsDNA. L2pB1 cells are potent antigen-presenting cells and a dramatic increase of circulating L2pB1 cells in lupus-prone BXSB mice correlates with elevated serum titers of anti-dsDNA antibodies (Xuemei Z. 2009).

Weight of Evidence Summary

Biological Plausibility

KER	KE_{up}-KE_{down}	Plausibility	Rationales supported by literatures
KER 1	Binding, Estrogen receptor α in immune cells - Induction, GATA3 expression	Weak	In immune cells, this event is confirmed indirectly; using artificial STAT6-ER fusion protein.
KER 2	Induction, GATA3 expression - Increase, Th2 cells producing IL-4	Strong	XXXX
KER 3	Increase, Th2 cells producing IL-4 - Increase, anti-DNA antibody production from autoreactive B cell	Weak	XXXX
KER 4	Increase, anti-DNA antibody production from autoreactive B cell -	Strong	XXXX

Empirical Support

KER	Empirical support of KERs
MIE=>KE 1 Binding, Estrogen receptor α in immune cells leads to Induction, GATA3 expression	Empirical support of the MIE => KE1 is weak. Rationale

	MIE: XXX KE XX: XXXX
KE 1=> KE 2: Induction, GATA3 expression leads to Increase, Th2 cells producing IL-4	Empirical support of the KE 1=> KE 2 is strong. Rationale KE XX: XXXX AO: XXXX
KE 2=> KE 3: Increase, Th2 cells producing IL-4 leads to Increase, anti-DNA antibody production from autoreactive B cell	Empirical support of the KE 2=> KE 3 is weak. Rationale KE XX: XXXX AO: XXXX
KE 3=>AO: Increase, antibody production from anti-DNA antibody production from autoreactive B cell leads to Exacerbation, systemic lupus erythematosus (SLE)	Empirical support of the KE 3 => AO is strong. Rationale KE XX: XXXX AO: XXXX

Quantitative Consideration

KER1

CD4^{+}T cell expressed GATA3 mRNA cultured with 10^{-9} M (272.4 pg/mL) concentrations of 17β-estradiol for 12-16 hr (Lambert KC. 2005).

BPA (0.1 mM) also indirectly induced GATA3 expression of Th cells, and this effect is mediated by dendritic cells exposed to BPA for 24 hr (Guo H. 2010). Naïve Th cells increased GATA3 expression cultured with dendritic cells exposure of BPA (0.1 mM) for 7 days.

KER2

Pre-stimulation 16 hr of 17β-estradiol (the concentration 10^{-9} M = 272.4 pg/mL) increased IL-4 secretion from CD4^{+}T cell (Lambert KC. 2005).

KER3

PBMCs or B cells were cultured for 7 days with 17β-estradiol (10^{-8} mol/L) and then, IgG and IgM production were increased up to about 150% (PBMC) and 200% (B cells) (Kanda N. 1999).

KER4

XXXX

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Appendix 1

List of MIEs in this AOP

Event: 1710: Binding to estrogen receptor (ER)-α in immune cells

Short Name: Binding to estrogen receptor (ER)-α

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	MolecularInitiatingEvent

Stressors

Name
Bisphenol A
17beta-Estradiol
Propylpyrazoletriol

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

E_2 activates ERα and ERβ with the same affinity although they share only 56% similarity in their ligand binding domains (Monroe DG. 2005, Papoutsi Z. 2009). Exposure E_2 induced thymic atrophy, and changing T-cell phenotype (decreasing double positive ($CD4^+CD8^+$) T cell and increasing double negative ($CD4^-CD8^-$) T cell) in thymus (Okasha SA. 2001).

BPA binds to both ERα and ERβ, and ERα binding affinity of BPA is lower than that of ERβ (Takayanagi S. 2006). While these bindings are less than 2000-fold affinity compared to the binding of estradiol to estrogen receptors (Krishnan AV. 1993).

Propylpyrazoletriol (PPT) is an ERα-selective agonist, which shows 410-fold selectivity for ERα as compared with ERβ (Kraichely DM. 2000, Li J. 2006). Li et al (2006) demonstrated that ovariectomized mice exposed PPT induced severe thymic atrophy, changing T-cell phenotype (CD4/CD8 phenotype profile) in thymus, and a reduction of mature B cell number in spleen. Since these effects by PPT were equal to or greater than E2, ERα plays the predominant role in the upregulation of immune responses.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	

Since ERα expresses in the cells of a vast variety of (vertebrate) species (Maria B. 2015) and there is common functionality in the immune systems of at least humans and mice, this AOP might be applicable to many mammal species, including humans and rodents. The estrogen receptors are composed of several domains important for hormone binding, DNA binding, dimer formation, and activation of transcription (Green S. 1986, Kumar V. 1986, Warnmark A. 2003). Interspecies sequence identities for the entire ERα are 88.5% (human-mouse), 87.5% (human-rat), and 97.5% (mouse-rat). For the ligand binding domain (ERα-LBD) alone, the interspecies sequence identities are 95.5% (human-mouse), 95.1% (human-rat), and 99.2% (mouse-rat) (White R. 1987).

ERα is widely expressed in most tissue types including most immune cells in males and females (Couse JF. 1997, Chelsea C. 2017). The ERs' expression patterns and functions vary in a receptor subtype, cell- and tissue-specific manner. In the adult human, large-scale sequencing approaches show that ERα mRNA is detected in numerous human tissues, with the highest levels in the uterus, liver, ovary, muscle, mammary gland, pituitary gland, adrenal gland, spleen and heart, and at lower levels in the prostate, testis, adipose tissue, thyroid gland, lymph nodes and spleen (Fagerberg L. 2014, Sayers EW. 2012) (www.ncbi.nlm.nih.gov/UniGene).

Estrogen level is higher in women than men. Ordinary estrogen levels in women are 20-30 pg/mL during diestrus, 100-200 pg/mL during estrus, and 5000-10000 pg/mL during pregnancy (Offner H. 2000). Therefore, the influence of ligand binding to ERα in immune cells is expressed more strong in women than men, especially high estrogen level period.

Key Event Description

ERα is expressed in all vertebrates (Eick GN. 2011). ERα was discovered in the late 1960s and was cloned and characterized in 1985 (Melissa C. 2011). ERα is expressed in a variety of immunocompetent cells, including thymocytes, CD4+ (Th1, Th2, Th17, and Tregs) and CD8+ cells and macrophages (Melissa C. 2011, Salem ML. 2004, Robinson DP. 2014). One study examined ERα expression in resting and activated PBMC subsets and found that ERα was expressed at higher levels in thymocytes, CD4+ T cells than B cells (Melissa C. 2011). ERα is a nuclear hormone transcription factor that classically binds with ligand (stressors), further stabilizing dimers that subsequently bind estrogen response elements (ERE) or non-ERE to transactivate or suppress specific target

genes (Parker MG. 1993, Goldstein RA. 1993, Sasson S. 1991, Brandt ME. 1997, Carolyn MK. 2001).

How it is Measured or Detected

The binding affinities of E_2 and BPA for ERα can be confirmed by radio receptor assay, and its dimer dissociation is measured using size exclusion chromatography (Brandt ME. 1997, Takayanagi S. 2006, OECD TG440 [*in vivo*] and TG455 [*in vitro*]). While the binding affinities of PPT for ERα was determined by competitive radiometric binding assays by chemiluminescence (Kraichely DM. 2000, Carlson KE. 1997).

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List of Key Events in the AOP

Event: 1711: Induction of GATA3 expression

Short Name: Induction of GATA3 expression

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	KeyEvent

Stressors

Name
17beta-Estradiol
Bisphenol A
4-Hydroxytamoxifen

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Evidence for Perturbation by Stressor

17beta-Estradiol

Expression of GATA3 was induced in $CD4^+$T cells treated with E_2 at a concentration of 10^{-9} M (272.4 pg/mL) for 12-16 hours (Lambert KC. 2005). GATA3 expression has potential to induced IL-4 production in $CD4^+$T cell. In contrast, expression of T-bet was decreased, which means E_2 skew the immune system from a Th1 to a Th2 profile (Lambert KC. 2005).

Bisphenol A

GATA3 expression is induced in Th cells primed by dendritic cells exposed to BPA (Guo H. 2010). Purified naive T cells were cultured and expanded under Th1 culture conditions in the presence or absence of 0.3 µM 4-HT (Research Biochemicals Institute) for 2 weeks starting from days 1, 7, 14, or 21 (Kurata H. 1999).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence

All life stages

Life Stage	Evidence

Sex Applicability

Sex	Evidence
Mixed	

Involvement of GATA3 in Th2 cell development through ER is common in humans, rodents, and other mammalian species (Ho IC. 2009). protein sequence conservation between all six vertebrate members (mouse, human, dog, cow, armadillo, capuchin and opossum) identifies GATA3 as having the highest sequence similarity with both its GATA paralogs and orthologs, suggesting that it may be closest to the ancestral mammalian GATA factor (Tremblay M. 2018).

Key Event Description

Naïve CD4 T cells can differentiate into several different types of T helpers, and Th2 cells, capable of producing IL-4, IL-5 and IL-13, are involved in humoral immunity against extracellular pathogens and in the induction of asthma and other allergic diseases. It was reported that GATA-3 promotes Th2 responses through three different mechanisms (Zhu J. 2006). Cell fate determination in each lineage requires at least two types of transcription factors: the master regulators (GATA3) as well as the signal transducers and activator of transcription (STAT) proteins (Zhu J. 2010). A direct role in bridging distant regulatory elements has been demonstrated for GATA3 at Th2 cytokine loci (Spilianakis and Flavell, 2004). GATA3 is the Th2 master regulator (Zhu J 2010, Sung-Yun. 2004, Zhu J. 2004, Zheng W. 1997, Zhang DH. 1997), but it also plays important roles in multiple steps of CD4 T cell development (Ho IC. 2009). GATA3 can act as pioneer factors by initiating local chromatin opening and allowing the recruitment of other transcription factors to regulatory elements (Spilianakis and Flavell, 2004). Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells (Zhu J. 2004, Pai SY. 2004). GATA-3 mRNA expression also increased in patients with SLE, compared with the healthy control groups (Zheng H. 2015, Sonia GR. 2012).

How it is Measured or Detected

GATA3 mRNA in CD4 T cells can be detected by Real-time PCR (RT-PCR) (Lambert KC. 2005, Kurata H. 1999, Zhu J. 2001).

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Event: 1712: Increase of Th2 cells producing IL-4

Short Name: Increase of Th2 cells producing IL-4

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	KeyEvent

Stressors

Name
17beta-Estradiol
Bisphenol A

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T-helper 2 cell

Organ term

Organ term
immune system

Evidence for Perturbation by Stressor

17beta-Estradiol

In vitro, the addition of E2 significantly increased IL-4 secretion from ERα-replete CD4+T cells, while this effect was abrogated in ERα-deficient CD4+T cells. (Lambert KC. 2005).

Bisphenol A

Mouse lymphocytes stimulated with a massive amount of BPA (50 µM) were Th2 polarized, with prominent elevation of IL-4 as well as IL-10 (Lee J. 2010). Similarly, BPA enhanced IL-4 production in antigen-activated T cells by ELISA or RT-PCR, although the concentrations of BPA that they utilized (10-50 µM) were high (Lee MH. 2003). In this experiment, IL-4 level is confirmed baseline when treated with anti-CD4 mAb. Exposure to BPA in adulthood mice promoted antigen-stimulated levels of IL-4, IL-10, and IL-13, but not IFN-γ (Huimin Y. 2008).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Mixed	

Production of IL-4 from Th2 is common in humans, rodents, and other mammalian species.

Key Event Description

In naive CD4+ T cells, T cell expansion shifts toward a Th2 phenotype that produces Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13, thereby increasing antibody production from autoantibody-producing B cells. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, meanwhile Th1 cells produce IL-12, TNF-α, and IFN-γ. During Th2 polarization, IL-4 produced by Th2 cell. IL-12 plays a central role in promoting the differentiation of naive CD4+ T cells into mature Th1 effector cells. Secretion of IL-10 from Th2 has been suggested to downregulate the DC-derived IL-12 production and lead to a Th2 differentiation (Aste-Amezaga M. 1998). Th2 cells produce IL-4, which stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. The receptor for IL-4 is IL-4Rα, which expresses in B cells. IL-4 also plays an important role in the development of certain immune disorders, particularly allergies and some autoimmune diseases and especially when there is Th2 polarization. Th2 cells from GATA3 and STAT6 knockout animals showed reduction in IL-4 production (Zhu J. 2004, Pai SY. 2004).

How it is Measured or Detected

The levels of IL-4 in the cell supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), cytometric bead array (CBA) kits, or immunoblot analysis (Lee MH. 2003, Huimin Y. 2008, Lee J. 2010), and mRNA levels of IL-4 in the cells were assayed by reverse transcription–polymerase chain reaction (RT-PCR) (Lee MH. 2003, Lee J. 2010).

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Event: 1713: Increase of anti-DNA antibody from autoreactive B cell

Short Name: Increase of autoantibody production

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	KeyEvent

Stressors

Name
17beta-Estradiol
Bisphenol A
Diethylstilbestrol

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term

B cell

Organ term

Organ term

immune system

Evidence for Perturbation by Stressor

17beta-Estradiol

BPA as well as E_2 and diethylstilbestrol (DES) enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of ED was more prominent on aged BWF1 mice developing lupus nephritis. (Yurino H. 2004).

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 or B2 cells, B1 cells were prepared from peritoneal cells and B2 cells from spleen, B1 or B2 cells were cultured in the presence of endocrine disruptors (E_2: 100 nM, DES: 100 nM, BPA: 1 µM) for 4 days (Yurino H. 2004).

Direct exposure of PBMCs from SLE patients to E_2 induces secretion of anti-dsDNA antibodies and enhances the secretion of Igs, in particular IgG (Kanda N. 1999).

In both (NZB×NZW) F1 and MRL/lpr mice, estrogen treatment exacerbates the lupus disease, with augmented levels of autoantibodies against dsDNA and phospholipids as well as formation of circulating immune complexes (Grimaldi CM. 2002, Peeva E. 2000).

Hybridomas generated from E_2-treated mice express high-affinity, unmutated anti-DNA antibodies, indicating that naı¨ve B cells that are normally deleted or anergized are rescued from tolerance induction (Bynoe MS. 2000). E_2 treatment resulted in a rise in anti-DNA serum titers and in Ig deposition in renal glomeruli (Bynoe MS. 2000).

Bisphenol A

BPA as well as E_2 and diethylstilbestrol (DES) enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of ED was more prominent on aged BWF1 mice developing lupus nephritis. (Yurino H. 2004).

In a murine model of SLE, BPA increased the number of B cells producing autoantibodies, and IgM antibody secretion by B1 cells was augmented (Yurino H. 2004).

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 or B2 cells, B1 cells were prepared from peritoneal cells and B2 cells from spleen, B1 or B2 cells were cultured in the presence of endocrine disruptors (E_2: 100 nM, DES: 100 nM, BPA: 1 µM) for 4 days (Yurino H. 2004).

Diethylstilbestrol

BPA as well as E_2 and diethylstilbestrol (DES) enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of ED was more prominent on aged BWF1 mice developing lupus nephritis. (Yurino H. 2004).

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 or B2 cells, B1 cells were prepared from peritoneal cells and B2 cells from spleen, B1 or B2 cells were cultured in the presence of endocrine disruptors (E_2: 100 nM, DES: 100 nM, BPA: 1 µM) for 4 days (Yurino H. 2004).

In both (NZB×NZW) F1 and MRL/lpr mice, estrogen treatment exacerbates the lupus disease, with augmented levels of autoantibodies against dsDNA and phospholipids as well as formation of circulating immune complexes (Grimaldi CM. 2002, Peeva E. 2000).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence

All life stages

Life Stage	Evidence

Sex Applicability

Sex	Evidence
Mixed	

Antibody production from B cells is common in humans, rodents, and other mammalian species. Since almost experiment are performed in female, it is considered that this event in SLE are noted more frequently in females.

Key Event Description

The receptor for IL-4 is IL-4Rα, which expresses in B cells. IL-4 produced by Th2 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Anti-DNA antibodies are produced from autoreactive B cell. In murine models, addition of estrogen or prolactin can lead to an autoimmune phenotype with an increase in mature high-affinity autoreactive B cells (Daniel P. 2011).

How it is Measured or Detected

[*in vivo* assay]

NZB/W F1 mice are used as model of SLE (Wu WM. 2000). BALB/c R4Ag-gamma 2b transgenic mice are used for evaluation of autoreactive B cells (Peeva E. 2005). These mice are administrated of the estrogen antagonist tamoxifen. Disruption of ERα (Bynote KK. 2008, Isenberg DA. 2007) and ovariectomy of NZB/W F1 mice are used as model of estrogen dysfunction (Daniel P. 2011). Survival and glomerulonephritis of these animals were evaluated.

Using female NZB/WF1 mice, silastic implants containing the powdered form of endocrine disruptors were placed subcutaneously on the back of ovariectomized mice. The implants were left in situ for 3 to 4 months and blood samples were collected periodically, and anti-DNA antibody was measured in ELISA using dsDNA (Yurino H. 2004).

[*in vitro* assay]

The amounts of anti-dsDNA, anti-glomerular antigens (GA), total IgG and IgM in the culture supernatants were measured by ELISA (Kanda N. 1999, Wu WM. 2000, Yurino H. 2004, Gabriela T. 2019, John LS. 2008, Wang Y.1996). Proliferative responses PBMCs or B cells were measured by [3H]-thymidine uptake, and the cell viability was assessed by a trypan blue exclusion test (Kanda N. 1999). Fluorescence activated cell sorting (FACScan) was used for the quantitated of total B cells and CD5+B cells expression in spleen and in peritoneal exudates or B cell subset analysis (Wu WM. 2000, Peeva E. 2005). Plaque forming cell (PFC) assay using autologous bromelain-treated erythrocytes (Br-RBC) was conducted to examine the effect of EDs on autoantibody production by B1 cells (Yurino H. 2004).

Enzyme-linked immunospot (ELISPOT) analysis confirmed a significant increase in the number of high-affinity anti-DNA antibody-secreting B cells in the spleens of E2-treated mice (Bynoe MS. 2000).

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List of Adverse Outcomes in this AOP

Event: 1714: Exacerbation of systemic lupus erythematosus (SLE)

Short Name: Exacerbation of SLE

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	AdverseOutcome

Stressors

Name
17beta-Estradiol
Bisphenol A

Biological Context

Level of Biological Organization
Individual

Evidence for Perturbation by Stressor

17beta-Estradiol

The NZB/W F1 mouse is the oldest classical model of lupus generated by the F1 hybrid between the NZB and NZW strains. In both NZB/W F1 and MRL/lpr mice, estrogen treatment exacerbates the lupus disease (Grimaldi CM. 2002, Peeva E. 2000). In postmenopausal women there was an increase in number of mild flares in women receiving estrogen supplementation suggesting that the addition of estrogen to a low estrogen state enhances flare rate (Buyon JP. 1998).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Mixed	

Exacerbation of SLE is common in humans and rodents, and is considered likely to occur in other animal species, as well. SLE is an autoimmune disease that occurs primarily in women (9:1 compared to men) (Rider V. 2001). SLE is an autoimmune disease that affects predominantly women during reproductive years, and its evolution is altered by hormonal events such as menses, menopause, and especially pregnancy (Luis JJ. 2014). The incidence of SLE is markedly increased in females of child-bearing age (Grainne M. 2013). Th1/Th2 shift is one of the most important immunologic changes during gestation. It is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses. For this reason, Th1-mediated diseases, such as rheumatoid arthritis, tend to improve, while Th2-mediated diseases, such as SLE tend to worsen during pregnancy (Doria A. 2006).

Female MRL/lpr mice that developed lymphadenopathy and a lupus-like disease also exhibited a 50% higher mortality rate than males at 5 months of age. In (NZB×NZW) F1 mice too, females develop signs of SLE several months before males, with severe autoimmune hemolytic anemia, glomerulonephritis, and autoantibodies to single-stranded DNA, doublestranded DNA, and histones (Carlsten H. 1992).

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Melissa, and Gary 2011). Low serum levels (60-100 pg/mL or 0.26-0.43 nM) of estradiol have been shown to increase Th1 T-cell development in vitro through an ERα mediated mechanism (Maret A. 2003). In contrast of SLE exacerbated by Th2, treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated autoimmune diseases (multiple sclerosis; MS, rheumatoid arthritis; RA, and experimental autoimmune encephalomyelitis; EAE, etc.) caused by Th1, while high doses (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset polarized T-cells to a Th2 phenotype in the EAE model (Bebo BF. 2001).

Key Event Description

SLE is an autoimmune disease characterized by overproduction of a variety of anti-cell nuclear and other pathogenic autoantibodies. It is characterized by B-cell hyperactivity, polyclonal hypergammaglobulinemia, and glomerulonephritis as immune complex deposition. Once SLE is suspected, the initial evaluation should include an antinuclear antibody (ANA) test. This is a highly sensitive test, with positive results in about 94% of patients with SLE. However, it also has low specificity, and may be positive in healthy patients. If ANA results show a 1:40 titer or higher, more specific tests should be performed, including measurement of anti–double-stranded DNA (anti-dsDNA), anti-Smith, anti-RNP, anticardiolipin, beta-2 glycoprotein antibodies and lupus anticoagulant; elevated levels of one or more of these biomarkers increase the likelihood of SLE (Nguyet-Cam VL. 2016). In the Systemic Lupus International Collaborating Clinics 2012 classification for SLE, biopsy-proven lupus nephritis plus positive ANA or anti-dsDNA is sufficient to fulfil SLE classification criteria (Bernard T. 2017). SLE is the prototypic multisystem autoimmune disorder with a broad spectrum of clinical presentations encompassing almost all organs and tissues including skin, kidney, heart, lungs, and joints. The pathogenesis of SLE includes both genetic and environmental components with female sex strongly influencing pathogenesis. These factors lead to an irreversible break in immunological tolerance manifested by immune responses against endogenous nuclear antigens (Daniel P. 2011).

It has been determined in a murine model of SLE that ERα is required for disease progression and that ERα deficiency impedes the course of the disease (Bynote KK. 2008). There is increased ERα mRNA expression in PBMCs of SLE patients (Inui A. 2007). It is considered that MIE affect later events and result in SLE.

How it is Measured or Detected

[*in vivo* assay]

Murine lupus models such as New Zealand Black (NZB)×New Zealand White (NZW) F1 (NZB/W F1), NZB.H-2bm12, NZB×SWR F1 (SNF1), MRL.lpr/lpr, and BXSB mice have led to a better understanding of the pathogenic mechanisms of lupus. All of these species of mice develop anti-dsDNA antibody, which is a characteristic of lupus, and die of uremia in early life. Among these murine lupus models, the natural course of NZB/W F1 mice is closer to human lupus than MRL.lpr/lpr and BXSB mice (Zhang DH. 1997, Pai SY. 2004, Daniel P. 2011).

For the disease onset, mice can monitor by proteinuria levels, body weights, blood urea nitrogen and appearance over time. (Gabriela T. 2019, John LS. 2008, Wang Y.1996). The major cause of death in the NZB/W F1 female is chronic glomerulonephritis with heavy mesangial deposits, tubular cast formation, proliferation of glomerular cells, prominent crescent formation, and a significant periglomerular and interstitial monocytic infiltrate. Extraglomerular renal deposits of IgG2a and C3 are present in the peritubular tissue and arterioles, and increase in frequency with age. Histological alterations in the kidney were assessed by Hematoxylin Eosin (H&E) and Periodic acid-Schiff (PAS) staining, expression of IgG and C3 was detected by immunohistochemistry (Gabriela T. 2019, Brian S. 1978).

To examine the relationship between oral contraceptive (OC) use and the development of SLE, analyzed data (1976 - 1990) from the Nurses' Health Study cohort. The questionnaire used to assemble biennially the group sought information on a variety of health conditions and exposures, such as use of OCs, use of post-menopausal hormones (PMH), current and past cigarette smoking habits and other health practices. Incidence of SLE was defined by; 1) strict American College of Rheumatology (ACR) classification criteria (> or = 4 ACR criteria), 2) > or = 4 ACR criteria and any physician's diagnosis, 3) > or = 4 ACR criteria and diagnosis by an ACR-certified rheumatologist, 4) > or = 3 ACR criteria, or 5) diagnosis by a physician even if the patient did not meet the ACR criteria. (Bertsias G. 2012, Sanchez-Guerrero J.1997).

Typical clinical symptoms include combinations of renal disease, swollen joints, skin rash, hematologic disorders, respiratory, and neurologic dysfunction.

Regulatory Significance of the AO

There are concerns about the increase in autoimmune diseases caused by estrogen-like substances, and its accurate in vitro toxicity assessment system is required in international regulations. The OECD has published a revised version of the guidance document on standardized test guidelines for evaluating ED (OECD. 2019).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2020: Binding to estrogen receptor (ER)-α leads to Induction of GATA3 expression

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

XXXX

Key Event Relationship Description

The hormone binding domain (HBD) of the ERα is required not only for binding ligand but also to form stable homodimers of the protein and mediate transcriptional activation by the receptor. There are two ligand-dependent signaling pathway. One is “classical” and the other is “tethered” pathway. A direct genomic interaction occurs between the ER ligand complex and specific sequences of DNA known as estrogen response elements (ERE) (Parker MG. 1993, Goldstein RA. 1993, Sasson S. 1991, Brandt ME. 1997). Transcriptional activation by ERα is mediated by two distinct activation functions: the constitutively active AF-1 domain, located in the N-terminal domain of the receptor protein, and the ligand-dependent AF-2 domain, located in the C-terminal domain of the receptor protein (Delaunay F. 2000). This is called “classical” signaling pathway. In addition to above classical mechanism, ligand-activated ERα interact with other transcription factor complexes and bind to non-EREs by attaching to other transcription factors and not with ERE directly. (Carolyn MK. 2001). This is also called “tethered” signaling pathway. The transcription factors GATA3 and STAT6 are essential for the establishment and/or maintenance of these interactions (Spilianakis and Flavell, 2004). In the tethered pathway, STAT6-ER fusion protein induce GATA-3 mRNA expression. Furthermore, in mammary gland but not in immune cells, GATA3 and ERα regulate each other and, along with FOXA1, can nucleate a remodeling complex at heterochromatic enhancer regions of ERα target genes, leading to the opening and epigenetic marking of sites for active transcription (Eeckhoute J. 2007, Kong SL. 2011). Alone, FOXA1 or ERα are not sufficient to fully open the chromatin, supporting a bona fide pioneer activity for GATA3 (Eeckhoute J. 2007, Kong SL. 2011).

Evidence Supporting this KER

Biological Plausibility

STAT6-ER fusion protein (STAT6:ER) induce expression of GATA-3 mRNAs in presence of 4-Hydroxytamoxifen (4-HT), estrogen analogue (Kurata H. 1999, Zhu J. 2001). Furthermore, A constitutively active form of Stat6 (STAT6VT) introduced GATA3 expression and resulted in both Th2 differentiation and enhanced cell expansion without IL-4 (Zhu J. 2001, Horiuchi S. 2011). CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under ThN conditions with added IL-4 (Zhu J. 2001). Therefore, it is considered that activated STAT6 after ligand-biniding to ERα induce GATA3 expression in immune cells.

Empirical Evidence

Expression of GATA3 was induced in T cells treated with E_2 at a concentration of 10^{-9} M (272.4 pg/mL) for 12-16 hours (Lambert KC. 2005). In contrast, expression of T-bet was decreased, which means E_2 skew the immune system from a Th1 to a Th2 profile. Stat6:ER Th1 cells expressed significant amounts of both GATA3 mRNAs in a 4-HT-dependent manner (Kurata H. 1999, Zhu J. 2001). Constitutively activated Stat6 (Stat6VT) is primed under null Th cell (ThN) conditions in the absence of human (h)IL-4. The expression level of Gata3 in this primed cells are checked by RT-PCR (Zhu J. 2001).

M12.4.1 cells, transfected with the luciferase reporter gene by inserting three copies of human STAT6 binding site oligonucleotide, are used nuclear extracts and electrophoretic mobility shift assay (EMSA) with 1 μM 4-HT. STAT6:ER DNA-binding activity is strongly and rapidly (within 1 hr) induced after addition of 4-HT to these cells. BA/F3 cells prepared as the same manner are stimulated with 1 μM 4-HT for 24 h at 37°C. The cells were harvested and assayed for luciferase activities using a Luciferase Assay Kit (Kamogawa Y. 1998).

Uncertainties and Inconsistencies

The “tethered” pathway is confirmed indirectly using artificial STAT6-ER fusion protein but not endogenous STAT6. It remains unknown whether the “classical” pathway is utilized after binding to ERα in immune cells.

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

XXXX

KE XX:

XXXX

Time-scale

XXXX

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during the menstrual cycle and gestation. Immune activity shifts across the menstrual cycle, with higher follicular-phase Th1 cell activity and higher luteal-phase Th2 cell activity (Tierney KL. 2015). This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria A. 2006). The effects of ERα signaling on T cells appear to be estrogen-dose dependent, i.e., low doses of estrogen stimulate a Th1 response, but higher doses promote a Th2 response (Priyanka HP. 2013).

Known Feedforward/Feedback loops influencing this KER

XXXX

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Relationship: 2021: Induction of GATA3 expression leads to Increase of Th2 cells producing IL-4

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

XXXX

Key Event Relationship Description

Intrachromosomal interactions in the Th2 cytokine locus may form the basis for the coordinated transcriptional regulation of cytokine-encoding genes by the Th2 locus control region (Spilianakis and Flavell, 2004). During Th2 cell differentiation, binding patterns of PcG and TrxG proteins are dynamically changed at the *Gata3* gene locus, and these epigenetic changes result in GATA3 protein upregulation, which consequently induces chromatin remodeling at the Th2 cytokine gene loci, including *Il4*, *Il5*, and *Il13* (Ansel KM. 2006, Horiuchi S. 2011).

Evidence Supporting this KER

Biological Plausibility

Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells. Th2 cells from both knockout animals showed reduction in IL-4 production. (Zhu J. 2004, Pai SY. 2004).

The GATA3 expression induced by TNF-α was enhanced in the presence of BPA. However, the T-bet expression did not change when tested at various culture conditions (Guo H. 2010, Uemura Y. 2008). IL-4 may serve multiple roles in the development of lupus: it may enhance autoantibody production via its direct B-cell effects (Ram RS. 2003).

Empirical Evidence

The proliferation of Stat6:ER Th2 cells was enhanced in a dose-dependent manner on days 10 and 31 after polarization by [^{3}H]thymidine incorporation (the effective concentration of 4-HT was between 0.08 and 2 μM, and the toxic concentration was greater than 5 μM) (Kurata H. 1999, Zhu J. 2001). Purified naive T cells were activated and infected with RV-Stat6:ER. The cells were cultured and expanded under Th culture conditions in the presence or absence of 0.3 μM 4-HT (Research Biochemicals Institute) for 2 weeks starting from days 1, 7, 14, or 21 and the cells were analyzed for cytokine (IL-4) expression by flow cytometer analysis of intracellular cytokine production or cytokine ELISA (Kurata H. 1999, Zhu J. 2001). CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under null Th cell (ThN) conditions with added with IL-4 (Zhu J. 2001).

Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells from GATA-3-deficient (FF and FF cre) mice (Sung-Yun. 2004, Zhu J. 2004). Antigen-specific immune response is evaluated with lymphocyte from FF and FF cre mice injected with KLH, and cytokine production was measured by sandwich ELISA (Sung-Yun. 2004).

Quantitative Understanding of the Linkage

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham M. 2011). When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ.

Response-response relationship

MIE:

XXXX

KE XX:

XXXX

Time-scale

XXXX

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during gestation. This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria et al. 2006).

Known Feedforward/Feedback loops influencing this KER

XXXX

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Relationship: 2022: Increase of Th2 cells producing IL-4 leads to Increase of autoantibody production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	adjacent	Moderate	Moderate

Key Event Relationship Description

During process of B cell maturation, the autoreactive B cell which has high-affinity for DNA are normally silenced by anergy, and activated by stimulation with antigen independent CD40 ligand (CD154) or IL-4 from Th2 cells. The receptor for IL-4 is IL-4Rα, which expresses in B cells. In the development of T-cell dependent antibody producing cells, the interaction between IL-4 and its receptor stimulates B-cells to mature (proliferate, switch immunoglobulin classes). As a result, production of anti-DNA antibody from activated autoreactive B cells in increased.

Evidence Supporting this KER

Biological Plausibility

Lack of ERα, in either male or female mice, did not increase B cell precursors (Smithson G. 1998). Restoration of estradiol in ovariectomized NZB/W F1 mice reestablished high numbers of autoantibody-producing (DNA-specific) B cells, and thereby suggests a pathogenic role of estrogen in lupus (Daniel P. 2011).

Anergic B cells, dsDNA-specific models, can be stimulated by IL-4 specific antibody in vitro, suggesting that they are capable of responding to T-cell-derived signals (Acevedo-Suarez CA. 2005, Noorchashm H. 1999, Mandik-Nayak L. 2000, Eris JM. 1994).

Transfer of either IL-4-stimulated splenocytes from 5-mo-old NZB/W F1 mice into NZB/W F1 mice of the same age enhanced the production of IgG anti-dsDNA Ab. Consistently, administration of mAb against IL-4 before the onset of lupus was effective in preventing the onset of lupus nephritis (Nakajima A. 1997).

Empirical Evidence

The administration of the estrogen antagonist tamoxifen diminishes anti-DNA antibody levels by ELISA as well as decreases percentages of total B cells and CD5+ B cells by FCM (Wu WM. 2000). Tamoxifen blocks estrogen-induced B cell maturation but not survival (Peeva E. 2005). ERα deficiency in (NZB×NZW) F1 female mice downregulated levels of anti-dsDNA IgG antibodies,

and the absence of ERα In (NZB×NZW) F1 males resulted in decreased anti-dsDNA antibodies (Bynote KK. 2008).

Uncertainties and Inconsistencies

Estrogen upregulates CD40L (CD154) on T cells from SLE patients (Desai-Mehta A. 1996, Li X. 2006). Anti-CD40L antibodies downregulate CD86 expression on normal and SLE B lymphocytes, blockade of CD86 only diminishes anti-DNA antibody production by SLE B cells (Nagafuchi H. 2003). Moreover, mice overexpressing CD40L develop a lupus-like disease with high levels of antibodies to nuclear antigens, DNA, and histones, as well as glomerulonephritis (Higuchi T. 2002). Activation of autoreactive B cell may be involved in stimulation not only IL-4, but also CD40 ligand (CD154) of Th2 cell as well as the other immune cells.

B1 cells from aged mice exhibited increased expression of ERα mRNA compared to young mice (Yurino H. 2004). Since the ER of B cell is also expressed, there may be a direct route that does not go through Th2.

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

XXXX

KE XX:

XXXX

Time-scale

XXXX

Known modulating factors

XXXX

Known Feedforward/Feedback loops influencing this KER

XXXX

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Relationship: 2023: Increase of autoantibody production leads to Exacerbation of SLE

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

XXXX

Key Event Relationship Description

The presence of many autoantibodies is a hallmark of SLE. In particular, autoantibodies directed to double-stranded DNA (dsDNA) are characteristic (Isenberg DA. 2007). SLE patients appear to produce significant amounts of the anti-dsDNA autoantibodies that cause the disease. Anti-dsDNA antibody exists even in healthy people, but in SLE patients, an increase in anti-dsDNA antibody has been observed and is also used for definitive diagnosis of SLE. Activation of autoantibody-producing B cells only serves to exacerbate that condition.

Evidence Supporting this KER

Biological Plausibility

SLE has been seen to flare up during pregnancy (Petri M. 1991). The aberrant T cell dysfunction in SLE is also associated with high levels of autoantibodies (Crispin JC. 2010).

Premenopausal women receiving low estrogen containing birth control pills did not have an increased flare rate compared to women receiving placebo suggesting that adding estrogen to an already high estrogen state had no effect on disease (Buyon JP. 1996).

Empirical Evidence

In a study to investigate a novel subpopulation of B-1 cells and its roles in murine lupus, anti-double-stranded DNA (anti-dsDNA) autoantibodies were preferentially secreted by a subpopulation of CD5+ B-1 cells that expressed programmed death ligand 2 (L2pB1 cells) (Xuemei Z. 2009). A substantial proportion of hybridoma clones generated from L2pB1 cells reacted to dsDNA. L2pB1 cells are potent antigen-presenting cells and a dramatic increase of circulating L2pB1 cells in lupus-prone BXSB mice correlates with elevated serum titers of anti-dsDNA antibodies (Xuemei Z. 2009).

Uncertainties and Inconsistencies

Stat6-deficient New Zealand Mixed (NZM) 2328 mice display a significant reduction in incidence of kidney disease, with a dramatic increase in survival, despite the presence of high levels of anti-dsDNA Abs same like the wild-type NZM 2328 animals (Chaim O. 2003). In NZM 2410 mice, STAT6 deficiency or anti-IL-4 Ab treatment decreases type 2 cytokine responses and ameliorates kidney disease, particularly glomerulosclerosis, despite the presence of high levels of IgG anti-dsDNA Abs same like the wild-type littermates or PBS-treated controls (Ram RS. 2003). Anti-dsDNA antibodies are not what we think they are, as they may be antibodies operational in quite different biological contexts, although they bind dsDNA by chance. This may not mean that these antibodies are not pathogenic but they do not inform how they are so (Ole PR. 2019). In other words, might be that the high levels of anti-dsDNA Abs does not always exacerbate SLE.

Quantitative Understanding of the Linkage

Response-response relationship

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham M. 2011). When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular type autoimmune diseases (multiple sclerosis; MS, rheumatoid arthritis; RA, and experimental autoimmune encephalomyelitis; EAE, etc.) caused by Th1 rather than SLE. Treatment with low serum levels (60-100 pg/mL or 0.26-0.43 nM) of estradiol increased Th1 T-cell development in vitro by acting through an ERα mediated mechanism (Maret A. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated autoimmune diseases caused by Th1, while high dose levels (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE. (Bebo BF. 2001, Korn-Lubetzki I. 1984).

Time-scale

XXXX

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during the menstrual cycle and gestation. Immune activity shifts across the menstrual cycle, with higher follicular-phase Th1 cell activity and higher luteal-phase Th2 cell activity (Tierney KL. 2015). This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria A. 2006). Incidence of flare in patients with SLE is increased during pregnancy and within the 3-months postpartum (Amanda E. 2018).

Known Feedforward/Feedback loops influencing this KER

XXXX

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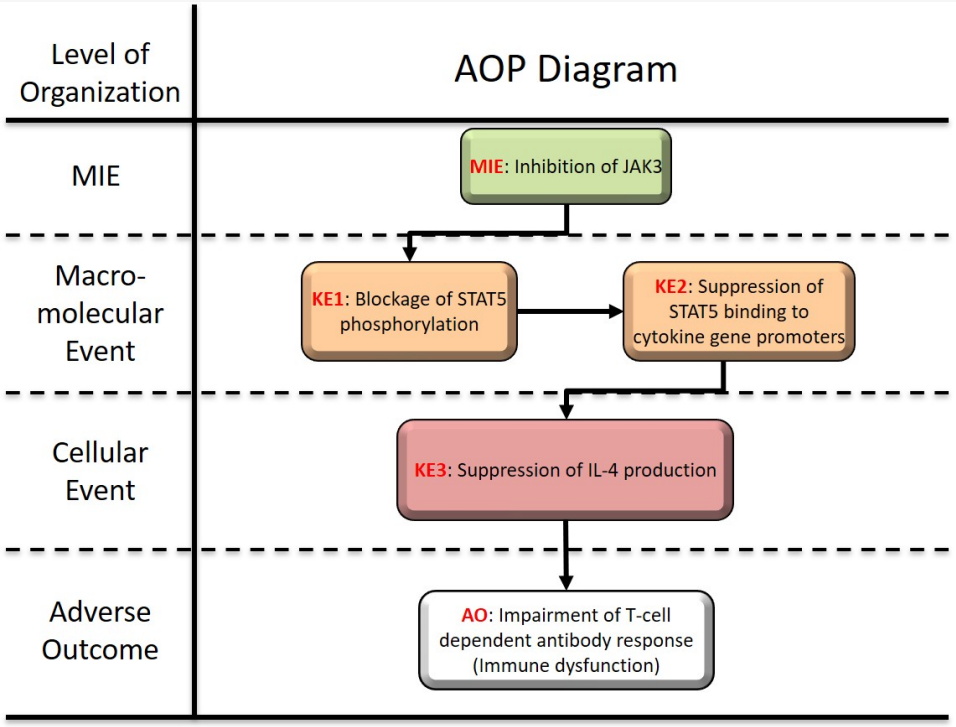
AOP315

AOP ID and Title:

AOP 315: Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response
Short Title: Immune dysfunction induced by JAK3 inhibition

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.74	Included in OECD Work Plan

Abstract

Signal transduction between immune-related cells depends in many cases on cytokines. The transduction involves cell surface cytokine receptors as well as direct cell-to-cell interaction. Cytokines influence the movement, proliferation, differentiation, and activation of lymphocytes and other leukocytes in a variety of ways. Some cytokine receptors require an activation step through a Janus-kinase (JAK)/signal transducer and activator of transcription (STAT) system. When cytokines bind to specific cytokine receptors, the receptors form dimers, which more closely resemble JAK molecules. JAK is activated and phosphorylates adjacent cytokine receptors. STATs bind to the phosphorylated receptor sites and are in turn phosphorylated by the activated JAK. The phosphorylated STAT is dimerized and translocated into the nucleus. There it binds to the promoter regions of cytokine genes, which initiates the transcription of these genes in the nucleus.

In mammals, four JAK families of enzymes (JAK1, JAK2, JAK3, and TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) are utilized by more than 50 cytokines and growth factors to mediate intracellular signaling. In particular, pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6, IL-13, IL-21, and IL-23 have been implicated in inflammatory diseases that utilize the JAK pathway. In addition, TH2 derived cytokines, including IL-31 and thymic stromal lymphopoietin (TSLP), are ligands for murine and human sensory nerves. These cytokines have critical roles in evoking itchiness. Because these cytokines also interact with JAK, several JAK inhibitors have received a lot of attention recently as therapeutic agents for major inflammatory diseases and pruritic diseases.

This proposed AOP consists of JAK3 inhibition as a MIE, blockade of STAT5 phosphorylation as the first key event (KE1), suppression of STAT5 binding to the promoter regions of cytokine genes as KE2, suppression of IL-4 production as KE3, and suppression of T cell

dependent antibody response (TDAR) as an AO. This AOP especially focuses on the inhibition of JAK3, which is required for signal transduction by cytokines through the common γ chain of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In the proposed AOP, JAK3 selective inhibitors that include PF-06651600 (CAS No: 1792180-81-4) and the 4-aminopiperidine-based compound RB1 are stressors. STAT5 that is phosphorylated by JAK3 forms a homo-dimer that translocate to the nucleus and induces expressions of genes, such as IL-4. Therefore, JAK3 inhibition leads to the suppressed binding of STAT5 to the promoter regions of cytokine genes and the subsequent suppression of IL-4 production. Thus, JAK/STAT regulation plays an important role in the TDAR. TDAR is frequently affected by immunosuppressive conditions and is a major endpoint in many preclinical immunotoxicity studies.

Background

Although many stressors inhibit JAK3 activity, this AOP is based on immunosuppression caused by the recently developed and highly selective JAK3 inhibitors PF-06651600 and RB1. A significant body of scientific literature has been published concerning these two inhibitors. We look forward to future amendments to this AOP with up-to-date information on other stressors, which will clarify the link between inhibition of JAK activity and impairment of TDAR.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1715	Inhibition of JAK3	Inhibition of JAK3
	KE	1716	Blockade of STAT5 phosphorylation	STAT5 inhibition
	KE	1717	Suppression of STAT5 binding to cytokine gene promoters	Suppression of STAT5 binding to cytokine gene promoters
	KE	1718	Suppression of IL-4 production	Suppression of IL-4 production
	AO	1719	Impairment of T-cell dependent antibody response	Impairment, TDAR

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition of JAK3	adjacent	Blockade of STAT5 phosphorylation	High	High
Blockade of STAT5 phosphorylation	adjacent	Suppression of STAT5 binding to cytokine gene promoters	High	High
Suppression of STAT5 binding to cytokine gene promoters	adjacent	Suppression of IL-4 production	High	High
Suppression of IL-4 production	adjacent	Impairment of T-cell dependent antibody response	High	High

Stressors

Name	Evidence
PF-06651600 (CAS No 1792180-81-4),	High
RB1	High

Overall Assessment of the AOP

JAKs are a family of nonreceptor tyrosine kinases and consist of four members: JAK1, JAK2, JAK3, and Tyk2 (Johnston, et al. 1994). All four mediate signals initiated by cytokines through interactions with receptors for IL-2, IL-5, IL-7, IL-9, and IL-15 via the common γ chain (Witthuhn, et al. 1994). Different studies have shown that JAK3 is widely expressed in different organs (Witthuhn, et al. 1994). Previous studies with IL-2Rγ-null mice showed that JAK3 is related to the development of spontaneous inflammatory bowel disease symptoms (Miyazaki, et al. 1994). Moreover, abnormal activation of JAK3 was associated with human hematology (Ihle, et al. 1997), indicating that a tight balance of its activity is essential for normal hematopoietic development.

Although JAK1, JAK2, and Tyk2 are widely expressed, JAK3 is predominantly expressed in hematopoietic cells and is associated only with th common γ chain of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (Nosaka, et al. 1995). IL-4 is a very well-known cytokine that is crucial in the

polarization of naïve T cells to type 2 helper T cells. IL-4 plays a major role in the growth and proliferation of many immune cells, such as natural killer (NK) cells and T cells (Dhupkar and Gordon 2017). Homozygous mutant mice harboring a disrupted JAK3 gene display profound reductions in thymocytes and severe B cell and T cell lymphopenia, similar to severe combined immunodeficiency disease (SCID), and functionally deficient residual T cells and B cells. Thus, JAK3 plays a critical role in γ chain signaling and lymphoid development.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

The proposed AOP involves inhibition of JAK activity, which leads to suppression of TDAR independent of life stage, sex, or age. Since JAK3 inhibitors (PF-06651600, RB1) are currently under phase 2 clinical evaluation for the treatment of rheumatoid arthritis, the AOP appears to be applicable to all life stages. JAK3 inhibitor-induced outcomes in humans are mimicked by similar responses in a variety of animal models, including non-human primates and rodents. Thus, immunosuppression induced by inhibition of JAK3 activity is considered to occur across a variety of mammalian species. For example, PF-06651600 was reported to reduce paw swelling with an unbound EC50 of 169 nM in rat adjuvant-induced arthritis. Similarly, PF-06651600 significantly reduced disease severity in an experimental autoimmune encephalomyelitis mouse model at 30 or 100 mg/kg or prophylactically at 20 and 60 mg/kg. PF-06651600 will be evaluated in clinical trials (Telliez, et al. 2016).

Essentiality of the Key Events

MIE: Inhibition of JAK3

JAK3 was initially identified (Johnston, et al. 1994, Witthuhn, et al. 1994) in studies designed to identify the JAK family member involved in the signaling of a group of cytokines with shared utilization of the γ chain first identified in the IL-2 receptor complex. It was subsequently demonstrated that JAK3 physically associates with the γ chain and is activated in a receptor complex that also contains JAK1, which associates with the ligand-specific α or β chain of the receptors (Miyazaki, et al. 1994). JAK3 is somewhat unique within the JAK family in that it is predominantly expressed in hematopoietic cells and is only activated in response to cytokines that use the γ chain (Ihle, et al. 1997). The phenotype of the JAK3 deletion mice is striking, with a range of deficiencies that collectively constitute SCID (Nosaka, et al. 1995, Thomis, et al. 1995). At the same time, two groups identified individuals that lacked JAK3 and exhibited somatically acquired SCID (Macchi, et al. 1995, Russell, et al. 1995). One of the most striking components of the phenotype are the dramatic reductions in both the T and B-cell lineages. Comparable reductions are seen in mice that lack IL-7 (von Freeden-Jeffry, et al. 1995), the IL-7 receptor α chain (Peschon, et al. 1994), or the γ chain. Despite the reduced numbers, the cells that do develop are phenotypically normal. These results are consistent with the hypothesis that activation of JAK3 is critical in the expansion, but not differentiation, of early lymphoid lineage-committed cells. In addition to the reduced numbers, the differentiated lymphoid cells that are generated fail to respond to the spectrum of cytokines that utilize the γ chain and activate JAK3 normally. In addition, there are other examples of JAK3 mutant mice. Primary immunodeficiencies (PIDs) are inborn errors that cause developmental and/or functional defects in the immune system (Picard, et al. 2015). PIDs are usually rare and monogenic. They present clinically with a broad array of phenotypes, including increased susceptibility to infection. One of the most deadly categories of PID is SCID. SCID is invariably caused by severe developmental and/or functional defects of T lymphocytes. However, SCID may also present with variable defects of B and/or NK cells. The B6.Cg-Nr1d1tm1Ven/LazJ mouse line harbors a spontaneous mutation in JAK3, which generates the SCID phenotype (Robinette, et al. 2018). STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells (Rani and Murphy 2016, Wittig and Groner 2005). The activation of STAT5 is transient and tightly regulated in normal cells (Quezada Urban, et al. 2018). The transcription factor STAT5 is expressed in all lymphocytes and plays a key role in multiple aspects of lymphocyte development and function (Owen and Farrar 2017). STAT5 was initially identified as a transcription factor activated by prolactin in mammary gland epithelial cells (Schmitt-Ney, et al. 1992, Wakao, et al. 1992). Subsequent studies identified STAT5 binding activity in T cells (Beadling, et al. 1994). Other authors described that the expression of STAT5 in multiple cell types and its' activation by a number of cytokines, including the common γ-chain-dependent cytokines IL-2, IL-4, IL-7, IL-13, and IL-15 (Lin, et al. 1995).

KE	description
KE1: Blockade of STAT5 phosphorylation	STAT5 refer to two proteins that share 94% structural homology and are transcribed from separate genes, STAT5A and STAT5B. Binding of these extracellular ligands to their target receptors induces the activation of receptor-associated JAK kinases that phosphorylate key tyrosine residues within the receptor, providing docking sites for the SRC homology 2 (SH2) domains of the inactive cytoplasmic STAT5 monomers. STAT5 is then phosphorylated at specific tyrosine residues, either Y694 (STAT5A) or Y699 (STAT5B) of the C-

	terminus. Subsequently, STAT5 undergoes a conformational change and phosphorylated STAT5 monomers form either homo- or hetero-STAT5X-STATX dimers through reciprocal phosphotyrosine–SH2 interactions (Cumaraswamy, et al. 2014, Tothova, et al. 2021). This means that STAT5 will never be activated without this phosphorylation step.
KE2: Suppression of STAT5 binding to cytokine gene promoters	Activated STAT5 dimers translocate to the nucleus, where they bind to STAT5 DNA response elements inducing transcription of genes involved in proliferation, cell differentiation, inflammation (cytokines) and cell survival. Since the STAT5 monomer does not bind directly to the DNA element, inhibiting the STAT5 phosphorylation step suppresses STAT5 activity.
KE3: Suppression of IL-4 production	The observation that STAT5 is activated by multiple cytokines in T cells suggests that it might play a critical role in the development and/or function of these cells. Disruption of the Stat5a gene or Stat5b gene reportedly resulted in relatively modest phenotypes. For example, Stat5a-/- mice displayed defects in mammary gland development and lactation, while Stat5b-/- mice displayed defects in response to growth hormone in male mice and NK cell proliferation (Imada, et al. 1998, Liu, et al. 1997). To determine whether combined deletion of Stat5a and Stat5b might result in more profound immunodeficiencies, subsequent studies deleted the first coding exons of both Stat5a and Stat5b. This intervention resulted in the production of truncated forms of STAT5a and STAT5b, which acted as functional hypomorphs. These mice had surprisingly mild defects in lymphocyte development, although T cells were grossly dysfunctional as they could no longer proliferate in response to IL-2 (Moriggl, et al. 1999, Teglund, et al. 1998). Finally, complete deletion of Stat5a and Stat5b using Cre-LoxP approaches demonstrated that STAT5a and STAT5b are absolutely required for lymphocyte development, as Stat5a/b-/- mice had profound blocks in lymphocyte development, which mimicked that observed in Il7r-/- mice (Cui, et al. 2004, Yao, et al. 2006). These studies definitively demonstrated the retention of appreciable STAT5 function in STAT5 hypomorph mice. Thus, T cell damage due to STAT5 deficiency or inactivation leads to suppression of the production of cytokines such as IL-4.

AOP: Impairment of T cell dependent antibody response (Immune dysfunction)

Weight of Evidence Summary

T cell development is mainly regulated by the JAK-STAT system. JAK3 deficiency in T cells induces multiple types of immunosuppression, including TDAR.

JAK3-deficient mice reportedly displayed profound reductions in thymocytes and severe B cell and T cell lymphopenia, similar to SCID disease. The residual T cells and B cells were functionally deficient (Peschon, et al. 1994).

Mice lacking JAK3 also showed a severe block in B cell development at the pre-B stage in the bone marrow. In contrast, although the thymuses of these mice were small, T cell maturation progressed relatively normally. In response to mitogenic signals, peripheral T cells in JAK3-deficient mice did not proliferate and secreted small amounts of IL-4. These data demonstrate that JAK3 is critical for the progression of B cell development in the bone marrow and for the functional competence of mature T cells (Nosaka, et al. 1995).

Furthermore, the abnormal architecture of lymphoid organs suggested the involvement of JAK3 in epithelial cells. T cells that developed in the mutant mice did not respond to IL-2, IL-4, or IL-7 (Ito, et al. 2017).

PF-06651600 and RB1 specifically inhibit JAK3 with over 100-fold preference over JAK2, JAK1, and TYK2 in kinase assays. Reduced inflammation and associated pathology have been described in collagen-induced arthritis mice. Importantly, the administration of PF-06651600 or RB1 results in decreased pro-inflammatory cytokines and JAK3 and STAT phosphorylation in mice. The findings suggest that the inhibition of JAK3/STAT signaling is closely correlated with the induction of multiple types of immunosuppression, including TDAR.

Quantitative Consideration

KER1 (MIE => KE1)

Treatment with the highly selective JAK3 inhibitor PF-06651600 or RB1 suppresses the complex formation of STAT5 in the nucleus. IL-2 stimulates STAT5 and induces tyrosine phosphorylation of STAT5 (Wakao, et al. 1995). RB1 inhibits the phosphorylation of STAT5 elicited by IL-2, as evidenced by an IC50 value of 31 nM in the peripheral blood mononuclear cells (PBMCs) of humans. PBMCs isolated from the buffy coats of healthy volunteers by density gradient centrifugation on Lymphoprep were cultured in complete RPMI 1640 medium (containing 10% fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin) plus 10 µg/mL lectin phytohemagglutinin (PHA) for 3 days. The cells were then treated with recombinant human IL-6 (400 ng/mL), recombinant human IL-2 (100 ng/mL), or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL) at 37°C for 20 min. To terminate the stimulation, the cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 min. The cells were incubated with anti-pSTAT3 and anti-CD4 antibodies, or anti-pSTAT5 and anti-CD4 antibodies at 4°C overnight, washed twice with PBS, and analyzed with by flow cytometry (Ju, et al. 2011).

The fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes was observed to increase upon incubation of peripheral blood with IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM fo two rats). Additionally, the effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T cells was evaluated. Parallel with the results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 127 nM in human lymphocytes (Ito, et al. 2017).

KER2 (KE1 => KE2)

STAT5 can be activated and phosphorylated by cytokines, such as IL-2 and IL-15. Tyrosine phosphorylation of STAT5 is important for the dimerization of STAT5 (Wakao, et al. 1995). The STAT5 dimer has an identical DNA binding specificity and immunoreactivity.

KER3 (KE2 => KE3)

STAT5 is phosphorylated by JAK kinases, allowing its dimerization and translocation into the nucleus where it can bind to its specific DNA binding sites. Electrophoretic mobility shift assay (EMSA) data revealed that IL-2 activation induced STAT5 dimerization and DNA binding to the gamma interferon activated site (GAS) motif in the IL-4 receptor alpha promoter region (John, et al. 1999). Other EMSA data showed that dexamethasone (10^{-6} M) inhibited STAT5 DNA binding in mononuclear cells in a dose-dependent fashion at dexamethasone concentrations of 10^{-8} to 10^{-7} M (Bianchi, et al. 2000). Dexamethasone could inhibit tyrosine phosphorylation, and nuclear translocation of STAT5 in primary T cells. The mechanism of inhibition involved suppression of IL-2 receptor and JAK3 expression.

KER4 (KE3 => AO)

Binding of IL-4 to the T cell receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

In co-cultured human T and B cells stimulated with anti-CD3 monoclonal antibody, the calcineurin inhibitors (CNIs) FK506 and cyclosporin A (CsA) lowered the levels of T cell cytokines, including IL-2 and IL-4, and inhibited IgM and IgG production in a dose-dependent manner (Heidt, et al. 2010).

The collective results demonstrate the quantitative relationships between the inhibition of IL-4 by specific antibodies or CNI and suppression of antibody production.

1. Support for Biological Plausibility of KER	
MIE => KE1: Inhibition of JAK3 to blockade of STAT5 phosphorylation	Biological Plausibility of the MIE => KE1 is Strong. Rationale: Administration of PF-06651600 or RB1 results in decreased pro-inflammatory cytokines and JAK3 and STAT phosphorylation in mice.
KE1 => KE2: Blockade of STAT5 phosphorylation to suppression of STAT5 binding to cytokine gene promoters	Biological Plausibility of the KE1 => KE2 is Strong. Rationale: STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells. STAT5 is activated by phosphorylation of a single constituent tyrosine residue (Y694) and is negatively regulated by dephosphorylation. A wide variety of growth factors and cytokines can activate STAT5 through the JAK-STAT pathway. The activation of STAT5 is transient and tightly regulated in normal cells.
KE2 => KE3: Suppression of STAT5 binding to cytokine gene promoters to impairment of T cell dependent antibody response (Immune dysfunction)	Biological Plausibility of the KE2 => KE3 is strong. Rationale: In response to mitogenic signals, peripheral T cells in JAK3-deficient mice did not proliferate and secreted small amounts of IL-4. These data demonstrate that JAK3 is critical for the progression of B cell development in the bone marrow and for the functional competence of mature T cells.
KE3 => AO: Suppression, IL-4 production to impairment, T cell dependent Antibody response	Biological Plausibility of the KE3 => KE4 is strong. Rationale: In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells provide help for B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to the impairment of TDAR.
2. Support for Essentiality of AOP	Rationale for Essentiality of KEs in the AOP is strong:
3. Empirical Support for KERs	
MIE => KE1: Inhibition of JAK3 to blockade of STAT5 phosphorylation	Empirical Support of the MIE => KE1 is strong. Rationale: Treatment with the highly selective JAK3 inhibitor PF-06651600 or RB1 suppresses the complex formation of STAT5 in the nucleus. IL-2 stimulates STAT5 and induces tyrosine phosphorylation of STAT5. RB1 inhibits the

	phosphorylation of STAT5 elicited by IL-2, as evidenced by an IC50 value of 31 nM in the peripheral blood mononuclear cells (PBMCs) of humans. PBMCs isolated from the buffy coats of healthy volunteers by density gradient centrifugation on Lymphoprep were cultured in complete RPMI 1640 medium (containing 10% fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin) plus 10 µg/mL lectin phytohemagglutinin (PHA) for 3 days. The cells were then treated with recombinant human IL-6 (400 ng/mL), recombinant human IL-2 (100 ng/mL), or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL) at 37°C for 20 min. To terminate the stimulation, the cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 min. The cells were incubated with anti-pSTAT3 and anti-CD4 antibodies, or anti-pSTAT5 and anti-CD4 antibodies at 4°C overnight, washed twice with PBS, and analyzed with by flow cytometry. The fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes was observed to increase upon incubation of peripheral blood with IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM for two rats). Additionally, the effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T cells was evaluated. Parallel with the results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 127 nM in human lymphocytes.
KE1 => KE2: Blockade of STAT5 phosphorylation to suppression of STAT5 binding to cytokine gene promoters	Empirical Support of the KE1 => KE2 is strong. Rationale: STAT5 can be activated and phosphorylated by cytokines, such as IL-2 and IL-15. Tyrosine phosphorylation of STAT5 is important for the dimerization of STAT5. The STAT5 dimer has an identical DNA binding specificity and immunoreactivity.
KE2 => KE3: Suppression of STAT5 binding to cytokine gene promoters to impairment of T cell dependent antibody response (Immune dysfunction)	Empirical Support of the KE2 => KE3 is strong. Rationale: STAT5 is phosphorylated by JAK kinases, allowing its dimerization and translocation into the nucleus where it can bind to its specific DNA binding sites. Electrophoretic mobility shift assay (EMSA) data revealed that IL-2 activation induced STAT5 dimerization and DNA binding to the gamma interferon activated site (GAS) motif in the IL-4 receptor alpha promoter region. Other EMSA data showed that dexamethasone (10^{-6} M) inhibited STAT5 DNA binding in mononuclear cells in a dose-dependent fashion at dexamethasone concentrations of 10^{-8} to 10^{-7} M. Dexamethasone could inhibit tyrosine phosphorylation, and nuclear translocation of STAT5 in primary T cells. The mechanism of inhibition involved suppression of IL-2 receptor and JAK3 expression.
KE3 => AO: Suppression, IL-4 production to impairment, T cell dependent Antibody response	Empirical Support of the KE3 => KE4 is strong. Rationale: Binding of IL-4 to the T cell receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR. In co-cultured human T and B cells stimulated with anti-CD3 monoclonal antibody, the calcineurin inhibitors (CNIs) FK506 and cyclosporin A (CsA) lowered the levels of T cell cytokines, including IL-2 and IL-4, and inhibited IgM and IgG production in a dose-dependent manner. The collective results demonstrate the quantitative relationships between the inhibition of IL-4 by specific antibodies or CNI and suppression of antibody production.

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Appendix 1

List of MIEs in this AOP

Event: 1715: Inhibition of JAK3

Short Name: Inhibition of JAK3

Key Event Component

Process	Object	Action
regulation of binding	tyrosine-protein kinase JAK3	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	MolecularInitiatingEvent

Stressors

Name
PF-06651600 (CAS No 1792180-81-4), RB1

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus rattus	Rattus rattus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

JAKs are a family of nonreceptor protein tyrosine kinases that are critical for cytokine-receptor-binding-triggered signal transduction through STAT to the nuclei of cells. In mammals, the JAK1, JAK2, and TYK2 kinases are ubiquitously expressed. In contrast, the expression of JAK3 is more restricted. It is predominantly expressed in hematopoietic cells and is highly regulated by cell development and activation (Gaffen, et al. 1995, Xu, et al. 1996). JAK3 is solely activated by type I cytokine receptors, featuring a common γ-chain subunit that is activated by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-7 (Peschon, et al. 1994). Mutations in either the γ chain or JAK3 have been identified as a cause of SCID in humans, which manifests as a depletion of T, B, and NK cells with no other defects (Darnell 1997, Decker, et al. 1997).

Loss-of-function mutations in JAK3 cause autosomal recessive SCID. Defects in this form of SCID are restricted to the immune system, which leads to the development of immunosuppressive JAK inhibitors.

Key Event Description

Janus tyrosine kinase (JAK) 3 is a member of the JAK family that is constitutively associated with the Box-1 region of the cytokine receptor intracellular domain. JAK3 is activated upon ligand-induced receptor dimerization (Stahl, et al. 1994).

The PF-06651600 selective JAK3 inhibitor is undergoing phase 2 clinical evaluation for use in treating rheumatoid arthritis. This compound inhibits JAK3 kinase activity with an IC50 of 33.1 nM (IC50 > 10000 nM). It lacks activity against JAK1, JAK2, or TYK2 (Telliez, et al. 2016, Thorarensen, et al. 2017). The RB1 novel and highly selective JAK3 inhibitor blocks JAK3 kinase in vitro and abrogates functional activity in various cell types (Pei, et al. 2018). When orally administered to mice, RB1 mediate the JAK-STAT pathway and reduces the clinical and microscopic manifestations of paw damage in collagen-induced arthritis mice.

How it is Measured or Detected

Enzymatic activities against JAK1, JAK2, JAK3, and TYK2 were examined using a Caliper Mobility Shift Assay. In the presence of an ATP concentration at Km for ATP for each JAK isoform, RB1 inhibited JAK3 kinase activity with an IC50 value of 40 nM without inhibiting JAK1, JAK2, or TYK2 (IC50 > 5000 nM) (Gianti and Zauhar 2015). The PF-06651600 JAK3 inhibitor displays potent inhibitory activity with an IC50 of 33.1 nM (IC50>10 000 nM), with no activity against JAK1, JAK2, and TYK2. PF-06651600 inhibits the phosphorylation of STAT5 elicited by IL-2, IL-4, IL-7, and IL-15 with an IC50 of 244, 340, 407, and 266 nM, respectively (Telliez, et al. 2016).

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List of Key Events in the AOP

Event: 1716: Blockade of STAT5 phosphorylation

Short Name: STAT5 inhibition

Key Event Component

Process	Object	Action
protein dephosphorylation	signal transducer and transcription activator STAT	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide Pimozide

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

STAT5 is expressed in hematopoietic cells, including T cells and B cells from humans, rodents, and other mammalian species (Thibault, et al. 2016).

Key Event Description

The STAT family of proteins regulate gene transcription upon activation. The proteins rely on cytokine signaling and a number of growth factors through the JAK/STAT pathway (Kisseleva, et al. 2002). STAT activation is regulated by phosphorylation of protein monomers at conserved tyrosine residues, followed by binding to phospho-peptide pockets and subsequent dimerization (Gianti and Zauhar 2015). STAT5 has been implicated in cell growth and differentiation. STAT5 was originally purified and cloned from mammary epithelial cells in sheep and identified as a signal transducer that confers the specific biological responses of prolactin (Wakao, et al. 1992, Xu, et al. 1996).Thus, STAT5 proteins function as signal transduction molecules in the cytoplasm and as transcription factors upon translocation to the nucleus.

How it is Measured or Detected

Phosphorylation of STAT5 tyrosine can be detected by specific antibodies using several detection systems, including flow cytometry. In one study, phosphorylated STAT5 expression was measured in T lymphocytes, and MFIs were reported for each subset (Osinalde, et al. 2017). A cell-permeable non-peptidic nicotinoyl hydrazone compound selectively targets the SH2 domain of STAT5 (IC50 = 47 µM against STAT5b SH2 domain EPO peptide binding activity), with markedly less recognition of the SH2 domain of STAT1, STAT3, or Lck (IC50 >500 µM). The compound was reported to block STAT5/STAT5 DNA binding activity in K562 nuclear extract and inhibit IFN-α-stimulated STAT5 tyrosine phosphorylation in Daudi cells, with no effect on STAT1 or STAT3 (Muller, et al. 2008).

Tyrosine phosphorylation of STAT5 induced by IL-2 has been analyzed using an anti-STAT5 antibody. In the study, this antibody immunoprecipitated STAT5 (p94 kDa). Peripheral blood lymphocytes were untreated (control) or treated with IL-2, IL-4, or IL-15 for 15 min. The extracts were incubated with biotinylated oligonucleotide bound to streptavidin-coated agarose. The agarose beads were washed and the eluted protein was immunoblotted with an antibody to STAT5 (Stahl, et al. 1994).

Other authors described the inhibition of JAK3 kinase activity by PF-06651600, followed by inhibition of the phosphorylation of STAT5 elicited by IL-2, IL-4, IL-7, and IL-15 with IC50 values of 244, 340, 407, and 266 nM, respectively (Telliez, et al. 2016).

Pimozide is a specific inhibitor of STAT5 phosphorylation. Pimozide decreased the survival of chronic myelogenous leukemia cells resistant to kinase inhibitors (Nelson, et al. 2011). IL-2 markedly stimulated STAT5 phosphorylation in PBMCs from patients with chronic kidney disease (CKD). Pretreatment with pimozide (3 µM) dramatically suppressed IL-2-induced STAT5 phosphorylation, indicating that it is a potent blocker of IL-2-stimulated STAT5 phosphorylation in PBMCs from CKD patients.

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Event: 1717: Suppression of STAT5 binding to cytokine gene promoters

Short Name: Suppression of STAT5 binding to cytokine gene promoters

Key Event Component

Process	Object	Action
negative regulation of DNA binding	protein-DNA complex	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculoides	Mus musculoides	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

STAT5 is expressed in hematopoietic cells, such as T and B cells from humans, rodents, and other mammalian species (Gilmour, et al. 1995).

Key Event Description

IL-2 and other cytokines rapidly activate JAK1 and JAK3 (Beadling, et al. 1994) in peripheral blood lymphocytes. The activation of JAK kinases and STAT proteins by IL-2 and IFN-α does not include the T cell antigen receptor in human T lymphocytes (Beadling, et al. 1994). After activation of JAKs, latent STAT transcription factors induce dimeric STAT proteins (Gaffen, et al. 1995). These proteins then translocate to the nucleus, where they bind to and regulate the transcriptional activation of the promoters of target genes. Dimeric STAT proteins can bind to the palindromic gamma interferon-activated (GAS) sequence TTCNmGAA, where m is 3 for all the STATs, except STAT6. The latter can additionally bind to GAS motifs. The m for STAT6 denotes 4 (Darnell 1997, Decker, et al. 1997, Ihle 1996, Leonard and O'Shea 1998).

How it is Measured or Detected

EMSA using nuclear extracts and specific oligonucleotides, including transcription factor binding sites, such as cytokine-inducible SH2-containing protein (CIS) gene promoters, are useful to evaluate DNA binding activity (Johnston, et al. 1995). Activated STAT5 binds to specific DNA-probes in splenocytes (Liu, et al. 2010). A cell-permeable non-peptidic nicotinoyl hydrazone compound selectively targets the SH2 domain of STAT5 (IC50 = 47 μM against STAT5b SH2 domain EPO peptide binding activity), with markedly less recognition of the SH2

domain of STAT1, STAT3, or Lck (IC50 > 500 µM). This compound inhibited STAT5/STAT5 DNA binding activity in K562 nuclear extract and inhibited IFN-α-stimulated STAT5 tyrosine phosphorylation in Daudi cells, but not STAT1 or STAT3 (Muller, et al. 2008).

Nuclear extracts were prepared from untreated YT cells or cells treated with recombinant IL-2 (2 nM) for 30 min at 37°C. EMSA was performed using glycerol-containing 5% polyacrylamide gels (29:1) containing 0.5× Tris-borate-EDTA buffer. For supershift assays, nuclear extracts were preincubated for 10 min with antibodies against STAT5. Oligonucleotide sequences from PRRIFV have been used as probes (Maeshima, et al. 2012). Other authors described a supershift ESMA that involved preincubating whole-cell extract with 3 µL of pan-STAT5 antiserum that recognizes both STAT5a and STAT5b. Electrophoresis was carried out at room temperature using 5% or 6% polyacrylamide gels (Heidt, et al. 2010).

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Event: 1718: Suppression of IL-4 production

Short Name: Suppression of IL-4 production

Key Event Component

Process	Object	Action
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tofacitinib (CP690,550)

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

T cell

Organ term

Organ term

immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In one study, only 1% of CD4 T cells from STAT5a-/- mice primed with soluble anti-CD3 and anti-CD28 with IL-2 produced IL-4, whereas 10.5% of control C57BL/6 CD4 T cells produced IL-4 (Cote-Sierra, et al. 2004).

Cells from STAT5A-deficient mice or cells treated with phospho-STAT5 peptide are defective in Th2 differentiation. STAT5A single-deficient mice showed impaired Th2 differentiation. Reconstituting STAT5A by retroviral infection restored the capacity of cells to induce IL-4 (Kagami, et al. 2001).

IL-2 directly activates STAT5A and STAT5B. T cells from mice deficient in either STAT5A or STAT5B did not show a dramatic change in T cell proliferation, but cells from mice in which both had been knocked out proliferated poorly in response to IL-4 (Moriggl, et al. 1999).

Key Event Description

IL-4 is a mammalian protein found in *Homo sapiens*. IL-4 is pivotal in shaping the nature of immune responses. Upon activation, naïve periph CD4+ T cells begin to synthesize and secrete cytokines. Type 2 helper cells (Th2 cells) produce IL-4, IL-5, IL-6, and IL-13. IL-4 is a 15-kD polypeptide with pleiotropic effects on many cell types. In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th cells. Th2 cells assist B cells in promoting class switching from IgM to IgG1 and IgE (Choi and Reiser 1998).

STAT5 phosphorylation facilitates the dimerization of STAT5, transport to the nucleus, and gene regulation (Levy and Darnell 2002). DNaseI hypersensitivity sites II (HSII) and III (HSIII) in intron 2 have been identified in several regions of the Il4/Il13 locus. STAT5A binding to sites nea HSII and HSIII could provide a mechanism through which STAT5A mediates IL-4 gene accessibility and participates in the induction of IL-4 production (Zhu, et al. 2003). The CD3 antibody-induced phosphorylation of STAT5 can be downregulated by tofacitinib, suggesting that JAK inhibition by tofacitinib can downregulate STAT5-dependent cytokine signaling. Tofacitinib was shown to abrogate anti-CD3-induced STAT5 activation in CD4+ T cells and inhibit IL-4 production from CD4+ T cells (Migita, et al. 2011).

How it is Measured or Detected

In one study, CD4+ T cells were stimulated with CD3 monoclonal antibodies in the presence or absence of tofacitinib (CP-690550) for 48 h. Supernatants were collected and the levels of IL-4 production were measured by ELISA (Migita, et al. 2011). The authors also extract total R after 8 h or 24 h of stimulation and measured IL-4 mRNA expression was measured by real-time PCR (Migita, et al. 2011).

In another study, flow cytometry analysis involving intracellular staining was used to measure cytosolic IL-4 content in stimulated cells (Zhu, e 2001). Relative gene expression levels were determined by quantitative RT-PCR using Taqman Gene Expression primer probe sets and ABI PRISM 7700 or 7900 Taqman systems (Applied Biosystems). The comparative threshold cycle method and internal controls (cyclophillin or β were used to normalize the expression of target gene (IL-4) (Ghoreschi, et al. 2011).

Cytokine content was quantified in appropriately diluted samples in duplicate using ELISA kits to test matched antibody pairs with biotin-

horseradish peroxidase-streptavidin detection and 3,3',5,5'-tetramethylbenzidine substrate. ELISA plates were scanned using the UVmax plate reader (Molecular Devices) using SOFT max software (Dumont, et al. 1998).

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List of Adverse Outcomes in this AOP

Event: 1719: Impairment of T-cell dependent antibody response

Short Name: Impairment, TDAR

Key Event Component

Process	Object	Action
T cell activation involved in immune response		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	AdverseOutcome

Stressors

Name
Cyclosporin, FK506, Basiliximab, PFOA (perfluorooctanoic acid)
Tacrolimus (also FK506)

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNI-induced impairment of TDAR has been demonstrated in rodent studies. In one study, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino, et al. 1987). Likewise, oral administration of FK506 to rats over a 4-week period reduced the production of both anti-KLH-IgG and IgM after subcutaneous immunization with KLH (Ulrich, et al. 2004). Other authors described that treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkeys resulted in reduction of serum SRBC-specific IgM and IgG (Gaida, et al. 2015). As for humans, in vitro experiments showed that treatment with FK506 or CsA of PBMCs from blood bank donors suppressed the production of IgM and IgG specific to T cell dependent antigens (Heidt, et al. 2010). In SKW6.4 cells (IL-6 dependent, IgM-secreting, human B cell line) cultures, FK506 or CsA suppressed the production of IgM in the presence of T cell activation (Sakuma, et al. 2001). Considering that FK506 and CsA reduce T cell derived IL-2, these findings strongly suggest that impairment of TDAR following reduced production of IL-2 occurs at least in common among humans, monkeys, and rodents.

Yang et al. (2002b) exposed male C57BL/6 mice to a single concentration (0.02%) of PFOA in the diet for 16 days. TDAR was measured after inoculating PFOA-treated mice with horse red blood cells intravenously on day 10; serum levels of horse red blood cell-specific IgM and IgG in response to the immunization were significantly decreased (Yang, et al. 2002).

The suppression of TDAR in adult C57BL/6 female mice has been observed in several studies. NOEL of 1.88 mg/kg/d and LOEL of 3.75 mg/kg/d were identified for PFOA administered in drinking water for over 15 days (Dewitt, et al. 2008).

The suppression of TDAR in adrenalectomized or sham-operated C57BL/6N female mice was observed when PFOA was provided in drinking water for 10 days at doses of 0, 3.75, 7.5, or 15 mg/kg/d. TDAR was determined as the primary antibody response to the T cell dependent antigen in SRBCs. The day after exposure ended, SRBCs were introduced intravenously and SRBC-specific IgM was measured 5 days later (DeWitt, et al. 2009).

Key Event Description

The production of antibodies to T cell-dependent antigens is a coordinated process involving B cells, antigen-presenting cells, and T cell derived cytokines. The B cells are stimulated to proliferate and differentiate. The TDAR might be altered if any of these cell populations are affected.

IL-2 and IL-4 are produced and secreted by helper T cells. Both are important in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation. Both events induce and enhance TDAR. IL-2 promotes differentiation of B cells, which stimulates differentiation of activated T cells to Th2 cells. The suppressed production of IL-2 and IL-4 impairs TDAR (Justiz Vaillant and Qurie 2020).

A mutant form of human IL-4, in which the tyrosine residue at position 124 is replaced by aspartic acid (hIL-4Y124D), was reported to specifically block IL-4 and IL-13-induced proliferation of B cells. In addition, hIL-4Y124D also strongly inhibited both IL-4- or IL-13-induced IgG4 and IgE synthesis in cultures of PBMCs, or highly purified sIgD+ B cells cultured in the presence of anti-CD40 monoclonal antibodies. IL-4 may be necessary to produce antibodies and to proliferate in B cells. The mutation of IL-4 may impair TDAR (Aversa, et al. 1993).

IL-4 stimulates B cells to proliferate, switch immunoglobulin classes, and differentiate to plasma and memory cells. Suppressing the production of these B cell related cytokines appears to impair TDAR, as evident from the results of FK506 treatment (Heidt, et al. 2010).

STAT5 is able to inhibit peroxisome proliferator activated receptor (PPAR)-regulated gene transcription. Conversely, ligand-activated PPAR can inhibit STAT5-regulated transcription. As a peroxisome proliferator, perfluorooctanoic acid (PFOA) induces PPARs. The suppression of TDAR has been observed with a no observable effect level (NOEL) of 1.88 mg/kg/d and lowest observed adverse effect level (LOEL) of 3.75 mg/kg/d for PFOA administered in drinking water over 15 days (Dewitt, et al. 2008). The increase in PPAR expression induced by PFOA may inhibit STAT5-regulated transcription, which is important for IL-4 production in TDAR.

How it is Measured or Detected

TDAR can be examined in vivo and in vitro. In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with ELISA (Onda, et al. 2014) or with a plaque-forming cell (PFC) assay.

To assess keyhole limpet hemocyanin (KLH) antigen-specific T cell proliferation, 1×10^5 CD4+ T cells were co-cultured with 2×10^5

autologous PBMCs in 96-well plates in the presence of KLH. Cells were cultured for 5 or 7 days before being pulsed with 0.5 µCi ^{3}[H]-thymidine (PerkinElmer) for 18 h. The cells were harvested using a 96-well cell FilterMate harvester. ^{3}[H]-thymidine incorporation in CD4+ T cell response to biopharmaceuticals was measured by liquid scintillation counting using a TopCount NXT (Schultz, et al. 2017).

In another in vivo study, rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH. Rat serum was examined for T cell dependent, antigen-specific IgM and IgG levels by ELISA (Ulrich, et al. 2004).

Other authors repeatedly administered CNIs, including FK506 and CsA, to mice orally for 4 days and immunized with sheep red blood cells (SRBCs). Spleen cells were examined using a PFC assay (Kino, et al. 1987). Antigen-specific plaque-forming splenocytes were reduced at doses of 3.2, 10, 32, and 100 mg/kg of FK506 or 32 and 100 mg/kg CsA.

In another study, cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBCs. Serum was examined for anti-SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Gaida, et al. 2015).

In the final in vivo study cited here, mice were exposed to a single pharyngeal aspiration of 1,2:5,6-Dibenzanthracene, after which the supernatants of splenocytes were cultured for 24 h in the presence of lipopolysaccharide and assayed using a mouse IgM or IgG matched pairs antibody kit (Smith, et al. 2010).

For in vitro studies, total IgM and IgG levels in culture supernatants are often measured after polyclonal T cell activation rather than after antigen stimulation in immune cell cultures.

In one study, T and B cells isolated from human PBMCs were co-cultured with CNIs for 9 days in the presence of polyclonal T cell stimulation. The supernatants were examined for IgM and IgG levels by ELISA. Treatment with FK506 or CsA reduced the levels of IgM and IgG at concentrations of 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) or 50 and 100 ng/mL (41.6 and 83.2 nM), respectively (Heidt, et al. 2010).

In another study, SKW6.4 IL-6-dependent IgM-secreting human B cells were cultured for 4 days with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. IgM produced in the culture supernatants was measured by ELISA. FK506 or CsA reduced the levels of IgM at concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma, et al. 2001).

Regulatory Significance of the AO

TDAR is considered to be the most important endpoint of immunotoxicity, because T cells, B cells, and antigen-presenting cells, such as dendritic cells, are involved in inducing and developing TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

The ICH S8 immunotoxicity testing guideline on pharmaceuticals recommends that TDAR can be evaluated whenever the target cells of immunotoxicity are not clear based on pharmacology and findings in standard toxicity studies. For the assessment of pesticides, the United States Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC.

Finally, the draft Food and Drug Administration guidance of nonclinical safety evaluation for immunotoxicology recommends the TDAR assay.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2024: Inhibition of JAK3 leads to STAT5 inhibition

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Nelson et al. reported that a membrane proximal region of the interleukin-2 receptor gamma c chain sufficient for Jak kinase activation and induction of proliferation in T cells (Nelson, et al. 1996). Furthermore, Kirken RA et al. demonstrated that activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and STAT5 recruitment by a COOH-terminal region of the IL-2 receptor beta-chain (Kirken, et al. 1995). Therefore, STAT activation is regulated by JAK via phosphorylation. Thus, JAK inhibitors commonly interfere with STAT activation.

Evidence Supporting this KER

STAT5 refer to two proteins that share 94% structural homology and are transcribed from separate genes, STAT5A and STAT5B. Binding of these extracellular ligands to their target receptors induces the activation of receptor-associated JAK kinases that phosphorylate key tyrosine residues within the receptor, providing docking sites for the SRC homology 2 (SH2) domains of the inactive cytoplasmic STAT5 monomers. STAT5 is then phosphorylated at specific tyrosine residues, either Y694 (STAT5A) or Y699 (STAT5B) of the C-terminus. Subsequently, STAT5 undergoes a conformational change and phosphorylated STAT5 monomers form either homo- or hetero- STAT5X-STATX dimers through reciprocal phosphotyrosine–SH2 interactions (Cumaraswamy, et al. 2014, Tothova, et al. 2021). This means that STAT5 will never be activated without this phosphorylation step by JAK3.

Biological Plausibility

STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis, of hematopoietic and immune cells (Wakao, et al. 1992). STAT5 is activated by JAK3 phosphorylation of a single tyrosine residue (Y694).

Empirical Evidence

GM-CSF-induced phosphorylation of STAT5 is inhibited by the RB1 selective JAK3 inhibitor. This suggests that JAK3 inhibition downregulates STAT5-dependent cytokine signaling (Al-Shami, et al. 1998).

Quantitative Understanding of the Linkage

The fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes was reportedly increased upon incubation of peripheral blood with IL-2. Peficitinib is a pan-JAK family inhibitor that can inhibit STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM for two rats). The effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T cells has been evaluated. In parallel with results obtained from rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after the addition of IL-2, but peficitinib inhibited STAT5 phosphorylation in a dose-dependent manner with a mean IC50 of 127 nM in human lymphocytes (Gianti and Zauhar 2015).

Response-response relationship

MIE:

Dose-response analysis of the effects of RB1 on JAK3 kinase activity showed that RB1 inhibits JAK3 kinase activity in a dose-dependent manner with an IC50 value of 40 nM, without inhibiting JAK1, JAK2, or TYK2 (Pei, et al. 2018).

Normal rats were administered peficitinib at 10 and 20 mg/kg. Thirteen hours later, the animals were bled and STAT5 phosphorylation was assessed. IL-2-induced STAT5 phosphorylation of CD3-positive lymphocytes in peripheral blood from the peficitinib-treated rats was suppressed by 37% at a dose of 10 mg/kg and 78% at 20 mg/kg (Gianti and Zauhar 2015).

Time-scale

The enzymatic activities against JAK1, JAK2, JAK3, and TYK2 were immediately tested in CTLL-2 cells using a Caliper Mobility Shift Assay with an ATP concentration at Km (Pei, et al. 2018). CTLL-2 cells were treated with 10 µM adenosine (plus coformycin) for 15 min at 37°C and then stimulated with IL-2 (10 U/mL) for different lengths of time (5 min-12 h). Adenosine dramatically decreased dose-dependent STAT5A/B tyrosine phosphorylation in response to IL-2 over the entire 12 h time course (Zhang, et al. 2004).

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Relationship: 2025: STAT5 inhibition leads to Suppression of STAT5 binding to cytokine gene promoters

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding

Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High	Quantitative Understanding
AOP Name	**Adjacency**		**Weight of Evidence**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Key Event Relationship Description

STAT proteins bind with their SH2 domains (which are located between amino acids 600 and 700) to phosphorylated tyrosine residues of transmembrane receptors (Heim, et al. 1995, Stahl, et al. 1995). Once STATs are bound to the receptors, the receptor-associated Jak kinases phosphorylate them on a single tyrosine residue located carboxy terminal of the SH2 domain. Changing this tyrosine to phenylalanine results in STATs that are no longer functional (Shuai, et al. 1993). Two STATs dimerize through specific reciprocal SH2–phosphotyrosine interaction and translocate to the nucleus. After translocation into the nucleus, STATs bind DNA response elements in promoters of target genes. The putative DNA-binding domain lies between amino acids 400 and 500. After DNA binding STATs interact directly or indirectly with the RNA polymerase II complex. The DNA sequence elements in the promoters of genes that bind STAT proteins can be classified in two groups. The prototype of the first class is the interferon-stimulated response element (ISRE).

The second class comprises the GAS-like response elements. STAT5 homodimers have been shown to bind to at least one of the GAS-like elements (Heim 1996).

Evidence Supporting this KER

The observation that STAT5a/STAT5b/double KO mice are defective in IL-2-induced IL-2Rα expression, suggested that STAT5 is essential for this expression (Kim, et al. 2001, Moriggl, et al. 1999).

In another study, CD25 associated with the intermediate affinity IL-2Rβγ subunits to form the high-affinity heterotrimeric IL-2Rαβγ. In response to ligation with IL-2, signaling of the complex through the IL-2Rβγ chains resulted in the phosphorylation of STAT5 (Waldmann 2006).

STAT5a/b mutant peripheral T cells in mice are profoundly deficient in proliferation and fail to undergo cell cycle progression or to express genes controlling cell cycle progression. STAT5 proteins are essential mediators of IL-2 signaling in T cells (Willerford, et al. 1995).

IL-2 binding to CD25 triggers the grouping with IL-2Rβ and γ chains, leading to signal transduction through STAT5, mitogen-activated protein kinase, and phosphoinositide 3-kinases (PI3Ks) (Fujii, et al. 1995, Ravichandran and Burakoff 1994, Remillard, et al. 1991). Within all T cell populations, IL-2 signaling appears to be primarily mediated through phosphorylation of STAT5 (Hirakawa, et al. 2016).

Biological Plausibility

Upon T cell receptor stimulation, IL-2/STAT5 signaling promotes T cell differentiation. This is the first key step in generating effector T cells that can target pathogens (Liao, et al. 2013).

Increasing the concentrations of IL-2 to superphysiological levels (1000 units/mL), which would eliminate the required upregulation of the IL-2 receptor α chain, also failed to induce a proliferative response in cells from Stat5a/b mutant mice (Willerford, et al. 1995).

Splenic lymphocytes from STAT5a/b, but not STAT5a or STAT5b, mutant mice failed to significantly respond to increasing concentrations of IL-2 in the presence of anti-CD3 (Moriggl, et al. 1999).

Empirical Evidence

Reversible protein phosphorylation plays a key role in IL-2 receptor-mediated activation of JAK3 and STAT5 in lymphocytes (Ross, et al. 2010).

In another study, adenosine was shown to act through A2 receptors and associated cAMP/protein kinase A-dependent signaling pathways to activate Src homology region 2 domain-containing phosphatase-2 (SHP-2) and cause STAT5 dephosphorylation. The dephosphorylation resulted in reduced IL-2R signaling in T cells (Zhang, et al. 2004).

Quantitative Understanding of the Linkage

CD2 signaling of human PBMCs results in activation of the -3.6-kb IFN-γ promoter. In contrast, mutation of the -3.6-kb STAT5 site attenuates promoter activity. Functional activation is accompanied by STAT5A, but scant STAT5B nucleoprotein binds to the STAT5 binding site on the IFN-γ promoter, as determined by competition and supershift assays. Western and fluorescence-activated cell sorting analyses revealed increased phospho-STAT5 following CD2 signaling (Gonsky, et al. 2004).

Response-response relationship

Inhibition of phosphatase activity by calyculin A treatment of YT cells resulted in a significant induction of serine phosphorylation of JAK3 and STAT5, and serine/threonine phosphorylation of IL-2Rβ. Moreover, inhibition of protein phosphatase 2 (PP2A) diminished IL-2-induced tyrosine phosphorylation of IL-2Rβ, JAK3, and STAT5, and abolished STAT5 DNA binding activity (Ross, et al. 2010).

Known modulating factors

As a property of STAT, it is known that DNA binding ability is acquired by forming a dimer, and it is considered that a modifying factor does not intervene in that respect.

Known Feedforward/Feedback loops influencing this KER

IL-2 acts on the same cell that secretes the cytokine. For instance, IL-2 produced by T cells operates on the same T cells that produce this cytokine, or on neighboring cells. With the highest levels in secondary lymphoid organs, IL-2 is believed to act in an autocrine or paracrine manner to support effector and memory CD8 T cell differentiation (Kalia and Sarkar 2018).

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DOI: 10.4049/jimmunol.173.2.932.

Relationship: 2026: Suppression of STAT5 binding to cytokine gene promoters leads to Suppression of IL-4 production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

A STAT5 binding site (TTCATGGAA) has been identified in intron 2 of the Il4 gene, near HSII (Hural, et al. 2000). Another potential STAT5 binding site (TTCTAAGAA) is conserved between mice and humans, and is located near HSIII. STAT5A binds to the sites near HSII and HSIII, which could provide a mechanism through which STAT5A mediates Il4 gene accessibility and participates in the induction of IL-4 production. Enhanced STAT5 signaling results in a larger proportion of cells producing IL-4. A consensus STAT site that preferentially associates with STAT5 contributes to its enhancer activity in mast cells. The intron element plays a role in acquiring and/or maintaining the IL-4 gene locus in a demethylated state in IL-4-producing cells.

Constitutively active STAT5A (STAT5A1*6) restores the capacity to produce IL-4 in cells primed under Th2 conditions in the absence of IL-2, suggesting that STAT5 activation plays a critical role in Th2 differentiation (Zhu, et al. 2003, Zhu, et al. 2004). Additionally, IL-2 critically regulates Th2 differentiation in a STAT5-dependent manner, acting early at the locus encoding IL-4Ra to induce expression of this receptor (IL-4Rα) (Liao, et al. 2008) and later to open chromatin accessibility at the Th2 locus, which encodes IL-4 and IL-13 (Cote-Sierra, et al. 2004).

The development of Th2 cells was reportedly impaired in STAT5a-/-CD4+ T cells, even in the presence of IL-4. Retrovirus-mediated expression of STAT5A restored Th2 cell differentiation in STAT5a-/-CD4+ T cells. Th2 cell-mediated immune responses were diminished in STAT5a-/- mice. When stimulated with anti-CD3 mAb, CD4+ T cells that produced IL-4, but not IFN-γ (Th2 cells), were significantly decreased in STAT5a-/- mice compared with those in wild-type mice, suggesting that STAT5A plays a regulatory role in T helper cell differentiation (Kagami, et al. 2001).

Evidence Supporting this KER

IL-2 stabilizes the accessibility of the Il4 gene. STAT5, a key transducer of IL-2 function, binds to sites in the second intron of the Il4 gene (Cote-Sierra, et al. 2004).

5C.C7 cells infected with a retrovirus expressing a constitutively active form of STAT5A (STAT5A1*6) were shown to be primed for IL-4 production.

STAT5a/b mutant peripheral T cells in mice are profoundly deficient in proliferation and fail to undergo cell cycle progression or to express genes controlling cell cycle progression. STAT5 proteins are essential mediators of IL-2 signaling in T cells (Willerford, et al. 1995).

IL-2 is one of the earliest cytokines produced by activated T cells and mediates its actions primarily through the activation of STAT5 proteins. A STAT5-chromatin immunoprecipitation assay (ChIP) was performed using chromatin from freshly isolated CD4 T cells to identify in vivo IL-2-activated STAT5 gene targets. The immunoprecipitated chromatin yielded a number of distinct clones based on sequencing. One clone mapped to chromosome 16 152,916 to 153,096 upstream of the C-MAF gene, and contained a consensus GAS motif (Rani, et al. 2011).

Heat map analysis of expression profiles of IL-2 regulated genes (sorted by superenhancer binding scores for STAT5, from strongest to

weakest) revealed that STAT5-bound superenhancer-containing genes were highly induced by IL-2 (Li, et al. 2018).

Cells primed under Th2, but not Th1, conditions showed an association of STAT5A with HSII and HSIII. In addition, cells infected with the STAT5A1*6 retrovirus acquired IL-4-producing capacity, and STAT5 was associated with DNA elements near HSII and HSIII (Zhu, et al. 2003).

CD4+ T cell-mediated allergic inflammation was reportedly diminished in STAT5A-deficient (STAT5a-/-) mice. Furthermore, Th2 cell differentiation was also impaired in STAT5a-/- mice, even when purified CD4+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of IL-4 (Kagami, et al. 2001).

Biological Plausibility

Th2 cell differentiation from antigen-stimulated splenocytes was significantly decreased in STAT5a-/- mice as compared with that in wild-type mice. The intrinsic expression of STAT5a in CD4+ T cells is required for Th2 cell differentiation and STAT5a is involved in the development of CD4+CD25+ immunoregulatory T cells that modulate T helper cell differentiation toward Th2 cells (Kagami, et al. 2001).

IL-4 production was reportedly induced by STAT5 phosphorylation. STAT5 phosphorylation facilitates STAT5 dimerization, transport to the nucleus, and gene regulation (56-Levy-2002). PPARs are members of the nuclear hormone receptor superfamily. STAT5 is able to inhibit PPAR-regulated gene transcription. Conversely, ligand-activated PPAR can inhibit STAT5-regulated transcription. STAT5 and PPAR disparate pathways are subject to mutually inhibitory crosstalk. The extent of the inhibitory crosstalk was dependent on the relative expression levels of each transcription factor (Shipley and Waxman 2004).

Empirical Evidence

When stimulated with anti-CD3 mAb, CD4 T cells that produced IL-4, but not IFN-γ (Th2 cells), were significantly decreased in STAT5a-/- mice as compared with those in wild-type mice. In contrast, CD4 T cells that produced IFN-γ, but not IL-4 (Th1 cells), were significantly increased in STAT5a-/- mice, and T helper cell differentiation was biased toward Th1 cells in STAT5a-/- mice (Kagami, et al. 2001).

In another study, BALB/c mice were exposed to PFNA (0, 1, 3, or 5 mg/kg/day) for 14 days. Exposure to PFNA led to a decrease in the weight of lymphoid organs. Cell cycle arrest and apoptosis were observed in the spleen and thymus following PFNA exposure. PFNA reduced the production of IL-4 by splenic lymphocytes and was associated with increases in messenger RNA (mRNA) of PPAR (Fang, et al. 2008). In a related study using male Sprague-Dawley rats given the same PFNA doses for the same duration, similar effects were observed on body and thymus weights and mRNA of PPARα.

Other authors described that cells infected with STAT5A retrovirus acquired the capacity to produce IL-4 when cultured in the presence of anti-IL-4; the strength of STAT5 signaling correlated with the percentage of IL-4 producers observed in the primed cell population (Zhu, et al. 2003).

STAT5 interacts with transcriptional regulatory regions and regulates T cell differentiation by enhancing key genes (Adamson, et al. 2009). Th2 differentiation in both mouse and human CD4 T cells is critically dependent on IL-2 (Ben-Sasson, et al. 1990, McDyer, et al. 2002).

Uncertainties and Inconsistencies

GAS is a STAT3-target gene, therefore STAT3 could regulate IL-4 production (Campia, et al. 2015). Additionally, Lederer et al. demonstrated that STAT6 binds to a sequence in the IL-4 promoter (Lederer, et al. 1996).

Quantitative Understanding of the Linkage

$CD4^+$ T cell blasts from BALB/c mice were cultured in the presence or absence of the antioxidant N-acetylcysteine (NAC). T cells preferentially followed a Th2 differentiation pathway. Treatment of $CD4^+$ T cell blasts with 10 mM NAC increased Th1 cytokine production and decreased IL-4 production as compared to untreated controls. T cells treated with NAC also showed decreased levels of phosphorylated STAT5 (Shatynski, et al. 2012).

Mycophenolic acid (MPA) treatment dramatically reduced STAT5 phosphorylation, without affecting the expression of CD25 and the levels of IL-2 (He, et al. 2011). Significantly lower concentrations of IL-4 were detected in the supernatants of MPA (5 μM)-treated T cells (Liu, et al. 2013).

Response-response relationship

Once STATs are recruited to the activated JAK/receptor complex and are tyrosine phosphorylated within the SH2 domain by JAKs, they form dimers and/or tetramers, translocate to the nucleus, and associate with promoter regions, such as gamma activated sequence (GAS) elements. STAT dimers can bind to GAS DNA sequences (TTCN3GAA) to induce transcription. The STAT5 dimers can also form tetramers through interactions between residues (I28, F81, and L82) in their N-terminal regions. These STAT5 tetramers bind to pairs of GAS motifs separated by a linker of 6–22 nucleotides (Lin, et al. 2012). Mutational studies have demonstrated that STAT5 is important for IL-2-induced gene expression. The interaction of STATs with gene promoters can enhance the expression of its target genes (Able, et al. 2017).

It was reported that while the wild-type construct displayed 4.6-fold IL-2 inducibility in YT cells, selective mutation of GAScI (M1), GASn (M2), and GAScII (M3) motifs modestly lowered IL-2 inducibility (M1 1.7-fold, M2 2.9-fold, M3 1.6-fold, respectively). Double mutation of GAScI and GASn (M4) or GASn and GAScII (M5) more potently decreased IL-2 inducibility, and simultaneous mutation of GAScI and GAScII (M6) or of all the GAS motifs (M7) abrogated IL-2 inducibility (M4 1.2-fold, M5 1.4-fold, M6 1.0-fold, M7 1.0-fold, respectively). These results suggest that all the GAS motifs are required for maximal IL-2 inducibility, including IL-4 induction (Kim, et al. 2001).

Time-scale

A STAT5 binding site (TTCATGGAA) has been identified in intron 2 of the Il4 gene. HS V (also known as CNS2) is a 3' enhancer in the Il4

locus. HS V is essential for IL-4 production by Tfh cells. Mice lacking HS V display marked defects in Th2 humoral immune responses, as evidenced by abrogated IgE and sharply reduced IgG1 production in vivo. HS V-deficient (ΔV) mice displayed complete abrogation of IgE production despite only mild reduction in Th2 responses. HS V-deficiency affected Il4 transcription in T cells naïve T cells lacking the HS V (CNS2) region were completely unable to produce Il4 transcripts following ex vivo stimulation with anti-CD3 and anti-CD28 antibodies for 180 min. In a similar time course assay (240 min), in vitro differentiated Th2 cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin showed only a 50% reduction in Il4 transcription (Vijayanand, et al. 2012).

Phosphorylation of STAT5 was reportedly decreased by nearly two-fold in NOX2-deficient T cells as compared to that in wild-type controls by intracellular staining 12 and 24 h after activation with immobilized anti-CD3 and soluble anti-CD28. PCR analysis also revealed decreases in Il4 and Il4rα mRNA expression in NOX2-deficient T cells (Shatynski, et al. 2012).

Known modulating factors

Adenosine can inhibit IL-2-dependent proliferation of CTLL-2 T cells. This inhibition was reportedly associated with a reduction in tyrosine phosphorylation of STAT5A and STAT5B, which was mediated by the activation of a protein tyrosine phosphatase (PTP). The PTP Src homology region 2 domain-containing phosphatase-2 (SHP-2) was implicated in STAT5A/B dephosphorylation because adenosine strongly increased tyrosine phosphorylation of SHP-2 and the formation of complexes consisting of SHP-2 and STAT5 in IL-2- stimulated CTLL-2 T cells. In contrast, adenosine did not affect the phosphorylation status of the upstream kinases JAK1 or JAK3. The inhibitory effect of adenosine on STAT5A/B phosphorylation was mediated through cell surface A_{2a} and A_{2b} receptors, and involved associated cAMP/protein kinase A (PKA)-dependent signaling pathways (Zhang, et al. 2004).

Known Feedforward/Feedback loops influencing this KER

STAT5 can upregulate a number of molecules, including cytokine-inducible SH2 proteins (CIS family, also referred to as the SOCS or SSI family) (Yasukawa, et al. 2000). Some CIS family proteins might be involved in the cross-regulation of cytokine networks and may regulate Th1 and Th2 cell differentiation (Dickensheets, et al. 1999, Losman, et al. 1999). CIS1, a prototype of CIS family proteins, is induced by STAT5 and inhibits STAT5 activation by blocking the interaction between STAT5 and cytokine receptors (Yasukawa, et al. 2000). Thus, CIS1 seems to function in classical negative feedback of STAT5 signaling.

IL-2 acts on the same cell that secretes the cytokine. For instance, IL-2 produced by T cells operates on the same T cells that make this cytokine or on nearby cells. With the highest levels in secondary lymphoid organs, IL-2 is believed to act in an autocrine or paracrine manner to support effector and memory CD8 T cell differentiation (Kalia and Sarkar 2018). IL-2Rα expression is triggered by antigens, mitogen lectins, or antibodies to the TCR through STAT5. These signals also result in the secretion of IL-2, which in turn can increase and prolong IL-2Rα expression, thus acting as a positive feedback regulator of its own high-affinity receptor (Waldmann 1989). Therefore, STAT5 deficiency disrupted T cell function.

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Relationship: 2027: Suppression of IL-4 production leads to Impairment, TDAR

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

The effects of FK506 on serum concentrations of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over 4 weeks and immunized with KLH (Ulrich, et al. 2004). The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt, et al. 2010, Sakuma, et al. 2001). In thymectomized mice, the development of KLH-specific effector CD4 T cells was reportedly reduced and these cells were suppressed in their production of IL-4 (Bradley, et al. 1991). The effects of FK506 and CsA on the production of IL-2 have been demonstrated using mice and human cells. These facts suggest that there are no species differences between humans and rodents in the inhibition of IL-4 production and TDAR induction.

Key Event Relationship Description

IL-2 induces T cell proliferation Therefore, the suppression of IL-2 production leads to the impairment of TDAR. The IL-2-JAK3-STAT5 axis regulates Th1 cell differentiation, suggesting that IL-2 mediated JAK3-STAT5 signaling may generically operate in the production of Th1-related cytokines (Shi, et al. 2008).

IL-2 is produced and secreted by helper T cells. IL-2 has important roles in the development of TDAR. IL-2 promotes differentiation of B cells by stimulating differentiation of activated T cells to Th2 T cells. Therefore, suppressed production of IL-2 impairs T cell dependent antibody production.

In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

T cells, B cells, and antigen-presenting cells, such as dendritic cells, are involved in the induction and development of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

After treatment with FK506 or CsA, production of IL-2, IL-4, and other cytokines decreases in T cells (Dumont, et al. 1998, Dumont, et al. 1998). This reduces stimulation of B cells as well as proliferation, activation, and class switching, leading to impairment of TDAR. Therefore, FK506 and CsA are potent inhibitors of T cell dependent antibody production. Suppression of the production of these B cell related cytokines appears to be the main factor in the impairment of TDAR (Heidt, et al. 2010).

Evidence Supporting this KER

In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

Biological Plausibility

FK506 and rapamycin suppress the mRNA expression levels of IL-2 and IL-4 in T cells, which stimulate the proliferation of B cells (Heidt, et al. 2010).

Several in vivo studies in rodents have shown decreased TDAR following treatment with FK506 (Kino, et al. 1987, Ulrich, et al. 2004). In vitro tests examined antibody production in blood samples obtained from blood bank donors and PBMCs treated with FK506 and CsA. The suppressed production of immunoglobulin (Ig) M and G antibodies to T cell dependent antigens was demonstrated (Heidt, et al. 2010).

T cells, B cells, and antigen-presenting cells, such as dendritic cells, are involved in the induction and development of TDAR. Thus, changes in any of these immune cell populations can influence TDAR. However, concerning the suppression of humoral immunity induced by the inhibition of CN phosphatase activity, CNIs do not affect B cells directly. Rather, the effect is indirect via T cells. FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B-cell response by FK506 and CsA appears solely due to inhibition of T helper cells (Heidt, et al. 2010).

Therefore, it is concluded that decreased amounts of IL-4, in addition to IL-2, secreted from helper T cells, is the main factor in the suppression of TDAR.

Empirical Evidence

Empirical support for the suppression of IL-4 production leads to impairment, and the T cell dependent antibody response is strong.

Rationale

In CD3/PMA activated human T cells, FK506 suppressed the production of IL-2, IL-4, and IFN-γ at concentrations of 1.2 to 12.5 nM and

inhibited the expression of IL-2, IL-4, and IFN-γ mRNA at concentrations of 10 nM (Dumont, et al. 1998).

After 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced. The FK506 levels were 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) and the CsA levels were 50 and 100 ng/mL (41 and 83 nM) (Heidt, et al. 2010).

After a 4-day culture of SKW6.4 IL-6-dependent IgM-secreting human B cells and anti-CD3/CD28 stimulation of the PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 and 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma, et al. 2001).

Rats were treated with FK506 for over 4 weeks and immunized with KLH. The serum concentrations of anti-KLH IgM and IgG were reduced at a dose of 3 mg/kg/day (Ulrich, et al. 2004).

In vitro suppression of T cell derived cytokines and T cell dependent antibody production or antibody production after polyclonal T cell stimulation showed similar dose responses to CNIs. Time gaps were found between these two KEs, which showed earlier onset of cytokine production and delayed onset of antibody production.

Uncertainties and Inconsistencies

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Additional suppression of other immune functions may also be possible.

Quantitative Understanding of the Linkage

CsA treatment achieved 100% maximal inhibition of the ex vivo IL-2 response on Days 0, 9, and 16. CsA treatment achieved 82 [± 10]%, 68 [± 25]%, and 82 [± 9]% maximal inhibition of the ex vivo IL-4 response on Days 0, 9, and 16, respectively.

Response-response relationship

In a rat T cell proliferation assay, IL-2-induced T cell proliferation was inhibited by peficitinib in a concentration-dependent manner with an IC50 of 10 nM and by tofacitinib with a similar IC50 of 24 nM (Gianti and Zauhar 2015). In addition, cynomolgus monkeys treated with CsA showed suppression of IL-2 and TDAR using SRBCs in a dose-dependent manner (Gaida, et al. 2015).\

In the human T-B-cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the mRNA levels of T cell cytokines at 8 h post-stimulation including IL-2 and IL-4 at 1.0 ng/mL (1.24 nM) FK506 or 100 ng/mL (90.7 nM) CsA, and inhibited IgM and IgG productions after 9 days at 0.3 and 1.0 ng/mL FK506 and 50 and 100 ng/mL CsA (Heidt, et al. 2010).

Time-scale

In human T cell culture, suplatast tosilate (an inhibitor of the production of cytokines by Th2 cells) inhibited IL-4 production after 3 days and antigen-specific IgE production after 10 days (Taiho 2013).

Other authors described that in human T-B-cell co-cultures, FK506 and CsA lowered the mRNA levels of IL-2 and IL-4 at 8 h post-stimulation and inhibited IgM and IgG production after 9 days (Heidt, et al. 2010).

Treatment with CsA (50 mg/kg) twice daily in cynomolgus monkeys resulted in reduction of IL-4 cytokine production from PMA/ionocycin stimulation of whole blood starting on day 0 and continuing through the end of the study on day 16. CsA treatment achieved 82 [±10]%, 68 [± 25]%, and 82 [± 9]% 100% maximal inhibition of ex vivo IL-4 response on days 0, 9, and 16. SRBC-specific IgM and IgG were significantly lower in animals dosed with CsA than in animals dosed with the vehicle control on days 9, 12, and 16 post-immunization. There was ≥80% or greater reduction in SRBC-specific IgM on days 9–16. SRBC-specific IgG was decreased by ≥95% on days 9–16 (Gaida, et al. 2015). This was similar to the degree of inhibition observed in rats using an KLH immunization model (Smith, et al. 2003).

Known modulating factors

Treatment with CsA (cyclosporin A) at 50 mg/kg BID (bis in die) resulted in reduction of IL-2, IL-4 cytokine production from PMA/ionomycin stimulation of whole blood in synomolgus monkey starting on Day 0 and continuing through the end of study on Day 16. In addition, Tacrolimus concentration was 1.0 ng/ml. Tacrolimus inhibited IL-2 and IL-4 mRNA levels. Glycosylation-inhibiting factor (GIF) secreted from CD4 cells suppressed IL-4 mRNA levels of the same cells during the initial 24 h of CD3/CD28 stimulation.

Known Feedforward/Feedback loops influencing this KER

B cells are required for the generation and / or maintenance of Th2 responses. Germinal center B cells regulate Th2 development through an IL-4 dependent process. Type 2 immunity and allergic responses are initiated by T cells and DCs, this response may be sustained and potentially amplified by an IL-4-driven feedback loop between Ag-specific T and B cells (Harris, et al. 2005).

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厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

Bhas42細胞形質転換試験法のTG開発

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研究要旨

Bhas42 細胞形質転換試験法（Bhas 42 CTA）は、化学物質の非遺伝毒性発がん性を遺伝毒性発がん性と区別して検出できる OECD 唯一の試験法（GD231）である。OECD では、化学物質の非遺伝毒性発がん性（NGTxC）検出を目的とした IATA（OECD　NGTxC・IATA）開発が 2016 年から行われており、2020 年には Expert working group として NGTxC・IATA 構築の方針が国際合意され、レビュー論文を公表した。その国際合意のレビューに従い、Mode of action (MoA) を構成する各 Key event 及びそれらに対応した 13 の Assay block において、各種試験法の選出、Assay description の作成及び評価を行っている。その中で、Bhas 42 CTA が含まれる“Cell Transformation”（Block 3）及び、わが国でも NGTxC 検出法として研究されてきた“Gap Junction”(Block 4)　について、NGTxC・IATA の Assay の選出及び評価を行った。その結果、本年度は NGTxC・IATA の Block 3 及び Block 4 での Assay の選出、Assay description の作成ならびに評価が完了し、Block 3 は Bhas 42 CTA を含む 3 種の Cell transformation assay がランク A に評価された。Block 4 については、2 種の Assay がランク B、1 種がランク C の評価となり、それらの Block 3 及び Block 4 の評価結果は NGTxC・IATA の全体会議で報告された。

A. 研究目的

Bhas 42 CTA は、神奈川県政策局の重点基礎研究事業として開発した細胞形質転換試験法であり、化学物質の非遺伝毒性発がん性を遺伝毒性発がん性と区別して検出できる OECD 唯一の試験法（GD231）である。OECD では、非遺伝毒性発がん性検出を目的とした IATA（OECD　NGTxC・IATA）開発が行われており、NGTxC のメカニズムをもとに MoA が議論されている。そこで、NGTxC 検出を目的として開発した Bhas 42 CTA の NGTxC・IATA 構築への貢献に基づき、Bhas 42 CTA の OECD テストガイドライン（TG）の開発に繋げることを目的とする。

B. 研究方法

OECD では、 NGTxC・IATA の開発が 2016 年から行われている。NGTxC・IATA では MoA の議論に基づき IATA 構築の方針が国際合意され、2020 年は Expert working group としてレビュー論文を公表した。NGTxC・IATA のレビューに従い、発がんモデルと AOP にもとづく 13 の Key event に対応する 13 の Assay block を立ち上げ、各 Block は定量的に評価可能な Assay

を選出し、それらの詳細な情報をとりまとめ Assay description を作成及び評価を行うこととした。各 Assay block における Assay の選出、Assay description の作成及び評価は、以下の Step 1 及び Step 2 により行った。Step 1 ではメンバーにより提案された Assay の中から定量的に評価可能な Assay を選出し、Assay ごとに詳細な情報をとりまとめた Assay description を作成した。続いて Step 2 では作成者以外のメンバーが Assay description の評価案を作成し、その評価案をもとに、Assay block のメンバー全体で協議し、合意したものを Assay block からの提案 Assay とその評価結果として Expert working group の全体会議に報告した。

担当した Block 3 の“Cell transformation”と Block 4 の“Gap Junction”において、Block 3 では Bhas 42 CTA の Assay description を他のメンバー1 名とともに作成し、その他のメンバー2 名が評価案を作成した。また、Balb c/3T3 cell transformation assay（Balb 3T3 CTA）については、Block 3 のメンバー2 名により作成された Assay description の評価案作成者として他のメンバー1 名とともに評価を行った。SHE cell transformation assay（SHE CTA）についても、同様に 2 名のメンバーが Assay description を作成し、その他のメンバー2 名が評価案を作成した。それらの 3 種の Cell transformation assay について、Block 3 全体で協議し評価結果とした。

Block 4 では、予てからわが国でも NGTxC 検出法として研究されてきた Dye transfer assay と Inhibition of metabolic co-operation assay を提案するとともに、Inhibition of metabolic co-operation assay である Metabolic cooperation assay with (HGPRT+&-) V79 cells (using 6-TG)の Assay description を作成した。2 種の Dye transfer assay は、他のメンバー2 名がそれぞれ Assay description を作成し、相互に Assay の評価案を作成した。それらの評価案は Block 4 全体で協議し評価結果とした。

以上の Block 3 及び Block 4 の評価結果は、Expert working group の全体会議で報告された。

なお、当研究は、倫理審査及び COI の指導・管理に該当しない。

C. 研究結果

Block 3 の“Cell Transformation”では、SHE Cell Transformation Assay (SHE CTA)、Bhas 42 CTA 、Balb 3T3 CTA 、 Balb c/3T3 transformics assay が評価対象となった。SHE CTA 及び Bhas 42 CTA のみが、NGTxC・IATA の全ての Assay 候補の中で OECD のガイダンスドキュメントとして掲載済みの Assay である。Bhas 42 CTA 及び SHE CTA は、各種性能及び再現性等の検証データに基づき Assay description が作成され、評価案の担当者により各評価項目において A 評価を得た後、Block 3 メンバーの全会一致でランク A の合意を得た。一方、Balb 3T3 CTA については、2 step assay と 1 step assay が開発されていることから、いずれのプロトコールを Assay description に記載し評価を行うかについて議論した。その結果、かつて ECVAM から OECD に提案され評価が中断された 1 step assay が評価プロトコールとなった。同プロトコールに基づくトランスクリプトミクス解析法の Balb c/3T3 transformics assay も、当初は Balb 3T3 CTA とは別に Assay description が作成されたが、検証等の完成度が低いことから最終的には Balb 3T3 CTA の optional assay として Balb 3T3 CTA の Assay description に組み込まれ、Balb 3T3 CTA として評価が行われた。Balb 3T3 CTA では、NGTxC 対象化合物で

のデータ、性能評価及び再現性検証の化合物数も限定的であることなどから、各評価項目は A と B が混在し、ランキング評価についても Block メンバー内での議論を要したが、最終的には Balb 3T3 CTA（Balb c/3T3 transformics assay 含む）はランク A で合意された。したがって、3 種の Cell transformation assay は全てランク A の評価となり、10 月に開催された Expert working group の全体会議での報告に至った。

Block 4 の“Gap Junction”では、提案した Dye transfer assay と Inhibition of metabolic co-operation Assay について、該当する 3 種の Assay（Metabolic cooperation assay with (HGPRT+&-) V79 cells (using 6-TG)、Gap junction dye transfer assay – combined、Gap junction multiparametric scrape loading-dye transfer assay (mSLDT)）を他のメンバーと共に選出した。Metabolic cooperation assay with (HGPRT+&-) V79 cells (using 6-TG)は、467 化合物で結果と 166 化合物での性能評価について総説（ATLA）が我が国から報告されており、それをもとに Assay description の作成を行った。他の 2 種の Assay は、他の 2 名のメンバーが 1 種ずつ Assay description を作成した。それらの評価結果は、Metabolic cooperation assay with (HGPRT+&-) V79 cells (using 6-TG)については、感度（49%）及び特異性（63%）が十分でなく室間再現性評価の結果が未投稿のためランク B となった。Gap junction dye transfer assay – combined は、ECVAM のプロトコールではあるが、17 化合物のデータのみで検証データが無いためランク C となった。mSLDT は 328 化合物での結果と IARC グループ 1 及び 2 の 72 化合物での性能評価が報告されており、感度は 75%以上であるが特異性が 45%と低値であることに加え、室間再現性評価が未実施であることからランク B となった。これら Block 4 の 3 種 Gap junction assay の評価結果についても、10 月の Expert working group の全体会議での報告に至った。

D. 考察

Block 3（Cell transformation assay）及び Block 4（Gap junction assay）での Assay の選出、Assay description の作成ならびに評価を行った結果、Bhas 42 CTA を含む Block 3 の Cell transformation assay は 3 種ともランク A の高評価を得た。一方、Block 4 の Gap junction assay は OECD での Assay 性能評価に対応した検証データが不足しており、ランクは B または C の評価となった。今後は、他の Block からも多数の NGTxC・IATA assay の候補について評価結果が報告される計画であるが、Assay の各種検証及び性能評価の完成度は Block 内のみならず、Block 間においても格差が生じるものと考えられることから、すべての Block での Assay 評価が一通り完了した時点で、13 の Block 間での評価の一貫性を確認し整合性を図る工程が重要になると考えられた。

E. 結論

NGTxC・IATA の Block 3（Cell transformation assay）及び Block 4（Gap junction assay）での Assay の選出、Assay description の作成ならびに評価が完了し、Block 3 は Bhas 42 CTA を含む全てランク A として Expert working group の全体会議に報告された。Block 4 は、2 種の Assay がランク B、1 種がランク C であり、これらについても全体会議に報告された。

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G. 知的財産権の出願・登録状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）
OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

光毒性のAOP 及び IATA の開発

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研究要旨

光線過敏症とは特定の化学物質摂取後、太陽光への曝露によって惹起される皮膚及び眼における異常反応である。光線過敏症は医薬品のみならず、食品及び化粧品等が原因となる場合も報告されており、新規化合物ならびに製品開発における光毒性リスクの回避は重要な課題となっている。本研究では *in vitro* 光化学的試験方法である ROS アッセイを主軸とした AOP を作成するため、光毒性物質の光生物化学的ならびに光化学的特性を精査することで光毒性反応機序のさらなる解明を行った。その研究の一環として、ラット凍結皮膚を人工膜に代替した *in vitro* 皮膚透過性試験及び ROS assay から得られたデータを併せて用いた光毒性リスク予測を行い、提案した評価系の適用可能性のさらなる精査ならびにヒトに対する外挿可能性を検証した。

また、OECD 専門家とともに、光安全性の AOP 及び IATA 案の作成を進めた。

A. 研究目的

地球上に降り注ぐ太陽光は生態系に多くの恵みを与え、太陽光のうち UVC 領域は DNA 損傷などの有害事象を惹起するものの、高エネルギーな短波長光を除けば本来は人体に対してほとんど無害である。しかし、ヒトに投薬された医薬品が体内で光と相互作用を起こすことによって、主に皮膚や眼において炎症、角質化、色素沈着などの有害反応を誘発することがあり、これを広義の光毒性と定義する。対象となるのが薬剤であった際にはこの有害事象を薬剤性光線過敏症と呼称するが、医薬品以外でもこれまでに多くの食品、化粧品等においても同様の光毒性反応が認められている。近年のオゾン層破壊に伴う紫外線量の著しい増加の背景もあって光毒性リスクへの注目が高まっており、光毒性発症機序解明ならびに評価系開発が精力的に進められている。ICH S10 ガイドラインでは、化合物の i) 光反応性及び ii) 露光部位（皮膚や眼）への分布が光毒性発現に重要な因子として明記されている。当研究室では既に光化学的評価方法として reactive oxygen species (ROS) assay を開発し、本データと皮膚内動態情報の組み合わせることで信頼性ある光安全性評価が可能となることを明らかにした。この知見を検証すべく、本研究では ROS assay による光化学的特性及び Franz 型拡散セルを用いた化学物質の *in vitro* 皮膚内動態のデータを統合的に解析することで経皮適用化合物の光毒性リスクを効果的に予測できるかを検証し、その予測データを用いることで動物実験代替法の開発を指向した検討を実施した。本研究では動物皮膚の代わりに人

工膜を活用した実験を推進した。また、検証結果を基に光毒性に関する AOP ならび光安全性評価に関する IATA 案構築を試みている。

B. 研究方法

B.1. 光安全性評価ツールの予測精度

B.1.1. ROS アッセイ

研究分担者らが既に公表している ROS assay 推奨プロトコルに基づき、acridine (ACD)、furosemide (FSM)、hexachlorophene (HCP)、8-methoxypsoralen (MOP)、norfloxacin (NFX) 及び promethazine (PMZ) について ROS assay を行った。疑似太陽光の照射には Xe arc lamp を備えた Atlas Suntest CPS+ (Atlas Material Technologies LCC、Chicago. IL. USA) 用いた。Atlas Suntest CPS+ では短波長の UV をカットし実際の太陽光を模すためのフィルターを用いて CIE85/1989 daylight simulation requirement に良好に対応した疑似太陽光を照射している。照射強度は 250 W/m^2 に設定し 1 h 照射を行った。Atlas Suntest CPS+ 内の温度は 28°C に保った。

ROS assay は被験物質が疑似太陽光照射下で産生する活性酸素種のうち singlet oxygen 及び superoxide anion を測定する試験法であり、被験物質の光反応性を評価する試験法である。Singlet oxygen は imidazole を singlet oxygen の accepter に用いて、*p*-nitrosodimethylaniline (RNO) 水溶液の 440 nm における吸光度変化からその評価を行った。まず、被験物質 (200 μM)、RNO (50 μM) 及び imidazole (50 μM) を含む 20 mM NaPB (pH7.4) 200 μL を 96-well microplate (旭硝子、東京; code number: 3881-096; clear, untreated, flat-bottom) に分注し、析出物の有無を確認後、440 nm の吸光値を SAFIRE microplate spectrophotometer (TECAN, Mannedorf, Switzerland) を用いて測定した。その後、プレートを reaction container に入れ石英の蓋を装着し、擬似太陽光を 1 h 照射した。照射後のプレートを振盪した後、440 nm の吸光値を再び測定した。Superoxide anion は nitroblue tetrazolium chloride (NBT) の還元によって生成される nitroblue diformazan を 560 nm における吸光度変化より評価した。被験物質 200 μM、NBT 50 mM を含む 20 mM NaPB (pH7.4) 200 μL を 96-well microplate に分注し、析出物の有無を確認後、560 nm の吸光値を SAFIRE microplate spectrophotometer を用いて測定した。その後、プレートを reaction container に入れ石英の蓋を装着し、擬似太陽光を 1 h 照射した。照射後のプレートを振盪した後、560 nm の吸光値を再び測定した。

B.1.2. *In vitro* 皮膚内動態実験

上記 5 種の被験物質について、フランツ型拡散セルを用いて人工膜 Strat-M における *in vitro* 皮膚透過性試験を実施した。まず、二層膜モデルとして選択した Strat-M® の角質模倣部位の除去を行った。Franz 型拡散セルに Strat-M® を角質模倣部位が上面になるように装着した。セットした Strat-M® に methanol 1 mL を添加し 25°C に保った温浴内にて 30 min 静置した。その後、取り外した膜表面の有機溶媒をふき取り、大気圧及び 25°C 環境下で膜の重量変化がなくなるまで乾燥した。ドナー側に被験物質 (各 1 mg/mL) を入れ、経時的に皮膚を透過したレセプター液中の被験物質の量を High performance liquid chromatography equipped with electrospray ionization mass spectrometry (HPLC/ESI-MS) にてモニタリングし、*in vitro* 皮膚透過性のデータを得た。得られたデータを基に定常状態における各

被験物質の皮膚内濃度 (C_{ss}) を算出した。得られた C_{ss} の値と光化学的特性データを併せて考慮することで光毒性予測を実施した。

B.1.3.ラット *in vivo* 光毒性試験

前日に腹部を剃毛した雄性ラットに各被験物質 (10 mg/site) を塗布し、塗布後 3 h で black light にて UVA (30 J/cm^2) を照射した。照射終了後 24 h に色差計にて皮膚表面の色調を計測し、光毒性の指標とした。

B.2. AOP 及び IATA 案の作成

OECD の専門家の協力を得て、光安全性の AOP 及び IATA 案の作成を進めた。

C. 研究結果

C.1. 光安全性評価ツールの予測精度

被験物質である ACD、FSM、HCP、MOP、NFX 及び PMZ の光毒性リスクを予測し、本評価系の適用可能性を精査した。Strat-M® を二層膜モデル、30 min の間 methanol 処置した Strat-M® を単層膜モデルとして用い、レセプター液中に到達した被験物質量を経時的に測定することにより各被験物質のそれぞれの膜に対する透過性を評価した (Table 1)。本試験では、 Strat-M® の薄さを鑑みて試験開始から 8 h で膜透過速度定常状態に達すると考えた。全被験物質の透過量について、単層膜モデルの方が二層膜モデルと比較して高かった。二層膜及び単層膜モデルのどちらにおいてもレセプター液中 PMZ 量は全被験物質中最も高く、試験開始 8 h 後における値はそれぞれ 0.4 及び 1.3 μg/cm^2 であった。被験物質の累積量プロファイルに基づき、P_{TLM} は 5.0×10^{-5} (ACD)、5.0×10^{-5} (FSM)、6.0×10^{-5} (HCP)、4.0×10^{-5} (MOP)、2.0×10^{-5} (NFX) 及び 1.0×10^{-4} (PMZ) cm/s であり、P_{OLM} は 4.0×10^{-4} (ACD)、1.7×10^{-4} (FSM)、1.8×10^{-4} (HCP)、1.1×10^{-4} (MOP)、1.2×10^{-4} (NFX) 及び 3.9×10^{-4} (PMZ) cm/s であった．全被験物質の P_{OLM} は P_{TLM} と比較して高く、その差は 2.9 から 7.4 倍であった。二層膜モデルに対する透過性は Clog *P* 値が -0.78 と被験物質中で唯一負の値を持つ NFX が最も低

Table 1 Integrated date with photoreactivity and skin deposition

	ACD	FSM	HCP	MOP	NFX	PMZ
ROS assay [a)]						
$^1O_2$ ($\Delta A_{440\,nm} \cdot 10^3$)	271	250	335	120	251	127
O_2^- ($\Delta A_{560\,nm} \cdot 10^3$)	288	151	N.D.	92	158	100
In vitro skin permeation test: C_{ss} (μg/mL)						
Strat-M®	23.4	19.6	20.5	9.4	5.9	38.1
Rat skin	69.1	2.8	57.3	50.1	3.2	59.2
In vivo cassette-dosing pharmacokinetic study						
C_{max} (μg/g skin)	15.9	4.4	23.8	10.4	5.9	19.0
MRT (h)	6.4	10.0	8.5	8.8	5.6	9.5

The risk level was divided into two grades. Black cells indicate high risk for phototoxicity. [a)] $\Delta A_{440\,nm}$ and $\Delta A_{560\,nm}$ represent a decrease in $A_{440\,nm}$ and increase in $A_{560\,nm}$, respectively.

かった。HCP 及び PMZ の Clog *P* 値はそれぞれ 7.0 及び 4.6 であり、Clog *P* を基準とした脂溶性は HCP が最も高い一方で、PMZ の P_{TLM} は HCP より高く全被験物質中最も高値であった。これら Strat-M® に対する各被験物質の透過性を基に Css を算出したところ、ACD、FSM、HCP、MOP、NFX 及び PMZ の C_{ss} はそれぞれ 23.4、19.6、20.5、9.4、5.9 及び 38.1 μg/mL であった。すなわち、PMZ が最も高く、次いで ACD、HCP 及び FSM の順であった。MOP 及び NFX のCss は他の被験物質と比較し低かった。

UV/VIS 吸収スペクトル測定及び ROS assay を 6 種被験物質に対して実施することで、それらの光化学的特性を評価した。全被験物質は UV 領域に強い吸収を持ち、その範囲で吸収が極大となる波長での MEC 値は 19,500 (ACD)、14,900 (FSM)、11,200 (HCP)、24,900 (MOP)、24,900 (NFX) と 6,600 (PMZ) $M^{-1}\cdot cm^{-1}$ であった。つまり、6 種類の被験物質は高い光励起性を持つと判断できる。ROS assay において、HCP を除く全被験物質が擬似太陽光照射により産生した singlet oxygen 及び superoxide anion はガイドラインによって定められた criteria を超えていた。HCP は singlet oxygen の産生量のみ criteria を超え、その産生量は全被験物質中最も多かった。したがって、6 種の被験物質全てが criteria を超えており、光反応性を持つことが明らかになった。特に、ACD は singlet oxygen 及び superoxide anion のどちらの産生量も非常に多く、被験物質中で最も高い光反応性を有していた。

提案した光安全性評価系の適用可能性の検証には、本評価系により予測した結果と実際に生体内で起こる光毒性反応との関連性を評価する必要がある。したがって、各被験物質の *in vivo* 光毒性を評価すべく、ラットを用いた *in vivo* 光毒性試験を行った。一般に、露光部に分布した光毒性陽性化合物から産生された ROS 及び光励起された化合物自身の一方もしくは両方が細胞膜障害を引き起こし、発赤や紅斑を主徴とする光刺激反応を呈する。本試験では、UVA 照射前後のラット腹部の皮膚表面における色調変化を客観的に評価することで光毒性、特に光刺激性の強さを評価した。全被験物質は UVA 照射により有意に皮膚表面の色調が変化した。つまり、6 種の被験物質が光毒性反応を誘発した。UVA 照射群では、全 6 種被験物質で Δa 値が正の変化を示した。この結果は塗布部の赤みが UVA 照射により増したこと示しており、単回投与により紅斑反応を誘発することを認めた。つまり、全被験物質は光刺激性を有することが明らかとなった。特に、ACD 及び HCP 投与群の色調変化が他の 4 種被験物質と比較して大きかった。

以上、被験物質の *in vitro* 皮膚滞留性及び光反応性の統合的な解析により予測した光毒性リスク及び *in vivo* 光毒性の強さを以下に示す。

Predicted phototoxic risk:

ACD＞HCP＞PMZ＞FSM＞MOP＞NFX

Observed phototoxicity:

ACD≒HCP＞PMZ＞MOP＞FSM＞NFX

予測した光毒性リスクと *in vivo* 光毒性の順は良い対応を示した。人工膜を用いた統合的光安全性評価法は精度良く被験物質の光毒性リスク予測ができ、実験動物を用いずとも光安全性評価が可能であることを示唆した。

C.2. AOP 及び IATA 案の作成

2019 年 6 月に OECD TG495 としてガイドライン化された ROS assay を主軸として、新たに光安全性評価のための IATA 構築を進めている。既に OECD の専門家会議にて共有し、専門家より修正に資する重要なコメントを頂いている。ICH S10 において推奨されているストレテジーをベースとし、(i) 被験物質の光化学的特性評価、(ii) 光生物化学的特性評価、そして (iii) 皮膚や眼への移行性・滞留性等体内動態評価の 3 段階のスクリーニングによる tiered approach を案として提示した。

D. 考察

人工膜を用いた *in vitro* 皮膚透過性試験及び ROS assay により構成された光安全性評価系は化合物の光毒性リスクを適切に予測可能であった。さらには予測結果のヒトへの外挿可能性においても良い知見が得られた。以上より、*in vivo* 試験に依存しない本光安全性評価系は将来的に光安全性の高い新規化合物創製に貢献すると期待する。

また、構築した IATA 案については今後当該領域のエキスパートから頂いたコメントを基に修正していく予定である。特に decision tree の構築を強く求められているので、draft を作成して関係者間で慎重な協議を進めている。専門家の意見を反映しつつ完成に向けて改訂作業を進めていく。

E. 結論

ROS assay を主軸とした光安全性評価系は化合物の光毒性リスクを適切に予測可能であった。従来法ではラットから摘出した皮膚組織を用いていたが、本検討では安価な人工膜を活用しており、これによって完全な *in vitro* 評価系となった。以上より、動物実験を用いない本評価系は新規物質創製時において効率的かつ信頼性の高い光安全性評価を実施可能であり、将来的に本評価法が光安全性の高い新規化合物創製に貢献すると期待する。これらの知見は構築中の IATA にも導入し、信頼性ある光安全性評価系を提示していく。

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G. 知的財産権の出願・登録状況

1. 特許取得
   特になし
2. 実用新案登録
   特になし
3. その他
   特になし

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

発達神経毒性に起因する行動解析に関する情報収集

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研究要旨

本研究は、化学物質やその混合物の安全性を評価するための国際的な合意を推進する経済協力開発機構（OECD: Organisation for Economic Co-operation and Development）の試験法ガイドライン（TG:Test Guideline）プログラム各国調整官作業グループ（WNT: Working Group of National Co-ordinators of the TGs programme）において、日本で開発された種々の TG やガイダンス文書（GD：Guidance Document）、毒性発現経路（AOP：Adverse Outcome Pathway）などの世界各国が必要とする成果物を公定化させるとともに、他国が提案する OECD 大型プロジェクトに関与し、その成果物に日本の主張を反映させ、その成果を化学物質の審査及び製造等の規制に関する法律（化審法）や毒物及び劇物取締法（毒劇法）などの我が国の厚生行政に反映させることを目的とする。

分担研究として、これまでの国内外における発達神経毒性評価の現状について情報収集を行うとともに課題を抽出した。具体的には、実験動物（げっ歯類）を対象とした発達神経毒性評価の現状、特に用いられる行動解析および関連するガイドラインについて調査を行った。得られた論文はリスト化し、現状把握のための資料とした。また、OECD で検討されている発達神経毒性の GD に関連する提案資料への意見募集に適切な意見を返すことで、関連する GD の開発に寄与した。

A. 研究目的

本分担研究では、これまでの国内外における発達神経毒性評価の現状、特に行動解析および関連するガイドラインについて情報収集を行うとともに課題を抽出する。加えて、OECD で検討されている発達神経毒性の GD の開発に寄与することを目的とする。

B. 研究方法

B.1. 発達神経毒性評価のための行動解析に関する情報収集

これまでの国内外における発達神経毒性評価の現状について情報収集を行うとともに課題を抽出した。まず、発達神経毒性試験における現状把握のために、米国環境保護庁（EPA）および経済協力開発機構（OECD）の発達神経毒性（Developmental neurotoxicity, DNT）テストガイドラインにおける、行動解析に関する情報について内容を確認し、比較した。また、発達神経毒性評価の現状についての文献調査には、医学・生物学分野の学術文献検索データベースである PubMed および MEDLINE を用いた。

＜文献検索に用いたキーワード＞

mice, rats, rodents, neurodevelopmental, developmental, neurotoxicity, test guideline

検索後、タイトル、雑誌情報、アブストラクトを確認し、下記（1）~（3）の内容を含む文献を選択した。

（1）げっ歯類（マウス、ラット）を用いた実験報告
（2）化学物質曝露による影響評価
（3）曝露時期、投与期間、用量等の実験条件や、解析に用いた行動試験の具体的な記載

B.2. OECD からの意見募集への対応

JaCVAM 発達神経毒性（DNT）資料編纂委員会にオブザーバーとして参画するとともに、公定化に向けて進行中の in vitro DNT ガイダンス文書の提案資料に対して、OECD からの意見募集に適切な意見を返した。

（倫理面への配慮）

本研究は、動物を用いない調査研究である。

C. 研究結果

C.1. 情報収集

EPA および OECD の発達神経毒性に関するテストガイドラインを確認し、げっ歯類に対する行動解析に関する内容を中心に比較を行った結果を表 1 に示した。発達神経毒性に関連する OECD ガイドライン（TG426）は、全般に EPA ガイドライン（OPPTS 876.6300）に沿った内容であり、EPA のガイドラインに比べ詳細に記載されていた。試験項目の内容に関しては、大きく分けて「行動発生」、「自発運動量」、「運動および感覚機能」、「学習および記憶」における推奨検査項目や実験条件について述べられていた。しかしながら、実施する行動評価の選択については具体的な記載はなく、明記されているのは評価の対象となる機能および試験を行う際の推奨日齢のみであった。また、脳高次機能に関する試験項目としては「学習および記憶」が該当するが、試験方法の記述は曖昧な表現にとどまっていた。さらに、行動試験を行うにあたり、解析環境の記載については、具体的な説明が乏しいことや、特定の試験を組み合わせた評価系を構築するなど、標準化（統一化）されたプロトコールではないことも明らかとなった。

PubMed を用い、OECD ガイドライン（TG426）が制定された 2007 年から、2021 年までの範囲で文献数調査を行ったところ、検索キーワード「“developmental neurotoxicity”」の検索条件では総文献数が 1001 件となり、その報告数は年々増加していた。一方で、「"developmental neurotoxicity" AND "test guideline"」の検索条件では 8 件と、ほとんど該当しないことが分かった。MEDLINE による文献検索にて収集した学術論文（95 報）については、オープンフィールド試験における活動量の評価、モリス水迷路における学習・記憶の評価が多くの文献で使用されていたが、その他、行動解析項目としての共通性はあまり存在しなかった。また、現行のガイドライン（OECD あるいは EPA）に準拠して行われたと記載のあるものは 2 件のみであった（66, 78）。なお、検索キーワードとアブストラクトをもとに選抜した論文は、文献管理ソフトである EndNote（ユサコ株式会社）にてリスト化し、発達神経毒性評価に係る毒性情報として資料とした（添付資料１：本文中括弧内の数字は添付資料 1 の論文番号を示す）。

C.2. OECD からの意見募集への対応

JaCVAM 発達神経毒性資料編纂委員会のオブザーバーとして委員会に参画するとともに、in vitro DNT ガイダンス文書（Guidance on the Interpretation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Assays for Use in Integrated Approaches for Testing and Assessment (IATA)）および Case Study に関して、OECD 事務局に提出するコメント募集に応じた。神経行動毒性の評価系、特に in vivo 試験を行っている立場から、本ガイダンスの改善点・懸念点について以下のコメントを行った。

1．提案されている in vitro 試験バッテリー（Developmental Neurotoxicity In Vitro Battery (DNT IVB)）により in vivo 発達神経毒性を予測するにあたって、化学物質影響の種差（中枢移行性等）、性差に関する言及がないこと。
2．in vivo 試験で観察される神経回路の機能変化や、顕在化する行動影響に関して、in vitro 試験において得られたデータはどこまで対応しうるのか、あるいは妥当性があるのか、具体的な言及を追加すること。
3．被験物質の複合曝露による、相加・相乗的影響についての懸念。
4．げっ歯類の代替としてのゼブラフィッシュによる解析について、検出系の施設間差、試験プロトコールの統一性（個体数や分析法）を議論すること。
5．今後、DNT IVB で種々の化学物質について検討する際に、in vitro 試験で確認されなかった影響が in vivo 試験のみで確認された場合の解釈について。
6．行動毒性においては、in vitro 試験の複数のアッセイを組み合わせたとしても予測が困難な部分があり、in vivo 脳高次機能影響（学習や記憶）の評価は現状難しいと考えられるが、その際に、最終的に in vivo アッセイで補強すべき役割について議論を追加すること。

D. 考察

OECD ガイドラインは 2007 年に制定（最終化）され、EPA ガイドラインの制定（1998 年）から比較的時間が経っている。そのため、DNT 試験に関する評価方法について長く議論され、多くの改善が加えられたことが、試験の信頼性および再現性に大きく寄与していると考えられる。一方で、検査の概要や該当する種々の検査例が詳細であるため、複雑化してしまった部分もある。実際に、PubMed および MEDLINE による検索キーワードを絞った調査からは、学術ベースで行われている発達神経毒性研究において、OECD あるいは EPA ガイドラインに準拠した文献は少なく、今回選定しなかった論文についても、ほとんど該当しないと予想された。現行ガイドラインに準拠した発達神経毒性試験の実施に当たっては、上述した試験項目の多さや複雑さ、加えて試験実施にあたりコスト面が課題になると考えられた。

また、ガイドラインにおける行動解析の検出方法を見ると、化学物質が及ぼし得る脳高次機能に対する試験の必要性は認識されているが、未だ不十分であると考えられた。スループット性への影響やコスト面を考慮する必要はあるが、学習・記憶以外の脳高次機能の評価として、OECD ガイドライン上も追加可能としている社会性に関する行動試験（ホームケージ試験、ソーシャルインタラクションテスト、マウス超音波発声（USV）等）の評価も重要であると考えられる。この点、論文調査にて収集した文献では、上述した社会性の評価が可能な行動試験を採用している文献が多く

存在し（11, 18, 26, 28, 32, 33, 34, 37, 57, 59, 72, 91）、現行のガイドラインを補強する評価手法として、その重要性を示唆するものであると考えられた。

さらに、動物の行動は様々な環境条件（順化期間、温度、湿度、照度、環境音、試験実施時間等）の変化に大きな影響を受ける可能性がある。文献情報からも、これら環境条件は具体的記載がない、あるいは実験室ごとに独自に設定しており、異なる傾向にあることが明らかになった。OECD ガイドラインには、実験・解析環境について詳細には記載していないため、この点、検査手順の違いにより、試験施設間での行動影響の検出感度に差が生じるおそれがあると考えられた。

以上より、発達神経毒性、特に行動解析を評価する上で、考慮しなければならない課題としては、

・学習、記憶以外の脳高次機能の評価項目の検討

・安定性、再現性のある実験を実現するため、環境条件によって影響受ける測定誤差の低減

・頑強性を保ちながらも、標準化されたプロトコールの確立

等が考えられた。

OECD からの意見募集（in vitro DNT ガイダンス文書）に関しては、ガイダンス文書自体が、in vitro 試験の有用性を示す内容でまとめられていることに変わりはないが、本来げっ歯類を主とした動物試験を代替する評価手法として、現行の in vivo 試験との対応や、解決すべき課題についての言及が少なく感じられ、in vivo 試験と in vitro 試験で行われる評価手法と得られる結果のブリッジングの点で、さらに議論を深める必要があると考える。

E. 結論

本研究で得られた発達神経毒性の文献情報や、OECD からの提案資料に対するコメント募集への参画による、国際貢献を通して得られた情報は、今後も、OECD プロジェクトに日本の意見や結果を反映させるために重要であり、引き続き厚生労働行政に活用できるよう調査を進めていく必要があると考えられる。

F. 研究発表

F.1. 論文発表

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F.2 学会発表

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2. 菅野 純、北嶋 聡、相﨑 健一、齊藤 洋克、 種村 健太郎、肺の遺伝子発現応答と毒性機序予測解析、第 48 回日本毒性学会学術年会(2021.7.8)、神戸

G. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

表 1 . EPA および OECD ガイドラインにおける行動解析に関する項目の比較

EPA DNT guideline	OECD DNT guideline (TG426)
供試動物：ラットを推奨	供試動物：ラットを推奨
供試動物数 (同腹児数調整)：♂4 +♀4 匹/腹	供試動物数 (同腹児数調整)：♂4 +♀4 匹/腹
【行動試験項目】	**【行動試験項目】**
自発運動：♂10+♀10 匹/群	行動発生：♂1+♀1 匹/腹 (♂20+♂20 匹/群)
出生後(PND)13、17、21、60±2 日に実施	離乳前に実施
聴覚性驚愕試験：♂10+♀10 匹/群	立ち直り反射、正向反射、自発運動
離乳時(PND21) およびPND60 前後	自発運動：♂1+♀1 匹/腹 (♂20+♀20 匹/群)
	出生後 (PND)13、17、21、60-70 日に実施
	運動および感覚機能：
	♂1+♀1 匹/腹 (♂20+♀20 匹/群)
	思春期・若齢期 (PND60-70)に実施
	伸筋突伸反応、立ち直り反射、聴覚性驚愕反応、誘発電位
	学習および記憶試験：
	♂1+♀1 匹/腹 (♂20+♀20 匹/群)
	離乳後(PND25±2)、PND60-70 に実施
	受動回避、位置遅延見本合わせ、嗅覚条件付け、モリス水迷路、ビール迷路、シンシナティ迷路、放射状迷路、T字型迷路

添付資料１　発達神経毒性評価に係る毒性情報（行動解析を中心とした研究論文）

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厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）
OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

腎障害・線維化の分子メカニズムに関する研究

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研究要旨

腎障害が生じた後に尿細管の再生機構が破綻した場合、不可逆的な慢性腎臓病へと移行する。これまでの研究により、我々は腎虚血再灌流障害モデルラットにおいて再生機構の破綻した尿細管にはCD44が発現する可能性を明らかにしており、尿細管の再生機構破綻をKey Event、CD44発現をエンドポイントとした腎障害・腎線維化のAOP開発の可能性を見出している。本研究では、CD44 のエンドポイントとしての汎用性を検証するため、障害機序の異なる3種類の腎障害物質をラットに投与してCD44発現を病理組織学的に解析した。

アロプリノール及びバンコマイシンを投与したラット腎臓では線維化領域の増加が認められた。線維化病変の尿細管は拡張あるいは萎縮しており、これらの尿細管はCD44に陽性を示した。一方、ピューロマイシンを投与したラット腎臓においては、尿細管障害は認められたものの線維化病変及びCD44陽性尿細管は確認されなかった。これらの結果は、CD44 は線維化病変内の再生機構の破綻した尿細管に発現することを示唆するものであり、さらに再生機構の破綻をKey Eventとした腎障害・腎線維化のAOPにおけるCD44のエンドポイントとしての汎用性を支持するものと考えられた。

A. 研究目的

腎障害が生じた後に尿細管の再生機構が破綻した場合、線維化を伴う不可逆的な慢性腎臓病へと移行する。我々はこれまで尿細管の再生機構破綻をKey Eventとした腎障害・腎線維化の AOP 作製に資するデータを得ることを目的とし、研究を行ってきた。そのなかで、腎虚血再灌流障害モデルラットの腎臓では再生機構の破綻した尿細管においてCD44が発現している可能性を明らかにした（厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）OECDプログラムにおいてTGとDAを開発するためのAOPに関する研究)。よって、CD44 を測定可能なエンドポイントとしたAOP開発の可能性を見出した。

本研究では再生機構の破綻した尿細管において発現するCD44のエンドポイントとしての汎用性を検証するため、障害機序の異なる3種類の腎障害物質を用いて腎障害・腎線維化モデルラットを作製し、CD44の発現を検索した。

B. 研究方法

実験1：6週齢の雄性SDラットを3群に配し（n=5)、媒体である0.5%メチルセルロースもしくはアロプリノール（APL）を 100及び150 mg/kg体重（5 mL/kg体重）の用量

で 1 日 1 回、28 日間反復強制経口投与した。体重測定を週に 1 回行い、最新体重に基づいて投与容量を算出した。最終投与 1 日後にイソフルラン深麻酔下において腹大動脈から採血した後、放血により安楽死させて剖検を行った。剖検時に腎臓を摘出して重量を測定した後、一部を 10%中性緩衝ホルマリンにて固定し、残りの組織は液体窒素にて瞬間凍結もしくは OCT コンパウンドにて凍結ブロックを作製して-80℃にて保存した。得られた血液サンプルを常温下で遠心（3000 rpm、15 分）して血清を分離し、尿素窒素（BUN）及びクレアチニン（sCre）の値を測定した。また、10%中性緩衝ホルマリンで固定した腎臓組織を用いて定法に従いパラフィン包埋、薄切し、HE 染色及び膠原線維を赤色に染色するシリウスレッド染色を施して病理組織学的検索を行った。さらに免疫組織学的解析のため、組織標本を抗原賦活化処置としてクエン酸バッファ（pH6.0）に浸漬してオートクレーブ処置し（121℃、15 分）、3% H_2O_2/メタノールにて内因性ペルオキシダーゼを除去した。引き続き、非特異反応を除去するため 10%正常ヤギ血清を用いてブロッキング処置を施した後､抗 CD44 抗体（ポリクローナル, x10000, Abcam）を 4℃にて一晩インキュベートし、二次抗体（ポリマー法：ヒストファインシンプルステイン）を室温下で 30 分インキュベートした。ジアミノベンジジンにて反応を可視化し、ヘマトキシリンにより核染色を行った。統計学的解析として、体重、血液生化学的検査及び腎重量のデータについて一元配置分散分析（ANOVA）を実施した後に Dunnett 法による多重検定を行った。有意水準は 0.05 に設定した。

実験 2：6 週齢の雄性 SD ラットを 3 群に配し（n=5）、媒体である生理食塩水もしくはバンコマイシン（VAN）を 200 及び 400 mg/kg 体重（10 mL/kg 体重）の用量で 1 日 1 回、28 日間反復腹腔内投与し、実験 1 と同様の測定及び解析を実施した。

実験 3：6 週齢の雄性 SD ラットを 3 群に配し（n=5）、媒体である生理食塩水もしくはピューロマイシン（PAN）を 8 及び 12 mg/kg 体重（5mL/kg 体重）の用量で 1 日 1 回、28 日間反復静脈内投与し、実験 1 及び 2 と同様の測定及び解析を行った。

（倫理面への配慮）

動物の数は最小限にとどめ、実験は国立医薬品食品衛生研究所の実験動物取扱い規定に基づき、動物の苦痛を最小限とするよう配慮して行った。

C. 研究結果

実験 1：実験結果を図 1 に示す。APL 投与群では実験期間を通して体重増加抑制が認められ、APL 100 mg/kg 群では実験開始 1 から 2 週後に、APL 150 mg/kg 群では実験開始 1 から 4 週後にかけて対照群と比較して有意な低値を示した。血清生化学的検査では、BUN 及び sCre ともに APL 100 mg/kg 群から増加傾向を示し、APL 150 mg/kg 群では有意な増加を認めた。腎重量の測定では、APL 100 及び 150 mg/kg 群ともに絶対及び相対重量が用量依存性を伴って有意に増加した。病理組織標本を用いたシリウスレッド染色による線維化の評価では、APL 100 及び 150 mg/kg 群ともに間質の膠原線維の明らかな増加を認めた。膠原線維の増加した領域では尿細管は拡張あるいは萎縮しており、これらの尿細管

は免疫染色にて CD44 陽性を示した。

実験 2：実験結果を図 2 に示す。体重測定において VAN 投与の影響は認められなかった。血清生化学的検査では、BUN の有意な高値が VAN 200 及び 400 mg/kg 群に、sCre の有意な高値が VAN 400 mg/kg 群に認められた。腎重量測定では、VAN 400 mg/kg 群において絶対及び相対重量の有意な増加が認められた。シリウスレッド染色では VAN 200 及び 400 mg/kg 群ともに軽度な膠原線維の増加が認められ、膠原線維の増加した領域における尿細管は拡張あるいは萎縮しており、免疫染色にて CD44 陽性を示した。

実験 3：実験結果を図 3 に示す。体重測定において PAN 投与の影響は認められなかった。血清生化学的検査では、PAN 12 mg/kg 群において BUN の有意な低値が認められた。腎重量測定では、PAN 12 mg/kg 群において相対重量の有意な増加が認められた。PAN 8 及び 12 mg/kg 群の腎臓では糸球体及び尿細管障害を示唆する変化がみられたものの、シリウスレッドによる線維化の評価において間質の膠原線維の明らかな増加は認められず、拡張/萎縮尿細管も観察されなかった。また CD44 の免疫染色においても、陽性を示す尿細管は認められなかった。

D. 考察

APL 投与ラット腎臓においては広範な線維化病変が認められ、病変内の尿細管は拡張あるいは萎縮していた。この線維化病変内の尿細管の形態学的な特徴は虚血再灌流障害による腎線維化モデルラットでみられたものと一致していた（Matsushita K. et al., J. Appl. Toxicol. 2021; 41(4): 607-617）。この拡張/萎縮尿細管は免疫染色において CD44 に明らかな陽性を示した。VAN 投与ラット腎臓においても軽度な線維化が認められた。APL 投与ラット腎臓と同様に、VAN 投与ラット腎臓においても線維化病変内の尿細管は拡張あるいは萎縮しており、CD44 の発現が認められた。PAN 投与ラットの腎臓では、典型病変である糸球体障害及びそれに続発するとされる尿細管障害が認められたものの、間質の膠原線維の明らかな増加はみられず、拡張/萎縮尿細管も認められなかった。これらの結果は PAN 投与ラット腎臓では尿細管障害が生じているものの、その再生機構は破綻していないことを示唆していると考えられた。また、PAN 投与ラット腎臓では CD44 陽性尿細管は認められなかった。

以上の結果は、CD44 は線維化病変内の再生機構の破綻した尿細管に発現するということを示唆するものであり、さらに CD44 の測定可能なエンドポイントとしての汎用性を支持するものと考えられた。

E. 結論

本研究結果は、CD44 は再生機構の破綻した尿細管に発現することを示唆しており、さらに尿細管の再生機構破綻を Key Event とした腎障害・腎線維化の AOP における CD44 の測定可能なエンドポイントとしての汎用性を支持するものと考えられた。

F. 研究発表

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F.2 学会発表

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G. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
該当なし
2. 実用新案登録
該当なし
3. その他
該当なし

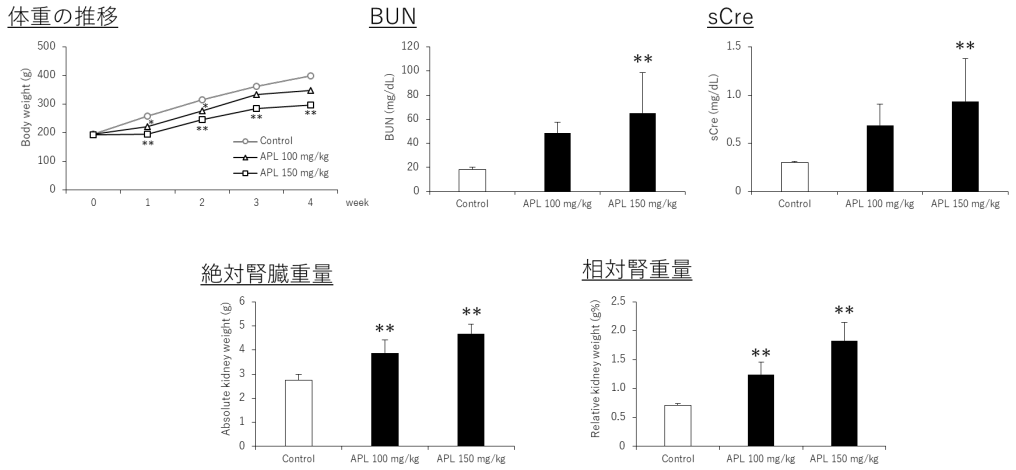

図1. アロプリノール（APL）投与ラットにおける体重の推移、血中尿素窒素（BUN）、血清クレアチニン（sCre）及び腎重量。*, **: $p < 0.05$, 0.01 vs. Control。

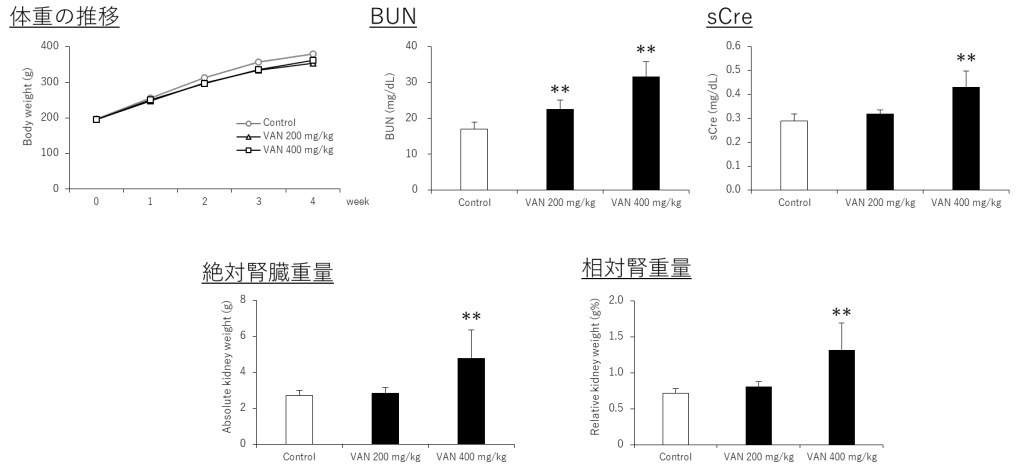

図2. バンコマイシン（VAN）投与ラットにおける体重の推移、血中尿素窒素（BUN）、血清クレアチニン（sCre）及び腎重量。**: $p < 0.01$ vs. Control。

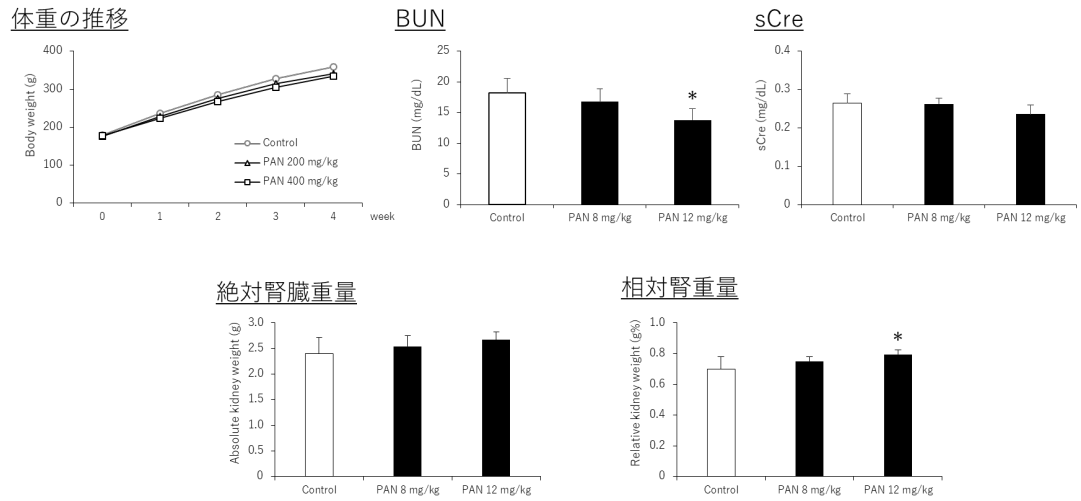

図3. ピューロマイシン（PAN）投与ラットにおける体重の推移及び腎重量。*: $p < 0.05$ vs. Control。

厚生労働行政推進調査事業費補助金（化学物質リスク研究事業）

OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

毒性等情報収集調査

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研究要旨

近年 OECD では、AOP (Adverse Outcome Pathway)に基づいて *in silico*、*in vitro*、*in vivo* の情報を組み合わせて化学物質の有害性を評価する Integrated Approaches to Testing and Assessment (IATA)及び Defined Approach (DA)の開発が進められている。厚生労働科学研究化学物質リスク研究事業における化学物質の有害性評価の迅速化・高度化・標準化に関する研究及び化学物質の新たなリスク評価手法の開発に関する研究の成果を、AOP の開発や代替試験法の公定化、IATA のコンセプトに基づいた化学物質のリスク評価の促進、規制・ガイドラインの新設や見直し等に反映させていくためには、当該研究事業で取得された新規有害性評価系のデータをとりまとめ、化学物質情報や毒性情報などとともに統合して利活用することが求められる。そこで本研究では、平成 30 年度に開始された厚生労働科学研究化学物質リスク研究事業公募型研究 4 課題について様々な観点から整理する。また、OECD IATA Case Studies Project を対象に AOP を用いた IATA 実践例を調査する。以上を今後の研究開発や行政導入の参考情報とする。

研究協力者

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A. 研究目的

近年 OECD では AOP に基づいて *in silico*、*in vitro*、*in vivo* の情報を組み合わせて化学物質の安全性を評価する統合的アプローチ（IATA）の開発が進められている。そのワークフローは、以下のように整理される。

① 課題を設定する。規制上のニーズ、制約、許容される不確実性を整理する。

② 対象の化学物質について既存情報（*in vivo, in vitro*, *in silico* 等）を収集し、Weight of Evidence (WoE)により統合的に解析し、規制上の結論を得る。

③ 既存情報が不十分な場合は、効率的な試験戦略を立て、試験により新規の情報を取得し、規制上の決定を下す。

IATA 実施において、AOP は種々の情報の因果関係を明確化し、結論の導出に必要な情報の同定に役立つと期待されている。

さらに OECD では、より透明性と客観性が高く標準化された IATA を構築することを目的として確定的アプローチ (DA)の検討が進められている。DA に必要なデータやその重みづけをサポートするのが AOP であると考えられている。

国際的には、IATA や DA の行政的な実運

用へ向けて、種々のケーススタディを実施して、得られた経験・教訓を整理してガイダンスの開発が進められている。今後は、動物実験への依存度を軽減しつつ、化学物質が発現しうるヒトへの毒性を効率的かつ高精度で予測するために、IATA や DA に基づいてヒト健康リスク評価のストラテジーを進化させる必要がある。

厚生労働科学研究化学物質リスク研究事業では、化学物質の有害性評価の迅速化・高度化・標準化に関する研究、新たなリスク評価手法の開発に関する研究を推進し、成果をあげてきた。今後、これらの成果を、規制・ガイドラインの新設や見直し、さらには日常生活に利用される種々の化学物質のリスク評価等に反映させていくためには、当該研究事業で開発された新規有害性評価系のデータを、化学物質情報や関連物質の毒性情報などとともに統合して利活用することが求められる。そのためには、各研究課題の成果を整理してデータを集積するとともに、体系的にデータを解析し、不足する情報の同定や研究事業で開発された評価系の有効利用による規制判断の支援や評価・試験の戦略を立てることが望まれるところである。

そこで、本研究では、厚生労働科学研究化学物質リスク研究事業公募型研究でこれまでに得られた成果の基礎試験データを集積し、IATA のコンセプトに基づいた安全性評価・審議の支援、評価・試験戦略の策定に必要な情報収集の効率化や AOP 開発に役立てることを目的とする。

B. 研究方法

B.1.化学リスク研究事業総合報告書の調査

以下の平成 30 年度開始の厚生労働科学研究化学物質リスク研究事業［公募型］計 4 課題の令和 2 年度報告書を入手して[*1]、毒性エンドポイントと解析の対象化学物質、評価系の構築状況ならびに試験結果を精査して Excel 形式で整理した。さらに、各研究課題の分担研究について、AOP の構築とテストガイドライン化へ向けた位置づけを整理した。

[*1]https://mhlw-grants.niph.go.jp/

１）令和 2 年度　化学物質の動物個体レベルの免疫毒性データ集積とそれに基づく Multi-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化（H30-化学-一般-001）

２）令和 2 年度　家庭用品化学物質が周産期の中枢神経系に及ぼす遅発性毒性の評価系作出に資する研究（H30-化学-一般-003）

３）令和 2 年度　生体影響予測を基盤としたナノマテリアルの統合的健康影響評価方法の提案（H30-化学-一般-004）

４）令和 2 年度　血液中の核酸をバイオマーカーに用いた化学物質の高感度な有害性評価に資する研究（H30-化学-一般-002）

B.2. OECD IATA Case Studies Project ケーススタディの調査

AOP を構成する Molecular Initiating Event (MIE)や Key Event (KE)に基づく *in vitro* 試験バッテリーによる前身毒性予測事例など、AOP の IATA への活用に関する調査を行った。調査には、主に OECD IATA Case Studies Project における事例研究の公開資料[*2] を使用した。

[*2]http://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm

（倫理面への配慮）

本研究は動物を用いた研究を行わないため対象外である。

C. 研究結果及び考察

C.1. 各研究事業における新規有害性評価系の開発状況と AOP 開発へ向けた課題

各研究事業の分担研究ごとに、各分担研究課題、目的、研究対象物質、材料と方法、結論、の項目を設定し情報を整理した。さらにそれらを総合して AOP 開発へ向けた位置づけを整理し、課題を考察した。

１）“化学物質の動物個体レベルの免疫毒性データ集積とそれに基づく Multi-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化”においては、化学物質の免疫毒性評価法を 2 つ作出した。IL-2 Luc assay に関しては validation、peer review を経て、2020 年 11 月に SPSF を OECD に提出した。IL-1 Luc assay も validation を行い、report を作成した。また、上記の評価法の確立の過程で得た免疫毒性データを収集しデータベースを構築した。なお、本 assay と免疫毒性を関連付ける AOP は、“OECD プログラムにおいて TG と DA を開発するための AOP に関する研究”で開発を進めた。

２）“家庭用品化学物質が周産期の中枢神経系に及ぼす遅発性毒性の評価系作出に資する研究”では、家庭用品に含まれる化学物質について、妊婦（胎児）や小児を対象に、低用量暴露による遅発性の中枢神経系への影響を検討した。令和 2 年度は、アセフェート、塩化トリブチルスズ、ビスフェノール類などによる成熟後の行動、中枢神経系、脳回路機能への影響などを体系的に解析したほか、影響されるノンコーディング RNA を探索した。また、液性因子、DNA メチル化などの評価手法の構築を進めた。さらに、行動影響と海馬の遺伝子発現様式についてデータベース化を行ったほか、ガイドライン作出に向け、OECD や JaCVAM の協力のもと、標準プロジェクト化への調整を行った。

３）“生体影響予測を基盤としたナノマテリアルの統合的健康影響評価方法の提案”では、二酸化チタンのナノ粒子を被験物質として、(1) ナノマテリアルの *in vitro* 安全性評価法の高度化として評価準備を行った。また、(2) AOP の確立に向けて、新規評価項目を見出した。(3) 毒性試験データベースの構築に向けては、試験データや *in silico* 予測に用いる情報項目の性差を行った。さらに、(4) *in silico* 生体影響予測を組合せたナノマテリアルの統合的健康影響評価方法の構築を目指し、機械学習に用いる実測データの条件を決定した。

４）“血液中の核酸をバイオマーカーに用いた化学物質の高感度な有害性評価に資する研究”では化学物質の「次世代型」有害性評価による迅速化、高度化及び標準化に向け、化学物質ばく露後のマウスの血液中のエクソソーム RNA の網羅的解析により、標的臓器を特定し、更に毒性発現機序の解明を目指した。昨年度までに確立した標準化プロトコールを用いて、四塩化炭素投与による肝毒性を検出する新規バイオマーカーとして、エクソソーム中の small RNA の網羅的スクリーニングを行い、1318 個の新規バイオマーカー候補を得た。

また、5 種類のベンゾトリアゾール類それぞれに特異的なバイオマーカー候補となる 476 個の新規 small RNA を単離し、クラスタリングを行った結果、ベンゾトリアゾール 5 種を 2 群に層別できた。

今回、エクソソーム RNA を指標とした次世代型安全性評価法により、迅速かつ高感度に肝臓毒性を検出でき、評価法の有効性が確かめられた。

いずれの研究課題も毒性がよく知られている化合物（AOP に作用する stressor に相当）を用い、分子レベル、細胞レベル、

個体レベルでの解析（KE に相当）がバランスよく配置されていた。しかし、共通の課題として、*in vivo* 毒性との関連付けと、分子開始イベント（MIE）情報の不足が挙げられる。MIE は化学物質と生体分子との相互作用により、毒性発現に至る最初の引き金となる反応である。OECD での AOP 開発においては必須の情報であり、例えば”Histone deacetylase inhibition leading to testicular toxicity” (AOP212)のように、MIE に関する情報は AOP のタイトルに含められる。AOP 開発の促進のためには、毒性発現に寄与する標的分子を効率的に同定する研究手法の開発が求められる。

C.2. AOP ネットワークを用いた IATA の実例と課題

OECD IATA Case Studies Project では、AOP を全身毒性評価の IATA へ活用した事例の提案が増えつつある。AOP は、リードアクロスなどに毒性機序に基づく類似性仮説の構築に有用であることは、広く認識されている。AOP を構成する KE を測定する *in vitro* 試験は、毒性予測の不確実性を減少させ、信頼性を高める上で有効であると考えられている。

今回、2019 年に提出された「2-エチル酪酸の 90 日反復投与毒性予測」のケーススタディを題材として、AOP を活用する毒性予測の優位性と課題を調査した。本ケーススタディはリードアクロスにより 2-エチル酪酸の 90 日間経口曝露毒性を予測したもので、鎖長の異なる類似物質の毒性試験データと、提案中の AOP を統合した AOP ネットワークから選定した *in vitro* 試験と PBPK モデルの情報を総合的に判断して、肝臓の脂肪症リスクを導出している。使用された AOP は多因子の関与する複雑なネットワークを構成しており（図 1）、この内、幾つかの MIE と KE の試験を使用した。

OECD の専門家レビューでも AOP により高い信頼性で機序を決定できたことが指摘されており、MIE の活性化だけでは有害事象を引き起こすと断定できないことを提示し、さらなる根拠として KE の *in vitro* 試験による裏付けを行った点は高く評価された。

一方で、複雑な AOP から *in vitro* 試験を選定した根拠の明示、提案中で承認されていない AOP についての補足などが課題として指摘された。

また、予測の不確実性に寄与するものとして、毒性試験データのない類似物質や代謝物の毒性を無視したこと、エンドポイントの種差、AOP が未承認であること、*in vitro* 試験による不確実性が挙げられた。

AOP を活用した IATA 事例から、複雑な AOP ネットワークを成す事象を論じる場合、全ての MIE と KE の測定は不可能かつ不要である可能性が指摘され、一方で、MIE だけでは経路全体が活性化されるか判断できないことが明らかとなった。選定した *in vitro* 試験が AOP をどの程度網羅しているかが不確実性につながるが、必須の KE の特定や AOP の根拠が示されることで低減できるとしている。また、未承認の AOP を用いたが、*in vitro* 試験により類似物質の実測データ（陽性/陰性）を正しく予測できたことで、その不確実性を低減できたと評価された。

このケーススタディでは、複数の AOP を結び付けて AOP ネットワークを形成すると、複雑なエンドポイントを評価するための共通の有害事象につながるさまざまな MIE/KE を検討でき、より適切な *in vitro* 試験を根拠とともに選定できることにより、高い信頼性で作用機序を決定することができたと考えられる。

AOP を IATA に活用していくためには、上記を含む様々な経験や情報を関係者が共有することが有用である。AOP の研究は日々進められており、IATA への活用事例も蓄積されつつある。これらの情報は、本研究で蓄積された AOP に関する知見や評価手法の実用化に貢献することと期待している。今後は IATA Case Studies Project のさまざまな事例の調査を進め、AOP による毒性評価の優位性や課題、ノウハウを明らかにしていく。

D. 結論

厚生労働科学研究化学物質リスク研究事業の 4 課題を取り上げ、その進捗を整理して、AOP の構築に向けた課題を明らかにした。

また、OECD IATA Case Studies Project から、AOP を用いたケーススタディを取り上げ、その優位性や課題を整理した。この成果は、AOP の確立と AOP を用いた毒性評価の行政導入の検討に資するものであると期待している。

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F. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

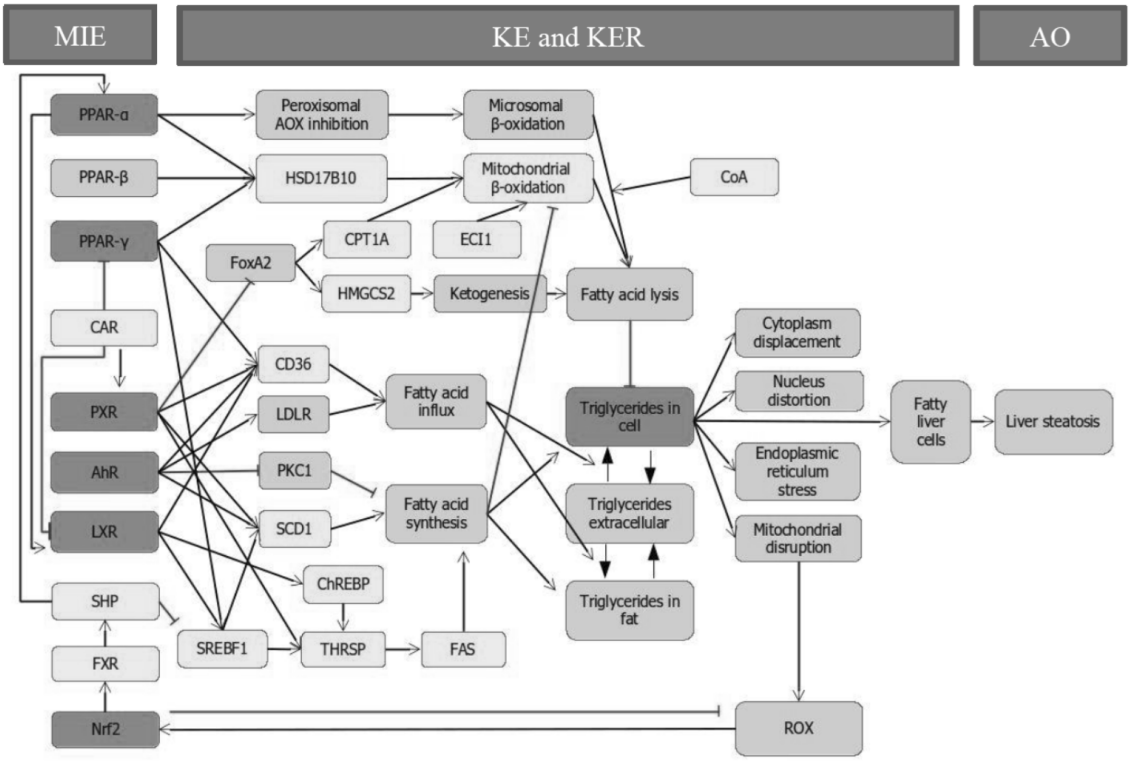

図1　肝脂肪症に関与するAOPネットワーク

別添５

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

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Kasamatsu T, Kitazawa A, Tajima S, Kaneko M, Sugiyama KI, Yamada M, Yasui M, Masumura K, Horibata K, Honma M.	Development of a new quantitative structure-activity relationship model for predicting Ames mutagenicity of food flavor chemicals using StarDrop™ auto-Modeller™.	*Genes Environ*	43(1)	16	2021
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Ambe K, Suzuki M, Ashikaga T, Tohkin M.	Development of quantitative model of a local lymph node assay for evaluating skin sensitization potency applying machine learning CatBoost.	*Regul Toxicol Pharmacol*	125	105019	2021
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Narita K, Okutomi H, Kawakami K, Sui H, Basketter D, Ashikaga T.	Behavior of Chemical Respiratory Sensitizers in *in Vitro* Methods for Skin Sensitization.	*AATEX*	26(1)	9-18	2021
Ashikaga T, Ambe K, Suzuki M, Kurimoto M, Yamada T, Tohkin M.	Establishment of a Threshold of Toxicological Concern Concept for Skin Sensitization by in Vitro/in Silico	*日本香粧品学会誌*	45(4)	331-335	2021
Masumoto M, Fukuda I, Furihata S, Arai T, Kageyama T, Ohmori K, Shirakawa S, Fukuda J.	Deep neural network for the determination of transformed foci in Bhas 42 cell transformation assay.	*Sci. Rep*	11(1)	23344	2021
Nishida H, Ohtake T, Ashikaga T, Hirota M, Onoue S, Seto Y, Tokura Y, Kouzuki H.	In chemico sequential testing strategy for assessing the photoallegic potential.	*Toxicology in Vitro*	77	105245	2021

Seto Y, Tonami R, Iyama Y, Sato H, Onoue S.	An approach to evaluate metabolite-related phototoxicity with combined use of photochemical properties and skin deposition.	*Toxicology Letters*	350	91-97	2021
Onoue S.	Establishment and international harmonization of photosafety testing strategy.	*Yakugaku Zasshi*	141	807-812	2021
Taquahashi Y, Saito H, Kuwagata M, Kitajima S.	Development of an inhalation exposure system of a pressurized metered-dose inhaler (pMDI) formulation for small experimental animals.	*Fundam Toxicol Sci.*	8	169-175	2021
Sasaki T, Saito H, Hiradate Y, Hara K, Tanemura K.	Behavioural effects in mice orally exposed to domoic acid or ibotenic acid are influenced by developmental stages and sex differences.	*Biochem Biophys Res Commun*	558	175-182	2021
Yamada T, Miura M, Kawamura T, Ushida K, Inoue K, Kuwagata M, Katsutani N, Hirose A.	Constructing a developmental and reproductive toxicity database of chemicals (DART NIHS DB) for integrated approaches to testing and assessment	*J. Toxicol. Sci*	46	531-538	2021
Yamada T, Kawamura T, Maruyama T, Kurimoto M, Yamamoto H, Katsutani N, Hirose A.	Quantitative structure-activity relationship and a category approach to support algal toxicity assessment of human pharmaceuticals	*Fundam. Toxicol. Sci*.	8	195-204	2021
Iso T, Shigeta Y, Murata Y, Hirose N, Inoue K, Yamada T, Hirose A, Matsumoto M.	Summary information of human health hazard assessment of existing chemical substances (VII)	*Bull. Natl Inst. Health Sci.*	139	71-78	2021
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Tanabe S, Hirose A, Yamada T.	Adverse Outcome Pathway on Histone deacetylase inhibition leading to testicular atrophy	*OECD Series on Adverse Outcome Pathways No. 17*			2021
Fujita M, Yamamoto Y, Wanibuchi S, Watanabe S, Yamaga H, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Takeuchi K, Kamiya K, Kawakami T, Kojima K, Sozu T, Kojima H, Kasahara T, Ono A.	The within- and between-laboratories reproducibility and predictive capacity of Amino acid Derivative Reactivity Assay using 4 mM test chemical solution: Results of ring study implemented at five participating laboratories	*Applied Toxicology*	42(2)	318-333	2022
Iso T, Natsume M, Murata Y, Shigeta Y, Hirose N, Umano T, Horibata K, Masumura K, Sugiyama K, Matsumoto M, Hirose A.	Absence of in vivo mutagenicity of 4,4'-oxybis (benzenesulfonohydrazide) in liver and glandular stomach of Muta™ Mouse.	*Fundamental Toxicological Sciences.*	9(2)	31-36	2022
Grúz P, Yasui M, Ukai A, Horibata K, Honma M, Sugiyama K.	Potent mutagenicity of an azide, 3-azido-1,2-propanediol, in human TK6 cells.	*Mutation Research*	876–877	503475	2022
Watanabe-Matsumoto S, Yoshida K, Meiseki Y, Ishida S, Yamada T.	A physiologically based kinetic modeling of ethyl tert-butyl ether in humans–An illustrative application of quantitative structure-property relationship and Monte Carlo simulation.	*J. Toxicol. Sci.*	47(2)	77-87	2022
山田 隆志	Cefic LRI/ILSI Europe Joint Workshopでの Carcinogen Dose Response Database for Threshold of Toxicological Concern （TTC）の概要ならびにTTCに関する近年の国際動向	*イルシー*	In press		2022

Imamura M, Yamamoto Y, Fujita M, Wanibuchi S, Nakashima N, Kojima H, Ono A, Kasahara T.	Applicability of ADRA (4 mM) for the prediction of skin sensitization by combining multiple alternative methods to evaluate key events	*J Appl Toxicol*.	in press		2022
Yamamoto Y, Fujita M, Watanabe S, Yamaga H, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Takeuchi K, Kamiya K, Kawakami T, Kojima K, Sozu T, Kojima H, Kasahara T, Ono A	Within- and between-laboratory reproducibility and predictive capacity of amino acid derivative reactivity assay (ADRA) using a 0.5 mg/mL test chemical solution: Results of the study for reproducibility confirmation implemented in five participating laboratories	*J Appl Toxicol*	in press		2022
Akane H, Toyoda T, Mizuta Y, Cho YM, Ide T, Kosaka T, Tajima H, Aoyama H, Ogawa K.	Histopathological and immunohistochemical evaluation for detecting changes in blood hormone levels caused by endocrine disruptors in a 28-day repeated-dose study in rats.	*J Appl Toxicol*	in press		2022

令和４年　　３月　３１日

厚生労働大臣 殿

機関名　国立医薬品食品衛生研究所

所属研究機関長　職　名　所長

氏　名　合田　幸広

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１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究　(21KD2003)

３．研究者名　(所属部署・職名)　安全性生物試験研究センター

(氏名・フリガナ)　平林　容子・ヒラバヤシ　ヨウコ

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入 (※1)		
	有　無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
その他、該当する倫理指針があれば記入すること (指針の名称：　　)	□　☑	□		□

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５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　　)
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当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑(有の場合はその内容：　　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和 4 年　　3月　31日

厚生労働大臣 殿

機関名　国立医薬品食品衛生研究所

所属研究機関長　職　名　所長

氏　名　合田　幸広

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３．研究者名　(所属部署・職名)　安全性予測評価部　主任研究官

(氏名・フリガナ)　小島　肇・コジマ　ハジメ

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入 (※1)		
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人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
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令和 4 年 3月 31 日

厚生労働大臣 殿

機関名 東京農業大学

所属研究機関長 職 名 学長

氏 名 江口 文陽

次の職員の令和３年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

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２．研究課題名 OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究 (21KD2003)

３．研究者名 (所属部局・職名) 応用生物科学部食品安全健康学科・教授

(氏名・フリガナ) 中江 大・ナカエ ダイ

４．倫理審査の状況

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３．研究者名　(所属部署・職名)　安全性生物試験研究センター病理部・部長

(氏名・フリガナ)　小川　久美子　・　オガワ　クミコ

４．倫理審査の状況

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(氏名・フリガナ)　豊田　武士・トヨダ　タケシ

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５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関:　　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由:　　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑(有の場合はその内容：　　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和 4 年　　3月　31日

厚生労働大臣 殿

機関名　国立医薬品食品衛生研究所

所属研究機関長　職　名　所長

氏　名　合田　幸広

次の職員の令和３年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究　(21KD2003)

３．研究者名　(所属部署・職名)　変異遺伝部　室長

(氏名・フリガナ)　堀端　克良・ホリバタ　カツヨシ

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入 (※1)		
	有　無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
その他、該当する倫理指針があれば記入すること (指針の名称：　　)	□　☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関:　　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由:　　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑(有の場合はその内容：　　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和 4 年　　3月　31日

厚生労働大臣 殿

機関名　国立医薬品食品衛生研究所

所属研究機関長　職　名　所長

氏　名　合田　幸広

次の職員の令和３年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究　(21KD2003)

３．研究者名　(所属部署・職名)　安全性予測評価部　室長

(氏名・フリガナ)　足利　太可雄・アシカガ　タカオ

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入 (※1)		
	有　無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
その他、該当する倫理指針があれば記入すること (指針の名称：　　)	□　☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関:　　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由:　　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑(有の場合はその内容：　　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和 4 年 4 月 21 日

厚生労働大臣 殿

機関名　神奈川県衛生研究所

所属研究機関長　職　名　所長

氏　名　多屋 馨子

次の職員の令和３年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究　(21KD2003)

３．研究者名　(所属部署・職名)　神奈川県衛生研究所　理化学部・　主任研究員

(氏名・フリガナ)　大森　清美　・　オオモリ　キヨミ

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入 (※1)		
	有　無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
その他、該当する倫理指針があれば記入すること (指針の名称：　)	□　☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関:　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由:　)
当研究に係るＣＯＩについての指導・管理の有無	有 ☑　無 □（有の場合はその内容：　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和４年４月14日

厚生労働大臣 殿

機関名　静岡県立大学

所属研究機関長　職　名　学長

氏　名　尾池 和夫

次の職員の令和３年度厚生労働行政推進調査事業費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究　(21KD2003)

３．研究者名　(所属部署・職名)　静岡県立大学 薬学部 教授

(氏名・フリガナ)　尾上 誠良（オノウエ サトミ）

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	☑	□	☑	静岡県立大学動物実験委員会	□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□	☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関:　　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由:　　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑(有の場合はその内容:　　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和 4 年　　3月　　31日

厚生労働大臣 殿

機関名　国立医薬品食品衛生研究所

所属研究機関長　職　名　所長

氏　名　合田　幸広

次の職員の令和３年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究　(21KD2003)

３．研究者名　(所属部署・職名) 毒性部　研究員

(氏名・フリガナ) 齊藤 洋克・サイトウ ヒロカツ

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入 (※1)		
	有　無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
その他、該当する倫理指針があれば記入すること (指針の名称：　　)	□　☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他 (特記事項)

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関:　　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由:　　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑(有の場合はその内容:　　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和 4 年　　3月　31日

厚生労働大臣 殿

機関名　国立医薬品食品衛生研究所

所属研究機関長　職　名　所長

氏　名　合田　幸広

次の職員の令和３年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究　(21KD2003)

３．研究者名　(所属部署・職名)　安全性生物試験研究センター 病理部・主任研究官

(氏名・フリガナ)　松下　幸平・マツシタ　コウヘイ

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□	☑	□		□
遺伝子治療等臨床研究に関する指針	□	☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	☑	□	☑	国立医薬品食品衛生研究所	□
その他、該当する倫理指針があれば記入すること (指針の名称：　　)	□	☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関:　　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由:　　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑(有の場合はその内容：　　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和 4 年　　3月　31日

厚生労働大臣 殿

機関名　国立医薬品食品衛生研究所

所属研究機関長　職　名　所長

氏　名　合田　幸広

次の職員の令和３年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究　(21KD2003)

３．研究者名　(所属部署・職名)　安全性予測評価部　第四室　室長

(氏名・フリガナ)　山田　隆志・ヤマダ　タカシ

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入 (※1)		
	有　無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	□　☑	□		□
遺伝子治療等臨床研究に関する指針	□　☑	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　☑	□		□
その他、該当する倫理指針があれば記入すること (指針の名称：　　)	□　☑	□		□

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ☑　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ☑　無 □(無の場合はその理由:　　)
当研究機関におけるＣＯＩ委員会設置の有無	有 ☑　無 □(無の場合は委託先機関:　　)
当研究に係るＣＯＩについての報告・審査の有無	有 ☑　無 □(無の場合はその理由:　　)
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑(有の場合はその内容：　　)

(留意事項)　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。