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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

令和元年度 分担研究報告書

免疫毒性のAOP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 4 種の AOP の開発を行った。

すでに AOP wiki に登録済みである「Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response」については、今年度外部レビューに進み、外部レビュアーの指摘に基づいて AOP 案の修正を行った。

昨年度 OECD に SPSF（Standard Project Submission Form）を提出し、計画が承認された以下 3 種の AOP はコーチ制となり、それぞれのコーチとともに AOP の作成を行った。

「Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease」については、提出した AOP 案に対するコーチのコメントに基づいて修正案を作成中である。

「Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus」については、コーチと 2 回の web 会議を行い、AOP 案の修正を行っている。

「Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response」については、コーチと 3 回の会議を行い、AOP 案の修正を行っている。

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を開発し、ネットワーク化する必要がある。日本主導でAOPを開発することにより、世界中の人々の化学物質による免疫毒性被害の防止に貢献するだけでなく、日本の研究レベルの高さを世界にアピールすることを目的とする。

B. 研究方法

AOP案の作成は、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検討小委員会に委託した（研究分担者も本小委員会の委員として活動）。それぞれ開発進度に合わせて、OECDより任命された外部レビュアーあるいはコーチと意見交換を行い、最終化に向けて修正を行う。

（倫理面への配慮）

本研究は動物実験を含め新たな実験は行わないため、倫理的問題は無いと考える。

C. 研究結果

「Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response」は、カルシニューリン阻害によりIL-2および４の産生抑制が生じ、最終的にTDAR (T-cell dependent antibody response)の阻害となるとするAOPである。本AOPについては、内部レビュアーによるマイナーコメントへの対応を行った結果、2019年6月にOECDパリ本部にて開催されたEAGMST (Advisory Group on Molecular Screening and Toxicogenomics)会議において、外部レビュー移行となった。その後外部レビュアーから詳細なコメントがあり、それらに基づいてAOPの修正を行った。主な論点とその対応を以下に示す。本AOPのMIE (Molecular Initiating Point)はカルシニューリンの阻害であり、免疫抑制剤であるFK506やサイクロスポリンをストレッサーとして挙げていたが、一般化学物質も例示すべきとの意見に従い、IC50は高くなるもののケンペロールなどカルシニューリン阻害活性がある化学物質を追加した。また、IL-2とIL-4の産生抑制がAO (Adverse Outcome)であるTDRAにつながるとした点について、IL-4の抑制がIgEクラスのみの産生を抑制するデータであったことから、関係性が弱いとの指摘があった。そこでIL-4を削除してIL-2のみの記載にすることも検討したが、その後の文献調査により、IL-4の抑制によるIgMおよびIgGクラスの抑制も明らかとなったことから、IL-4の産生抑制もTDARの抑制要因として残すこととした。

「Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease」は、樹状細胞に存在するTLR (Toll-like receptor) 7/8への結合が、Th17によるIL-17の過剰発現を誘導し、乾癬様の皮膚疾患を生じさせるというAOPである。本AOPについては、提出したAOP案に対するコーチのコメントへの対応案を作成した。主な論点とその対応を以下に示す。当初、TLR (Toll Like Receptor)7/8への刺激が、樹状細胞のIL-23の過剰産生につながるとしていたが、より一般的な炎症のKE (Key Event)をその間に入れることで規制での活用においてより重要性が高くなるという指摘があり、樹状細胞の活性化をKEとして追加することを検討している。また、例えば環境汚染物質などの潜在的なストレッサーを考慮するべきとの指摘については、TLR 7/8のリガンドとして知られている一本鎖RNAやグアノシンなどの低分子化合物から構造的特徴が見いだせないか検討中である。

「Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus」は、様々なタイプの免疫細胞に存在するERの活性化がTh2タイプのサイトカイン(IL-4)の異常亢進を誘導し、自己免疫疾患であるSLE（全身性エリテマトーデス）を増悪させるというAOPである。本AOPについては、これまでコーチとの2回の会議を経て、AOP案の修正を行っており、主な論点とその対応は以下の通りである。AOとしてのSLEの増悪について具体的な測定指標が明確になっていないという指摘については、ヒトやSLEモデルマウスでのglomerulonephritis（糸球体腎炎）の診断法（診断や剖検所見）を記載することを提案した。またAOに相当するin vitroの試験法は見いだせなかったため、その旨記載することとした。

「Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response」は、JAK3の阻害によりL-2産生が抑制され、最終的にTDARの阻害となるというAOPである。本AOPについては、これまでコーチと3回の会議を行い、AOP案の修正を行っている。主

な議論と対応を以下に示す。JAKおよびSTATには様々なファミリーがあり、その組み合わせも様々であるが、本AOPにおいてJAK3-STAT5に限定しているのはダイアグラムをシンプルにするためである。またストレッサーを特異性の高いものに限定しているのも、他のJAKおよびSTATファミリーを阻害するストレッサーを除くためである。さらに先行しているカルシニューリン阻害のAOPとAOをなるべく共通にしているのは、将来AOPネットワークを構築することを目指しているためである。

D. 考察

AOPの開発は最終化まで2回のレビュー（内部及び外部）を経る必要があり、平均約1000日を要すると言われる膨大で緻密な作業である。そこで新たにコーチ制が導入され、レビューの効率化が図られている。一方で、コーチあるいはレビュアーの負担の大きさから、EAGMSTでは将来的には独立したジャーナルでのレビューも検討されている。こうした流動的な状況においても、本研究では、1つが外部レビューに入り、3つがコーチによる指導を受けながらAOPの開発を進めている。

今回、多くの指摘を受けたのが、記載したストレッサーが少なく、行政的活用という観点から汎用性に欠けるのではないかというものである。AOPを作成する際には、そのメカニズムが量的関係も含めて詳細に解明されていることが必要であるが、その条件を満たしつつ汎用性を高めるには、今後Read acrossの適用や、AOPを共通のKEあるいはAOでつなぐ、いわゆるAOPネットワークの構築が必要となると考えられた。JAK3阻害に関するAOと、カルシニューリン阻害のAOを同じ「TDARの抑制」としたのは、将来のネットワーク化を想定したためであり、TLR刺激のAOPにIncreased Pro-inflammatory mediatorsというより一般的なKEを挿入することの検討も、他のAOPとネットワーク化するためである。

免疫毒性は極めて複雑な毒性であり、エビデンスに基づいたAOPを多数作り、それらをネットワーク化することで全体像を明確にすることが重要と考える。免疫毒性に関するAOPは未だ承認されたものはないが、日本が大きく貢献できるよう引き続き取り組んでいく。

E. 結論

今年度は開発中の4種のPのうち一つが外部レビューに進み、残りの3つもコーチとともに開発を進めた。

F. 研究発表

F.1. 論文発表

なし

F.2. 学会発表

1. 足利太可雄: 免疫毒性ＡＯＰ開発が目指すもの, 第 26 回日本免疫毒性学会学術年会（2019.9.10, 北九州）.
2. Suzuki M, Ambe K, Tohkin M, Yamada T, Ashikaga T: Development of in silico prediction model for skin sensitization using the alternative tests dataset, 情報計算化学生物学会(CBI 学会)2019 年大会(2019.10.22-24, 東京）
3. 成田和人, 奥富弘子, 川上久美, 須井哉, 足利太可雄: 呼吸器感作性物質評価に対する h-CLAT の有用性検討, 日本動物実験代替法学会第 32 回大会, （2019.11.21, つくば）
4. 足利太可雄: 呼吸器感作性物質評価に関

する h-CLAT の有用性検討, 日本動物実験代替法学会第 32 回大会（2019.11.22, つくば）

5. Ashikaga T, Narita K, Okutomi H, Kawakami K, Sui H: Effectiveness of h-CLAT, an In Vitro Skin Sensitization Test Method, in Evaluating Respiratory Sensitizers, 59th Annual Meeting of SOT, (2020.3.15-19, Anaheim,CA,USA)

H. 添付資料

1. AOP:154
   Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response
2. AOP:313
   Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease
3. AOP:314
   Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus
4. AOP:315
   Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response

G. 知的財産権の出願・登録状況

なし

AOP ID and Title:

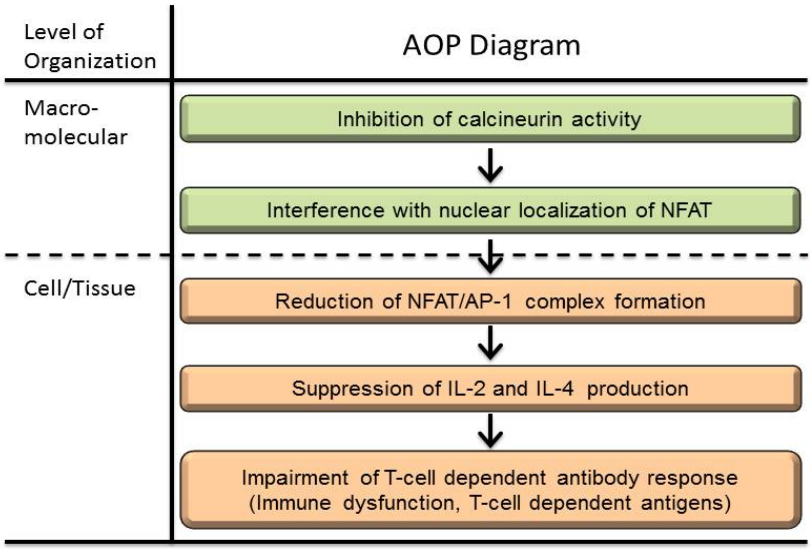
SNAPSHOT

Created at: 2020-05-18 09:20

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 kinds of T-cell derived immune functions to induce compromised

host. Among the affected immune functions. T-cell dependent antibody response (TDAR) is the 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 of CNIs 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 expresses in cells among vast variety of species, this AOP might be applicable to many mammal species, including humans and rodents.

Background

Although there are stressors that inhibit CN activity, this AOP is mainly based on an understanding of immunosuppression caused by FK506 and FKBP12 complexes, 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 (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 complex formation
4	KE	1202	Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)	Suppression, IL-2 and IL-4 production
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-4 production	High	High

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Suppression, IL-2 and IL-4 production (https://aopwiki.org/relationships/1510)	adjacent	Impairment, T-cell dependent antibody response	High	High

Stressors

Name	Evidence
Tacrolimus	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.

CN phosphatase activity is inhibited when stressors bind to Calcineurin-A (CnA) with immunophilins, which interferes with the nuclear localization of NFAT, a substrate of 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 expresses in cells 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

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
Mixed	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 Cyclosporine A (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, TDAR 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 on 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 commonality 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; 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 localization of NFAT and resultant binding with AP-1 at the promoter region of the T cell cytokine genes. NFAT has NLS and NES among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS is exposed and the NES 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).
KER3	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 from 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).

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 one of the calcineurin inhibitiors. CsA-treatment is reported to suppresses 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). 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). 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
KER1	Inhibition, calcineurin activity leads to interference, nuclear localization of NFAT	Moderate	CN phosphatase activity is inhibited by CNI of FK506 with IC50 values of 0.5 nM (FK506) and 5nM (CsA) after 1 hours treatment (Fruman et al.1992). 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 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). The experiment of gel mobility shift assay 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 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 assay 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.

KER3	Reduction, NFAT/AP-1 complex formation leads to suppression, IL-2 and IL-4 production	Moderate	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 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). 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 in a dose-dependent (10 nM) manner (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.Time lag is found between the two KEs; 2 hours for KE2 and 22 to 48 hours for KE3.

KER4	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 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). 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). 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 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). 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

There have been no literature available to show 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 with 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)

CN is expressed in T cells as well as other types of immune cells like B cells and dendritic cells. CNIs suppress many kinds of immune functions leading to increased susceptibility to infections and decreased hyper immune reactions such as rejection and GVHD. Among these, TDAR is considered to be the important endpoint of immunotoxicity, because 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.

Moreover, when evaluating the immunotoxicity of pharmaceuticals, the ICH S8 immunotoxicity testing guideline recommends that TDAR be evaluated whenever the target cells of immunotoxicity are not clear based on pharmacology and findings in standard toxicity studies.

The draft FDA guidance of nonclinical safety evaluation for immunotoxicology is recently issued (2020) and recommends TDAR assay. Because TDAR is a common secondary assay that requires functionality of several key immune cell subtypes (e.g., antigen-presenting cells, T-helper cells, B cells).

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using sheep red blood cells.

As a part of an IATA of immunotoxicology, the present AOP could be used to predict whether or not a compound that potentially acts on T cells could also affect TDAR. On the other hand, it would be inappropriate to use the present AOP alone as an alternative to TDAR measurement in the ICH S8 or US EPA OPPTS 870.7800 immunotoxicity testing guideline.

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Appendix 1

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

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)

Term	Scientific Term	Evidence	Links
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
Mixed	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 Ki inhibition of CN 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 the 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
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 (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
Mixed	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 followed by nuclear localization. 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 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).

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 a gel mobility shift assay 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
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 (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
Mixed	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).

NFAT is known to bind cooperatively at the promoters of Interleukin-4 (IL-4) and other T-cell cytokines as well as that of IL-2 (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

Process	Object	Action
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
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)
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

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 (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 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).

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 in a dose-dependent (10 nM) manner (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 (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)	AdverseOutcome

Stressors

Name
Tacrolimus
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
Mixed	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

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 of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

Moreover, ICH S8 immunotoxicity testing guideline on pharmaceuticals recommends that TDAR be evaluated whenever the target cells of immunotoxicity are not clear based on pharmacology and findings in standard toxicity studies. For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC.

The draft FDA guidance of nonclinical safety evaluation for immunotoxicology recommends TDAR assay.

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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 Dependent Antibody Response (https://aopwiki.org/aops/154)	**adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	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
Mixed	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).

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 with the 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 the PPIase activity and the inhibition of activity that they indicate are unrelated 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 exposes nuclear localization signal (NLS) and covers 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 interfering with nuclear localization.

Evidence Supporting this KER

Biological Plausibility
The molecular structures and functions of CN and NFAT are evident based on sufficient scientific findings 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 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).

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 flowcytometer. 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).

There have been no literature available to compare directly the dose response of inhibition of CN phosphatase activity with that of 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	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)

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).

Nuclear localization of NFAT results in the NFAT binding 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 among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS is exposed and the NES 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 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.

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).

The experiment of gel mobility shift assay 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 with FK506 at the concentration within the concentration 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, the gel mobility shift assay 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 assay 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 Dependent Antibody 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
Mixed	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 binds to form 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 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).

Decreased formation of NFAT/AP-1 complex at the promoter region of IL-2 genes in the nucleus of T cells following lowed nuclear localization of NFAT by calcineurin inhibitor (CNI) treatment reduces 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 leads to inhibit translocation of NFAT to the nucleus. 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 Dependent Antibody 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)
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
Mixed	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 one of the calcineurin inhibitiors. CsA-treatment is reported to suppresses 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:

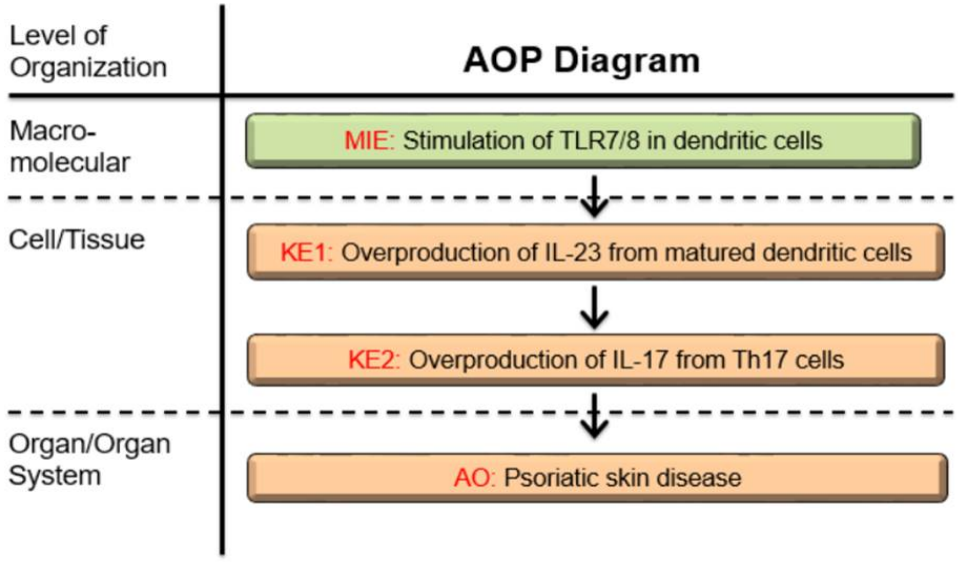
SNAPSHOT

Created at: 2020-05-18 09:22

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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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 in dendritic cells (https://aopwiki.org/events/1706)	Stimulation of TLR7/8
2	KE	1707	Overproduction of IL-23, matured dendritic cells (https://aopwiki.org/events/1707)	Overproduction of IL-23
3	KE	1708	Overproduction of IL-17 from Th17 cells (https://aopwiki.org/events/1708)	Overproduction of IL-17
4	AO	1709	Psoriatic skin disease (https://aopwiki.org/events/1709)	Skin disease

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Stimulation, TLR7/8 in dendritic cells (https://aopwiki.org/relationships/2017)	adjacent	Overproduction of IL-23, matured dendritic cells	High	High
Overproduction of IL-23, matured dendritic cells (https://aopwiki.org/relationships/2018)	adjacent	Overproduction of IL-17 from Th17 cells	High	High
Overproduction of IL-17 from Th17 cells (https://aopwiki.org/relationships/2019)	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 (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

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 in dendritic cells (https://aopwiki.org/events/1706)

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 (https://aopwiki.org/aops/313)	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
dendritic cell

Organ term

Organ term
immune system

Domain of Applicability

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, CD4/CD8DN DC and pDC express TLR7 in mice (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 (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).

Stimulation of blood DCs with self-RNA–LL37 complexes induces a robust TNF-α response (Hänsel et al. 2011). DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

How it is Measured or Detected

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 in luciferase production relative to medium control (Gibson et al. 2002). R848 (0.001-10 μg/mL) induced NF-κB activation in HEK293 cells transfected with human TLR8 is detected in the same manner (Jurk et al. 2002).

IFN-α in cell-free supernatants collected after imidazoquinoline stimulation to human PBMC and/or pDC-enriched cells is detected by ELISA (Gibson et al. 2002).

TNF-α and IL-6 in cell-free supernatants collected after RNA-LL37 stimulation to mDCs were measured by ELISA (Ganguly et al. 2009). mDCs were also stained with fluorochrome-labeled anti-CD80, anti-CD86, and anti-CD83 antibodies and analyzed by flow cytometry (Ganguly et al. 2009).

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List of Key Events in the AOP

Event: 1707: Overproduction of IL-23, matured dendritic cells (https://aopwiki.org/events/1707)

Short Name: Overproduction 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 (https://aopwiki.org/aops/313)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
dendritic cell

Organ term

Organ term
immune system

Domain of Applicability

pDCs were prepared from mouse spleen, and cytokine production after culture with IMQ was measured. IFN-a production from splenic pDCs was induced by IMQ treatment. The production of IL-23, IL-6 and TNF-α was also induced by IMQ treatment. Although 4–8% of mPDCA-1$^-$ CD11c$^+$ DCs were contaminated in prepared mPDCA-1$^+$ pDC fraction, we confirmed that splenic mPDCA-1$^-$ CD11c$^+$ DCs enriched to approximately 80% purity could not produce IL-23 and TNF-α by IMQ stimulation. In Tlr7-/- splenic pDCs, these cytokines (IFN-α, IL-23, IL-6 and TNF-α) 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-a, IL-23, IL-6 and TNF-α from pDCs existing in the skin in vivo (Ueyama et al. 2014).

Key Event Description

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).

How it is Measured or Detected

IL-23 in cell-free supernatants collected after R848 stimulation to moDCs is detected by ELISA (Schwarz et al. 2013). Expression of IL-23 mRNA in R848-stimulated moDCs is measured by qRT-PCR (Schwarz et al. 2013).

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Event: 1708: Overproduction of IL-17 from Th17 cells (https://aopwiki.org/events/1708)

Short Name: Overproduction of IL-17

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 (https://aopwiki.org/aops/313)	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

Key Event Description

Psoriasis has been known to play a major role in the pathogenesis of T cell dysfunction, particularly overactivation of the Th17 pathway. Th17 cells are a subset of CD4 positive T helper cells newly found in 2005 as a cell population different from Th1 and Th2 (Lisa C. et al. 2007).

Serum IL-17 levels in psoriasis patients are significantly higher than in healthy individuals, and the neutralizing antibody Brodalumab against the IL-17A receptor has been shown to be effective in treating psoriasis (Gilliet et al. 2004). Furthermore, because the antibody preparations against IL-17 (Ixéquizumab [John K. et al. 2002]), Sequinumab (Szeimies et al. 2004)) are used for the treatment of psoriasis, the Th17 pathway for pathogenesis is considered to play an important role.

Psoriatic 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 dermal T cells Less up-regulation. Cheuk et al. 2013

IL-22, produced mainly by lesional epidermal CD4 T cells, is associated with the activation of keratinocytes and the formation of epidermal thickening, a prominent morphological feature of psoriasis. The lesional epidermal 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 the recruitment and activation of leukocytes to the skin.(Cheuk et al. 2013)

How it is Measured or Detected

Flow cytometric analysis of psoriatic skin biopsy showed increased frequency of IL-17 + and IL-22 + CD4 + T cells, and IL-17 secretion was significantly increased. CD4 + cells making IL-17 or IL-22 expressed IL-23R, and the frequency of IL-17 +, CCR6 + and CCR4 + T cells increased. The frequency of IL-17 + and IL-22 + CD4 + T cells was increased compared with normal skin, and the proportion of IL-22 positive IL-17 + cells was high. There was also an increase in IL-22 producing cells (Th-22 cells) that do not produce IL-17 or IFNγ. (Benham et al. 2013)

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List of Adverse Outcomes in this AOP

Event: 1709: Psoriatic skin disease (https://aopwiki.org/events/1709)

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 (https://aopwiki.org/aops/313)	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.)

Key Event Description

In psoriasis vulgaris, the S100 protein family psoriasin (sorazine) and koebnerisin (kebneridine) are overexpressed, and the epidermal antimicrobial peptide induced by IL-17 functions itself as a chemotactic factor and cytokine. It recruits CD4 + T cells and neutrophils to exacerbate inflammation. (Kanagawa Psoriasis Treatment Study Group)

A biopsy of the skin area of psoriasis and surrounding normal skin was performed, and immunohistological examination of the sections was performed. In psoriatic lesions, the number of activated dendritic cells was increased, and CD1a-positive Langerhans cells in the epidermis and CD83-positive CD1a-negative Langerin-negative CD11c-positive dermal dendritic cells in the epidermis boundary were observed. In normal skin,

the number of wet cells was the same as in the lesion, but CD3-positive T lymphocytes were significantly less than in the lesion.

In the normal area, inflammatory keratin K6 and K16-positive keratinocytes were found, and the transcription factor C / EBPβ, which is normally expressed only in the granular layer of the normal epidermis, was expressed in the entire epidermis as in the lesion. This suggests that early inflammatory changes have already occurred in normal 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

A biopsy of the skin area and the surrounding normal skin of a patient with psoriasis vulgaris was performed, and immunohistological studies were performed using dendritic cell surface markers and lymphocyte surface markers as primary antibodies in the sections. In the vicinity of the psoriatic lesion, an increased number of activated dendritic cells was observed, and CD1a-positive Langerhans cells in the epidermis and CD83-positive CD1a-negative Langerin-negative CD11c-positive dermal dendritic cells in the epidermis boundary were observed. In normal skin, the number of wet cells was the same as in the lesion, but CD3-positive T lymphocytes were significantly less than in the lesion. In the normal area, inflammatory keratin K6 and K16-positive keratinocytes were found, and the transcription factor C / EBPβ, which is normally expressed only in the granular layer of the normal epidermis, was expressed in the entire epidermis as in the lesion. This suggests that early inflammatory changes have already occurred in normal 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)

Serum amyloid A: SAA was measured in 35 psoriasis patients and healthy humans. DNA microarray analysis in lesions of psoriasis patients showed that SAA levels were about 5 times higher than normal skin. The average SAA in psoriasis patients was 19.1 ug / ml, and the SAA after treatment was significantly reduced to an average of 6.9 ug / ml. There was a correlation between SAA and psoriasis severity score (PASI). In addition, amyloid A deposition, which is thought to be the result of prolonged chronic inflammation, was observed in the skin-stained section of the psoriatic lesion skin area. (J Dermatolog Treat. 2013; 24 (6): 477-80)
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.)

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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 Overproduction of IL-23 (https://aopwiki.org/relationships/2017)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	**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 and$CD1c^+$ 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 and$CD1c^+$ 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 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).

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: Overproduction of IL-23 leads to Overproduction of IL-17 (https://aopwiki.org/relationships/2018)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	**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: Overproduction of IL-17 leads to Skin disease (https://aopwiki.org/relationships/2019)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease (https://aopwiki.org/aops/313)	**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:

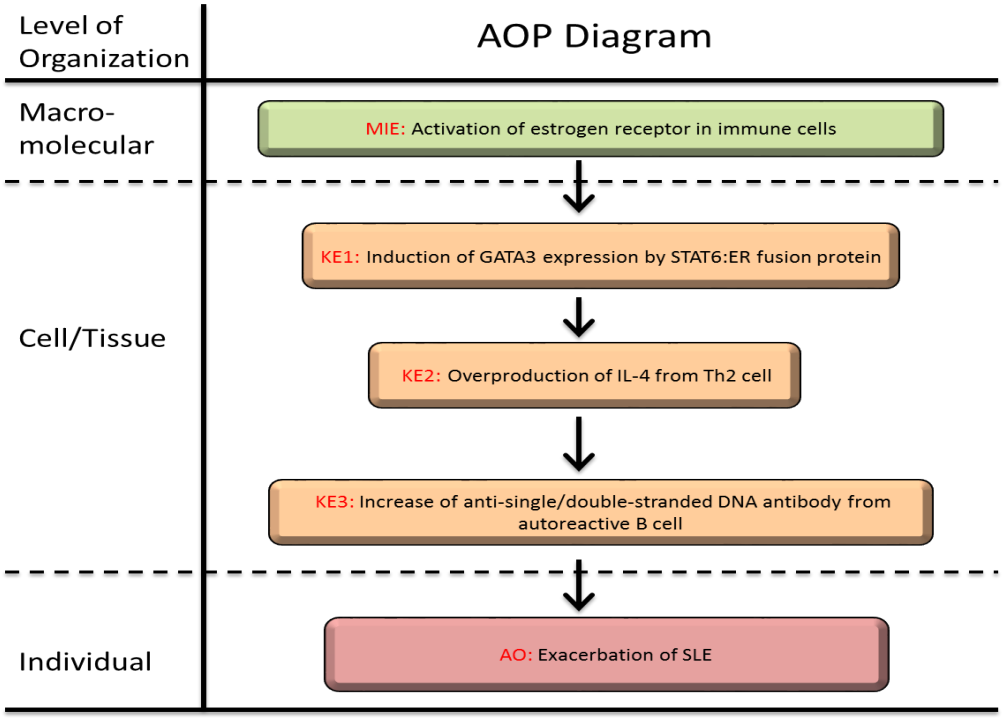
SNAPSHOT

Created at: 2020-05-18 09:23

AOP 314: Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus

Short Title: Exacerbation of SLE by activation of estrogen receptor

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	1.73	Included in OECD Work Plan

Abstract

This AOP describes the linkage between the activation of estrogen receptor (ER)α and the exacerbation of the autoimmune disease systemic lupus erythematosus (SLE). 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.

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. ERs can be activated with exogenous and endogenous estrogens. Also, there are numerous xenoestrogens that exist in the environment and imitate estrogen. Bisphenol A is an example of a xenoestrogen that is considered an endocrine disrupting compound (EDC).

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Binding of ER in immune cells by a xenoestrogen or endogenous ER 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. Hyperactivation of ERα skew the immune system from a T helper 1 (Th1) to a Th2 profile and exacerbates autoimmune diseases and allergic diseases.

Complexes formed by the binding of ERα to stressors such as estrogen or EDC transport into cell nuclei, where they activate the transcription of specific genes. Excessive ERα-activation promotes the differentiation of naive CD4+ T cells into mature Th2 cells. This pathway leads to the overproduction of the cytokine interleukin-4 (IL-4) from Th2 cells and anti-single/double-stranded DNA antibody from autoreactive B cell are increased, which results in the adverse outcome 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 activation of ERα in immune cells leading to exacerbated SLE.

Background

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. ERs are involved in a wide range of physiological function. Women generally exhibit a stronger response to a variety of antigens including ER ligands than men, which is perhaps one reason that they are more prone to develop autoimmune and allergic diseases such as SLE in greater severity than men. This AOP could be helpful to assess the type of Th2 dominant autoimmune disorders

Humans and mammals have two ligand-activated transcription factors that bind estrogen, encoded by separate genes, estrogen receptor alpha (ESR1/ERα) and estrogen receptor beta (ESR2/ERβ) (Maria, B. 2015). 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). 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). In the same data sets, human ERβ mRNA is primarily detected in the lung and testis. There is increased ERα and decreased ERβ mRNA expression in PBMCs of SLE patients (Inui A. 2007). Although ERs are widely expressed in most tissue types, including most immune cells, this AOP mainly addresses hyperactivation of ERα in immune cells.

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). This AOP describes events occurring when high levels of estrogen shift the Th1/Th2 balance toward increased Th2 activity

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1710	Activation of estrogen receptor in immune cells (https://aopwiki.org/events/1710)	Activation of estrogen receptor
	KE	1711	Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/events/1711)	Induction of GATA3 expression
	KE	1712	Overproduction of IL-4 from Th2 cell (https://aopwiki.org/events/1712)	Overproduction of IL-4
	KE	1713	Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/events/1713)	Increase of autoantibody production

Sequence	Type	Event ID	Title	Short name
	AO	1714	Exacerbation of systemic lupus erythematosus (https://aopwiki.org/events/1714)	Exacerbation of SLE

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells (https://aopwiki.org/relationships/2020)	adjacent	Induction of GATA3 expression by STAT6:ER fusion protein	Moderate	Moderate
Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/relationships/2021)	adjacent	Overproduction of IL-4 from Th2 cell	Moderate	Moderate
Overproduction of IL-4 from Th2 cell (https://aopwiki.org/relationships/2022)	adjacent	Increase of anti-single/double-stranded DNA antibody from autoreactive B cell	Moderate	Moderate
Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/relationships/2023)	adjacent	Exacerbation of systemic lupus erythematosus	Moderate	Moderate

Stressors

Name	Evidence
Estrogen	High
Bisphenol A	Moderate

Overall Assessment of the AOP

The immune system is the most complex and sophisticated in the body's defense mechanisms . Estrogen plays a role in controlling the immune balance. Hyperactivation of ERα can skew the immune system from a Th1 to a Th2 profile. This Th1/Th2 shift is one of the most important immunologic changes during gestation and occurs due to a progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress Th1-mediated responses and stimulate Th2-mediated responses (Doria A. 2006). Incidence of flare in patients with SLE is increased during pregnancy and within the 3-months postpartum (Amanda E. 2018). Thus, ERα activation can potentially induce immunoactivation-derived adverse outcomes, one effect of which could be exacerbation of SLE. The present AOP focused on ERα activation-induced exacerbation of SLE.

In general, ERα is activated when bound to a stressor, which subsequently binds to estrogen response elements (EREs) to transactivate or to suppress specific target genes. 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. We have identified a number of key events (KE) along this pathway and used these key event relationships (KER) to create an AOP that describes the activation of ERα leading to exacerbation of SLE.

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). While BPA binds in some assays with less than 2000-fold affinity compared to the binding of estradiol to estrogen receptors, it still has dramatic effects (Krishnan AV. 1993). Since each KE is quantifiable and shows similar dose responses with the stressors in vitro, the activation of ER leading to exacerbation of SLE comprise a suitable AOP. Additionally, each KER is based on sufficient scientific evidence and exhibits no contradiction with dose response of adjacent KE.

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.

Essentiality of KEs – what would be good is to have a table listing references that have demonstrated occurrence of individual KEs and their relationship with the AO.

Evidence assessment – here listing knockout or overexpression studies that intervene with a KE to show its essentiality to the AO

Quantitative assessment – if you have this informaiton

[Otsubo2]We will reconsider it and revise later.

[SH3]It seems like KE1 is not needed as it is not described much.

[Otsubo4]We want to discuss about it in WebEX meeting.

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 (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Sex Applicability

Sex	Evidence
Mixed	High

The proposed AOP describes the activation of ERα leading to exacerbation of SLE is dependent on estrogen level, which means it varies with life stage, sex, and age. SLE frequently develops or progresses when sympathoadrenomedullary and gonadal hormone levels are altered during pregnancy, the postpartum period, or menopause as well as during exposure to estrogen and includes the risk of preeclampsia or premature birth (Wilder RL. 1999, Whitelaw DA. 2008). 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).

Since stressor-induced outcomes in humans are mimicked by similar responses in rodents, Th2 dominant conditions induced by activation of ERα is considered likely to occur in a variety of mammalian species.

Essentiality of the Key Events

Stressor , MIE and later events: ER knock out (KO) mice

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).

The NZB/W F1 mouse is the oldest classical model of lupus generated by the F1 hybrid between the NZB and NZW strains. Both NZB and NZW display limited autoimmunity, while NZB/W F1 hybrids develop severe lupus-like phenotypes comparable to that of lupus patients. SLE in the NZB/W F1 strain is strongly biased toward females, and this is at least in part due to estrogen levels. Indeed, 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 cellsDNA-specific B cells, and thereby suggests a pathogenic role of estrogen in lupus (Daniel P. 2011).

In females of the lupus-prone NZB/NZW F1 strain, disruption of estrogen receptor-α (ERα or Esr1) both attenuated glomerulonephritis and increased survival. ERα deficiency also retarded development of anti-histone/DNA antibodies, suggesting that ERα promotes loss of immunologic tolerance. The presence of many autoantibodies is a hallmark of SLE. In particular, autoantibodies directed to double-stranded DNA (dsDNA) are characteristic (Isenberg DA. 2007). ERα deficiency in NZB/NZW F1 males increased survival and reduced anti-dsDNA antibodies, suggesting that ERα also modulates lupus in males (Bynote KK. 2008).

KE1 and later events: Stat6 KO mice

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)

KE1 and later events: GATA3 KO mice

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, IL-5, IL-13, and IL-10 production. Conversely, IFN-γ production was increased even under Th2 conditions (Zhu J. 2004, Pai SY. 2004).

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Appendix 1

List of MIEs in this AOP

Event: 1710: Activation of estrogen receptor in immune cells (https://aopwiki.org/events/1710)

Short Name: Activation of estrogen receptor

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	MolecularInitiatingEvent

Stressors

Name
Estrogen
Bisphenol A

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 (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

ERα is mainly expressed in uterus, prostate (stroma), ovary (theca cells), testes (Leydig cells), epididymis, bone, breast, various regions of the brain, liver, and white adipose tissue (Dahlman-Wright K. 2006). ERs is widely expressed in most tissue types including most immune cells (Couse JF. 1997). ERα and ERβ show a high degree of similarity when compered at the amino acid level (Dahlman-Wright K. 2006). Interspecies sequence identities for the entire ERα receptor 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 found in female reproductive organs, yet is robustly expressed in kidney, liver, heart, and lungs in males and females, as well as on most immune cells (Chelsea C. 2017).

Key Event Description

Estrogen receptor alpha (ERα) was discovered in the late 1960s and was cloned and characterized in 1985 (Melissa C. 2011). ERα and ERβ show a high degree of similarity when compared at the amino acid level (Dahlman-Wright K. 2006). 17β-estradiol (E2) 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). The hormone binding domain of the estrogen receptor is required not only for binding estradiol but also to form stable homodimers of the protein and mediate transcriptional activation by the receptor. A direct genomic interaction occurs between the estrogen receptor (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). In addition to above classical mechanism, ERα is also able to play roles both in ER binding and transcriptional activation; phosphorylation of ER and other proteins involved in transcriptional activation with cellular amounts of coactivators and adaptor proteins (Carolyn MK. 2001).

ERs are expressed in a variety of immunocompetent cells, including CD4+ (Th1, Th2, Th17, and Tregs) and CD8+ cells and macrophages (Salem ML. 2004, Robinson DP. 2014). One recent study examined ERα expression in resting and activated PBMC subsets and found that ERα was expressed at higher levels in CD4+ T cells than B cells (Melissa C. 2011).

How it is Measured or Detected

Recombinant human estrogen receptor hormone-binding domain (HBD) fragment is isolated from Escherichia coli. Purified HBD peptide is assayed for their ability to bind estradiol, [3H] estradiol binding using low concentrations (0.15 nM), by Radioreceptor Assay. Moreover HBD dimer dissociation is measured using size exclusion chromatography (Brandt ME. 1997).

On the other hand, a conditionally active form of STAT (the signal transducers and activator of transcription) 6 by fusing the HBD of a modified form of the mouse estrogen receptor (ER) gene is prepared as STAT6-ER fusion protein (STAT6:ER). 4-Hydroxytamoxifen (4-HT), estrogen analogue, (Research Biochemicals Institute, Natick, MA) was used to activate STAT6 fusion protein. 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 4HT. STAT6:ER DNA-binding activity is strongly and rapidly (within 1 hr) induced after addition of 4HT to these cells. BA/F3 cells prepared as the same manner are stimulated with 1 μM 4HT for 24 h at 37°C. The cells were harvested and assayed for luciferase activities using a Luciferase Assay Kit (Promega, Madison, WI). (Kamogawa et al. 1998).

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List of Key Events in the AOP

Event: 1711: Induction of GATA3 expression by STAT6:ER fusion protein (https://aopwiki.org/events/1711)

Short Name: Induction of GATA3 expression

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Involvement of GATA3 and STAT6 in Th2 cell development through ER is common in humans, rodents, and other mammalian species (Ho IC. 2009). A constitutively activated form of Stat6 introduced into CD4 T cells resulted in both Th2 differentiation and enhanced cell expansion. Stat6 is not only necessary but also sufficient to drive IL-4-mediated Th2 differentiation and cell expansion in naive CD4 T cells (Zhu J. 2001). CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under ThN conditions with added with IL-4 (Zhu J. 2001).

Key Event Description

Transcription factors are critical for Th cell differentiation and cytokine production. Cell fate determination in each lineage requires at least two types of transcription factors: the master regulators as well as the signal transducers and activator of transcription (STAT) proteins (Zhu J. 2010). The ability of STAT6: ER to induce a Th2 phenotype correlates with the induction of GATA-3 mRNA expression. 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).

How it is Measured or Detected

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. GATA-3 mRNAs can be measured using RNase protection assay in developing Th1 cells. RNase protection assay was performed with RiboQuant multiprobe kit (PharMingen) following the manufacturer's method using GATA-3. Stat6:ER Th1 cells expressed significant amounts of both GATA-3 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).

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Event: 1712: Overproduction of IL-4 from Th2 cell (https://aopwiki.org/events/1712)

Short Name: Overproduction of IL-4

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T-helper 2 cell

Organ term

Organ term
immune system

Key Event Description

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. IL4 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.

How it is Measured or Detected

Purified naive T cells were activated and infected with RV-Stat6:ER. The 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 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).

Single-cell suspensions of lymph nodes removed from BALB/c mice 7 days after priming with KLH absorbed to aluminium hydroxide adjuvant in the footpads, were prepared and cultured in vitro with KLH in the absence or presence of either BPA (0.1, 1, 10, 50 and/or 100 μM) or NP. After 4 days, the levels of IL-4 and IFN-γ in the cell supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) and mRNA levels of IL-4, IL-6 and IL-10 in the cells were assayed by reverse transcription–polymerase chain reaction (RT–PCR) (Lee MH. 2003). To evaluate the effects of exposure to BPA in adulthood, male Leishmania major– susceptible BALB/c and –resistant C57BL/6 mice were subcutaneously injected with BPA (0.625, 1.25, 2.5 and 5 μmol) dissolved in corn oil 1 week before being infected with L. major. A single cell suspension containing splenocytes from each mouse was incubated in 24-well tissue-culture plates in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were stimulated with L. major antigen (3 μg/mL) during the cultivation. Culture supernatants were collected 48 hr later. Concentrations of IL-4, IL-10, IL-13, and IFN-γ in culture supernatants were determined using CBA kits (Huimin Y. 2008).

Th2 cell-related cytokine (IL-4 and -10) in BPA (50 μM)-stimulated primary cultured mouse lymphocytes were evaluated using immunoblot analysis and reverse-transcription polymerase chain reaction (RT-PCR) (Lee et al. 2010).

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Event: 1713: Increase of anti-single/double-stranded DNA antibody from autoreactive B cell (https://aopwiki.org/events/1713)

Short Name: Increase of autoantibody production

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
B cell

Organ term

Organ term
immune system

Key Event Description

In the development of T-cell dependent antibody producing cells, the interaction between IL-4 and its receptor delivers the first signal for switching to IgE. IL-4 produced by Th2 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. The engagement of CD40 on B cells by CD154 (CD40L) expressed on T cells and DC provides the second signal required for switching to IgE.

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 et al. 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 et al. 2009).

Bisphenol-A (BPA) as well as E2 and DES enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of EDs was more prominent on aged BWF1 mice developing lupus nephritis. B1 cells from aged mice exhibited increased expression of ERα mRNA compared to young mice (Yurino H. 2004).

How it is Measured or Detected

For the detection of anti-DNA antibodies in serum of female NZB/W F1 mice administrated of the estrogen antagonist tamoxifen, enzyme-linked immunosorbent assay (ELISA) was carried out. For the quantitated of total B cells and CD5+B cells expression in spleen and in peritoneal exudates were analyzed with fluorescence activated cell sorting (FACScan) (Wu et al. 2000). For the B cell subset analysis (including immature (transitional T1 and T2) and mature (MZ and follicular)) in BALB/c R4Ag-gamma 2b transgenic mice administrated the tamoxifen were performed with FACScan (Peeva et al. 2005).

In another study, used ERα deficiency in NZB/W F1 mice, autoantibody (anti-dsDNA antibodies) development and concentration was assessed by ELISA using serum isolated from blood collected monthlyvia (Bynote et al. 2008).

Using female NZB/WF1 mice, silastic implants containing the powdered form of endocrine disruptors were placed subcutaneously on the back of ovariectomized mice, and 3 to 4 months blood samples were collected peritoneal. 4 months after implantation, peritoneal lavage cells and splenic cells were obtained from mice. Anti-DNA antibody was measured in ELISA using ssDNA for the culture supernatant of and dsDNA for the serum. To examine the effect of EDs on autoantibody production by B1 cells, a PFC assay using autologous bromelain-treated erythrocytes (Br-RBC) was conducted. To evaluate autoantibody (IgG) production including plaque forming cell (PFC) assay for anti-RBC Ab. It has been reported

that B1 cells produce autoantibody against phosphatidylcholine expressed on bromelain-treated red blood cells (Br-RBC) using PFC assay (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 (E2: 100 nM, DES: 100 nM, BPA: 1 μM) for 4 days. The amount of total IgM and IgM anti-DNA Ab in the culture supernatant was measured by ELISA. Expression level of ERα and ERβ genes in B

cells was examined by RT-PCR and quantitative real-time PCR analysis (Yurino H. 2004).

For the investigate the in vitro effects of 17β-estradiol (E2) on spontaneous immunoglobulin production by human PBMCs, PBMCs from healthy human volunteers were cultured with E2. Levels of IgG and IgM and cytokine activity were measured by ELISA. Proliferation was determined by [3H]-thymidine uptake. The cell viability was assessed by a trypan blue exclusion test (Kanda et al. 1999).

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List of Adverse Outcomes in this AOP

Event: 1714: Exacerbation of systemic lupus erythematosus (https://aopwiki.org/events/1714)

Short Name: Exacerbation of SLE

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

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 et al., 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 et al., 2014). The incidence of SLE is markedly increased in females of child-bearing age (Grainne et al., 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 systemic lupus erythematosus (SLE) tend to worsen during pregnancy (Doria et al., 2006).

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 immune complex deposition. Epstein– Barr virus (EBV) has been identified as a possible factor in the development of lupus. Over 100 drugs have been reported to cause drug-induced lupus (DIL), including a number of the newer biologics and antiviral agents. Although the pathogenesis of DIL is not well understood, these drugs may alter gene expression in CD4+ T cells by inhibiting DNA methylation and induce over-expression of lymphocyte function-associated antigen 1, thus promoting autoreactivity. Generally, sunlight is the most obvious environmental factor that may exacerbate SLE. High estrogen levels and BPA-induced ER activation skewed T cells toward a Th2 phenotype, thereby inducing hyperactivity by B-cells, which leads to exacerbation of SLE. T cell dysfunction is a characteristic of SLE, which is also associated with high levels of autoantibodies (Crispin et al. 2010).

How it is Measured or Detected

Most of the mouse models of lupus produce autoantibodies and develop immune complex glomerulonephritis. For the disease onset, mice can monitor by proteinuria levels, body weights, blood urea nitrogen and appearance over time. Additionally, serum levels of anti-dsDNA, anti-glomerular antigens (GA), total IgG can measure by ELISA. (Gabriela et al., 2019, Yurino et. al.,2004, John et. al.,2008, Wang et. al.1996).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2020: Activation of estrogen receptor leads to Induction of GATA3 expression (https://aopwiki.org/relationships/2020)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

Stressors bind to the ERs in immune cells, a ligand-activated transcription factor that regulates transcription of target genes in the nucleus or located in or adjacent to the plasma membrane (Deroo BJ. 2006). ERα is a nuclear hormone transcription factor that classically binds ligand stressors estrogen or EDC, further stabilizing dimers that subsequently bind estrogen response elements to transactivate or suppress specific target genes.

Evidence Supporting this KER

Biological Plausibility

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). Naive Th cells primed by BPA/TNF-α-matured DCs differentiated into Th2 cells with characteristically high IL-5/IFN-γ, IL-10/IFN-γ, and IL-13/IFN-γ ratios. However, the IFN-γ production was not affected at all, thus indicating that

Th2 bias was induced by enhanced Th2 cytokine production (Guo H. 2010, Uemura Y. 2008). Also, dendritic cells exposed to BPA (100 nM) and TNF-α produced high levels of IL-10 relative to IL-12, and this induced Th2 deviation (Liu Y. 2009).

Uncertainties and Inconsistencies
Dendritic cells exposed to human exposure-relevant concentrations of BPA (10-100 nM) preferentially skewed T cells toward a Th2 phenotype. Th cells were primed by BPA/TNF-α-DCs. The administration of 17β-estradiol enhanced the differentiation of dendritic cells and increased IFN-γ production by dendritic cells in C57BL/6 mice.

Quantitative Understanding of the Linkage

Response-response relationship
When estrogen levels are low, T cell expansion shifts toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and experimental autoimmune encephalomyelitis (EAE) rather than SLE.

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 et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, while high doses (>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 model (Bebo et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

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 et al. 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 et al. 2006).

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Relationship: 2021: Induction of GATA3 expression leads to Overproduction of IL-4 (https://aopwiki.org/relationships/2021)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

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).

Evidence Supporting this KER

Biological Plausibility

IFN-γ is noticeably reduced in pregnant women compared with non-pregnant women or in response to high levels of estrogen (Kruse et al. 2000). Thus, pharmacological or pregnancy levels of estrogen may skew the immune system from a Th1 to a Th2 profile (Ebru et al. 2011). 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). 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 MH. 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 et al. 2008).

Empirical Evidence

The proliferation of Stat6:ER Th1 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).

Uncertainties and Inconsistencies

The essential transcription factors of Th2 are GATA-3 and STAT5. Activation of GATA-3 and STAT5 induce IL-4 production in naïve CD4 T cells. IL-4-mediated STAT6 activation promotes Th2 differentiation (Kaplan MH. 1996, Shimoda K. 1996, Takeda K. 1996).

Quantitative Understanding of the Linkage

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 disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). 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 et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, 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 et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

IL-4 may serve multiple roles in the development of lupus: it may enhance autoantibody production via its direct B-cell effects, protect against autoimmunity via its T-cell suppressor effect, or perpetuate tissue damage via its direct effects on target organs (Ram Raj Singh 2003).

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).

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Relationship: 2022: Overproduction of IL-4 leads to Increase of autoantibody production (https://aopwiki.org/relationships/2022)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

The receptor for IL-4 is IL-4Rα, which expresses in B cells. Th2 cells secrete cytokines IL-4 that upregulate antibody formation via B cells. Naive B cells that have not yet encountered antigen express immunoglobulin M and immunoglobulin D on their surface. During an immune response, B cells can express different immunoglobulin heavy chain isotypes sharing the same variable–diversity–joining (VDJ) region. This isotype-switching recombination allows a B-cell clone to produce antibodies with the same specificity for antigens but with different effector functions. To switch to a particular isotype, a B cell needs two signals, one cytokine-dependent and the other CD40-dependent. In B cells, estrogen-mediated events could occur through the CD40/CD40L costimulatory pathway. Estrogen can also enhance differentiation of immature DCs into mature functional DCs and regulate the expression of cytokines and chemokines such as IL-6, IL-10, CXCL8, and CCL2 (Liu Y. 2009, Guo H. 2010). This increase the number of B cells producing autoantibodies.

Evidence Supporting this KER

Biological Plausibility
Lack of ERα, in either male or female mice, did not increase B cell precursors (Smithson G. 1998).

Estrogen upregulates CD40L on B and T cells from SLE patients (Desai-Mehta A. 1996, Li X. 2006), and CD40L expression on B cells is increased two-fold in SLE patients (Díaz-Alderete A. 2004). Whereas 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). It is possible that this estrogen modulated elevation in CD40/CD40L crosstalk as well as stimulation via CD86 synergizes in the exacerbation of SLE by promoting autoantibody secretion as well as activation of T cells (Karpuzoglu E. 2011). 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 et al. 2004).

Direct exposure of PBMCs from SLE patients to 17β-estradiol induces secretion of anti-dsDNA antibodies and enhances the secretion of Igs, in particular IgG (Kanda et al. 1999).

Empirical Evidence
CD23 on M12.4.1 cells, transfected with the luciferase reporter gene by inserting three copies of human STAT6 binding site oligonucleotide, is up-regulated with treatment1 μM 4HT for 16 hr (Kamogawa et al. 1998).

The production of IgA and IgG2a was increased in B cells from mice fed BPA (Goto et al. 2007). Similarly, in mice exposed prenatally to BPA and then immunized in adulthood with hen egg lysozyme (HEL), the anti-HEL IgG2a measured three weeks later was elevated (Yoshino et al. 2004). These Ig can be measured by ELISA. 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 et al. 2000). Tamoxifen Blocks Estrogen-Induced B Cell Maturation but not survival (Peeva et al. 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 et al. 2008).

Quantitative Understanding of the Linkage

Response-response relationship
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 disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). 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 et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, 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 et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

Time-scale
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).

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Relationship: 2023: Increase of autoantibody production leads to Exacerbation of SLE (https://aopwiki.org/relationships/2023)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of estrogen receptor in immune cells leading to exacerbation of systemic lupus erythematosus (https://aopwiki.org/aops/314)	**adjacent**	**Moderate**	**Moderate**

Key Event Relationship Description

SLE patients appear to produce significant amounts of the anti-double-stranded DNA (anti-dsDNA) autoantibodies that cause the disease. 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 et al., 1991). 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 (Carlsten H. 1992).

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. 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).

Murine lupus models such as NZB×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 (Zhang DH. 1997, Pai SY. 2004). All of these species of mice develop immunoglobulin G (IgG) 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. The administration of the estrogen antagonist tamoxifen diminishes immune complex deposition in the kidneys and increases survival. Renal disease was evaluated by the development of albuminuria and histological changes in the kidney (Wu et al. 2000).

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).

Empirical Evidence

Estrogen enhances anti-double-stranded DNA antibody and IgG, IgM production by PBMCs. PBMCs or B cells were cultured for 7 days with E2 (10^{-8} mol/L). Theamounts of total IgG and IgM in the supernatants were measured by ELISA. Proliferative responses PBMCs or B cells were measured by [3H]-thymidine (Kanda N. 1999).

Quantitative Understanding of the Linkage

Response-response relationship

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 disease such as multiple sclerosis (MS) and EAE rather than SLE.

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham and Gilkeson, 2011). 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 et al. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated disease, 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 et al. 2001). High levels of estrogen during pregnancy have been reported to ameliorate T cell mediated diseases such as multiple sclerosis (Korn-Lubetzki et al. 1984).

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 et al. 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., et al. 2006).

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AOP ID and Title:

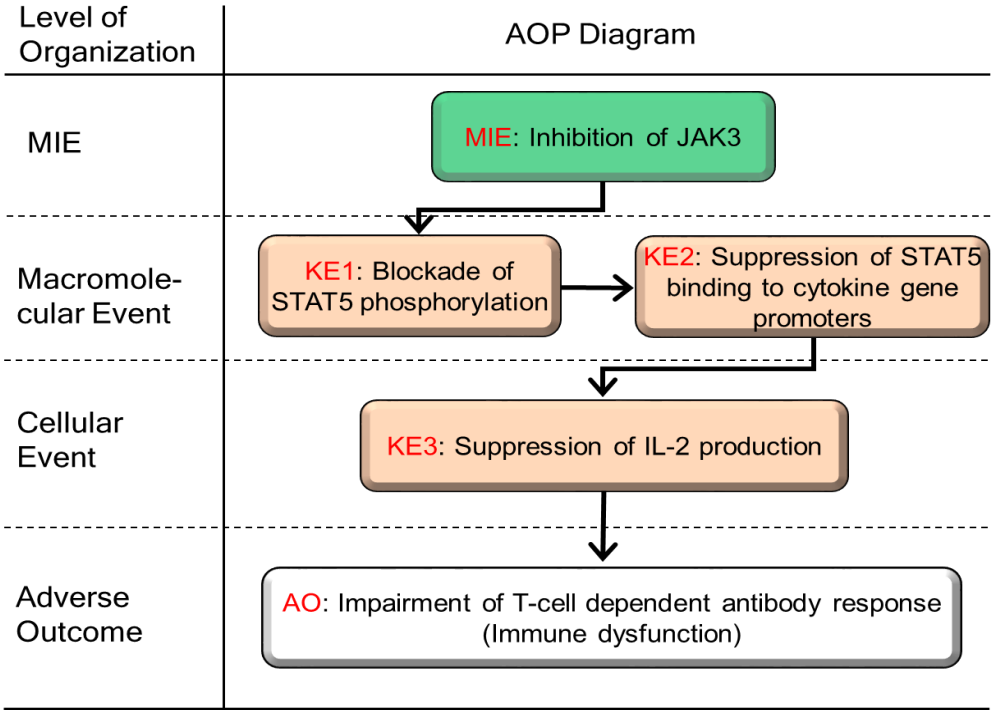
SNAPSHOT

Created at: 2020-05-18 09:23

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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Abstract

Signal transduction between immune-related cells depends in many cases on cytokines and takes place via 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 receptors for cytokines require and activation step through a Janus-kinase (JAK) Signal Transducers and Activator of Transcription (STAT) system. When cytokine binds to its specific cytokine receptors, the cytokine receptors form dimers, which more closely resemble the JAK molecules. The JAK then activates to phosphorylate adjacent cytokine receptors. STATs bind to the phosphorylated sites of the receptors and

are then phosphorylated by the activated JAK. The phosphorylated STAT is dimerized to be translocated into nucleus and bind to promoter regions of cytokine genes, which starts transcription of cytokine genes in the nucleus.

In mammals, four JAK families of enzymes (JAK1, JAK2, JAK3, TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, 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 and have a critical function that evokes itchiness. Because these cytokines also interact with JAK, several JAK-inhibitors have received a lot of attention recently as a therapeutic agent for major inflammatory diseases and pruritic diseases. However, immunotoxic events due to inhibition of the JAK pathway have yet to be examined.

This AOP focuses on the inhibition of JAK3, which is required for signal transduction by cytokines through the common gamma (γ) chain of the interleukin receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. This AOP proposes JAK3 inhibition as an MIE that leads to suppression of T cell-dependent antibody response (TDAR) as an AO. TDAR is frequently affected under immunosuppressive conditions and is a major endpoint in many preclinical immunotoxicity studies. In this proposed AOP, JAK3 selective inhibitors (e.g. PF-06651600, RB1) are stressors, blockade of STAT5 phosphorylation is KE1, suppression of STAT5 binding to the promoter regions of cytokine genes is KE2, and subsequent suppression of IL-2 production is KE3.

Background

Although there are numerous stressors that inhibit JAK3 activity, this AOP is based on immunosuppression caused by recently developed, highly selective JAK3 inhibitors PF-06651600 and RB1, about 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 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 (https://aopwiki.org/events/1715)	Inhibition of JAK3
	KE	1716	Blockade of STAT5 phosphorylation (https://aopwiki.org/events/1716)	STAT5 inhibition
	KE	1717	Suppression of STAT5 binding to cytokine gene promoters (https://aopwiki.org/events/1717)	Suppression of STAT5 binding
	KE	1718	Suppression of IL-2 production (https://aopwiki.org/events/1718)	Suppression of IL-2 production
	AO	1719	Impairment of T-cell dependent antibody response (https://aopwiki.org/events/1719)	Impairment, TDAR

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition of JAK3 (https://aopwiki.org/relationships/2024)	adjacent	Blockade of STAT5 phosphorylation	High	High
Blockade of STAT5 phosphorylation (https://aopwiki.org/relationships/2025)	adjacent	Suppression of STAT5 binding to cytokine gene promoters	High	High

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Suppression of STAT5 binding to cytokine gene promoters (https://aopwiki.org/relationships/2026)	adjacent	Suppression of IL-2 production	High	High
Suppression of IL-2 production (https://aopwiki.org/relationships/2027)	adjacent	Impairment of T-cell dependent antibody response	High	High

Overall Assessment of the AOP

Janus kinases (JAKs) are a family of nonreceptor tyrosine kinase and consists of four members: JAK1, JAK2, JAK3, and Tyk2 (1). All four members 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 (2). Previous studies with IL-2Rγ-null mice showed that JAK3 is related to the development of spontaneous IBD symptoms (3). Moreover, abnormal activation of JAK3 was associated with human hematological (4), indicating that a tight balance of its activity was essential for normal hematopoietic development.Janus kinases (JAKs) are a family of nonreceptor tyrosine kinase and consists of four members: JAK1, JAK2, JAK3, and Tyk2 (1). Different studies have shown that JAK3 is widely expressed in different organs (2). Previous studies with IL-2Rγ-null mice showed that JAK3 is related to the development of spontaneous IBD symptoms (3). Moreover, abnormal activation of JAK3 was associated with human hematological (4), indicating that a tight balance of its activity was essential for normal hematopoietic development.

Although JAK1, JAK2, and Tyk2 are each widely expressed, JAK3 is predominantly expressed in hematopoietic cells and is known to associate only with the common γ (γc) chain of the interleukin-2 (IL-2), IL-4, IL-7, IL-9, and IL-15 receptors (5). Homozygous mutant mice in which the JAK3 gene had been disrupted were generated by gene targeting. JAK3-deficient mice had profound reductions in thymocytes and severe B cell and T cell lymphopenia similar to severe combined immunodeficiency disease (SCID), and the residual T cells and B cells were functionally deficient. Thus, JAK3 plays a critical role in γc 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 (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)

Sex Applicability

Sex	Evidence
Mixed	High

This proposed AOP involves inhibition of JAK activity leading to suppression of TDAR and is not dependent on life stage, sex, or age. Since JAK3 inhibitors (PF-06651600, RB1) are currently under a phase 2 clinical evaluation to treat rheumatoid arthritis, the AOP appears to be applicable to all life stages. Since JAK3 inhibitor-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 JAK3 activity is considered to occur across a variety of mammalian species.

Essentiality of the Key Events

MIE and later events: JAK3-knockout (KO) mice

JAK3 was initially identified (1,2) in studies to identify the JAK family member that was involved in the signaling of a group of cytokines that shared in common the utilization of the γc chain first identified in the interleukin 2 (IL-2) receptor complex. It was subsequently demonstrated that JAK3 physically associates with the γc chain and is activated in a receptor complex that also contains JAK1, which associates with the ligand specific alpha or beta chain of the receptors (3). JAK3 is somewhat unique within the JAK family in that it is predominantly expressed in

hematopoietic cells and is only activated in the responses to cytokines that use the γc chain (4). The phenotype of the JAK3 deletion mice was quite striking and consisted of a range of deficiencies which collectively constituted SCID (5,6). At the same time, two groups identified individuals that lacked JAK3 and exhibited somatically acquired SCID (7,8). One of the most striking components of the phenotype is the dramatic reduction seen in both the T and B cell lineages. Comparable reductions are seen in mice that lack IL-7 (9), the IL-7 receptor alpha chain (10), or the γc chain. In spite of the reduced numbers, the cells that do develop are phenotypically normal. These results are consistent with the hypothesis that activation of JAK3 give it a critical role in the expansion but not the 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 γc chain and activate JAK3 normally.

Stressor: B6.Cg-Nr1d1tm1Ven/LazJ mouse

The B6.Cg-Nr1d1tm1Ven/LazJ mouse line harbors a spontaneous mutation in JAK3, which generates an SCID phenotype with an inability to generate antigen-independent professional cytokine-producing innate lymphoid cells (ILCs). Mechanistically, JAK3 deficiency blocks ILC differentiation in the bone marrow at the ILC progenitor (ILCP) and the pre-NK cell progenitor (pre-NKP). Similar phenomenon was further demonstrated by the pan-JAK inhibitor tofacitinib and specific JAK3 inhibitor PF-06651600. Both JAK-inhibitors impair the ability of human intraepithelial ILC1 (iILC1) to produce IFN-γ, without affecting ILC3 production of IL-22. Both inhibitors impaired the proliferation of iILC1 and ILC3 and differentiation of human ILC in vitro. These findings indicate that JAK3 deficiency blocks innate lymphoid cell development (11).

KE1 and later events: STAT5-KO mice

STAT5 plays a major role in regulating vital cellular functions such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells (12,13). STAT5 is activated by phosphorylation of a single tyrosine residue (Y694 in STAT5) and 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 (14).

The following phenotypes are observed in STAT5-KO mice:

The transcription factor STAT5 is expressed in all lymphocytes and plays a key role in multiple aspects of lymphocyte development and function (15). STAT5 was initially identified as a transcription factor activated by prolactin in mammary gland epithelial cells (16,17). Subsequent studies identified STAT5 binding activity in T cells (18), and it was later established that STAT5 was expressed in multiple cell types and activated by a number of cytokines, including the common gamma chain (γc)-dependent cytokines interleukin 2 (IL2), IL4, IL7, IL13, and IL15 (19).

STAT5 in T-cell development

The observation that STAT5 is activated by multiple cytokines in T cells suggested that it might play a critical role in the development or function (or both) of these cells. Disruption of Stat5a or Stat5b genes alone resulted in relatively modest phenotypes; for example, Stat5a-/- mice had defects in mammary gland development and lactation while Stat5b-/- mice had defects in response to growth hormone in male mice and natural killer cell proliferation (20,21). 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 that acted as functional hypomorphs. These mice too had surprisingly mild defects in lymphocyte development, although T cells were grossly dysfunctional, as they could no longer proliferate in response to IL2 (22,23). 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 (24,25). These studies definitively demonstrated that the STAT5 hypomorph mice retained significant STAT5 function.

Weight of Evidence Summary

Biological Plausibility

T-cell development is mainly regulated by JAK-STAT system, and JAK3 deficiency in T cells is known to induce multiple types of immunosuppression, including T cell-dependent antibody response (TDAR).

JAK3-deficient mice had profound reductions in thymocytes and severe B cell and T cell lymphopenia similar to SCID disease, and the residual T cells and B cells were functionally deficient (10).

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-2. 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 (5).

Furthermore, the abnormal architecture of lymphoid organs suggested the involvement of JAK3 in the function of epithelial cells. T cells developed in the mutant mice did not respond to either IL-2, IL-4, or IL-7 (26).

Specific JAK3 inhibitor PF-06651600 or RB1, which selectively inhibited JAK3 with an over 100-fold preference over JAK2, JAK1, and TYK2 in the kinase assay, displayed reduced inflammation and associated pathology in collagen-induced-arthritis mice. Importantly, with PF-06651600 or RB1 administration, pro-inflammatory cytokines and JAK3 and STATs phosphorylation decreased in mice, suggesting that the inhibition of JAK3/STAT signaling was closely correlated with induction of multiple types of immunosuppression, including TDAR .

Quantitative Consideration

KER1 (MIE=>KE 1)

Interleukin 2 (IL-2) activated STAT5 via distinct pathways (30).

IL-2 have been demonstrated to stimulate STAT5 and induce tyrosine phosphorylation of STAT5. Treatment of highly selective JAK3 inhibitors

(PF-06651600 or RB1) treatment clearly suppresses the complex formation of STAT5 in the nucleus.

Highly-selective JAK3 inhibitor RB1 inhibited the phosphorylation of STAT5 elicited by IL-2 at IC50 value of 31 nM in the raw peripheral blood mononuclear cells (PBMCs) of humans. PBMCs were isolated from the buffy coats of healthy volunteers using density gradient centrifugation on Lymphoprep. Cells were cultured in complete RPMI 1640 medium (containing 10% foetal bovine serum, 100 mg/ml streptomycin and 100 U/ml penicillin) plus 10 µg/ml lectin phytohemagglutinin (PHA) for 3 days and then treated with either recombinant human IL-6 (400 ng/ml), recombinant human IL-2 (100 ng/ml), or recombinant human GM-CSF (50 ng/ml) at 37 °C for 20 min. To terminate the stimulation, cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 minutes; cells were incubated with anti-pSTAT3 and anti-CD4 Abs, or anti-pSTAT5 and anti-CD4 Abs at 4 °C overnight, washed twice with PBS, and analysed with an flow cytometer (31).

Fluorescence intensity for phospho-STAT5 in CD3-positive lymphocytes increased 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. Paralleling results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2, but peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 127 nM in human lymphocytes (26).

KER2 (KE1 =>KE 2)

IL-2 activated STAT5 (30).

IL-2 have been demonstrated to stimulate STAT5 and induce tyrosine phosphorylation of STAT5. These IL-2-induced STATs have an identical DNA binding specificity and immunoreactivity.

KER3 (KE2 =>KE 3)

IL-2 activated STAT5 (30)

IL-2 have been demonstrated to stimulate STAT5 and induce tyrosine phosphorylation of STAT5. These IL-2-induced STATs have an identical DNA binding specificity and immunoreactivity.

Gel mobility shift assay showed that IL-2 activation induced STAT5 dimerization and DNA binding to gamma interferon-activated site (GAS) motif in IL-2 promoter region (32).

KER4 (KE3 =>AO)

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 (33). In addition, cynomolgus monkeys treated with CsA showed suppression of IL-2 and TDAR using sheep red blood cells with a dose dependent manner (34).

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 (35).

These results show the quantitative relationships between the inhibition of IL-2 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 (https://aopwiki.org/events/1715)

Short Name: Inhibition of JAK3

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 (https://aopwiki.org/aops/315)	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Molecular

List of Key Events in the AOP

Event: 1716: Blockade of STAT5 phosphorylation (https://aopwiki.org/events/1716)

Short Name: STAT5 inhibition

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 (https://aopwiki.org/aops/315)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Event: 1717: Suppression of STAT5 binding to cytokine gene promoters (https://aopwiki.org/events/1717)

Short Name: Suppression of STAT5 binding

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 (https://aopwiki.org/aops/315)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Event: 1718: Suppression of IL-2 production (https://aopwiki.org/events/1718)

Short Name: Suppression of IL-2 production

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 (https://aopwiki.org/aops/315)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

List of Adverse Outcomes in this AOP

Event: 1719: Impairment of T-cell dependent antibody response (https://aopwiki.org/events/1719)

Short Name: Impairment, TDAR

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 (https://aopwiki.org/aops/315)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

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 (https://aopwiki.org/relationships/2024)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	**adjacent**	**High**	**High**

Relationship: 2025: STAT5 inhibition leads to Suppression of STAT5 binding (https://aopwiki.org/relationships/2025)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	**adjacent**	**High**	**High**

Relationship: 2026: Suppression of STAT5 binding leads to Suppression of IL-2 production

(https://aopwiki.org/relationships/2026)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	**adjacent**	**High**	**High**

Relationship: 2027: Suppression of IL-2 production leads to Impairment, TDAR (https://aopwiki.org/relationships/2027)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response (https://aopwiki.org/aops/315)	**adjacent**	**High**	**High**