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OECDプロジェクトでの成果物を厚生労働行政に反映させるための研究

令和３年度 分担研究報告書

免疫毒性のAOP及びTG開発

研究分担者　足利太可雄
国立医薬品食品衛生研究所　安全性生物試験研究センター　安全性予測評価部　室長

研究要旨

日本免疫毒性学会 AOP 検討小委員会とともに、免疫毒性に関する 5 種の有害性発現経路（AOP: Adverse Outcome Pathway）の開発を行った。

昨年度外部レビューに進んだ「Inhibition of Calcineurin Activity Leading to impaired T-Cell Dependent Antibody Response (AOP154)」については、全ての評価プロセスが終了し、OECD Library において公開された。

AOP313, 314 及び 315 については、コーチ制による内部レビューを受けており、そのうち AOP315 については内部レビューが終了し、今後専門家による外部レビューに進む予定である。

本年度より新たに取り組むこととなった AOP277 については、external review に相当する scientific review に進んでいる。

A. 研究目的

免疫毒性は化学物質の安全性を評価するうえで重要な項目であり、その複雑さから IATA (Integrated Approaches to Testing and Assessment）による総合的な評価が必要と考えられている。IATA 確立のためには、免疫毒性に関する複数の AOP (Adverse Outcome Pathway)を開発し、ネットワーク化する必要がある。免疫毒性に関する各種 AOP を日本主導で開発することにより、将来の IATA 開発に大きく貢献するだけでなく、当該分野における日本の活動を世界にアピールすることを目的とする。

B. 研究方法

日本免疫毒性学会会員をメンバーとする同学会試験法委員会 AOP 検討小委員会に免疫毒性 AOP の開発を委託している（研究分担者も本委員会のメンバー）。文献調査の結果に基づいて、MIE (Molecular Initiating Event)、AO (Adverse Outcome)及びその間に介在する KE (Key Event)を定めて、OECD に指定された外部（または scientific）レビュアー及びコーチの指摘事項に対応することで開発を進めた。

（倫理面への配慮）
該当なし

C. 研究結果

「カルシニューリン阻害による T 細胞依存的抗体産生抑制 : AOP154」については、WNT/WPHA に提出したのち、ドイツからコメントがあり、AOP Wiki の改訂作業を行った。ドイツからの主な指摘は、TDAR (T cell Dependent Antibody Response)アッセイ

の方法をガイドラインごとに詳細に記載すること、本 AOP のみで免疫毒性試験が免除されることはなく、本 AOP は IATA 開発に利用されるべきであること、本 AOP の EU 地域での規制上の重要性についても記載することなどであった。指摘事項に対応し、OECD 事務局に改訂完了の連絡と著者回答ファイルを提出したところ、OECD 事務局から、本 AOP が WNT/WPHA で承認されたという通知があった。その後 OECD 事務局に著作権譲渡に関する著者全員の署名書類を提出し、2021 年 10 月 15 日に OECD iLibrary において公開された。

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

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

「JAK3 の阻害による T 細胞依存的抗体産生抑制 : AOP315」については、コーチに改訂完了の連絡と著者回答ファイルを提出し、その後コーチコメント受領した。コーチコメントに対する AOP Wiki 改訂作業を行い、再びコーチに改訂版を提出した。コーチからの主な指摘は、本 AOP の AO が AOP154 の AO と類似しているので同じイベントの場合は AOP154 の AO（KE984）にリンクさせ、別のイベントを作成したい場合は全体を修正すること、KER3 の定量的考察はデキサメタゾンがどのように STAT5DNA 結合を阻害するかをより詳細に説明すること、biological plausibility of KERs, essentiality of KEs 及び empirical support for KERs についてハンドブックに従ってサマリーテーブルを加えることなどであった。OECD より、外部レビューに入れる AOP があればチェックリストを提出して欲しいとの連絡を受けたコーチが、2021 年 11 月 8 日にチェックリストを OECD に提出したことから、今後外部レビューに進むことが想定される。AOP154 の場合外部レビュアーが決まってから OECD に承認されるまで約 1 年半かかっており、AOP315 も同程度の期間内の承認を目指す。

「IL-1 receptor 結合阻害 : AOP277」については、今年度より本分担研究に追加されたものであり、AOP 開発の引継ぎに関する web 会議を実施し、本 AOP の開発者である東北大学の相場節也先生、木村裕先生から説明を受けた。さらに Scientific レビュアーとの web 会議が行われ、review 結果について説明を受けた。その後 OECD から scientific review report を受領した。Review report における主な推奨事項は、IL-1R シグナルを阻害するストレッサーに特異抗体だけでなく化合物/医薬品を加えること、AP-1 など NF-kB が関与しない経路も考慮すること、T cell のタイプを明確にすること、増加する感染のタイプを明確にすることなどであった。現在対応案を作成中である。

D. 考察

開発中の AOP については、コーチ及び scientific reviewer のコメントに基づいて修正を行っている。IL-23 の機能の説明のよ

うに既存の情報を収集することで対応できるものもあるが、医薬品だけでなく化学物質のストレッサーを示すことなど情報がないものについては調査したことを示して納得いただく必要がある。また、AO が疾患の憎悪である AOP については、行政活用の観点からその汎用性に疑問が投げかけられており、すでに承認された AOP154 のように AO を TDAR (T cell Dependent Antibody Response)に変更するなど対応を検討する。今回 OECD に承認された AOP154 の KE の一つは IL-2 産生抑制であり、本年度日本から別途 OECD に提案した免疫毒性スクリーニング試験 IL-2 Luc assay のテストガイドライン化の理論的基盤となるため、その意義は大きいと考える。

E. 結論

AOP154 については全ての評価プロセスが終了し、OECD iLibrary において公開された。AOP313, 314 及び 315 については、コーチ制による internal review を受けており、そのうち AOP315 については今後外部レビューに進む予定である。本年度より新たに担当することとなった AOP277 については、external review に相当する scientific review に進んでいる。

F. 添付資料

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2. AOP154_AOP approved by WNT and WPHA
3. AOP313-2022-01-12
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測モデルの開発,日本薬学会第 142
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H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

OECD Series on Adverse Outcome Pathways No. 18

Adverse Outcome Pathway on inhibition of calcineurin activity leading to impaired T-cell dependent antibody response

Hiroyuki Komatsu,
Junichiro Sugimoto,
Ken Goto,
Kiyoshi Kushima,
Naohisa Tsutsui,
Shigeru Hisada,
Shiho Ito,
Tadashi Kosaka,
Takumi Ohishi,
Yasuharu Otsubo,
Yoshihiro Takahashi

https://dx.doi.org/10.1787/3c988dde-en

Adverse Outcome Pathway on Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response

Series on Adverse Outcome Pathways No. 18

AOP No. 154 in the AOP Wiki platform

Foreword

This Adverse Outcome Pathway (AOP) on Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response, has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Party of the National Coordinators for the Test Guidelines Programme (WNT). The AOP has been reviewed internally by the EAGMST, externally by experts nominated by the WNT, and has been endorsed by the WNT and the Working Party on Hazard Assessment (WPHA) on 22 July 2021.

Through endorsement of this AOP, the WNT and the WPHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The OECD's Chemicals and Biotechnology Committee agreed to declassification of this AOP on 7 October 2021.

This document is being published under the responsibility of the OECD's Chemicals and Biotechnology Committee.

The outcome of the internal and external reviews are publicly available respectively in the AOP Wiki and the eAOP Portal of the AOP Knowledge Base at the following links: [internal review] [external review].

Authors:

Hiroyuki Komatsu (1) Junichiro Sugimoto (1) Ken Goto (1) Kiyoshi Kushima (1) Naohisa Tsutsui (1) Shigeru Hisada (1) Shiho Ito (1) Tadashi Kosaka (1) Takumi Ohishi (1) Yasuharu Otsubo (1) Yoshihiro Takahashi (1)

(1) AOP Working Group, Testing Methodology Committee, The Japanese Society of Immunotoxicology

Corresponding author: Takumi Ohishi (*ohishi@bozo.co.jp*)

Abstract

Calcineurin (CN), a protein phosphatase, is known to impair immune function when its phosphatase activation is inhibited. The relationship between CN and immune functions is well understood, and immunosuppressants that work by inhibiting CN have been developed.

CN inhibitors (CNIs) inhibit CN phosphatase activity to suppress many kinds of immune functions and have been used to prevent hyper immune reactions such as rejection and graft versus host disease (GVHD), and treat autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CNIs are reported to induce immunosuppression-derived adverse effects such as increased frequency and/or severity of infections and increased tumor incidences. CNIs might affect several T-cell derived immune functions to induce compromised host. Among the affected immune functions, T-cell dependent antibody response (TDAR) is an important factor to resist infections and thought to be the useful endpoint on evaluating immunotoxicity of chemicals; therefore, this AOP describes the linkage between the inhibition of CN activity and impairment of TDAR.

CN activity is inhibited when stressors bind to CN with their respective immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes with activator protein-1 (AP-1) that bind at the site of IL-2, IL-4 and other T cell -derived cytokine promoters is reduced, thereby suppressing production of these cytokines. Among the affected cytokines from each of the helper T cell subsets, reduced production of IL-2 and IL-4 affects the proliferation and differentiation of B-cells to suppress TDAR.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for inhibition of CN activity leading to impaired TDAR.

Since CN is produced in a vast variety of species, this AOP might be applicable to many mammal species, including humans and rodents.

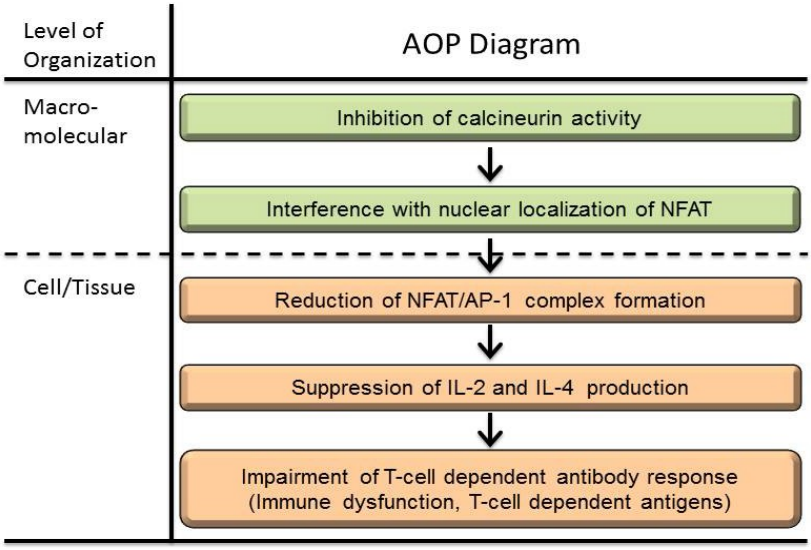
Contents

Background

Although there are other stressors that inhibit CN activity, this AOP is mainly based on an understanding of immunosuppression caused by the complex of FK506 and FKBP12 and cyclophilin and CsA, on which a significant body of scientific literature has been published.

We look forward to future amendments to this AOP with up-to-date information on other stressors, which will more clarify the linkage between inhibition of CN activity and impairment of TDAR.

Graphical Representation



Summary of the AOP

Events

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

Sequence	Type	EventID	Title	Short name
1	MIE	980	Inhibition, Calcineurin Activity (https://aopwiki.org/events/980)	Inhibition, Calcineurin Activity
2	KE	979	Interference, nuclear localization of NFAT (https://aopwiki.org/events/979)	Interference, nuclear localization of NFAT
3	KE	981	Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/events/981)	Reduction, NFAT/AP-1 complexformation
4	KE	1202	Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)	Suppression, IL-2 and IL-4production
5	AO	984	Impairment, T-cell dependent antibody response (https://aopwiki.org/events/984)	Impairment, T-cell dependent antibody response

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Calcineurin Activity (https://aopwiki.org/relationships/1508)	adjacent	Interference, nuclear localization of NFAT	Moderate	Moderate
Interference, nuclear localization of NFAT (https://aopwiki.org/relationships/1017)	adjacent	Reduction, NFAT/AP-1 complex formation	High	High
Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/relationships/1509)	adjacent	Suppression, IL-2 and IL-4production	High	High
Suppression, IL-2 and IL-4 production (https://aopwiki.org/relationships/1510)	adjacent	Impairment, T-cell dependentantibody response	High	High

Stressors

Name	Evidence
Tacrolimus (also FK506)	High
Cyclosporin	High
Pimecrolimus	High
Gossypol	Moderate
Kaempferol	Moderate
Dodecylbenzene sulfonate	Moderate
Dibefurin	Moderate
Ascomycin	Moderate
1,5-dibenzoyloxymethyl-norcantharidin	Moderate

Overall Assessment of the AOP

Inhibition of CN might induce suppression of cytokines production from all the T helper cell subsets as well as other immune functions of other immune cells. Suppression of cell-mediated immunity is involved in the pharmacology of preventing hyper immune reactions such as rejection and GVHD, and treatment of autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CN inhibition might induce immunosuppression-derived adverse outcomes. One of the effects is increased frequency and/or severity of infections. Compromised host might be related with impairment of multiple immune functions; however, impaired TDAR deems to be usually related. Moreover, TDAR is the frequently used measurable endpoint in immunotoxicity testing according the ICH S8 or US EPA OPPTS 870.7800 immunotoxicity testing guideline. Therefore, the present AOP focus on CN inhibition-induced impairment of TDAR.

When stressors bind to calcineurin-A (CnA) with immunophilin, CN phosphatase activity is inhibited. Immunophilins are composed of a family of highly conserved proteins that have the ability to bind immunosuppressive drugs. This interfere with the nuclear localization of NFAT, the substrate for CN. As a result, the formation of functional NFAT/ AP-1 complexes that bind at the site of IL-2, IL-4 and other cytokine promoters in each of the T helper cell subsets is reduced, thereby suppressing production of these cytokines. Among the affected cytokines TDAR is impaired mainly by the suppression of production of IL-2 and IL-4, which affect the proliferation and differentiation of B-cells to lower TDAR. We have identified a number of key events (KEs) along this pathway, and based on these key event relationships (KERs), created an AOP for inhibition of CN activity leading to impaired TDAR.

Since each KE involving MIE and AO is quantifiable, and shows similar dose responses with the CNIs in vitro, this AOP is useful for understanding immunosuppression due to inhibition of CN activity. In addition, each KER is based on sufficient scientific evidence and exhibits no contradiction with dose responses of adjacent KEs.

Since CN/NFAT system is conserved among vast variety of species and the function in immune system is common in at least human and mice, this AOP might be applicable to many mammalian species, including humans and rodents.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Macaca fascicularis	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Unspecific	High

The proposed AOP regarding inhibition of CN activity leading to impaired TDAR is not dependent on life stage, sex, or age. Since tacrolimus (FK506) ointment (Protopic) is approved for pediatric atopic dermatitis, the MOA for immunosuppression appears to be applicable to all life stages. The applicable state is considered supported by the draft FDA guidance for immunotoxicology that was recently issued (2020) indicating that “example of immunotoxicology testing could included TDAR assay” to address the concern of immunotoxicity in offspring in juvenile animal studies.

Since FK506 or CsA-induced outcomes in humans are mimicked by similar responses in a variety of animal models including non-human primates and rodents, immunosuppression induced by inhibition of CN activity is considered to occur across a variety of mammalian species.

In addition to the drugs, it is known that CN activity is suppressed by alkeylbenzene sulfonate (dodecylbenzene sulfonate) extracted from an acrylonitrile butadiene rubber (Ito et al. 2013), suggesting that the proposed AOP would be applicable to non-pharmacological agents.

For the chemicals such as pesticide, the TDAR assay is also recommended in the US EPA OPPTS 870.7800 immunotoxicity testing guideline.

Essentiality of the Key Events

Essentiality is supported by several knockout animals as follows.

Stage	Essentiality	Evidence	Supported by literatures
MIE and later	CnA-KO mice	Strong	The CN molecule consists of two regions, CnA and CnB, of which CnA exhibits phosphatase activity. In CnA-KO mice, T-cell proliferation in response to ovalbumin stimulation is lower than that for wild-type mice and is not complemented by normal antibody producing cells. In addition, when stimulated with ovalbumin, CnA-KO mice produce less IFN-γ, IL 2, and IL 4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to TNP-ovalbumin, which means that CnA deficiency affects only T cell-dependent antibody response (TDAR) (Zhang et al. 1996).
KE1 and later	NFAT-KO mice	Strong	The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly, moderately enhanced B- and T-cell responses, with bias towards Th2-cell response, decreased IFN-γ production in response to T-cell receptor (TCR) ligation, reduced proliferative responses by T cells, impaired repopulation of the thymus and lymphoid organs, impaired Th2- cell responses and IL-4 production, grossly impaired T-cell effector functions, profound defects in cytokine production and cytolytic activity, B-cell hyperactivity, impaired development of CD4 and CD8 single-positive cells, increased apoptosis of double-positive thymocytes, and mild hyperactivation of peripheral T cells. Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by CN. Suppression of T-cell-derived cytokines is noted both in CnA-knockout and NFAT-knockout mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system (Macian, 2005).
Stressor	FKBP12-KO mice	Moderate	FK506 induces suppression of immune responses; however, there is no literature showing a relationship of a relationship between FKBP12 knockout and the immune system in the FKBP12-KO mouse model. Steric structure of FKBP12/FK506 complex is considered the key factor for inhibition of CN phosphatase activity, but not for the enzymatic activities of FKBP12.

Weight of Evidence Summary

Biological Plausibility

T-cell functions are mainly regulated by the CN-NFAT system and suppression of CN activity in T cells is known to induce multiple types of immunosuppression, including T cell-dependent antibody response (TDAR).

Experiments with T cells indicate that TCR stimulation brings about increases in intracellular concentrations of Ca2+ that trigger CN activity, thereby inducing nuclear localization of substrate NFAT per dephosphorylation. The localized NFAT forms complexes with activator protein 1 (AP-1) at the promoter sites of the T cell cytokine genes and induces production of the cytokines.

CN phosphatase activity is known to be inhibited by the formation of immunophilin-CN inhibitor (CNI) complexes, such as CsA/cyclophilin complexes or FK506/FK506-binding protein (FKBP) 12 complexes. Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity, but there is no similarity between amino-acid sequences of the two classes of immunophilins. The three-dimensional structures of immunophilin complexes are essential to the inhibition of CN phosphatase activity, even though their enzymatic activities are not.

It is also known that one of the effects on immune function when CNI forms complexes with its respective immunophilin and inhibits CN activity is the suppression of IL-2 and other T-cell derived cytokine production. It is further known that inhibition of CN leads to suppression of TDAR because IL 2 and IL 4 mainly promote the proliferation, class switching, differentiation, and maturation of B-cells.

Furthermore, CN-NFAT also exists in B cells and it has been reported that CNIs do suppress production of certain cytokines from them. At the time of our review of the literature, however, we did not find any reports of a direct effect of CN inhibition on B cells, such as changes in proliferation, class switching, differentiation, or maturation of B cells.

Also, although CN-NFAT is known to exist in dendritic cells, natural killer T (NKT) cells, and other types of cells in which it regulates the expression of IL-2 receptors, there are no reports of effects on the production of T cell-dependent antibodies due to CNI-induced alteration in expression of IL-2 receptors in these cells.

CN-NFAT system-mediated immunosuppression is well understood based on the pharmacology of some CNI drugs (mostly FK506 and Cs A); therefore, AOP of CN inhibition-induced suppression of TDAR is useful for prediction of CN-mediated immunotoxicity.

KER	KEup-KEdown	Evidence	Rationales supported by literatures
KER 1	CN inhibitionto interference, NFAT nuclear translocation	Moderate	CN phosphatase activation through TCR stimulation dephosphorylates NFAT, thereby promoting nuclear localization of NFAT. CN phosphatase activity in T cells could be inhibited by CNI/immunophilin complexes, thus interfering with dephosphorylation and nuclear localization of NFAT. The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPlase activity, but modification of these functions is unrelated to inhibition of CN activity and thus thought to arise in the molecular structure of the complexes (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).
KER 2	Interference, nuclear localization NFAT to reduction, NFAT/AP-1 complex formation	Strong	CN activity dephosphorylates NFAT, thereby promoting its nuclear translocation. Nuclear-located NFAT binds with AP-1 at the promoter regions of the cytokine genes to promote T-cell cytokine production. Inhibition of dephosphorylation of NFAT by CNIs prevents nuclear export of NFAT and resultant binding with AP-1 at the promoter region of the T cell cytokine genes. NFAT has NLS and NES domains among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS domain is exposed whereas the NES domain is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000). CNIs interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).
KER 3	Reduction, NFAT/AP-1 complex formation to suppression of IL-2 and IL-4 production	Strong	NFAT/AP-1 complexes bind to the promoter regions of the cytokine genes, which promotes production of cytokines in T cells. Of these cytokines, IL-2 and IL-4 have a major role in promoting proliferation, maturation and class-switching of B cells, and development of TDAR. Reduction of NFAT/AP-1 complex formation in the nucleus due to inhibition CN activity by CNIs suppresses production of T-cell derived cytokines, including IL-2 and IL-4. T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015). Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001). Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).

KER 4	Suppression of IL-2 and IL-4 production to impaired TDAR	Strong	T cell-derived cytokines play important roles in TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation. Inhibition of CN activity by CNIs is known to suppress production of multiple cytokine species from T cells. Of these cytokines and receptors, suppression of IL-2 and IL-4 production mainly leads to impairment of TDAR. Suppressed production of other cytokines due to inhibition of CN activity exhibits only minor effects, if any, on TDAR. CsA is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in reduced productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015). Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production. Th2 cells produce cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 in Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells. IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one of the IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Empirical Support

KER	KEup-KEdown	Evidence	Empirical support of KERs
			CN phosphatase activity is inhibited with IC50 values of 0.5 nM (FK506) and 5nM (CsA) after 1 hours treatment (Fruman et al.1992).
KER 1	Inhibition, calcineurin activity leadsto interference, nuclear localization of NFAT	Moderate	Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at concentrations from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 μM (1000 nM) under the conditions of 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991).
			CN phosphatase activity and nuclear translocation of NFAT seems to be suppressed by CNIs at the similar ranges of doses and reaction times of 1 to 2 hours.

KER 2	Interference, nuclear localization of NFAT leads to reduction, NFAT/AP-1 complex formation	Strong	Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 μM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013) Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991). Gel mobility shift assays using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1μM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to bind IL-2 promoters (Flanagan et al. 1991). NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997). Quantitative data on NFAT/AP-1 complex formation in the nucleus is insufficient; however, inhibition of nuclear localization of NFAT and following NFAT/AP-1 complex formation in the nucleus are simultaneously detected by gel mobility shift assays at the concentration of FK506 within the range for inhibition of nuclear translocation of NFAT using imaging flowcytometry after 2 hours culture of T cells.
		Moderate	In NFATp- and NFAT4-deficient mice, cultured splenocytes boundanti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).
			In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 μg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 μg/mL (Yoshida et al. 2015).
			In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 μM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 μM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM for 4 h (Checker et al. 2012).
			Gel mobility shift assay revealed that treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders NFAT nuclear translocation and following formation of NFAT/AP-1 complexes in the nucleus (Flanagan et al. 1991).
KER	Reduction, NFAT/AP-1		Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993). In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at 10 nM (Dumont et al. 1998).

3	complex formation leads to suppression, IL-2 and IL-4 production		Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).
			In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).
			Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).
			In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003).
			Therefore, concentration of CNI needed for inhibition of NFAT/AP-1 complex formation in the nucleus is higher than that for inhibition of IL-2 and IL-4 production. A time lag is found between the two KEs; 2 hours for KE2 and 22 to 48 hours for KE3.

KER 4	Suppression ,IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response	Strong	Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015). In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4 weeks (Sanofi K.K. 2018). In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines such as IL-4 and IL-5 on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013). 1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010). In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH (keyhole limpet hemocyanine) and decrease in anti-KLH IgG(Alessandro, B. et al. 2003). FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a). In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998). Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004). Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b). After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2010). After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001). In vitro suppression of T-cell–derived cytokines and T-cell–dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two events, which showed earlier onset of cytokine production and delayed onset of antibody production.

Based on these findings of empirical support, each KE involving MIE and AO except for KE2 shows similar dose responses to the CNIs in vitro; however, culture time lag is noted, in that, 1 hour for MIE, 2 hours for KE1 and KE2, 22 to 24 hours for KE3 and more than days for AO.

Quantitative Understanding

KER1

No literature is available showing a clear quantitative relationship between the inhibition of CN phosphatase activity and nuclear translocation of NFAT; however, the dose responses of CN phosphatase activity and nuclear translocation of NFAT to CNI deem to be the same.

KER2:

Gel mobility shift assay of activated T cells showed that NFAT/AP-1 complexes are only found in nuclear extract, which indicates a strong relationship between the nuclear translocation of NFAT and simultaneous complex formation with AP-1 in the nucleus. CNI treatment clearly suppresses the complex formation of nuclear located NFAT and AP-1 in the nucleus, which also shows the solid relationship between these adjacent two KEs although quantitative data on suppressed NFAT/AP-1 complex formation is insufficient (Flaganan W.M. et al. 1991).

KER3:

The quantitative relationship between the decreased formation of NFAT/AP-1 complexes and the production of IL2/IL-4 formation induced by CNIs has not been reported.

However, as mentioned in the empirical support, nuclear localization of NFAT is strongly related to NFAT/AP-1 complex formation in the nucleus based on the fact that these two events are detected simultaneously by gel mobility shift assay, and the dose responses of IL2/IL-4 production and nuclear translocation of NFAT inhibited by CNI are similar; therefore, dose ranges of CNI in the inhibitions of IL2/IL-4 production and NFAT/AP-1 complex formation in the nucleus might also be the same.

In addition, T-5224 and ursolic acid inhibit AP-1 DNA binding activity or production of NF-κB, NFAT and AP-1, respectively, and both suppress the IL-2 and/or IL-4 production with dose dependent manner including the doses of inhibiting NFAT-AP-1 system (Yoshida et al. 2015, Checker et al. 2012).

KER4:

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).

Inhibition of IL-4 production in mice treated with oral administration of suplatast tosilate suppresses antigen-specific IgE production in a dose-dependent manner (Taiho Pharmaceutical 2013). In the inhibition of IL-4 production in human cell culture by suplatast tosilate at the concentration of 10 μg/mL for 10 days, antigen specific IgE production was suppressed from 56 to 72% and IL-4 production was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the levels of T-cell cytokines including IL-2 and IL-4 and inhibited IgM and IgG productions with a dose-dependent manner (Heidt S. 2010).

These results show the quantitative relationships between the inhibition of IL-4 or IL-2 by specific antibodies or CNI and suppression of antibody production.

Considerations for Potential Applications of the AOP (optional)

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

In AOP154, it was difficult to define susceptibility to infection as an AO for the AOP154, so TDAR, which is recommended as an indicator of immunosuppresoin in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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Appendix 1 - MIE, KEs and AO

List of MIEs in this AOP

Event: 980: Inhibition, Calcineurin Activity (https://aopwiki.org/events/980)

Short Name: Inhibition, Calcineurin Activity

Key Event Component

Process	Object	Action
binding	FK506-binding protein 15	increased
binding	FKBP12 (Arabidopsis thaliana)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	MolecularInitiatingEvent

Stressors

Name
Tacrorimus
Cyclosporin
Pimecrolimus
Dodecylbenzene sulfonate
Dibefurin
Gossypol
Ascomycin
Kaempferol
1,5-dibenzoyloxymethyl-norcantharidin
Tacrolimus (also FK506)

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

CN inhibitory activities (IC50) are shown in follows.

Tacrorimus: 0.4nM

Cyclosporin: 7nM

Pimecrolimus: 0.4 nM

Dodecylbenzene sulfonate 9.3 uM

Dibefurin: 44 uM

Gossypol: 17 uM

Ascomycin: 0.7 nM

1,5-dibenzoyloxymethyl-norcantharidin: 7 uM

Kaempferol: 51.3 uM

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus rattus	Rattus rattus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10117)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN is broadly distributed in T-cells, B cells, and throughout the body. The structure of CnA and CnB is highly conserved from yeasts to humans. Also highly conserved are the amino acid sequences of the catalytic and regulatory domains of CnA isoforms from different organisms (Kincaid. 1996).

As for immunophilins, of which complexes inhibit the CN activity, FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989a). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all living beings have PPIase activity (Wang P et al. 2005).

However, inhibition of CN phosphatase activity through immunophilin-CNI complex has been reported at least in rodents and humans.

Key Event Description

Calcineurin (CN) is a heterodimer that comprises a catalytic subunit (CnA), which handles phosphatase activity as well as calmodulin binding, and a Ca-binding regulatory subunit (CnB), which regulates intracellular calcium as well as CnA (Klee et al. 1988, Zhang et al. 1996). CnA, a 59kDa protein, has a serine-threonine phosphatase domain.

Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity (Barik. 2006) and an immunophilin-CN inhibitor (CNI) complex such as FKBP12- FK506 and cyclophilin-CsA binds directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which inhibits the phosphatase activity of CN without any contribution of PPIase activity (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

How it is Measured or Detected

Phosphatase activity can be measured using a phosphatase assay. CN, calmodulin, FK506, and FKBP are incubated together, and the phosphatase activity is measured at various concentrations of FKBP. Kinetic analysis of FKBP12 concentration-dependent phosphatase activity and calculation of the CN inhibition constant Ki by the FKBP12-FK506 complex are conducted. (Bram et al. 1993). Phosphatase activity of CN in the presence of cyclosporin A (CsA) , gossypol or dibefurin can also be determined in a similar manner (Sieber et al. 2009).

Immunophilin-CNI complexes directly inhibit phosphatase activity of CN, therefore, as a surrogate measurement of the CN activity, the binding of CsA with cyclophilin can be detected using an ELISA kit. Microtiter plates precoated with BSA and conjugated to cyclosporin are incubated with cyclophilin. Bound cyclophilin is then revealed by incubation with anti-cyclophilin rabbit antiserum followed by incubation with anti-rabbit globulin goat IgG coupled to alkaline phosphatase (Quesniaux et al. 1987).

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List of Key Events in the AOP

Event: 979: Interference, nuclear localization of NFAT (https://aopwiki.org/events/979)

Short Name: Interference, nuclear localization of NFAT

Key Event Component

Process	Object	Action
genetic interference	NFAT protein	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientifi cTerm	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

Key Event Description

The nuclear factor of activated T cells (NFAT) is a substrate of calcineurin (CN) (Rao et al. 1997). A NFAT has an N-terminal with a plurality of SP motifs rich in serine and proline, which are controlled by means of phosphorylation and dephosphorylation. There is a nuclear localization signal (NLS) held between these SP regions as well as a nuclear export signal (NES) in the N-terminal adjacent to the SP motifs (Beals et al. 1997, Zhu and McKeon 1999, Serfling et al. 2000). SP motifs ordinarily are phosphorylated, which covers the NLS and leaves the NES exposed, so that NFAT localizes in cytoplasm. When SP motifs are dephosphorylated by activated CN, the NLS is exposed and the NES is covered, thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999). When T-cell activation takes place, T-cell–receptor- mediated stimulus increases the intracellular concentration of calcium and activates a regulatory subunit (CnB), which subsequently induces a catalytic subunit (CnA) phosphatase activation, leading to dephosphorylation of NFAT thereby promoting nuclear localization of NFAT. CNI-immunophilin complexes inhibit CN phosphatase activation, thereby interfering with NFAT nuclear localization (Bhattacharyya et al.2011).

Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at concentrations from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 μM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013).

How it is Measured or Detected

Nuclear translocation of NFAT can be tested by imaging flowcytometer, in which lymphocytes are treated with fluorescence-labeled anti-NFAT antibody and DAPI (nuclear stain) and intracellular distribution of NFAT is analyzed by imaging flowcytometry with image analysis (Maguire O et al. 2013).

Interference with translocation of NFAT to the nucleus can be detected using gel mobility shift assays of nuclear or cytoplasmic extracts electrophoresed with end-labeled NFAT-binding site from human IL-2 enhancer (Flanagan et al. 1991).

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Event: 981: Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/events/981)

Short Name: Reduction, NFAT/AP-1 complex formation

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	NFAT activation molecule 1	decreased
cell activation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientifi cTerm	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Description

Activated nuclear factor of activated T cells (NFAT) that has localized to the nucleus binds cooperatively at the site of the Interleukin-2 (IL-2) promoter with activator protein-1 (AP-1), which is a heterodimer comprising a Fos and a Jun protein (Schreiber and Crabtree 1992, Jain et al. 1992), thereby inducing transcription of IL-2 (Jain et al. 1993). Interfered nuclear localization of NFAT, induced by FK506, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991).

Besides IL-2, NFAT is known to bind cooperatively at the promoters of other T-cell cytokines, such as Interleukin-4 (IL-4) (Macian et al. 2005).

Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991).

How it is Measured or Detected

Reductions in NFAT/AP-1 complex formation can be detected using a gel shift assay to test nuclear extracts from either stimulated or unstimulated Ar-5 T cells with radio-labelled NFAT binding oligonucleotide from murine IL-2 promoter. Anti-Fos and anti-Jun antibodies are used to examine NFAT/AP-1 complex formation (Jain et al. 1992).

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Event: 1202: Suppression, IL-2 and IL-4 production (https://aopwiki.org/events/1202)

Short Name: Suppression, IL-2 and IL-4 production

Key Event Component

Process	Object	Action
interleukin-2 production	interleukin-2	decreased
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin
Dexamethasone
Azathioprine
Methotrexate
Benzo(a)pyrene
Urethane
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
cynomolgu smonkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNIs suppress production of IL-2, IL-3, IL-4, IL-5, IFN-γ, Granulocyte Macrophage colony-stimulating Factor (GM-CSF), and other cytokines, as induced by CD2/CD3 or CD3/CD26 stimulation, in human peripheral blood mononuclear cells (PBMC) (Sakuma et al. 2001a). Also, CNIs (FK506 and CsA) suppress production of IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, Tumor Necrosis Factor-α, IFN-γ, and GM-CSF, as induced by CD3/PMA stimulation, in human PBMC (Dumont et al. 1998).

CNIs (FK506 and CsA) exhibit suppression of IL-2 production induced from mixed lymphocyte reactions in mice and humans (Kino, T et al. 1987a).

Treatment with CsA or 2C1.1 resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

These facts indicate that Calcineurin-NFAT system-mediated suppression of cytokines is commonly found in humans, monkey and mice.

Key Event Description

Production of T cell cytokines including Interleukin (IL)-2 and IL-4 is regulated by nuclear factor of activated T cells (NFAT)/ activator protein-1 (AP-1) complexes. Activated NFAT/AP-1 complex that bind at the site of the IL-2 and IL-4 promoters, thereby induces transcription of IL-2 and IL-4 (Jain et al. 1993). For IL-2, NFAT proteins are necessary for IL-2 gene expression and cooperation of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab

reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016).

Calcineurin inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) hinder the formation of the functional NFAT/AP-1 complexes by interfering with NFAT nuclear localization (Flanagan et al. 1991). Reduced binding of NFAT/AP-1 complexes at the promoter site of the IL-2 gene lowers the transcription of the mRNA of IL-2 and the following cytokine production.

Transcription of IL-4 is also inhibited by CNIs in the same manner as IL-2 (Dumont et al. 1998).

In CD3/ phorbol 12-myristate-13-acetate (PMA)-activated human T cells, FK506 suppressed production of IL-2, IL-4, and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at 10 nM (Dumont et al. 1998).

Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (KLH) (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).

Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

CNIs is considered to increase carcinogenicity through the suppression of IL-2 and IL-4 production.

- Renal transplant patients on immunosuppressive therapy were found to develop cancer within 10 years after surgery (Luster, M.I. et al. 1993).

In experimental animal studies, carcinogenicity of FK506 was reported as follows.

- In mice subjected to topical application testing, in which 100 μL of FK506 ointment was applied once daily for two years to roughly 40% of the total body area, an increased incidence of lymphoma was found in mice of the 0.1% ointment group showing high blood concentrations of the drug (Maruho Co., Ltd 2014).
- In hairless albino mice, virtually all of which developed skin tumors after a 40-week exposure to ultraviolet light, application of a 1% FK506 ointment reduced the time to outbreak of the skin tumors. (Maruho Co., Ltd 2014).

How it is Measured or Detected

Quantitation of cytokine content was done on appropriately diluted samples, run in duplicate, using Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) kits to test matched Antibody pairs with biotin-horseradish peroxidase-streptavidin detection and 3,3',5,5'-tetramethylbenzidine substrate. ELISA plates were scanned in a Molecular Devices UVmax plate reader (Menlo Park, CA), using SOFT max software (Molecular Devices) (Dumont et al. 1998).

Ex vivo whole blood stimulated cytokine (IL-2, IL-4, IL-5, and IL-17) production assay in the supernatants were determined using an electrochemiluminescent immunoassay from Meso Scale Discovery (MSD; Gaithersburg, MD) (Kevin, G. et al. 2014).

Total RNA was extracted using RNeasy mini kit (Qiagen, Chatsworth, CA) and quantitated by absorbance at 260 nm. Cytokine mRNAs were detected using a RiboQuant MultiProbe RPA system (PharMingen, San Diego, CA). Riboprobes were 32P-labeled and hybridized overnight with 10 to 30 mg of the RNA samples. The hybridized RNA was treated with RNase and purified according to the RiboQuant protocol. The samples were then electrophoresed in 6% polyacrylamide-Tris-borate-EDTA-urea gels using the Seqi -Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Hercules, CA), or minigels (Novex, San Diego, CA). The gels were dried, exposed and quantitated in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software (Dumont et al. 1998).

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List of Adverse Outcomes in this AOP

Event: 984: Impairment, T-cell dependent antibody response (https://aopwiki.org/events/984)

Short Name: Impairment, T-cell dependent antibody response

Key Event Component

Process	Object	Action
Immunosuppression		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	Adverse Outcome

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Rattus norvegicus	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
cynomolgus monkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence

All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNIs induced impairment of TDAR is demonstrated with rodent studies. That is, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino et al. 1987). Likewise, oral administration of FK506 to rats over a four-week period reduced production of both anti-KLH-IgG and IgM antibodies after subcutaneous immunization with KLH (Ulrich et al. 2004). Moreover, Treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum SRBC-specific IgM and IgG (Kevin, G. et al. 2014). As for humans, in vitro experiments showed that treatment with FK506 or CsA of peripheral blood mononuclear cells from blood-bank donors suppressed the production of IgM and IgG antibodies specific to T-cell–dependent antigens. (Heidt et al, 2009) Also, in SKW6.4 cells (IL-6–dependent, IgM-secreting, human B-cell line) cultures, FK506 or CsA suppressed the production of IgM antibodies in the presence of T-cell activation. (Sakuma et al. 2001b) Considering that FKF506 and CsA reduce T cell-derived cytokines including IL-2 and IL-4, these findings strongly suggest that impairment of TDAR following reduced production of such cytokines occurs at least in common among humans monkey and rodents.

Key Event Description

Antibody production to T-cell–dependent antigens is established through the coordination of B cells, antigen-presenting cells as well as T-cell–derived cytokines, which stimulate B cells to proliferate and differentiate. T-cell–dependent antibody response (TDAR) might be altered if any of these cell populations is affected.

Interleukin (IL)-2 stimulates B cells to proliferate through surface IL-2 receptors. IL-4 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Suppressing the production of these B-cell–related cytokines appears to impaire TDAR, as seen in the result of FK506 treatment (Heidt et al, 2009).

IL-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production. IL-2 promotes differentiation of B cells through IL-2 stimulates differentiation of the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs TDAR (Alberts et al. 2008).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) decrease in anti- keyhole limpet hemocyanine (KLH) immunoglobulin (Ig)G. (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene (DBA) exposure reduced total IgG antibody in spleen cell culture supernatants after in vitro stimulation with lipopolysaccharide (LPS) (Donna, C. et al. 2010).

Treatment with cyclosporin A (CsA) at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum sheep red blood cells (SRBC)-specific IgM and IgG (Kevin, G. et al. 2014).

After a 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41.6 and 83.2 nM) of CsA (Heidt et al, 2009).

After a 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated peripheral blood mononuclear cells (PBMC) culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.012 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.083 to 83.2 nM) of CsA (Sakuma et al. 2001b).

Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG was reduced at the dose level of 3 mg/kg/day (Ulrich et al. 2004).

Mice were treated with FK506 or CsA for 4 days, and immunized with SRBC, after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).

As immunosuppression-derived adverse outcomes by calcineurin inhibition, FK506 and CsA increase the frequency and/or severity of infections and allergic reactions impaired TDAR deems to be one of the causative factors for these side effects . Some clinical trials of FK506 and CsA revealed these adverse effects as follows.

- In clinical trials of renal transplantation using FK506 or CsA, opportunistic infections such as candida, cytomegalovirus and herpes simplex virus were reported (Ekberg et al. 2007).
- In recipients of liver transplants treated with FK506 or CsA, opportunistic infections such as cytomegalovirus, hepatitis C virus, hepatitis B and herpes simplex virus were reported (Fung et al. 1991).
- Cardiac transplant patients treated with cyclosporin developed pulmonary infections within the first year after surgery (Luster, M.I. et al. 1993).
- In patients of X-linked autoimmune enteropathy treated with CsA or FK506, serum levels of IgE developed extremely high during the immunosuppressive therapy (Kawamura et al. 1997).
- Renal transplant recipients treated with belatacepy/mycophenolate (MMF)/predonisone or FK506/MMF/prednisone showed significantly lower the geometric mean hemagglutination inhibition titer against influenza vaccine, hemagglutination-specific IgG and isotype IgG1 antibodies, and IgG-antibody secreting cells response (Gangappa et al. 2019).

How it is Measured or Detected

TDAR could be examined in vivo and in vitro.
In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with Enzyme-Linked ImmunoSorbent Assay (ELISA) or with a plaque-forming cell (PFC) assay.

- Rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH, after which the serum was examined for T-cell–dependent, antigen-specific, IgM and IgG levels using a Sandwich ELISA kit (Ulrich et al. 2004).
- Mice were repeatedly administered calcineurin inhibitors (CNIs) including FK506 and CsA orally for 4 days and immunized with SRBC, after which spleen cells were examined using a PFC assay (Kino et al. 1987).
- Cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBC, after which the serum was examined for Anti-SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Kevin, G. et al. 2014).
- Mice were exposed a single pharyngeal aspiration of DBA, after which supernatants of splenocytes cultured for 24 h in the presence of LPS and assayed using a mouse IgM or IgG matched pairs antibody kit (Bethyl Laboratories, Montgomery, TX) (Donna, C. et al. 2010).

For in vitro studies, total IgM and IgG levels in culture supernatant are often measured after polyclonal T-cell activation rather than measuring antigen stimulation in immune cell cultures.

- T cells and B cells isolated from human peripheral blood mononuclear cells (PBMC) were co-cultured with a CNIs for nine days in the presence of polyclonal–T-cell stimulation, after which supernatants were tested for immunoglobulin IgM and IgG levels using a Sandwich ELISA kit. Treatment with FK506 or CsA reduced the levels of IgM and IgG at the concentrations of 0.3 and 1.0 ng/mL or 50 and 100 ng/mL (Heidt et al, 2009).
  SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) were cultured with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. After culturing for four days, IgM produced in the culture

- supernatants was measured using an ELISA kit. FK506 or CsA reduced the levels of IgM at the concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma et al. 2001b).
- In order to examine class switching, T cells derived from human PBMCs were cultured with CNIs, and cytokine mRNA levels of Interferon-gamma, IL-2, IL-4, IL-5, IL-10, IL-13, and other B-cell–stimulatory cytokines produced in T cells were measured by quantitative PCR (Dumont et al. 1998).

Regulatory Significance of the AO

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

In AOP154, it was difficult to define susceptibility to infection as an AO for the AOP154, so TDAR, which is recommended as an indicator of immunosuppresoin in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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Appendix 2: List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1508: Inhibition, Calcineurin Activity leads to Interference, nuclear localization of NFAT (https://aopwiki.org/relationships/1508)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell DependentAntibody Response (https://aopwiki.org/aops/154)	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	ScientifiCTerm	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculoides	Mus musculoides	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=60742)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN is broadly distributed throughout the body, and the structure of CnA and CnB is highly conserved from yeasts to humans (Kincaid. 1993).

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells, and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all have PPIase activity (Wang P et al. 2005). They form binary complexes with their ligand cyclosporine A.

These facts indicate that CN and immunophilins are conserved among animals and plants although they show multiple physiological functions.

In addition, CNI/immunophilin complex-induced inhibition of CN phosphatase activity resulting in suppression of immune responses is found in humans and mice.

Key Event Relationship Description

The phosphatase activity of calcineurin (CN) is known to be inhibited by CN inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) through the formation of complexes with immunophilins.

Immunophilins of FK506-binding protein (FKBP) and cyclophilin bind with CNIs FK506 and CsA to form complexes, which inhibit CN activity (Barik. 2006).

While FKBP12, FKBP12.6, FKBP13, and FKBP52 are all part of the FK506-binding FKBP family, FKBP12 has a significant involvement in the mechanism of action for FK506-induced immunosuppression (Siekierka et al. 1989, Kang et al. 2008).

FKBP12 is a 12-kDa protein localized in cytoplasm and has been isolated from Jurkat T-cells as a receptor that binds to FK506 (Bram et al. 1993). FKBP12 has an FK506-binding domain (FKBD) that comprises 108 amino acids, and is expressed in T cells, B cells, Langerhans cells, and mast cells (Siekierka et al. 1990, Panhans-Gross et al. 2001, Hultsch et al. 1991).

Cyclophilin and FKBP both exhibit peptidyl propyl isomerase (PPIase) activity, but inhibition of PPIase activity is not related to CN regulation.

CN is a heterodimer that comprises a catalytic subunit (CnA) and a Ca-binding regulatory subunit (CnB). CnA handles phosphatase activity as well as calmodulin binding, and CnB regulates intracellular calcium and CnA (Klee et al. 1988, Zhang et al. 1996). CnA is a 59kDa protein with a serine-threonine phosphatase domain.

CNI-immunophilin complexes such as FK506/FKBP complexes and cyclophilin/CsA complexes bind directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which in turn inhibits phosphatase activity of CN (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

The nuclear factor of activated T cells (NFAT) is a substrate of CN (Rao et al. 1997).

When T-cell activation takes place, T-cell–receptor-mediated stimulus increases the intracellular concentration of calcium and activates CnB, which subsequently induces CnA phosphatase activation, leading to dephosphorylation of NFAT. In that process, dephosphorylated SP motifs expose the nuclear localization signal (NLS) and cover nuclear export signal (NES), thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

When CN activity is inhibited by the binding of immunophilin complexes, dephosphorylation does not occur in NFAT, thereby resulting in nuclear export.

Evidence Supporting this KER

Biological Plausibility

The molecular structures and functions of CN and NFAT are based on sufficient scientific evidence as mentioned above The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPlase activity, but the isomerase activity per se is not relevant for CN activity indicating that the latter is affected by the molecular structure of the complex (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

As mentioned above, inhibition of CN phosphatase activity interferes with the dephosphorylation of NFAT, which leads to the suppression of its nuclear localization.

Empirical Evidence

Much experimental data is available that supports the inhibition of CN activity induced by CNI/immunophilin complexes, which subsequently suppress nuclear localization of NFAT. In addition, CN phosphatase activity is inhibited by 24 hours treatment with CNI of FK506 and CsA with IC50 values of 0.5 and 5 nM, respectively (Fruman et al.1992).

Also, concentration-dependent reduction of in vitro nuclear localization of NFAT was evident using imaging flowcytometry at the maximum concentration of 1 μM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM (T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991).

These findings show that dose responses and temporality of MIE and KE1 seem to be the same.

Uncertainties and Inconsistencies

CN and NFAT are expressed in T cells and other immune cells including B cells, DC, and NKT cells and related to cytokine productions from these immune cells. Also, expression of IL-2 receptors (IL-2R) in DCs are lowered due to the inhibition of CN phosphatase activity by CNI treatment. Of these, reduced production of IL-2 and IL-4 from T cells plays a major role in suppression of TDAR due to lower proliferation, differentiation, and class switching of B cells. There have been no reports of CNI-induced reduction of cytokines other than IL-2 and IL-4 or reduced expression of IL-2R resulting in TDAR suppression.

FKBP12, a specific immunophilin that binds with FK506, is also an accessory molecule that binds to IP3 and Ryanodine receptors, both of which occur in Ca channels located on the membrane of the endoplasmic reticulum and participate in the regulation of intracellular Ca concentration. When binding with FK506, FKBP12 leaves these receptors to increase the influx of Ca2+ from the endoplasmic reticulum to cytoplasm, which should increase CN activity. Treatment with FK506, however, suppresses NFAT nuclear localization. In addition, FKBP12-knock out mice show no changes in immune function, including T-cell function. These facts suggest that the inhibition of CN-NFAT systems induced by FK506 treatment results from direct inhibition of CN phosphatase activity by FK506/FKBP12 complexes and not by affecting Ryanodine and IP3 receptors associated with FKBP12.

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

Dose-response analysis of the effects of FK506 on CN phosphatase activity in mast cell-derived KiSVMC4W cells transfected with human FKBP12 cDNA showed that increased expression of FKBP12 resulted in a greater than ten-fold increase in sensitivity to FK506-mediated inhibition, as indicated by an IC50 value of roughly 2 nM with linear inverse dose-response curve after 1 hour incuvation (Fruman et al.1995). Another phosphatase assay showed that FK506 inhibition of CN activity was concentration-dependent reverse sigmoidal and that IC50 values for CN inhibition were approximately 0.5 nM for FK 506 and 5 nM for CsA after 1 hour culture (Fruman et al.1992).

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing CNI concentrations from 0.1 nM (Jurkat human T cells) up to 1 μM (1000 nM) using imaging flowcytometry. Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again at maximal concentrations of 1 μM with minimum concentration of 10nM (Maguire et al. 2013).

So far, there is no evidence available that the dose response of inhibition of CN phosphatase activity is correlated with nuclear translocation of NFAT; however, the concentration ranges of CNIs for inhibition of CN phosphatase activity and nuclear translocation of NFAT seem to be the same range.

Time-scale

Inhibition of CN phosphatase activity was examined after 1 hour culture of T cells (Fruman et al.1995, Fruman et al.1992), and inhibition of nuclear translocation of NFAT was measured by imaging flowcytometry after 2 hour culture of T cells with CNI (Maguire et al. 2013).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1017: Interference, nuclear localization of NFAT leads to Reduction, NFAT/AP-1 complex formation (https://aopwiki.org/relationships/1017)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response (https://aopwiki.org/aops/154)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientifi cTerm	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents, and other mammalian species (Rao et al. 1997).
CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Relationship Description

Activated (dephosphorylated) nuclear factor of activated T cells (NFAT) is translocated into the nucleus through the molecular changes of exposing nuclear localization signal (NLS) and concomitant masking of nuclear export signal (NES) due to dephosphorylation of the SP motifs of NFAT. (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

In the nucleus NFAT binds with AP 1 at the IL-2 promoter region, (Schreiber and Crabtree 1992; Jain et al. 1992) and induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

Once CN phosphatase activity is inhibited, dephosphorylation of NFAT and subsequent nuclear localization of NFAT decreases, which results in a decrease of NFAT/AP-1 complex formation at the cytokine promoter sites (Rao et al. 1997).

Evidence Supporting this KER

Biological Plausibility

As has been mentioned, NFAT has NLS and NES domains among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS domain is exposed whereas the NES doman is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000).

It is well known from the experiments using CN inhibitors (CNIs) that interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).

In contrast to T cells, B-cell receptor-mediated increases in intracellular concentration of calcium in B cells leads to NFAT nuclear localization, thereby producing some classes of cytokines in the same manner as T-cells (Bhattacharyya et al.2011). However, there has been no report of any evidence that CNI acts directly on B cells to effect antibody production.

Expression of IL-2 receptors in dendritic cells and NKT cells is also reported to be regulated by this CN-NFAT system (Panhans-Gross A et al. 2001; Kim et al. 2010), but there is no report showing that CNIs suppress TDAR through the changes in IL-2R expression in these cells.

Empirical Evidence

The relationship of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

Imaging flowcytometry revealed that concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the maximum concentration of 1 μM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM (CD4+T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013).

Gel mobility shift assays using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1μM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991) NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997).

Quantitative understanding of NFAT/AP-1 complex formation in the nucleus is insufficient although nuclear NFAT/AP-1 complex formation was shown to be inhibited by FK506 at concentrations within the range of FK506 for the inhibition of nuclear translocation of NFAT.

Uncertainties and Inconsistencies

Nothing especially

Quantitative Understanding of the Linkage

Response-response relationship

The relationship of the interference of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing FK506 concentrations from 0.01nM (Jarkat T cells) up to 1 μM (1000 nM). Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again from 10nM to maximal concentrations of 1 μM (Maguire et al. 2013). Both parameters were measured after 2 hour culture of T cells with FK506.

KE2:

Reduction in generation of NFAT/AP-1 complexes can be detected using a gel shift assay (Rao et al. 1997, Jain et al. 1992, Jain et al. 1993).

Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991). As mentioned above, gel mobility shift assays also showed that NFAT/AP-1 complexes were formed only in the nucleus after T cell activation with unchanged preexisting NFAT in the cytoplasm and that treatment of T cells with 1μM FK506 led to decease the levels of NFAT/AP-1 complex (Jain et al. 1992).

These findings suggest that nuclear translocation of NFAT after T cell stimulation is strongly related to the complex formation with AP-1 in the nucleus, and FK506 was shown to inhibit NFAT/AP-1 complex formation in

the nucleus at the concentrations within the concentration range of FK506 for suppressing nuclear translocation of NFAT (Maguire et al. 2013).

Time-scale

Nuclear translocation of NFAT was shown to be inhibited in vitro using imaging flowcytometry after 2 hours culture of T cells with FK506 (Maguire et al. 2013), and gel mobility shift assays revealed the inhibition of nuclear translocation of NFAT and following complex formation with AP-1 within the nucleus after 2 hours culture of T cells with FK506 (Jain et al. 1992, Flanagan et al. 1991).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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11. Zhu, J. and McKeon, F. (1999). NF-AT activation requires suppression of Crm1-dependent export by calcineurin. Nature. 398(6724): 256-60.

Relationship: 1509: Reduction, NFAT/AP-1 complex formation leads to Suppression, IL-2 and IL-4 production(https://aopwiki.org/relationships/1509)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell DependentAntibody Response (https://aopwiki.org/aops/154)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1, IL-2 production and CD25 (IL-2R) up-regulation (Yoshida et al. 2015).
In splenic lymphocytes and/or CD4+ T cells, ursolic acid suppressed products of NF-kB, NFAT and AP-1, and inhibits secretion of IL-2 and IL-4, mRNA level of IL-2 and CD25 expression (Checker et al. 2012).
NFATp- and NFAT4-deficient mice indicate decreased production of IL-2 (Ranger et al. 1998).
NFAT/AP-1 complex formation in the nucleus was shown using murine and human T cells lines (Jain J et al. 1992). In addition to data on suppression of cytokine production by CNI in rodents, FK506 is reported to inhibit expression of both IL-2 and mRNA in human anti-CD3/PMA-activated cells (Dumont et al. 1998).

Key Event Relationship Description

Localized nuclear factor of activated T cells (NFAT) in the nucleus of T cells forms complexes with activator protein-1 (AP-1) at the Interleukin (IL)-2 promoter region (Schreiber and Crabtree 1992; Jain et al. 1992), which induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

For IL-2, NFAT proteins are necessary for IL-2 gene expression and interaction of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

Lowered nuclear localization of NFAT by calcineurin inhibitor (CNI) results in decreased formation of NFAT/AP-1 complex at the promoter region of IL-2 genes in the nucleus of T cells thereby reducing the transcription of IL-2 (Dumont et al. 1998). Production in T cells of IL-4 and other classes of cytokines is also suppressed in the same manner as IL-2 (Dumont et al. 1998).

Evidence Supporting this KER

Biological Plausibility

T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015).

Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001).

Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).

NFATp- and NFAT4-deficient mice indicate decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is generally accepted that NFAT, translocated to the nucleus after T-cell stimulation, binds with AP-1 to the promoter regions of the cytokine genes to mount transcription, which follows production of these T-cell–derived cytokines. Of these cytokines, IL-2 and IL-4 promote proliferation, maturation, and class-switching of B cells to enhance TDAR.

There is also sufficient evidence to support the hypothesis that CNI-induced decreases in T-cell–derived cytokine production is mediated through suppressed nuclear localization of NFAT, with a resultant decrease in the amount of NFAT/AP-1 complex binding to the promoter regions of T-cell-derived cytokines.

When stimulated with ovalbumin, calcineurin A (CnA)-knockout (KO) mice produce less Interferon (IFN)- γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to trinitrophenol-ovalbumin (Zhang et al. 1996).

The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly; moderately enhanced B- and T-cell responses, with bias towards Th2- cell responses; decreased IFN-γ production in response to TCR ligation; reduced proliferative responses by T cells; impaired repopulation of the thymus and lymphoid organs; impaired Th2-cell responses and IL-4 production; grossly impaired T-cell effector functions, with profound defects in cytokine production and cytolytic activity; B-cell hyperactivity; impaired development of CD4 and CD8 single-positive cells, with increased apoptosis of double-positive thymocytes; and mild hyperactivation of peripheral T cells (Macian, 2005).

Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by calcineurin (CN). Suppression of T-cell-derived cytokines is noted both in CnA-KO and NFAT-KO mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system.

FK506-FKBP12 complex decreased CN phosphatase activity, which inhibits. Because NF-ATp is an essential transcription factor regulating the IL-2 gene, FK506 ultimately blocks the T-cell response by inhibiting IL-2 transcription (Panhans-Gross A et al. 2001). FK506 inhibited IL-2 mRNA expression in anti-CD3/phorbol 12-myristate-13-acetate (PMA)-activated cells (Dumont et al. 1998).

These facts indicate that although NFAT is widely involved in the function of T cells, the effect of CNIs is to suppress production of some classes of T cell–derived cytokines through reducing the formation of NFAT/AP-1 complexes induced by inhibition of CN phosphatase activity.

Empirical Evidence

Empirical support of Reduction, NFAT/AP-1 complex formation leading to Suppression, IL-2 and IL-4 production is strong.

Rationale:

- In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 μg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 μg/mL (Yoshida et al. 2015).
- In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 μM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 μM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM for 4 h (Checker et al. 2012).
- In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is well established that inhibition of NFAT/AP-1 complex formation at the promoter sites reduces the production of T-cell–derived cytokines including IL-2 and IL-4, which are mainly involved in T-cell–dependent antibody response.

- NFAT/AP-1 complex formation is inhibited by CNI shown by gel shit mobility assay using human T cell line or CD4+ T cells from heathy donners after 2 hours treatment with cyclosporin A (CsA) at 1μM.. Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993).
- In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA in a dose-dependent (10 nM) manner after 3 day culture (Dumont et al. 1998).
- Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).
- In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).
- Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003).

Reduced nuclear translocation of NFAT followed by NFAT/AP-1 complex formation and suppression of IL-2/IL-4 productions are shown to occur under similar dose ranges and treatment duration.

Uncertainties and Inconsistencies

CNIs are reported to suppress IL-17 release from Th17 cells and development of Th17 cells from naïve T cells (Tsuda et al, 2012). On the other hand, Yadav reported that Th17 cells increased and Treg cells decreased in number and that the levels of RORC mRNA increased and those of FOXP3 decreased in renal transplanted patients with chronic calcineurin inhibitor toxicity (Yadav, 2015). From these findings, CNIs suppress the functions of Th17 and Treg cells, which enhance Th17 cells to develop chronic CNI toxicity.

FK506 suppresses expression of IL-2 receptor (IL-2R: CD25) and costimulatory molecules CD80 (B7.1)/CD40 in Langerhans cells (Panhans-Gross A et al. 2001).

In human NK cells, FK506 suppresses IL-2 responsive proliferation and cytokine production as well as lowers cytotoxicity directed toward K562 tumor cells (Kim et al. 2010). FK506 suppresses IL-2 production of NKT cell line DN32.D3 induced by stimulus from PMA/calcium -ionophore (van Dieren et al. 2010).

The relationship between these FK506-induced mechanisms and NFAT and contribution of those to TDAR are unclear.

In addition to NFAT/AP-1 complexes, NFAT forms complexes at the site of IL-3 and IL-4 enhancers with avian musculoaponeurotic fibrosarcoma oncogene homolog, early growth response 1, early growth response 4, interferon-regulatory factor 4, octamer-binding transcription factor, and other transcriptional partners to induce transcription of a variety of cytokines (Macian 2005). The production of cytokine induced by these transcriptional partners also suppressed by CNI; however, contribution of these additional transcription factors to TDAR is also unclear.

Quantitative Understanding of the Linkage

Response-response relationship

In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 at 80 μg/mL. On the other hand, T-5224 inhibits IL-2 production in a dose-dependent manner from 40, 60 and 80 μg/mL after 48 hours culture. T-5224 also inhibits CD25 (IL-2R) up-regulation at 80 μg/mL (Yoshida et al. 2015).

In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibits secretion of IL-2 and IL-4 at 0.5, 1 and 5 μM. In lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid also inhibits secretion of IL-2 and IL-4 at 5 μM. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM (Checker et al. 2012).

These findings showed that T-5244 and ursolic acid treated for 24 hours inhibit NFAT/AP-1 complex formation at a single concentration each and that these compounds suppress IL-2 and IL-4 production with dose dependent manner including the doses for inhibition of NFAT/AP-1 complex formation.

FK506 suppressed proliferation in human T cells induced by anti-CD3 mAb in the presence of adherent autologous peripheral blood mononuclear cells (mean IC50 = 0.06 nM). FK506 suppressed, in a dose-dependent (1.2 to 12.5 nM) manner after 22-24 hours culture, production of IL-2, IL-4, and IFN-γ by human T cells stimulated with anti-CD3 mAb in the presence of PMA, as well as inhibited, also in a dose-dependent (10 nM) manner, expression of IL-2, IL-4, and IFN-γ mRNA in anti-CD3/PMA- activated cells (Dumont et al. 1998). On the other hand, the quantitative data for the decreased formation of NFAT/AP-1 complexes by CNI is insufficient, although the formation was suppressed by FK506 at the concentration within the range needed for suppressed production of IL2/IL-4 by FK506 after 2 hours culture.

Time-scale

Inhibition of NFAT/AP-1 complex is detected by gel mobility shit assay after 2 hours culture with CNI; however, suppression of IL2/IL-4 could be measured after 22-48 hours in vitro culture.

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1510: Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response(https://aopwiki.org/relationships/1510)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell DependentAntibody Response (https://aopwiki.org/aops/154)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
cynomolgu smonkey	Macaca fascicularis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In cynomolgus monkeys, the effects of CsA on production of IL-2 and IL-4, and antigen-specific IgM and IgG in TDAR were demonstrated (Gaida K. 2015).

Suppressed IgE and antigen specific IgG1 productions by the blocking of IL-4 receptor were reported in mice using dupilumab (anti-IL-4/13R antibody) (Sanofi K.K. 2018).

Suppressed antigen specific IgE production by the inhibition of IL-4 production was reported in mice using suplatast tosilate (Taiho Pharmaceutical 2013).

Suppressed antigen specific IgE and IL-4 productions by the inhibition of IL-4 production were reported in human cell culture using suplatast tosilate(Taiho Pharmaceutical 2013).

The effects of FK506 on serum concentration of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over four weeks and immunized with KLH (Ulrich et al. 2004). The effects of FK506 and CsA on antigen-specific plaque-forming splenocytes have been demonstrated in mice treated with FK506 or CsA for 4 days and immunized with SRBC (Kino et al. 1987b).

The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt et al, 2009, Sakuma et al, 2001).

The effects of FK506 and CsA on production of IL-2 and IL-4 have been demonstrated using mice and human cells (Kino et al. 1987a, Dumont et al. 1998).

These facts suggest that there are no species differences between humans, monkeys and rodents in inhibitions of IL-2 and IL-4 production and TDAR induction.

Key Event Relationship Description

Interleukin (IL)-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of T-cell dependent antibody response (TDAR), both of which induces/enhances T cell dependent antibody production. IL-4 affects maturation and class switching of B cells as well as proliferation, IL-2 promotes differentiation of B cells through IL-2 receptors and stimulates the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs T cell dependent antibody production (Alberts et al. 2008).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR

T cell-derived cytokines play important roles in the development of TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production.

Thus, suppressing the production of IL-2, IL-4, and other cytokines in T cells reduces stimulation of B cells including proliferation, activation, and class switching, and leading to impairment of TDAR. Therefore, suppressing the production of these B-cell-related cytokines appears to be the main factor in impairment of TDAR by inhibitors of T-cell–dependent-antibody production.

Evidence Supporting this KER

Biological Plausibility

Cyclosporin A (CsA) is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in the reduction of the productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015).

It is established that IL-2 stimulates B cells to proliferate through the surface IL-2 receptors and that IL-4 stimulates B cells to proliferate, to induce class switch, and to differentiate into plasma and memory cells.

Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production.

Th2 cell produces cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 from Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells.

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). They suggest that the blocking of IL-2 signaling by anti-IL-2R antibody results in decreased rejection through the inhibition of the activation of antigen specific T cell with reduced antibody production.

FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Several in vivo studies in rodents showed decreased TDAR by the treatment of FK506 (Kino et al. 1987b, Ulrich et al. 2004). In in vitro tests examining antibody production in blood samples obtained from blood-bank donors, peripheral blood mononuclear cells (PBMC) treated with FK506 and CsA suppressed the production of IgM and IgG antibodies to T-cell dependent antigens (Heidt et al, 2009).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

However, as for the suppression of humoral immunity induced by the inhibition of calcineurin (CN) phosphatase activity, calcineurin inhibitors (CNIs) do not affect B cells directly but rather indirectly through T cells. That is, FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B cell response by FK506 and CsA appears due solely to inhibition of T helper cells (Heidt et al, 2010).

Therefore, it is concluded that decreased amounts of IL-2 and IL-4 secreted from helper T cells is the main factor for suppression of TDAR induced by CN phosphatase inhibition.

Empirical Evidence

Empirical support of the suppression, IL-2 and IL-4 production leads to impairment, T-cell dependent antibody response is strong.

Rationale:

- Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).
- In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018).
- In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013).
- In the clinical study of renal transplantation, basiliximab decreased incidence of acute rejection at 20 mg/kg (Novartis Pharma 2016). In human T cell culture immunized with PPD, basiliximab reduced activation of antigen specific T cell at the concentration of 300 ng/mL (Novartis Pharma 2016).
- In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998).
- FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a).
- After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2009).
- After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture

supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001).

- Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004).
- Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).
- 1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH and decrease in anti-KLH IgG(Alessandro, B. et al. 2003).

In vitro suppression of T-cell–derived cytokines and T-cell-dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two KEs, which showed earlier onset of cytokine production and delayed onset of antibody production.

Uncertainties and Inconsistencies

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Therefore, reduced production of both IL-2 and IL-4 might certainly induce suppression of TDAR; however, there remains some possibility of additional suppression of other immune functions.

Quantitative Understanding of the Linkage

Response-response relationship

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In the blocking of IL-4 receptor in mice by dupilumab (anti-IL-4/13R antibody) at 25 mg/kg of twice weekly subcutaneous administration for 4weeks, IgE production was suppressed to about 1/100 and antigen specific IgG1 production was suppressed to about 1/200 (Sanofi K.K. 2018).

In the inhibition of IL-4 production in mice by suplatast tosilate at 10, 20, 50 and 100 mg/kg of oral administration for 5 days, antigen specific IgE production was suppressed from about 1/10 to 1/100 (Taiho Pharmaceutical 2013). In human T cell culture by suplatast tosilate at the concentration of 10 μg/mL, antigen specific IgE production after 10 days was suppressed from 56 to 72% and IL-4 production after 3 days was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. That is, murine small resting B cells, cultured with irradiated hapten-specific TH1 clone, were induced to enter cell cycle at 2 days and to secret antibody at 5 days. An anti-IL-2 and anti-IL-2R antibodies completely inhibited this T-cell dependent antibody production (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the m-RNA levels of T-cell cytokines at 8h post-stimulation including IL-2 and IL-4 at 1.0ng/mL (1.24nM) FK506 or 100ng/mL (90.7nM) CsA and inhibited IgM and IgG productions after 9 days at 0.3 and 1.0ng/mL FK506 and 50 and 100ng/mL CsA (Heidt S. 2010).

Time-scale

In CsA-treatment for 24 days at 50 mg/kg BID, cynomolgus monkeys showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In human T cell culture, suplatast tosilate inhibits IL-4 production after 3 days and antigen specific IgE production after 10 days (Taiho Pharmaceutical 2013).

In the human T-B cell co-culture, CNIs of FK506 and CsA lowered the m-RNA levels of IL-2 and IL-4 at 8h post-stimulation and inhibited IgM and IgG productions after 9 days (Heidt S. 2010).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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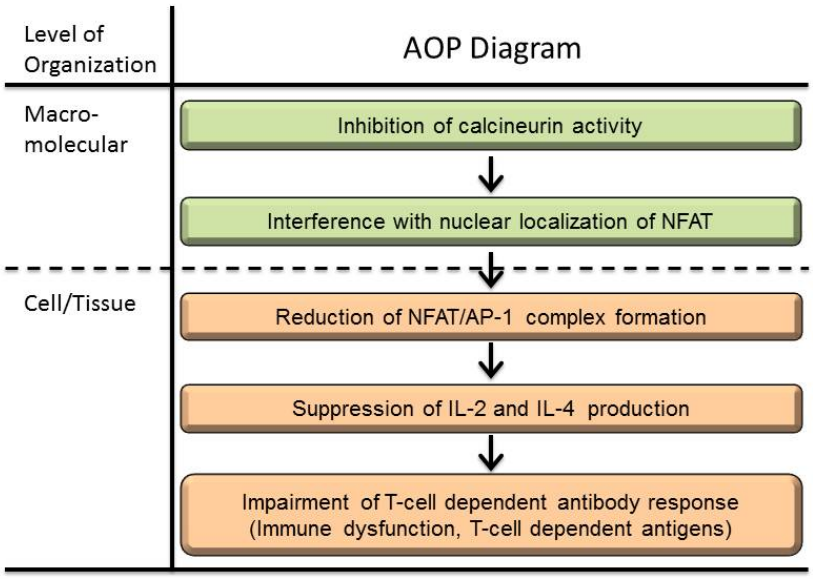
AOP ID and Title:

AOP 154: Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response
Short Title: Immunosuppression

Graphical Representation



Authors

Hiroyuki Komatsu (1) Junichiro Sugimoto (1) Ken Goto (1) Kiyoshi Kushima (1) Naohisa Tsutsui (1) Shigeru Hisada (1) Shiho Ito (1) Tadashi Kosaka (1) Takumi Ohishi (1) Yasuharu Otsubo (1) Yoshihiro Takahashi (1)

(1) AOP Working Group, Testing Methodology Committee, The Japanese Society of Immunotoxicology

Corresponding author: Takumi Ohishi (ohishi@bozo.co.jp)

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Abstract

Calcineurin (CN), a protein phosphatase, is known to impair immune function when its phosphatase activation is inhibited. The relationship between CN and immune functions is well understood, and immunosuppressants that work by inhibiting CN have been developed.

CN inhibitors (CNIs) inhibit CN phosphatase activity to suppress many kinds of immune functions and have been used to prevent hyper immune reactions such as rejection and graft versus host disease (GVHD), and treat autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CNIs are reported to induce immunosuppression-derived adverse effects such as increased frequency and/or severity of infections and increased tumor incidences. CNIs might affect several T-cell derived immune functions to induce compromised host. Among the affected immune functions, T-cell dependent antibody response (TDAR) is an important factor to resist infections and thought to be the useful endpoint on evaluating immunotoxicity of chemicals; therefore, this AOP describes the linkage between the inhibition of CN activity and impairment of TDAR.

CN activity is inhibited when stressors bind to CN with their respective immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes with activator protein-1 (AP-1) that bind at the site of IL-2, IL-4 and other T cell -derived cytokine promoters is reduced, thereby

suppressing production of these cytokines. Among the affected cytokines from each of the helper T cell subsets, reduced production of IL-2 and IL-4 affects the proliferation and differentiation of B-cells to suppress TDAR.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for inhibition of CN activity leading to impaired TDAR.

Since CN is produced in a vast variety of species, this AOP might be applicable to many mammal species, including humans and rodents.

Background

Although there are other stressors that inhibit CN activity, this AOP is mainly based on an understanding of immunosuppression caused by the complex of FK506 and FKBP12 and cyclophilin and CsA, on which a significant body of scientific literature has been published.

We look forward to future amendments to this AOP with up-to-date information on other stressors, which will more clarify the linkage between inhibition of CN activity and impairment of TDAR.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	980	Inhibition, Calcineurin Activity	Inhibition, Calcineurin Activity
2	KE	979	Interference, nuclear localization of NFAT	Interference, nuclear localization of NFAT
3	KE	981	Reduction, NFAT/AP-1 complex formation	Reduction, NFAT/AP-1 complex formation
4	KE	1202	Suppression, IL-2 and IL-4 production	Suppression, IL-2 and IL-4 production
5	AO	984	Impairment, T-cell dependent antibody response	Impairment, T-cell dependent antibody response

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Calcineurin Activity	adjacent	Interference, nuclear localization of NFAT	Moderate	Moderate
Interference, nuclear localization of NFAT	adjacent	Reduction, NFAT/AP-1 complex formation	High	High
Reduction, NFAT/AP-1 complex formation	adjacent	Suppression, IL-2 and IL-4 production	High	High
Suppression, IL-2 and IL-4 production	adjacent	Impairment, T-cell dependent antibody response	High	High

Stressors

Name	Evidence
Tacrolimus (also FK506)	High
Cyclosporin	High
Pimecrolimus	High
Gossypol	Moderate
Kaempferol	Moderate
Dodecylbenzene sulfonate	Moderate

Name	Evidence
Dibefurin	Moderate
Ascomycin	Moderate
1,5-dibenzoyloxymethyl-norcantharidin	Moderate

Tacrolimus (also FK506)

also known as FK506

Overall Assessment of the AOP

Inhibition of CN might induce suppression of cytokines production from all the T helper cell subsets as well as other immune functions of other immune cells. Suppression of cell-mediated immunity is involved in the pharmacology of preventing hyper immune reactions such as rejection and GVHD, and treatment of autoimmune and allergic disorders such as psoriasis and atopic dermatitis. On the other hand, CN inhibition might induce immunosuppression-derived adverse outcomes. One of the effects is increased frequency and/or severity of infections. Compromised host might be related with impairment of multiple immune functions; however, impaired TDAR deems to be usually related. Moreover, TDAR is the frequently used measurable endpoint in immunotoxicity testing according the ICH S8 or US EPA OPPTS 870.7800 immunotoxicity testing guideline. Therefore, the present AOP focus on CN inhibition-induced impairment of TDAR.

When stressors bind to calcineurin-A (CnA) with immunophilin, CN phosphatase activity is inhibited. Immunophilins are composed of a family of highly conserved proteins that have the ability to bind immunosuppressive drugs. This interfere with the nuclear localization of NFAT, the substrate for CN. As a result, the formation of functional NFAT/ AP-1 complexes that bind at the site of IL-2 , IL-4 and other cytokine promoters in each of the T helper cell subsets is reduced, thereby suppressing production of these cytokines. Among the affected cytokines TDAR is impaired mainly by the suppression of production of IL-2 and IL-4, which affect the proliferation and differentiation of B-cells to lower TDAR. We have identified a number of key events (KEs) along this pathway, and based on these key event relationships (KERs), created an AOP for inhibition of CN activity leading to impaired TDAR.

Since each KE involving MIE and AO is quantifiable, and shows similar dose responses with the CNIs in vitro, this AOP is useful for understanding immunosuppression due to inhibition of CN activity. In addition, each KER is based on sufficient scientific evidence and exhibits no contradiction with dose responses of adjacent KEs.

Since CN/NFAT system is conserved among vast variety of species and the function in immune system is common in at least human and mice, this AOP might be applicable to many mammalian species, including humans and rodents.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Macaca fascicularis	Macaca fascicularis	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

The proposed AOP regarding inhibition of CN activity leading to impaired TDAR is not dependent on life stage, sex, or age. Since tacrolimus (FK506) ointment (Protopic) is approved for pediatric atopic dermatitis, the MOA for immunosuppression appears to be applicable to all life stages. The applicable state is considered supported by the draft FDA guidance for immunotoxicology that was recently issued (2020) indicating that “example of immunotoxicology testing could included TDAR assay” to address the concern of immunotoxicity in offspring in juvenile animal studies.

Since FK506 or CsA-induced outcomes in humans are mimicked by similar responses in a variety of animal models including non-human primates and rodents, immunosuppression induced by inhibition of CN activity is considered to occur across a variety of mammalian species.

In addition to the drugs, it is known that CN activity is suppressed by alkeylbenzene sulfonate (dodecylbenzene sulfonate) extracted from an acrylonitrile butadiene rubber (Ito et al. 2013)., suggesting that the proposed AOP would be applicable to non-

pharmacological agents.

For the chemicals such as pesticide, the TDAR assay is also recommended in the US EPA OPPTS 870.7800 immunotoxicity testing guideline.

Essentiality of the Key Events

Essentiality is supported by several knockout animals as follows.

Stage	Essentiality	Evidence	Supported by literatures
MIE and later	CnA-KO mice	Strong	The CN molecule consists of two regions, CnA and CnB, of which CnA exhibits phosphatase activity. In CnA-KO mice, T-cell proliferation in response to ovalbumin stimulation is lower than that for wild-type mice and is not complemented by normal antibody producing cells. In addition, when stimulated with ovalbumin, CnA-KO mice produce less IFN-γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to TNP-ovalbumin, which means that CnA deficiency affects only T cell-dependent antibody response (TDAR) (Zhang et al. 1996).
KE1 and later	NFAT-KO mice	Strong	The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly, moderately enhanced B- and T-cell responses, with bias towards Th2-cell response, decreased IFN-γ production in response to T-cell receptor (TCR) ligation, reduced proliferative responses by T cells, impaired repopulation of the thymus and lymphoid organs, impaired Th2- cell responses and IL-4 production, grossly impaired T-cell effector functions, profound defects in cytokine production and cytolytic activity, B-cell hyperactivity, impaired development of CD4 and CD8 single-positive cells, increased apoptosis of double-positive thymocytes, and mild hyperactivation of peripheral T cells. Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by CN. Suppression of T-cell-derived cytokines is noted both in CnA-knockout and NFAT-knockout mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system (Macian, 2005).
Stressor	FKBP12-KO mice	Moderate	FK506 induces suppression of immune responses; however, there is no literature showing a relationship of a relationship between FKBP12 knockout and the immune system in the FKBP12-KO mouse model. Steric structure of FKBP12/FK506 complex is considered the key factor for inhibition of CN phosphatase activity, but not for the enzymatic activities of FKBP12.

Weight of Evidence Summary

Biological Plausibility

T-cell functions are mainly regulated by the CN-NFAT system and suppression of CN activity in T cells is known to induce multiple types of immunosuppression, including T cell-dependent antibody response (TDAR).

Experiments with T cells indicate that TCR stimulation brings about increases in intracellular concentrations of Ca2+ that trigger CN activity, thereby inducing nuclear localization of substrate NFAT per dephosphorylation. The localized NFAT forms complexes with activator protein 1 (AP-1) at the promoter sites of the T-cell cytokine genes and induces production of the cytokines.

CN phosphatase activity is known to be inhibited by the formation of immunophilin-CN inhibitor (CNI) complexes, such as CsA/cyclophilin complexes or FK506/FK506-binding protein (FKBP) 12 complexes. Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity, but there is no similarity between amino-acid sequences of the two classes

of immunophilins. The three-dimensional structures of immunophilin complexes are essential to the inhibition of CN phosphatase activity, even though their enzymatic activities are not.

It is also known that one of the effects on immune function when CNI forms complexes with its respective immunophilin and inhibits CN activity is the suppression of IL-2 and other T-cell derived cytokine production. It is further known that inhibition of CN leads to suppression of TDAR because IL-2 and IL-4 mainly promote the proliferation, class switching, differentiation, and maturation of B-cells.

Furthermore, CN-NFAT also exists in B-cells and it has been reported that CNIs do suppress production of certain cytokines from them. At the time of our review of the literature, however, we did not find any reports of a direct effect of CN inhibition on B-cells, such as changes in proliferation, class switching, differentiation, or maturation of B-cells.

Also, although CN-NFAT is known to exist in dendritic cells, natural killer T (NKT) cells, and other types of cells in which it regulates the expression of IL-2 receptors, there are no reports of effects on the production of T cell-dependent antibodies due to CNI-induced alteration in expression of IL-2 receptors in these cells.

CN-NFAT system-mediated immunosuppression is well understood based on the pharmacology of some CNI drugs (mostly FK506 and Cs A); therefore, AOP of CN inhibition-induced suppression of TDAR is useful for prediction of CN-mediated immunotoxicity.

KER	KE_{up}-KE_{down}	Evidence	Rationales supported by literatures
KER1	CN inhibition to interference, NFAT nuclear translocation	Moderate	CN phosphatase activation through TCR stimulation dephosphorylates NFAT, thereby promoting nuclear localization of NFAT. CN phosphatase activity in T cells could be inhibited by CNI/immunophilin complexes, thus interfering with dephosphorylation and nuclear localization of NFAT. The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPIase activity, but modification of these functions is unrelated to inhibition of CN activity and thus thought to arise in the molecular structure of the complexes (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).
KER2	Interference, nuclear localization NFAT to reduction, NFAT/AP-1 complex formation	Strong	CN activity dephosphorylates NFAT, thereby promoting its nuclear translocation. Nuclear-located NFAT binds with AP-1 at the promoter regions of the cytokine genes to promote T-cell cytokine production. Inhibition of dephosphorylation of NFAT by CNIs prevents nuclear export of NFAT and resultant binding with AP-1 at the promoter region of the T cell cytokine genes. NFAT has NLS and NES domains among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS domain is exposed whereas the NES domain is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000). CNIs interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).
	Reduction,		NFAT/AP-1 complexes bind to the promoter regions of the cytokine genes, which promotes production of cytokines in T cells. Of these cytokines, IL-2 and IL-4 have a major role in promoting proliferation, maturation and class-switching of B cells, and development of TDAR. Reduction of NFAT/AP-1 complex formation in the nucleus due to inhibition CN activity by CNIs suppresses production of T-cell derived cytokines, including IL-2 and IL-4. T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding

KER3	NFAT/AP-1 complex formation to suppression of IL-2 and IL-4 production	Strong	activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015). Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001). Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).
KER4	Suppression of IL-2 and IL-4 production to impaired TDAR	Strong	T cell-derived cytokines play important roles in TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation. Inhibition of CN activity by CNIs is known to suppress production of multiple cytokine species from T cells. Of these cytokines and receptors, suppression of IL-2 and IL-4 production mainly leads to impairment of TDAR. Suppressed production of other cytokines due to inhibition of CN activity exhibits only minor effects, if any, on TDAR. CsA is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in reduced productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015). Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody results in the decrease in T cell dependent antibody production. Th2 cells produce cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 in Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells. IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one the of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Empirical Support

KER	KE_{up}-KE_{down}	Evidence	Empirical support of KERs
			CN phosphatase activity is inhibited with IC50 values of 0.5 nM (FK506) and 5nM (CsA) after 1 hours treatment (Fruman et

KER1	Inhibition, calcineurin activity leads to interference, nuclear localization of NFAT	Moderate	al.1992). Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at concentrations from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991). CN phosphatase activity and nuclear translocation of NFAT seems to be suppressed by CNIs at the similar ranges of doses and reaction times of 1 to 2 hours.
KER2	Interference, nuclear localization of NFAT leads to reduction, NFAT/AP-1 complex formation	Strong	Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the concentration from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013). Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991). Gel mobility shift assays using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1µM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to bind IL-2 promoters (Flanagan et al. 1991). NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997). Quantitative data on NFAT/AP-1 complex formation in the nucleus is insufficient; however, inhibition of nuclear localization of NFAT and following NFAT/AP-1 complex formation in the nucleus are simultaneously detected by gel mobility shift assays at the concentration of FK506 within the range for inhibition of nuclear translocation of NFAT using imaging flowcytometry after 2 hours culture of T cells.
			In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998). In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 µg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 µg/mL (Yoshida et al. 2015). In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 µM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 µM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 µM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 µM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 µM for 4 h (Checker et al. 2012).

KER3	Reduction, NFAT/AP-1 complex formation leads to suppression, IL-2 and IL-4 production	Moderate	Gel mobility shift assay revealed that treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders NFAT nuclear translocation and following formation of NFAT/AP-1 complexes in the nucleus (Flanagan et al. 1991). Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993). In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at 10 nM (Dumont et al. 1998). Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018). In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010). Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014). In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003). Therefore, concentration of CNI needed for inhibition of NFAT/AP-1 complex formation in the nucleus is higher than that for inhibition of IL-2 and IL-4 production. A time lag is found between the two KEs; 2 hours for KE2 and 22 to 48 hours for KE3.
			Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015). In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018). In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines such as IL-4 and IL-5 on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013).

KER4	Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response	Strong	1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010). In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH (keyhole limpet hemocyanine) and decrease in anti-KLH IgG(Alessandro, B. et al. 2003). FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a). In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998). Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004). Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b). After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2010). After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001). In vitro suppression of T-cell–derived cytokines and T-cell–dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two events, which showed earlier onset of cytokine production and delayed onset of antibody production.

Based on these findings of empirical support, each KE involving MIE and AO except for KE2 shows similar dose responses to the CNIs in vitro; however, culture time lag is noted, in that, 1 hour for MIE, 2 hours for KE1 and KE2, 22 to 24 hours for KE3 and more than days for AO.

Quantitative Consideration

KER1

No literature is available showing a clear quantitative relationship between the inhibition of CN phosphatase activity and nuclear translocation of NFAT; however, the dose responses of CN phosphatase activity and nuclear translocation of NFAT to CNI deem to be the same.

KER2:

Gel mobility shift assay of activated T cells showed that NFAT/AP-1 complexes are only found in nuclear extract, which indicates a

strong relationship between the nuclear translocation of NFAT and simultaneous complex formation with AP-1 in the nucleus. CNI treatment clearly suppresses the complex formation of nuclear located NFAT and AP-1 in the nucleus, which also shows the solid relationship between these adjacent two KEs although quantitative data on suppressed NFAT/AP-1 complex formation is insufficient (Flaganan W.M. et al. 1991).

KER3:

The quantitative relationship between the decreased formation of NFAT/AP-1 complexes and the production of IL2/IL-4 formation induced by CNIs has not been reported.

However, as mentioned in the empirical support, nuclear localization of NFAT is strongly related to NFAT/AP-1 complex formation in the nucleus based on the fact that these two events are detected simultaneously by gel mobility shift assay, and the dose responses of IL2/IL-4 production and nuclear translocation of NFAT inhibited by CNI are similar; therefore, dose ranges of CNI in the inhibitions of IL2/IL-4 production and NFAT/AP-1 complex formation in the nucleus might also be the same.

In addition, T-5224 and ursolic acid inhibit AP-1 DNA binding activity or production of NF-κB, NFAT and AP-1, respectively, and both suppress the IL-2 and/or IL-4 production with dose dependent manner including the doses of inhibiting NFAT-AP-1 system (Yoshida et al. 2015, Checker et al. 2012).

KER4:

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).

Inhibition of IL-4 production in mice treated with oral administration of suplatast tosilate suppresses antigen-specific IgE production in a dose-dependent manner (Taiho Pharmaceutical 2013). In the inhibition of IL-4 production in human cell culture by suplatast tosilate at the concentration of 10 µg/mL for 10 days, antigen specific IgE production was suppressed from 56 to 72% and IL-4 production was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. T (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the levels of T-cell cytokines including IL-2 and IL-4 and inhibited IgM and IgG productions with a dose-dependent manner (Heidt S. 2010).

These results show the quantitative relationships between the inhibition of IL-4 or IL-2 by specific antibodies or CNI and suppression of antibody production.

Considerations for Potential Applications of the AOP (optional)

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and

immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

In AOP154, it was difficult to define susceptibility to infection as an AO for the AOP154, so TDAR, which is recommended as an indicator of immunosuppresoin in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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Appendix 1

List of MIEs in this AOP

Event: 980: Inhibition, Calcineurin Activity

Short Name: Inhibition, Calcineurin Activity

Key Event Component

Process	Object	Action
binding	FK506-binding protein 15	increased
binding	FKBP12 (Arabidopsis thaliana)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	MolecularInitiatingEvent

Stressors

Name
Tacrorimus
Cyclosporin
Pimecrolimus
Dodecylbenzene sulfonate
Dibefurin
Gossypol
Ascomycin
Kaempferol
1,5-dibenzoyloxymethyl-norcantharidin
Tacrolimus (also FK506)

Biological Context

Level of Biological Organization

Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

CN inhibitory activities (IC50) are shown in follows.

Tacrorimus: 0.4nM

Cyclosporin: 7nM

Pimecrolimus: 0.4 nM

Dodecylbenzene sulfonate 9.3 uM

Dibefurin: 44 uM

Gossypol: 17 uM

Ascomycin: 0.7 nM

1,5-dibenzoyloxymethyl-norcantharidin: 7 uM

Kaempferol: 51.3 uM

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus rattus	Rattus rattus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN is broadly distributed in T-cells, B-cells, and throughout the body. The structure of CnA and CnB is highly conserved from yeasts to humans. Also highly conserved are the amino acid sequences of the catalytic and regulatory domains of CnA isoforms from different organisms (Kincaid. 1996).

As for immunophilins, of which complexes inhibit the CN activity, FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989a). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all living beings have PPIase activity (Wang P et al. 2005).

However, inhibition of CN phosphatase activity through immunophilin-CNI complex has been reported at least in rodents and humans.

Key Event Description

Calcineurin (CN) is a heterodimer that comprises a catalytic subunit (CnA), which handles phosphatase activity as well as calmodulin binding, and a Ca-binding regulatory subunit (CnB), which regulates intracellular calcium as well as CnA (Klee et al. 1988, Zhang et al. 1996). CnA, a 59kDa protein, has a serine-threonine phosphatase domain.

Immunophilins are a general class of proteins that exhibit peptidyl-propyl isomerase (PPIase) activity (Barik. 2006) and an

immunophilin-CN inhibitor (CNI) complex such as FKBP12- FK506 and cyclophilin-CsA binds directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which inhibits the phosphatase activity of CN without any contribution of PPIase activity (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

How it is Measured or Detected

Phosphatase activity can be measured using a phosphatase assay. CN, calmodulin, FK506, and FKBP are incubated together, and the phosphatase activity is measured at various concentrations of FKBP. Kinetic analysis of FKBP12 concentration-dependent phosphatase activity and calculation of the CN inhibition constant Ki by the FKBP12-FK506 complex are conducted. (Bram et al. 1993). Phosphatase activity of CN in the presence of cyclosporin A (CsA) , gossypol or dibefurin can also be determined in a similar manner (Sieber et al. 2009).

Immunophilin-CNI complexes directly inhibit phosphatase activity of CN, therefore, as a surrogate measurement of the CN activity, the binding of CsA with cyclophilin can be detected using an ELISA kit. Microtiter plates precoated with BSA and conjugated to cyclosporin are incubated with cyclophilin. Bound cyclophilin is then revealed by incubation with anti-cyclophilin rabbit antiserum followed by incubation with anti-rabbit globulin goat IgG coupled to alkaline phosphatase (Quesniaux et al. 1987).

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List of Key Events in the AOP

Event: 979: Interference, nuclear localization of NFAT

Short Name: Interference, nuclear localization of NFAT

Key Event Component

Process	Object	Action
genetic interference	NFAT protein	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

Key Event Description

The nuclear factor of activated T cells (NFAT) is a substrate of calcineurin (CN) (Rao et al. 1997). A NFAT has an N-terminal with a plurality of SP motifs rich in serine and proline, which are controlled by means of phosphorylation and dephosphorylation. There is a nuclear localization signal (NLS) held between these SP regions as well as a nuclear export signal (NES) in the N-terminal adjacent to the SP motifs (Beals et al. 1997, Zhu and McKeon 1999, Serfling et al. 2000). SP motifs ordinarily are phosphorylated, which covers the NLS and leaves the NES exposed, so that NFAT localizes in cytoplasm. When SP motifs are dephosphorylated by activated CN, the NLS is exposed and the NES is covered, thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999). When T-cell activation takes place, T-cell–receptor- mediated stimulus increases the intracellular concentration of calcium and activates a regulatory subunit (CnB), which subsequently induces a catalytic subunit (CnA) phosphatase activation, leading to dephosphorylation of NFAT thereby promoting nuclear localization of NFAT. CNI-immunophilin

complexes inhibit CN phosphatase activation, thereby interfering with NFAT nuclear localization (Bhattacharyya et al.2011).

Concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at concentrations from 0.1 nM (Jurkat T cells) or 10nM (human CD4+ T cells) and up to 1 µM (1000 nM) under the conditions of 2 hours treatment (Maguire et al. 2013).

How it is Measured or Detected

Nuclear translocation of NFAT can be tested by imaging flowcytometer, in which lymphocytes are treated with fluorescence-labeled anti-NFAT antibody and DAPI (nuclear stain) and intracellular distribution of NFAT is analyzed by imaging flowcytometry with image analysis (Maguire O et al. 2013).

Interference with translocation of NFAT to the nucleus can be detected using gel mobility shift assays of nuclear or cytoplasmic extracts electrophoresed with end-labeled NFAT-binding site from human IL-2 enhancer (Flanagan et al. 1991).

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Event: 981: Reduction, NFAT/AP-1 complex formation

Short Name: Reduction, NFAT/AP-1 complex formation

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	NFAT activation molecule 1	decreased
cell activation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

T cell

Organ term

Organ term

immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Description

Activated nuclear factor of activated T cells (NFAT) that has localized to the nucleus binds cooperatively at the site of the Interleukin-2 (IL-2) promoter with activator protein-1 (AP-1), which is a heterodimer comprising a Fos and a Jun protein (Schreiber and Crabtree 1992, Jain et al. 1992), thereby inducing transcription of IL-2 (Jain et al. 1993). Interfered nuclear localization of NFAT, induced by FK506, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991).

Besides IL-2, NFAT is known to bind cooperatively at the promoters of other T-cell cytokines, such as Interleukin-4 (IL-4) (Macian et al. 2005).

Treatment of activated T cells with FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) for 2 hours hinders the formation of functional NFAT/AP-1 in the nucleus (Flanagan et al. 1991).

How it is Measured or Detected

Reductions in NFAT/AP-1 complex formation can be detected using a gel shift assay to test nuclear extracts from either stimulated or unstimulated Ar-5 T cells with radio-labelled NFAT binding oligonucleotide from murine IL-2 promoter. Anti-Fos and anti-Jun antibodies are used to examine NFAT/AP-1 complex formation (Jain et al. 1992).

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Event: 1202: Suppression, IL-2 and IL-4 production

Short Name: Suppression, IL-2 and IL-4 production

Key Event Component

Process	Object	Action
interleukin-2 production	interleukin-2	decreased
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin
Dexamethasone
Azathioprine
Methotrexate
Benzo(a)pyrene
Urethane
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
cynomolgus monkey	Macaca fascicularis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNIs suppress production of IL-2, IL-3, IL-4, IL-5, IFN-γ, Granulocyte Macrophage colony-stimulating Factor (GM-CSF), and other cytokines, as induced by CD2/CD3 or CD3/CD26 stimulation, in human peripheral blood mononuclear cells (PBMC) (Sakuma et al. 2001a). Also, CNIs (FK506 and CsA) suppress production of IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, Tumor Necrosis Factor-α, IFN-γ, and GM-CSF, as induced by CD3/PMA stimulation, in human PBMC (Dumont et al. 1998).

CNIs (FK506 and CsA) exhibit suppression of IL-2 production induced from mixed lymphocyte reactions in mice and humans (Kino, T et al. 1987a).

Treatment with CsA or 2C1.1 resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

These facts indicate that Calcineurin-NFAT system-mediated suppression of cytokines is commonly found in humans, monkey and mice.

Key Event Description

Production of T cell cytokines including Interleukin (IL)-2 and IL-4 is regulated by nuclear factor of activated T cells (NFAT)/ activator protein-1 (AP-1) complexes. Activated NFAT/AP-1 complex that bind at the site of the IL-2 and IL-4 promoters, thereby induces transcription of IL-2 and IL-4 (Jain et al. 1993). For IL-2, NFAT proteins are necessary for IL-2 gene expression and cooperation of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cells. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016).

Calcineurin inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) hinder the formation of the functional NFAT/AP-1 complexes by interfering with NFAT nuclear localization (Flanagan et al. 1991). Reduced binding of NFAT/AP-1 complexes at the promoter site of the IL-2 gene lowers the transcription of the mRNA of IL-2 and the following cytokine production.

Transcription of IL-4 is also inhibited by CNIs in the same manner as IL-2 (Dumont et al. 1998).

In CD3/ phorbol 12-myristate-13-acetate (PMA)-activated human T cells, FK506 suppressed production of IL-2, IL-4, and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at 10 nM (Dumont et al. 1998).

Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC_{25} and IC_{50} for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC_{25} and IC_{50} for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (KLH) (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).

Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).

CNIs is considered to increase carcinogenicity through the suppression of IL-2 and IL-4 production.

- Renal transplant patients on immunosuppressive therapy were found to develop cancer within 10 years after surgery (Luster, M.I. et al. 1993).

In experimental animal studies, carcinogenicity of FK506 was reported as follows.

- In mice subjected to topical application testing, in which 100 µL of FK506 ointment was applied once daily for two years to roughly 40% of the total body area, an increased incidence of lymphoma was found in mice of the 0.1% ointment group showing high blood concentrations of the drug (Maruho Co., Ltd 2014).
- In hairless albino mice, virtually all of which developed skin tumors after a 40-week exposure to ultraviolet light, application of a 1% FK506 ointment reduced the time to outbreak of the skin tumors. (Maruho Co., Ltd 2014).

How it is Measured or Detected

Quantitation of cytokine content was done on appropriately diluted samples, run in duplicate, using Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) kits to test matched Antibody pairs with biotin-horseradish peroxidase-streptavidin detection and 3,3',5,5'-tetramethylbenzidine substrate. ELISA plates were scanned in a Molecular Devices UVmax plate reader (Menlo Park, CA), using SOFT max software (Molecular Devices) (Dumont et al. 1998).

Ex vivo whole blood stimulated cytokine (IL-2, IL-4, IL-5, and IL-17) production assay in the supernatants were determined using an electrochemiluminescent immunoassay from Meso Scale Discovery (MSD; Gaithersburg, MD) (Kevin, G. et al. 2014).

Total RNA was extracted using RNeasy mini kit (Qiagen, Chatsworth, CA) and quantitated by absorbance at 260 nm. Cytokine mRNAs were detected using a RiboQuant MultiProbe RPA system (PharMingen, San Diego, CA). Riboprobes were 32P-labeled and hybridized overnight with 10 to 30 mg of the RNA samples. The hybridized RNA was treated with RNase and purified according to the RiboQuant protocol. The samples were then electrophoresed in 6% polyacrylamide-Tris-borate-EDTA-urea gels using the Seqi - Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Hercules, CA), or minigels (Novex, San Diego, CA). The gels were dried, exposed and quantitated in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using the ImageQuant software (Dumont et al. 1998).

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List of Adverse Outcomes in this AOP

Event: 984: Impairment, T-cell dependent antibody response

Short Name: Impairment, T-cell dependent antibody response

Key Event Component

Process	Object	Action
Immunosuppression		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:154 - Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	AdverseOutcome

Stressors

Name
Tacrolimus (also FK506)
Cyclosporin
1,2:5,6-dibenzanthracene
psychosocial stress

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
cynomolgus monkey	Macaca fascicularis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNIs induced impairment of TDAR is demonstrated with rodent studies. That is, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino et al. 1987). Likewise, oral administration of FK506 to rats over a four-week period reduced production of both anti-KLH-IgG and IgM antibodies after subcutaneous immunization with KLH (Ulrich et al. 2004). Moreover, Treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum SRBC-specific IgM and IgG (Kevin, G. et al. 2014). As for humans, in vitro experiments showed that treatment with FK506 or CsA of peripheral blood mononuclear cells from blood-bank donors suppressed the production of IgM and IgG antibodies specific to T-cell–dependent antigens. (Heidt et al, 2009) Also, in SKW6.4 cells (IL-6–dependent, IgM-secreting, human B-cell line) cultures, FK506 or CsA suppressed the production of IgM antibodies in the presence of T-cell activation. (Sakuma et al. 2001b) Considering that FKF506 and CsA reduce T cell-derived cytokines including IL-2 and IL-4, these findings strongly suggest that impairment of TDAR following reduced production of such cytokines occurs at least in common among humans monkey and rodents.

Key Event Description

Antibody production to T-cell–dependent antigens is established through the coordination of B cells, antigen-presenting cells as well as T-cell–derived cytokines, which stimulate B cells to proliferate and differentiate. T-cell–dependent antibody response (TDAR) might be altered if any of these cell populations is affected.

Interleukin (IL)-2 stimulates B cells to proliferate through surface IL-2 receptors. IL-4 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Suppressing the production of these B-cell–related cytokines appears to impaire TDAR, as seen in the result of FK506 treatment (Heidt et al, 2009).

IL-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production. IL-2 promotes differentiation of B cells through IL-2 stimulates differentiation of the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs TDAR (Alberts et al. 2008).

In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) decrease in anti- keyhole limpet hemocyanine (KLH) immunoglobulin (Ig)G. (Alessandro, B. et al. 2003).

In female B6C3F1 mice, 1,2:5,6-dibenzanthracene (DBA) exposure reduced total IgG antibody in spleen cell culture supernatants after in vitro stimulation with lipopolysaccharide (LPS) (Donna, C. et al. 2010).

Treatment with cyclosporin A (CsA) at 50 mg/kg BID via oral gavage in cynomolgus monkey resulted in reduction of serum sheep red blood cells (SRBC)-specific IgM and IgG (Kevin, G. et al. 2014).

After a 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41.6 and 83.2 nM) of CsA (Heidt et al, 2009).

After a 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated peripheral blood mononuclear cells (PBMC) culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.012 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.083 to 83.2 nM) of CsA (Sakuma et al. 2001b).

Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG was reduced at the dose level of 3 mg/kg/day (Ulrich et al. 2004).

Mice were treated with FK506 or CsA for 4 days, and immunized with SRBC, after which antigen-specific plaque-forming splenocytes were reduced at dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).

As immunosuppression-derived adverse outcomes by calcineurin inhibition, FK506 and CsA increase the frequency and/or severity of infections and allergic reactions impaired TDAR deems to be one of the causative factors for these side effects . Some clinical trials of FK506 and CsA revealed these adverse effects as follows.

- In clinical trials of renal transplantation using FK506 or CsA, opportunistic infections such as candida, cytomegalovirus and herpes simplex virus were reported (Ekberg et al. 2007).
- In recipients of liver transplants treated with FK506 or CsA, opportunistic infections such as cytomegalovirus, hepatitis C virus, hepatitis B and herpes simplex virus were reported (Fung et al. 1991).
- Cardiac transplant patients treated with cyclosporin developed pulmonary infections within the first year after surgery (Luster, M.I. et al. 1993).
- In patients of X-linked autoimmune enteropathy treated with CsA or FK506, serum levels of IgE developed extremely high during the immunosuppressive therapy (Kawamura et al. 1997).
- Renal transplant recipients treated with belatacepy/mycophenolate (MMF)/predonisone or FK506/MMF/prednisone showed significantly lower the geometric mean hemagglutination inhibition titer against influenza vaccine, hemagglutination-specific IgG and isotype IgG1 antibodies, and IgG-antibody secreting cells response (Gangappa et al. 2019).

How it is Measured or Detected

TDAR could be examined in vivo and in vitro.

In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with Enzyme-Linked ImmunoSorbent Assay (ELISA) or with a plaque-forming cell (PFC) assay.

- Rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH, after which the serum was examined for T-cell–dependent, antigen-specific, IgM and IgG levels using a Sandwich ELISA kit (Ulrich et al. 2004).
- Mice were repeatedly administered calcineurin inhibitors (CNIs) including FK506 and CsA orally for 4 days and immunized with SRBC, after which spleen cells were examined using a PFC assay (Kino et al. 1987).
- Cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBC, after which the serum was examined for Anti-SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Kevin, G. et al. 2014).
- Mice were exposed a single pharyngeal aspiration of DBA, after which supernatants of splenocytes cultured for 24 h in the

presence of LPS and assayed using a mouse IgM or IgG matched pairs antibody kit (Bethyl Laboratories, Montgomery, TX) (Donna, C. et al. 2010).

For in vitro studies, total IgM and IgG levels in culture supernatant are often measured after polyclonal T-cell activation rather than measuring antigen stimulation in immune cell cultures.

- T cells and B cells isolated from human peripheral blood mononuclear cells (PBMC) were co-cultured with a CNIs for nine days in the presence of polyclonal–T-cell stimulation, after which supernatants were tested for immunoglobulin IgM and IgG levels using a Sandwich ELISA kit. Treatment with FK506 or CsA reduced the levels of IgM and IgG at the concentrations of 0.3 and 1.0 ng/mL or 50 and 100 ng/mL (Heidt et al, 2009).
- SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) were cultured with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. After culturing for four days, IgM produced in the culture supernatants was measured using an ELISA kit. FK506 or CsA reduced the levels of IgM at the concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma et al. 2001b).
- In order to examine class switching, T cells derived from human PBMCs were cultured with CNIs, and cytokine mRNA levels of Interferon-gamma, IL-2, IL-4, IL-5, IL-10, IL-13, and other B-cell–stimulatory cytokines produced in T cells were measured by quantitative PCR (Dumont et al. 1998).

Regulatory Significance of the AO

The ICH S8 guideline, which covers immunosuppression of small molecule drugs, determines the need for immunotoxicity studies by comprehensively evaluating the findings of pharmacology, changes in the immune system in repeated-dose toxicity studies, and other factors using a Weight of Evidence approach. If there is concern about immunotoxicity, the presence or absence of immunotoxicity should be determined using an in vivo test system capable of assessing the functional changes of predicted immunotoxic target cells. If immunotoxicity is observed, additional studies including in vitro assays or clinical evaluation should be considered to assess the risk of immunotoxicity in humans. Because TDAR involves many immune cell populations, including T cells, B cells, and antigen-presenting cells, evaluation of TDAR is recommended when there is concern about immunotoxicity but the immunotoxic target cells are unclear. The S8 guidelines list KLH, SRBC, and tetanus toxin as antigens for TDAR.

The draft FDA immunotoxicity testing guidance (2020) covers immunosuppressive and immunostimulatory drugs and biologics; evaluating immunosuppressive drugs in the draft FDA guidance is similar to that in the S8 guideline, with in vivo TDAR assays recommended when toxic target cells are unknown. The draft guidance states that TDAR assays using KLH as an antigen have been established in mice, rats, dogs, minipigs, and cynomolgus monkeys, but the use of SRBC and tetanus toxin as antigens is also acceptable.

For the assessment for pesticides, US EPA OPPTS 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC. The REACH guideline does not provide for immunotoxicity testing, but it provides triggers for conducting immunotoxicity testing.

The WHO/IPSS Immunotoxicity Risk assessment Guidance (2012) describes a strategy for assessing five categories of immunotoxicity risks, including immunosuppression. For risk assessment of immunosuppression, it calls for identification of immunosuppression risks, prediction of pathogenesis that may occur, and consideration of safety margins based on the WoE approach from human findings, infection resistance tests, immune function tests, general immune system assays, histopathological findings and organ weights in general toxicity studies, and hematological data.

The evaluation of immunotoxicity in F1 animals in the OECD Guidelines for Extended First Generation Reproductive and Developmental Toxicity Studies (TG443) requires that PFC and ELSA assays to measure primary IgM antibody production by TDAR using T-cell dependent antigens (SRBC, KLH, etc.) be performed. Furthermore, if changes are observed, the significance of the changes should be examined by comprehensively evaluating other data.

The outcomes of immunosuppression are susceptibility to infection and tumorigenesis, and the FDA guidance requires that immunosuppressive drugs be evaluated for carcinogenic risk using WoE approach based on the results of carcinogenicity and immunotoxicity studies. Meanwhile, the ICH S1B(R1) Draft Step 2 Guidelines for Carcinogenicity Testing calls for evaluation of carcinogenicity by WoE approach instead of rat carcinogenicity testing, because rodent carcinogenicity test models are less capable of detecting carcinogenicity. On the other hand, it is difficult to define susceptibility to infection as a measurable AO with a clear mechanism, because immune responses vary among pathogens. In fact, many immunotoxicity guidelines require that the risk of immunotoxicity be identified and assessed by evaluating immune functions.

In AOP154, it was difficult to define susceptibility to infection as an AO for the AOP154, so TDAR, which is recommended as an indicator of immunosuppresoin in many guidelines, was used as an AO. It is expected that several AOPs with TDARs as AOs will be developed, and based on these AOPs, it may be possible to develop an IATA to assess the risk of immunotoxicity characterized by TDARs.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1508: Inhibition, Calcineurin Activity leads to Interference, nuclear localization of NFAT

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Mus musculoides	Mus musculoides	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Sex **Evidence**

CN is broadly distributed throughout the body, and the structure of CnA and CnB is highly conserved from yeasts to humans (Kincaid. 1993).

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents and other mammalian species (Rao et al. 1997).

FKBP is found in a wide variety of organisms, from prokaryotes to multicellular organisms (Siekierka et al. 1989). Multiple subfamilies of FKBP have been reported, with at least eight types having been found in mammals. FKBP12 is reported to be expressed in B-cells, Langerhans cells, and mast cells as well as in T-cells of humans, mice and other mammalian species.

Cyclophilins have been found in mammals, plants, insects, fungi and bacteria. They are structurally conserved throughout evolution and all have PPIase activity (Wang P et al. 2005). They form binary complexes with their ligand cyclosporine A.

These facts indicate that CN and immunophilins are conserved among animals and plants although they show multiple physiological functions.

In addition, CNI/immunophilin complex-induced inhibition of CN phosphatase activity resulting in suppression of immune responses is found in humans and mice.

Key Event Relationship Description

The phosphatase activity of calcineurin (CN) is known to be inhibited by CN inhibitors (CNIs) such as FK506 and cyclosporin A (CsA) through the formation of complexes with immunophilins.

Immunophilins of FK506-binding protein (FKBP) and cyclophilin bind with CNIs FK506 and CsA to form complexes, which inhibit CN activity (Barik. 2006).

While FKBP12, FKBP12.6, FKBP13, and FKBP52 are all part of the FK506-binding FKBP family, FKBP12 has a significant involvement in the mechanism of action for FK506-induced immunosuppression (Siekierka et al. 1989, Kang et al. 2008).

FKBP12 is a 12-kDa protein localized in cytoplasm and has been isolated from Jurkat T-cells as a receptor that binds to FK506 (Bram et al. 1993). FKBP12 has an FK506-binding domain (FKBD) that comprises 108 amino acids, and is expressed in T cells, B cells, Langerhans cells, and mast cells (Siekierka et al. 1990, Panhans-Gross et al. 2001, Hultsch et al. 1991).

Cyclophilin and FKBP both exhibit peptidyl propyl isomerase (PPIase) activity, but inhibition of PPIase activity is not related to CN regulation.

CN is a heterodimer that comprises a catalytic subunit (CnA) and a Ca-binding regulatory subunit (CnB). CnA handles phosphatase activity as well as calmodulin binding, and CnB regulates intracellular calcium and CnA (Klee et al. 1988, Zhang et al. 1996). CnA is a 59kDa protein with a serine-threonine phosphatase domain.

CNI-immunophilin complexes such as FK506/FKBP complexes and cyclophilin/CsA complexes bind directly to CnA in the cell, causing steric hindrance of substrate binding to CN, which in turn inhibits phosphatase activity of CN (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

The nuclear factor of activated T cells (NFAT) is a substrate of CN (Rao et al. 1997).

When T-cell activation takes place, T-cell–receptor-mediated stimulus increases the intracellular concentration of calcium and activates CnB, which subsequently induces CnA phosphatase activation, leading to dephosphorylation of NFAT. In that process, dephosphorylated SP motifs expose the nuclear localization signal (NLS) and cover nuclear export signal (NES), thereby promoting nuclear localization of NFAT (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

When CN activity is inhibited by the binding of immunophilin complexes, dephosphorylation does not occur in NFAT, thereby resulting in nuclear export.

Evidence Supporting this KER

Biological Plausibility

The molecular structures and functions of CN and NFAT are based on sufficient scientific evidence as mentioned above The known mechanisms for inhibition of CN phosphatase activity by FK506, CsA, or other CNIs are initiated by the formation of complexes with their respective immunophilin species. Immunophilins are general classes of proteins that exhibit PPIase activity, but the isomerase activity per se is not relevant for CN activity indicating that the latter is affected by the molecular structure of the complex (Schreiber and Crabtree 1992, Liu et al. 1993, Bierer et al. 1993, Bram et al. 1993, Rao et al. 1997, Liu et al. 1991).

As mentioned above, inhibition of CN phosphatase activity interferes with the dephosphorylation of NFAT, which leads to the suppression of its nuclear localization.

Empirical Evidence

Much experimental data is available that supports the inhibition of CN activity induced by CNI/immunophilin complexes, which subsequently suppress nuclear localization of NFAT. In addition, CN phosphatase activity is inhibited by 24 hours treatment with CNI of FK506 and CsA with IC50 values of 0.5 and 5 nM, respectively (Fruman et al.1992).

Also, concentration-dependent reduction of in vitro nuclear localization of NFAT was evident using imaging flowcytometry at the maximum concentration of 1 µM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM (T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013). Interference with translocation of NFAT to the nucleus is also detected using gel mobility shift assay to test nuclear extracts and cytoplasmic extracts, in which the examined concentration of FK506 was 10ng/mL (Flanagan et al. 1991).

These findings show that dose responses and temporality of MIE and KE1 seem to be the same.

Uncertainties and Inconsistencies

CN and NFAT are expressed in T cells and other immune cells including B cells, DC, and NKT cells and related to cytokine productions from these immune cells. Also, expression of IL-2 receptors (IL-2R) in DCs are lowered due to the inhibition of CN phosphatase activity by CNI treatment. Of these, reduced production of IL-2 and IL-4 from T cells plays a major role in suppression of TDAR due to lower proliferation, differentiation, and class switching of B cells. There have been no reports of CNI-induced reduction of cytokines other than IL-2 and IL-4 or reduced expression of IL-2R resulting in TDAR suppression.

FKBP12, a specific immunophilin that binds with FK506, is also an accessory molecule that binds to IP3 and Ryanodine receptors, both of which occur in Ca channels located on the membrane of the endoplasmic reticulum and participate in the regulation of intracellular Ca concentration. When binding with FK506, FKBP12 leaves these receptors to increase the influx of Ca2+ from the endoplasmic reticulum to cytoplasm, which should increase CN activity. Treatment with FK506, however, suppresses NFAT nuclear localization. In addition, FKBP12-knock out mice show no changes in immune function, including T-cell function. These facts suggest that the inhibition of CN-NFAT systems induced by FK506 treatment results from direct inhibition of CN phosphatase activity by FK506/FKBP12 complexes and not by affecting Ryanodine and IP3 receptors associated with FKBP12.

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

Dose-response analysis of the effects of FK506 on CN phosphatase activity in mast cell-derived KiSVMC4W cells transfected with human FKBP12 cDNA showed that increased expression of FKBP12 resulted in a greater than ten-fold increase in sensitivity to FK506-mediated inhibition, as indicated by an IC50 value of roughly 2 nM with linear inverse dose-response curve after 1 hour incuvation (Fruman et al.1995). Another phosphatase assay showed that FK506 inhibition of CN activity was concentration-dependent reverse sigmoidal and that IC50 values for CN inhibition were approximately 0.5 nM for FK 506 and 5 nM for CsA after 1 hour culture (Fruman et al.1992).

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing CNI concentrations from 0.1 nM (Jurkat human T cells) up to 1 µM (1000 nM) using imaging flowcytometry. Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again at maximal concentrations of 1 µM with minimum concentration of 10nM (Maguire et al. 2013).

So far, there is no evidence available that the dose response of inhibition of CN phosphatase activity is correlated with nuclear translocation of NFAT; however, the concentration ranges of CNIs for inhibition of CN phosphatase activity and nuclear translocation of NFAT seem to be the same range.

Time-scale

Inhibition of CN phosphatase activity was examined after 1 hour culture of T cells (Fruman et al.1995, Fruman et al.1992), and inhibition of nuclear translocation of NFAT was measured by imaging flowcytometry after 2 hour culture of T cells with CNI (Maguire et al. 2013).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1017: Interference, nuclear localization of NFAT leads to Reduction, NFAT/AP-1 complex formation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

NFAT expresses in B cells, mast cells, neutrophils, granulocytes, dendritic cells, macrophages, and natural killer cells as well as T cells from humans, rodents, and other mammalian species (Rao et al. 1997).

CN-NFAT system functionality is common among mammalian species, including humans and rodents. It is also possible that FK506-induced interference with NFAT/AP-1 complex formation at the promoter site of the IL-2 gene is common among mammalian T cells, including those of humans and rodents (Flanagan et al. 1991).

Key Event Relationship Description

Activated (dephosphorylated) nuclear factor of activated T cells (NFAT) is translocated into the nucleus through the molecular changes of exposing nuclear localization signal (NLS) and concomitant masking of nuclear export signal (NES) due to dephosphorylation of the SP motifs of NFAT. (Matsuda and Koyasu 2000, Zhu and McKeon 1999).

In the nucleus NFAT binds with AP 1 at the IL-2 promoter region, (Schreiber and Crabtree 1992; Jain et al. 1992) and induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

Once CN phosphatase activity is inhibited, dephosphorylation of NFAT and subsequent nuclear localization of NFAT decreases, which results in a decrease of NFAT/AP-1 complex formation at the cytokine promoter sites (Rao et al. 1997).

Evidence Supporting this KER

Biological Plausibility

As has been mentioned, NFAT has NLS and NES domains among and adjacent to the N-terminal region rich in SP motifs, and once the SP region is dephosphorylated, the NLS domain is exposed whereas the NES doman is covered, which leads to translocation of NFAT into the nucleus (Matsuda and Koyasu 2000).

It is well known from the experiments using CN inhibitors (CNIs) that interference with the nuclear localization of NFAT in T cells leads to a reduction in the formation of NFAT/AP-1 complexes, thereby suppressing transcription of IL-2, IL-4, and a number of other cytokines (Maguire et al. 2013, Jain et al. 1992, Jain et al. 1993).

In contrast to T cells, B-cell receptor-mediated increases in intracellular concentration of calcium in B cells leads to NFAT nuclear localization, thereby producing some classes of cytokines in the same manner as T-cells (Bhattacharyya et al.2011). However, there has been no report of any evidence that CNI acts directly on B cells to effect antibody production.

Expression of IL-2 receptors in dendritic cells and NKT cells is also reported to be regulated by this CN-NFAT system (Panhans-Gross A et al. 2001; Kim et al. 2010), but there is no report showing that CNIs suppress TDAR through the changes in IL-2R expression in these cells.

Empirical Evidence

The relationship of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

Imaging flowcytometry revealed that concentration-dependent reduction of in vitro nuclear localization of NFAT was evident at the maximum concentration of 1 μM with minimal concentration of 0.1nM (Jurkat human T cell line) or 10nM ($CD4^+$T cells from whole blood) after 2 hours treatment of tacrolimus (Maguire et al. 2013).

Gel mobility shift assays using Ar-5 human T cells stimulated with cross-linked anti-CD3 antibody showed that NFAT/AP-1 (cFos and Jun) complexes were found only in the nuclear extract with preexisting NFAT in the cytoplasm after T cell stimulation and that the NFAT/AP-1 complexes in the nucleus decreased after 2 hours treatment with CsA at 1μM (Jain et al. 1992). Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991) NFAT/AP-1 complex formation was also reported to be inhibited by CNI (Rao et al. 1997).

Quantitative understanding of NFAT/AP-1 complex formation in the nucleus is insufficient although nuclear NFAT/AP-1 complex formation was shown to be inhibited by FK506 at concentrations within the range of FK506 for the inhibition of nuclear translocation

of NFAT.

Uncertainties and Inconsistencies

Nothing especially

Quantitative Understanding of the Linkage

Response-response relationship

The relationship of the interference of nuclear localization of NFAT leading to reduced NFAT/AP-1 complex formation bound at the promoter sites of cytokine genes in the presence of CNIs is well known as mentioned above.

KE1:

Dose-dependent interference with nuclear translocation of NFAT1 was observed with increasing FK506 concentrations from 0.01nM (Jarkat T cells) up to 1 µM (1000 nM). Higher concentrations induced cellular toxicity and resulted in cell death. Dose-dependent interference of nuclear NFAT1 translocation per CN inhibition was also observed in CD4+ T cells from healthy donors, again from 10nM to maximal concentrations of 1 µM (Maguire et al. 2013). Both parameters were measured after 2 hour culture of T cells with FK506.

KE2:

Reduction in generation of NFAT/AP-1 complexes can be detected using a gel shift assay (Rao et al. 1997, Jain et al. 1992, Jain et al. 1993).

Decreased NFAT translocated to the nucleus, induced by FK506 at 100ng/mL (124nM) or CsA at 500ng/mL (416nM) after 2 hours treatment, hinders the formation of the functional NFAT/AP-1 complexes necessary to binding at the site of IL-2 promoters (Flanagan et al. 1991). As mentioned above, gel mobility shift assays also showed that NFAT/AP-1 complexes were formed only in the nucleus after T cell activation with unchanged preexisting NFAT in the cytoplasm and that treatment of T cells with 1µM FK506 led to decease the levels of NFAT/AP-1 complex (Jain et al. 1992).

These findings suggest that nuclear translocation of NFAT after T cell stimulation is strongly related to the complex formation with AP-1 in the nucleus, and FK506 was shown to inhibit NFAT/AP-1 complex formation in the nucleus at the concentrations within the concentration range of FK506 for suppressing nuclear translocation of NFAT (Maguire et al. 2013).

Time-scale

Nuclear translocation of NFAT was shown to be inhibited in vitro using imaging flowcytometry after 2 hours culture of T cells with FK506 (Maguire et al. 2013), and gel mobility shift assays revealed the inhibition of nuclear translocation of NFAT and following complex formation with AP-1 within the nucleus after 2 hours culture of T cells with FK506 (Jain et al. 1992, Flanagan et al. 1991).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1509: Reduction, NFAT/AP-1 complex formation leads to Suppression, IL-2 and IL-4 production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1, IL-2 production and CD25 (IL-2R) up-regulation (Yoshida et al. 2015).

In splenic lymphocytes and/or CD4+ T cells, ursolic acid suppressed products of NF-kB, NFAT and AP-1, and inhibits secretion of IL-2 and IL-4, mRNA level of IL-2 and CD25 expression (Checker et al. 2012).

NFATp- and NFAT4-deficient mice indicate decreased production of IL-2 (Ranger et al. 1998).

NFAT/AP-1 complex formation in the nucleus was shown using murine and human T cells lines (Jain J et al. 1992). In addition to data on suppression of cytokine production by CNI in rodents, FK506 is reported to inhibit expression of both IL-2 and mRNA in human anti-CD3/PMA-activated cells (Dumont et al. 1998).

Key Event Relationship Description

Localized nuclear factor of activated T cells (NFAT) in the nucleus of T cells forms complexes with activator protein-1 (AP-1) at the Interleukin (IL)-2 promoter region (Schreiber and Crabtree 1992; Jain et al. 1992), which induces transcription of IL-2 (Jain et al. 1993). In addition to IL-2, NFAT localized in the nucleus of T cells also binds to the promoter region of the other classes of cytokines including IL-4 and IL-13.

For IL-2, NFAT proteins are necessary for IL-2 gene expression and interaction of NFAT with AP-1 is required for IL-2 gene transcription. For IL-4, At least five different NFAT sites have been described in the IL-4 promoter with at least three of them being composite sites binding NFAT and AP-1 (Macián et al. 2001).

Lowered nuclear localization of NFAT by calcineurin inhibitor (CNI) results in decreased formation of NFAT/AP-1 complex at the promoter region of IL-2 genes in the nucleus of T cells thereby reducing the transcription of IL-2 (Dumont et al. 1998). Production in T cells of IL-4 and other classes of cytokines is also suppressed in the same manner as IL-2 (Dumont et al. 1998).

Evidence Supporting this KER

Biological Plausibility

T-5224, a selective c-Fos/AP-1 inhibitor, inhibits the DNA-binding activity of AP-1 in primary murine T cells. T-5224 also inhibits CD25 (one of IL-2 receptors) up-regulation, IL-2 production, and c-Fos DNA-binding activity in mice (Yoshida et al. 2015).

Dexamethasone represses the IL-2 mRNA induction. glucocorticoid-induced leucine zipper (GILZ) is one of the most prominent

glucocorticoid-induced genes, and inhibited the induction of the NFAT reporter and interferes with the AP-1 component of the NFAT/AP-1 complex. GILZ also inhibits the IL-2 promoter (Mittelstadt et al. 2001).

Ursolic acid suppressed activation of three immunoregulatory transcription factors NF-kB, NFAT and AP-1. Treatment of lymphocytes and CD4+ T cells with ursolic acid inhibited secretion of IL-2 and IL-4 cytokines. Treatment of CD4+ T cells with ursolic acid suppressed mRNA level of IL-2. Treatment of lymphocytes with ursolic acid inhibited the upregulation of CD25 expression on T cells (Checker et al. 2012).

NFATp- and NFAT4-deficient mice indicate decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is generally accepted that NFAT, translocated to the nucleus after T-cell stimulation, binds with AP-1 to the promoter regions of the cytokine genes to mount transcription, which follows production of these T-cell–derived cytokines. Of these cytokines, IL-2 and IL-4 promote proliferation, maturation, and class-switching of B cells to enhance TDAR.

There is also sufficient evidence to support the hypothesis that CNI-induced decreases in T-cell–derived cytokine production is mediated through suppressed nuclear localization of NFAT, with a resultant decrease in the amount of NFAT/AP-1 complex binding to the promoter regions of T-cell-derived cytokines.

When stimulated with ovalbumin, calcineurin A (CnA)-knockout (KO) mice produce less Interferon (IFN)- γ, IL-2, and IL-4 than wild-type mice. However, primary antibody response in CnA-KO mice is normal in response to trinitrophenol-ovalbumin (Zhang et al. 1996).

The following phenotypes are observed in NFAT-KO mice: moderate hyperproliferation with splenomegaly; moderately enhanced B- and T-cell responses, with bias towards Th2- cell responses; decreased IFN-γ production in response to TCR ligation; reduced proliferative responses by T cells; impaired repopulation of the thymus and lymphoid organs; impaired Th2-cell responses and IL-4 production; grossly impaired T-cell effector functions, with profound defects in cytokine production and cytolytic activity; B-cell hyperactivity; impaired development of CD4 and CD8 single-positive cells, with increased apoptosis of double-positive thymocytes; and mild hyperactivation of peripheral T cells (Macian, 2005).

Therefore, the study of NFAT-KO mice shows that NFAT is involved in a wide range of immune responses, and some of these phenomenon are known to be regulated by calcineurin (CN). Suppression of T-cell-derived cytokines is noted both in CnA-KO and NFAT-KO mice, which indicates that the production of T-cell derived cytokines such as IL-2 and IL-4 is regulated by the CN-NFAT system.

FK506-FKBP12 complex decreased CN phosphatase activity, which inhibits. Because NF-ATp is an essential transcription factor regulating the IL-2 gene, FK506 ultimately blocks the T-cell response by inhibiting IL-2 transcription (Panhans-Gross A et al. 2001). FK506 inhibited IL-2 mRNA expression in anti-CD3/phorbol 12-myristate-13-acetate (PMA)-activated cells (Dumont et al. 1998).

These facts indicate that although NFAT is widely involved in the function of T cells, the effect of CNIs is to suppress production of some classes of T-cell–derived cytokines through reducing the formation of NFAT/AP-1 complexes induced by inhibition of CN phosphatase activity.

Empirical Evidence

Empirical support of Reduction, NFAT/AP-1 complex formation leading to Suppression, IL-2 and IL-4 production is strong.

Rationale

- In purified T cell from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 and CD25 (one of IL-2 receptors) up-regulation at 80 μg/mL, and IL-2 production in a dose-dependent manner from 40 to 80 μg/mL (Yoshida et al. 2015).
- In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 μM for 4 h. Secretion of IL-2 and IL-4 was inhibited in lymphocytes stimulated with concanavalin A for 24 h at concentrations of 0.5, 1 and 5 μM of ursolic acid, and lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h at concentration of 5 μM of ursolic acid. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 μM for 4 h. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 μM for 4 h (Checker et al. 2012).
- In NFATp- and NFAT4-deficient mice, cultured splenocytes bound anti-CD3 for 48 h indicates decreased production of Th1 cytokine including IL-2 (Ranger et al. 1998).

It is well established that inhibition of NFAT/AP-1 complex formation at the promoter sites reduces the production of T-cell–derived cytokines including IL-2 and IL-4, which are mainly involved in T-cell–dependent antibody response.

- NFAT/AP-1 complex formation is inhibited by CNI shown by gel shit mobility assay using human T cell line or CD4+ T cells from heathy donners after 2 hours treatment with cyclosporin A (CsA) at 1μM.. Preceding NFAT nuclear localization after T cell activation is suppressed with FK506 at the dose range of 0.01nM (Jarkat T cells) or 10nM (CD4+ T cells) to 1μM (Maguire et al. 2013), and NFAT nuclear localization and NFAT/AP-1 complex formation is shown to be strongly related (Jain et al. 1992, Jain et al. 1993).
- In CD3/PMA-activated human T cells, FK506 suppressed production of IL-2, IL-4, and IFN-γ at the concentrations of 1.2 to 12.5 nM after 22 to 24 hours culture as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA in a dose-dependent (10 nM) manner after 3 day culture (Dumont et al. 1998).

- Treatment with CsA completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells with an IC25 and IC50 for IL-2 production of 1.19 nM and 1.99 nM. Treatment with other immunosuppressant compounds (dexamethasone, azathioprine, methotrexate, benzo(a)pyrene and urethane) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations. Urethane, a weakly immunosuppressive chemical, was least potent in the assay, with an IC25 and IC50 for IL-2 secretion of 4.24 mM and 13.26 mM (D.M. Lehmann. et al. 2018).
- In female B6C3F1 mice, 1,2:5,6-dibenzanthracene exposure reduced production of IL-2 in spleen cell culture supernatants after in vitro stimulation with Concanavalin A or lipopolysaccharide (Donna, C. et al. 2010).
- Treatment with CsA at 50 mg/kg BID via oral gavage or 2C1.1 (a fully human anti-ORAI1 monoclonal antibody) at 25 mg/kg single IV resulted in reduction of IL-2, IL-4, IL-5, and IL-17 cytokine production from PMA/ionomycin stimulation of whole blood in the cynomolgus monkey (Kevin, G. et al. 2014).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) reduced IL-2 release in response to keyhole limpet hemocyanine (Alessandro, B. et al. 2003).

Reduced nuclear translocation of NFAT followed by NFAT/AP-1 complex formation and suppression of IL-2/IL-4 productions are shown to occur under similar dose ranges and treatment duration.

Uncertainties and Inconsistencies

CNIs are reported to suppress IL-17 release from Th17 cells and development of Th17 cells from naïve T cells (Tsuda et al, 2012). On the other hand, Yadav reported that Th17 cells increased and Treg cells decreased in number and that the levels of RORC mRNA increased and those of FOXP3 decreased in renal transplanted patients with chronic calcineurin inhibitor toxicity (Yadav, 2015). From these findings, CNIs suppress the functions of Th17 and Treg cells which enhance Th17 cells to develop chronic CNI toxicity.

FK506 suppresses expression of IL-2 receptor (IL-2R: CD25) and costimulatory molecules CD80 (B7.1)/CD40 in Langerhans cells (Panhans-Gross A et al. 2001).

In human NK cells, FK506 suppresses IL-2 responsive proliferation and cytokine production as well as lowers cytotoxicity directed toward K562 tumor cells (Kim et al. 2010). FK506 suppresses IL-2 production of NKT cell line DN32.D3 induced by stimulus from PMA/calcium -ionophore (van Dieren et al. 2010).

The relationship between these FK506-induced mechanisms and NFAT and contribution of those to TDAR are unclear.

In addition to NFAT/AP-1 complexes, NFAT forms complexes at the site of IL-3 and IL-4 enhancers with avian musculoaponeurotic fibrosarcoma oncogene homolog, early growth response 1, early growth response 4, interferon-regulatory factor 4, octamer-binding transcription factor, and other transcriptional partners to induce transcription of a variety of cytokines (Macian 2005). The production of cytokine induced by these transcriptional partners also suppressed by CNI; however, contribution of these additional transcription factors to TDAR is also unclear.

Quantitative Understanding of the Linkage

Response-response relationship

In purified T cells from male C57BL/6J mice, T-5224 (a selective c-Fos/AP-1 inhibitor) inhibits the DNA-binding activity of AP-1 at 80 µg/mL. On the other hand, T-5224 inhibits IL-2 production in a dose-dependent manner from 40, 60 and 80 µg/mL after 48 hours culture. T-5224 also inhibits CD25 (IL-2R) up-regulation at 80 µg/mL (Yoshida et al. 2015).

In splenic lymphocytes stimulated with concanavalin A for 24 h in C57BL/6 mice, ursolic acid suppressed products of NF-kB, NFAT and AP-1 at 5 µM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibits secretion of IL-2 and IL-4 at 0.5, 1 and 5 µM. In lymphocytes and CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid also inhibits secretion of IL-2 and IL-4 at 5 µM. In CD4+ T cells stimulated with anti-CD3/anti-CD28 mAb for 24 h, ursolic acid suppressed mRNA level of IL-2 at 5 µM. In lymphocytes stimulated with concanavalin A for 24 h, ursolic acid inhibited CD25 expression at 5 µM (Checker et al. 2012).

These findings showed that T-5244 and ursolic acid treated for 24 hours inhibit NFAT/AP-1 complex formation at a single concentration each and that these compounds suppress IL-2 and IL-4 production with dose dependent manner including the doses for inhibition of NFAT/AP-1 complex formation.

FK506 suppressed proliferation in human T cells induced by anti-CD3 mAb in the presence of adherent autologous peripheral blood mononuclear cells (mean IC50 = 0.06 nM). FK506 suppressed, in a dose-dependent (1.2 to 12.5 nM) manner after 22-24 hours culture, production of IL-2, IL-4, and IFN-γ by human T cells stimulated with anti-CD3 mAb in the presence of PMA, as well as inhibited, also in a dose-dependent (10 nM) manner, expression of IL-2, IL-4, and IFN-γ mRNA in anti-CD3/PMA- activated cells (Dumont et al. 1998). On the other hand, the quantitative data for the decreased formation of NFAT/AP-1 complexes by CNI is insufficient, although the formation was suppressed by FK506 at the concentration within the range needed for suppressed production of IL2/IL-4 by FK506 after 2 hours culture.

Time-scale

Inhibition of NFAT/AP-1 complex is detected by gel mobility shit assay after 2 hours culture with CNI; however, suppression of IL2/IL-4 could be measured after 22-48 hours in vitro culture.

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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Relationship: 1510: Suppression, IL-2 and IL-4 production leads to Impairment, T-cell dependent antibody response

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
cynomolgus monkey	Macaca fascicularis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In cynomolgus monkeys, the effects of CsA on production of IL-2 and IL-4, and antigen-specific IgM and IgG in TDAR were demonstrated (Gaida K. 2015).

Suppressed IgE and antigen specific IgG1 productions by the blocking of IL-4 receptor were reported in mice using dupilumab (anti-IL-4/13R antibody) (Sanofi K.K. 2018).

Suppressed antigen specific IgE production by the inhibition of IL-4 production was reported in mice using suplatast tosilate (Taiho Pharmaceutical 2013).

Suppressed antigen specific IgE and IL-4 productions by the inhibition of IL-4 production were reported in human cell culture using suplatast tosilate(Taiho Pharmaceutical 2013).

The effects of FK506 on serum concentration of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over four weeks and immunized with KLH (Ulrich et al. 2004). The effects of FK506 and CsA on antigen-specific plaque-forming splenocytes have been demonstrated in mice treated with FK506 or CsA for 4 days and immunized with SRBC (Kino et al. 1987b).

The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt et al, 2009, Sakuma et al, 2001).

The effects of FK506 and CsA on production of IL-2 and IL-4 have been demonstrated using mice and human cells (Kino et al. 1987a, Dumont et al. 1998).

These facts suggest that there are no species differences between humans, monkeys and rodents in inhibitions of IL-2 and IL-4 production and TDAR induction.

Key Event Relationship Description

Interleukin (IL)-2 and IL-4 are produced and secreted by helper T cells and play important roles in the development of T-cell dependent antibody response (TDAR), both of which induces/enhances T cell dependent antibody production. IL-4 affects maturation and class switching of B cells as well as proliferation, IL-2 promotes differentiation of B cells through IL-2 receptors and stimulates the activated T cell into T cell called Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs T cell dependent antibody production (Alberts et al. 2008).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR

T cell-derived cytokines play important roles in the development of TDAR. Among them, IL-2 promotes proliferation of B cells, and IL-4 affects maturation and class switching of B cells as well as proliferation, both of which induces/enhances T cell dependent antibody production.

Thus, suppressing the production of IL-2, IL-4, and other cytokines in T cells reduces stimulation of B cells including proliferation, activation, and class switching, and leading to impairment of TDAR. Therefore, suppressing the production of these B-cell-related cytokines appears to be the main factor in impairment of TDAR by inhibitors of T-cell–dependent-antibody production.

Evidence Supporting this KER

Biological Plausibility

Cyclosporin A (CsA) is known to be one of the calcineurin inhibitiors. CsA-treatment is reported to suppress the productions of IL-2 and IL-4 and result in the reduction of the productions of antigen-specific IgM and IgG in cynomolgus monkeys (Gaida K. 2015).

It is established that IL-2 stimulates B cells to proliferate through the surface IL-2 receptors and that IL-4 stimulates B cells to proliferate, to induce class switch, and to differentiate into plasma and memory cells.

Dupilumab is known as anti-IL-4/13 receptor (IL-4/13R) antibody. Dupilumab (Dupixent) reduces productions of immunoglobulin (Ig) E and antigen specific IgG1 in mice (Sanofi K.K. 2018). It suggests that the blocking of IL-4 signaling by anti-IL-4/13R antibody

results in the decrease in T cell dependent antibody production.

Th2 cell produces cytokines including IL-4. Suplatast tosilate (IPD) is known as an inhibitor of the production of IL-4 and IL-5 from Th2 cells and reduces the production of antigen specific IgE in human cell culture and mice (Taiho Pharmaceutical 2013). These findings suggests that the reduction of IL-4 production by the inhibitor of Th2 cell cytokines results in reduced production of IgE and/or IgG1 through inhibitions of maturation, proliferation and class switching of B cells.

IL-2 binds to IL-2 receptor (IL-2R) and acts on T cell. CD25 is one of IL-2R. Basiliximab (Simulect) is known as anti-CD25 antibody. Basiliximab binds to IL-2R and blocks IL-2 signaling. Clinical transplantation study of basiliximab reveals decreases in rejections. On the other hand, basiliximab inhibits the activation of antigen specific T cells (Novartis Pharma 2016). They suggest that the blocking of IL-2 signaling by anti-IL-2R antibody results in decreased rejection through the inhibition of the activation of antigen specific T cell with reduced antibody production.

FK506 and CsA suppress mRNA expression levels of cytokines in T cells including IL-2 and IL-4 that stimulate proliferation of B cells as well as B cell activation and class switching (Heidt et al, 2010).

Several in vivo studies in rodents showed decreased TDAR by the treatment of FK506 (Kino et al. 1987b, Ulrich et al. 2004). In in vitro tests examining antibody production in blood samples obtained from blood-bank donors, peripheral blood mononuclear cells (PBMC) treated with FK506 and CsA suppressed the production of IgM and IgG antibodies to T-cell dependent antigens (Heidt et al, 2009).

T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

However, as for the suppression of humoral immunity induced by the inhibition of calcineurin (CN) phosphatase activity, calcineurin inhibitors (CNIs) do not affect B cells directly but rather indirectly through T cells. That is, FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B cell response by FK506 and CsA appears due solely to inhibition of T helper cells (Heidt et al, 2010).

Therefore, it is concluded that decreased amounts of IL-2 and IL-4 secreted from helper T cells is the main factor for suppression of TDAR induced by CN phosphatase inhibition.

Empirical Evidence

Empirical support of the suppression, IL-2 and IL-4 production leads to impairment, T-cell dependent antibody response is strong.

Rationale

- Cynomolgus monkeys treated wth CsA at 50 mg/kg BID for 24 days suppression of IL-2, IL-4 and sheep red blood cell (SRBC)-specific IgM and IgG (Gaida K. 2015).
- In the allergen-induced pneumonia model in mice, dupilumab (anti-IL-4/13R antibody) reduced productions of IgE and antigen specific IgG1 at 25 mg/kg of twice weekly subcutaneous administration for 4weeks (Sanofi K.K. 2018).
- In mice immunized with dinitrophenyl antigen by i.p. injection, suplatast tosilate (an inhibitor of the production of cytokines on Th2 cell) reduced productions of antigen specific IgE at 10, 20, 50 and 100 mg/kg of oral administration for 5 days (Taiho Pharmaceutical 2013). In human cell culture immunized with Japanese cedar antigen, suplatast tosilate reduced productions of antigen specific IgE at the concentration of 10 μg/mL for 10 days (Taiho Pharmaceutical 2013).
- In the clinical study of renal transplantation, basiliximab decreased incidence of acute rejection at 20 mg/kg (Novartis Pharma 2016). In human T cell culture immunized with PPD, basiliximab reduced activation of antigen specific T cell at the concentration of 300 ng/mL (Novartis Pharma 2016).
- In CD3/phorbol 12-myristate-13-acetate-activated human T cells, FK506 suppressed production of IL-2, IL-4 and Interferon (IFN)-γ at the concentrations of 1.2 to 12.5 nM as well as inhibited expression of IL-2, IL-4 and IFN-γ mRNA at the concentrations of 10 nM. (Dumont et al. 1998).
- FK506 or CsA suppressed production of IL-2 in mouse mixed lymphocyte reaction (MLR) at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA as well as in human MLR at 0.1 to 10 nM of FK506 and 10 to 100 nM of CsA (Kino et al. 1987a).
- After 9-day culture of B cells and non-pre-activated T cell stimulationwith FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced at 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) of FK506 or 50 and 100 ng/mL (41 and 83nM) of CsA (Heidt et al, 2009).
- After 4-day culture of SKW6.4 cells (IL-6-dependent IgM-secreting human B-cell line) and anti-CD3/CD28 stimulated PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at the concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 or 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma et al, 2001).
- Rats were treated with FK506 for over four weeks and immunized with keyhole limpet hemocyanine (KLH), after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (Ulrich et al. 2004).
- Mice were treated with FK506 or CsA for 4 days, and immunized with sheep red blood cells (SRBC), after which antigen-specific plaque-forming splenocytes reduced at the dose levels of 3.2, 10, 32 and 100 mg/kg of FK506 or 32 and 100 mg/kg of CsA (Kino et al. 1987b).
- 1,2:5,6-dibenzanthracene single adoministration suppressed production of IL-2 and total IgG antibody in mice at the dose levels of 3 and 30 mg/kg(Donna, C. et al. 2010).
- In male CD-1 mice, chronic psychosocial stress (types of social outcome occurred: residents becoming subordinates) for 21 days reduced IL-2 release in response to KLH and decrease in anti-KLH IgG(Alessandro, B. et al. 2003).

In vitro suppression of T-cell–derived cytokines and T-cell-dependent antibody production or antibody production after polyclonal T-cell stimulation showed similar dose responses to CNIs. Time gaps were found, however, between these two KEs, which showed earlier onset of cytokine production and delayed onset of antibody production.

Uncertainties and Inconsistencies

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Therefore, reduced production of both IL-2 and IL-4 might certainly induce suppression of TDAR; however, there remains some possibility of additional suppression of other immune functions.

Quantitative Understanding of the Linkage

Response-response relationship

Cynomolgus monkeys treated wth CsA at 50 mg/kg BID showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In the blocking of IL-4 receptor in mice by dupilumab (anti-IL-4/13R antibody) at 25 mg/kg of twice weekly subcutaneous administration for 4weeks, IgE production was suppressed to about 1/100 and antigen specific IgG1 production was suppressed to about 1/200 (Sanofi K.K. 2018).

In the inhibition of IL-4 production in mice by suplatast tosilate at 10, 20, 50 and 100 mg/kg of oral administration for 5 days, antigen specific IgE production was suppressed from about 1/10 to 1/100 (Taiho Pharmaceutical 2013). In human T cell culture by suplatast tosilate at the concentration of 10 µg/mL, antigen specific IgE production after 10 days was suppressed from 56 to 72% and IL-4 production after 3 days was suppressed from 58 to 76% (Taiho Pharmaceutical 2013).

As for IL-2 and antibody production, in vitro T-cell-induced polyclonal B cell activation to produce antibody was inhibited with anti-IL-2 and anti-IL-2R antibodies. That is, murine small resting B cells, cultured with irradiated hapten-specific TH1 clone, were induced to enter cell cycle at 2 days and to secret antibody at 5 days. An anti-IL-2 and anti-IL-2R antibodies completely inhibited this T-cell dependent antibody production (Owens T, 1991).

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the m-RNA levels of T-cell cytokines at 8h post-stimulation including IL-2 and IL-4 at 1.0ng/mL (1.24nM) FK506 or 100ng/mL (90.7nM) CsA and inhibited IgM and IgG productions after 9 days at 0.3 and 1.0ng/mL FK506 and 50 and 100ng/mL CsA (Heidt S. 2010).

Time-scale

In CsA-treatment for 24 days at 50 mg/kg BID, cynomolgus monkeys showed suppression of IL-2 and IL-4 production and inhibition of SRBC-specific IgM and IgG in TDAR (Gaida K. 2015).

In human T cell culture, suplatast tosilate inhibits IL-4 production after 3 days and antigen specific IgE production after 10 days (Taiho Pharmaceutical 2013).

In the human T-B cell co-culture, CNIs of FK506 and CsA lowered the m-RNA levels of IL-2 and IL-4 at 8h post-stimulation and inhibited IgM and IgG productions after 9 days (Heidt S. 2010).

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is found.

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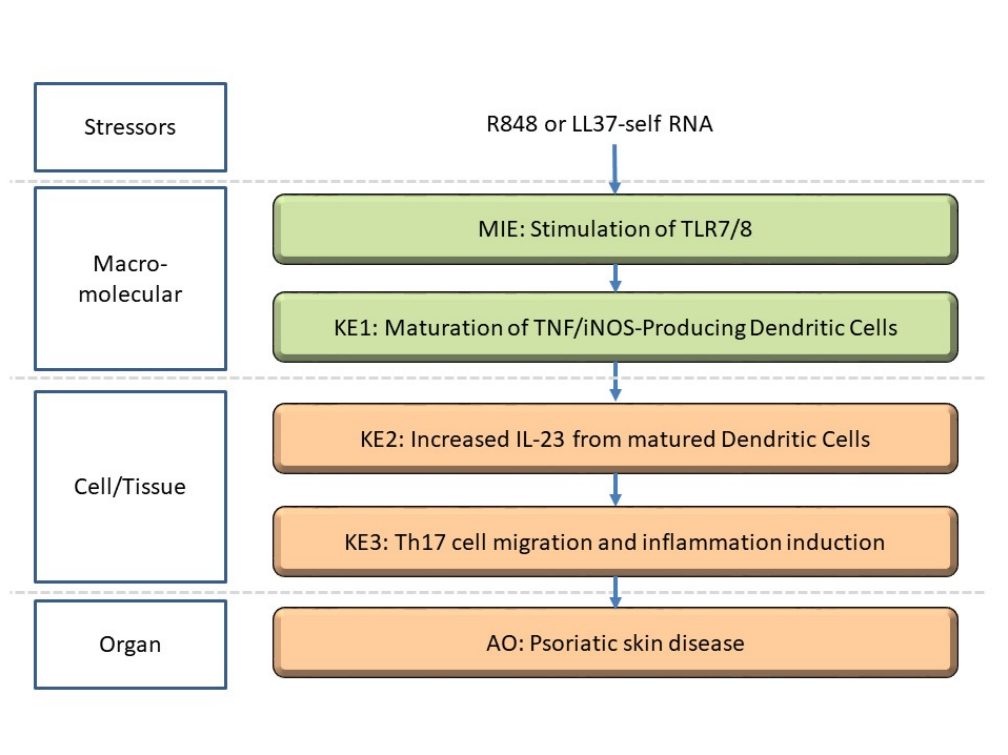
AOP ID and Title:

AOP 313: Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease
Short Title: Skin disease by stimulation of TLR7/8

Graphical Representation



Authors

Hiroyuki Komatsu (1) Takao Ashikaga (1) Tomoki Fukuyama (1) Ken Goto (1) Shinko Hata (1) Shigeru Hisada (1) Shiho Ito (1) Sumie Konishi (1) Tadashi Kosaka (1) Kiyoshi Kushima (1) Shogo Matsumura (1) Takumi Ohishi (1) Yasuharu Otsubo (1) Junichiro Sugimoto (1) Yasuhiro Yoshida (1)

(1) AOP Working Group, Testing Methodology Committee, The Japanese Society of Immunotoxicology

Corresponding author: Komatsu (hiroyuki-komatsu@cmic.co.jp)

Status

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Under development: Not open for comment. Do not cite	Under Development	1.75	Included in OECD Work Plan

Abstract

Toll-like receptor (TLR) 7 and TLR8 are pattern recognition receptors that are known to activate antiviral reaction of immune system, hyperactivation of which can lead to psoriatic skin disease when hyperactivation of them occurred. The relationship between TLR7/8 and immune functions is well understood, and antiviral compound that work by stimulating TLR7/8 have been developed. TLR7/8 agonists such as imidazoquinolin compounds stimulate these TLRs through the formation of homodimer. This signal activates the IL-23/IL-17 axis, which leads to psoriasis and other related skin diseases.

Activation of the IL-23 / IL-17 axis and causes abnormal proliferation and inflammation of the epidermis, which is a pathological condition of psoriasis. This AOP shows an association between TLR7 / 8 stimulation and psoriatic skin disease.

TLR7-mediated signaling in plasmacytoid dendric cells (pDC) is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response, secreting vast amounts of IFN type 1. Similarly, upon engagement of ligands in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation. IFN-α and TNF-α cooperatively mature myeloid dendritic cells. TLR7/8 agonist stimulates a specific population of inflammatory dermal dendritic cells referred as TNF and inducible nitric oxide synthase–expressing DCs (Tip-DCs) to produce IL-23 after maturation by enhanced transcriptional activity.

IL-23R is mainly expressed in Th17 cells. In chronic psoriasis, the cytokines IL-12 and IL-23 produced by resident DC are the main causes. Not only does the expression of IL-23 increases in the skin tissue of the lesion, Th17 cells also increase.

Mature Th17 cells are activated by IL-23 stimulation. Signaling through IL-23 produces cytokines IL-17 and IL-22 that mediate the psoriasis response and promote neutrophil migration into the epidermis, epidermal cell proliferation, and similar responses, which lead to the development of a psoriasis rash. In mice, psoriasis-like hyperplasia is induced by the application of IL-23 but does not occur in IL-17A and IL-22 KO mice, so IL-17A and IL-22 play an important role downstream of IL-23.

IL-17 receptor form heterodimers, and IL-17RA / IL-17RC appears in a variety of cells, including fibroblasts and epidermal cells. IL-17RE / IL-17RA expressed in epidermal cells and IL-17C binding are also important in the pathology of psoriasis. Immunohistochemically, IL-17A is expressed only in cells of the dermal papilla layer, while IL-17C is widely expressed in cells such as hyperproliferative overexpressed keratinocytes, leukocytes, and vascular endothelial cells. IL-17C produces keratinocytes by bacterial stimulation and further stimulates keratinocytes to induce the production of various cytokines and chemokines. Keratinocytes are known to be self-activated by IL-17C.

IL-17 and IL-22 secreted from Th17 act on keratinocytes, causing abnormalities in keratinocytes through the secretion of inflammatory cytokines, chemokines, growth factors, and antimicrobial peptides, and thereby exacerbating the skin symptoms of psoriasis.

The creation of this AOP began with an examination of important event relationships brought about by TLR7 / 8 activity due to environmental or genetic factors and resulting in abnormal differentiation of keratinocytes, which leads to thickening of the epidermis and its resultant autoimmune skin disease, psoriasis

Background

Psoriasis is an chronic autoimmune disease characterized by chronic epithelial inflammatory disease induced by environmental factors such as infection, stress, smoking or alcohol consumption as well as by genetic factors. The onset of psoriasis has been reported to be triggered by drugs and chemical substances use, including beta-blockers, chloroquine, lithium, ACE inhibitors, indomethacin, terbinafine, and interferon alpha. Diagnosis is based on the type and distribution of the lesions.

Psoriasis occurs when abnormal differentiation (keratosis) of keratinocytes leads to thickening of the epidermis. Patients often exhibit an erythema with a clear border and epidermal hyperplasia, stratum corneum hyperplasia, heterocytosis in the stratum corneum, mixed skin moist cells of neutrophilic granulocytes and T cells in the epidermis. Dendritic cells (DC) and macrophages are associated with silver-white plaque. Neutrophilic effusion (Munro microabscesses) are observed in the epidermis, and CD8+ T cells (Tc17) increase the expression of angiogenesis related genes.

The main therapeutic agents are mild topical treatments such as emollients, salicylic acid, coal tar preparations, anthralin, corticosteroids, vitamin D3 derivatives, retinoids, calcineurin inhibitors or tazarotene. UV therapy is also used for moderate or severe psoriasis. Widespread psoriasis is treated with systemic therapies such as immunomodulators methotrexate, cyclosporin, retinoids and other immunosuppressants used alone or in combination.

Although there are stressors that are well known to induce psoriasis-like skin inflammation in mice, this AOP is based primarily on an understanding of stimulation caused by imiquimod, resiquimod or LL37-selfRNA complexes, for which a significant body of scientific literature has been published.

As a test model for psoriasis, an Autoimmune skin disease, mouse tests that induce skin inflammation like psoriasis are frequently conducted using the imidazoquinoline derivative imiquimod. This AOP is primarily based on an understanding of stimuli caused by imiquimod, resiquimod, or LL37-selfRNA complexes.

Imiquimod is derived from imidazoquinoline and is often used to create mouse models. It is our hope that this AOP will contribute to greater knowledge about the development of psoriatic skin diseases that start from stimulation of TLR as well as the development of new treatment targets for psoriasis.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1706	Stimulation, TLR7/8	Stimulation of TLR7/8
2	KE	1822	Maturation of TNF/iNOS-Producing Dendritic Cells	Maturation, TNF/iNOS-Producing Dendritic Cells
3	KE	1707	Increase, IL-23 from matured dendritic cells	Increase of IL-23

Sequence	Type	Event ID	Title	Short name
4	KE	1708	Th17 cell migration and inflammation induction	Th17 cell migration and inflammation induction
5	AO	1709	Psoriatic skin disease	Skin disease

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Stimulation, TLR7/8	adjacent	Increase, IL-23 from matured dendritic cells	High	High
Increase, IL-23 from matured dendritic cells	adjacent	Th17 cell migration and inflammation induction	High	High
Th17 cell migration and inflammation induction	adjacent	Psoriatic skin disease	High	High

Stressors

Name	Evidence
Imiquimod	High
Resiquimod	High

Overall Assessment of the AOP

TLR7/8 is stimulated when imidazoquinolin compounds or stimilar agonists from homodimers TLR7-mediated signaling in plasmacytoid dendritic cells (pDC) is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response,thereby secreting large amounts of IFN-α. Similarly, the engagement of ligands in endosomescauses TLR8 initiate the MyD88-dependent pathway, culminating in synthesis and release of TNF-a and other proinflammatory mediators, via NF-κB activation.

IFN-α and TNF-α cooperatively mature myeloid dendritic cells. TLR7/8 agonist stimulates a specific population of inflammatory dermal dendritic cells referred as Tip-DCs to produce IL-23 after maturation by enhanced transcriptional activity.

Naive T cells differentiate into Naive Th17 by both IL-6 and TGF-β cells that express the transcription factors ROR-γt, ROR-α, and STAT3. These naive Th17 cells are self-activated by IL-21 in an autocrine manner and mature into Th17 cells which express IL-23 receptor on cell surface. Mature Th17 cells are activated by IL-23 stimulation. IL-23-mediated signal transduction produces cytokines IL-17.

IL-17 mediates the psoriasis response, promoting such activities as neutrophil migration to the epidermis,and proliferation of epidermal cells, which leads to the outbreak of psoriasis rash. Thus, psoriatic skin is induced mainly by overproduction of IL-17, which leads to a variety of adverse effects. We have identified a number of key events (KEs) along this pathway and created an AOP for stimulation of TLR7/8 that leads to psoriatic skin disease based on these key event relationships(KERs).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Mixed	High

The proposed AOP for psoriasis-like skin thickening resulting from abnormal differentiation of keratinocytes, starting with Toll-like receptor (TLR) 7/8 activity, is independent of life stage, gender, or age (Lowes et al. 2007). The pathogenesis of psoriasis, an

autoimmune disease, is genetically predisposed (3), but the autoantigen that causes psoriasis has not been identified (Zaba et al. 2008). Other causes of psoriasis are caused by external and internal triggers such as mild trauma, sunburn, infection, systemic drugs, and stress (Hansel et al. 2011). Stimulation of TLR7 / 8 releases INF-α and TNF-α in large amounts to produce IL-23, and Th17 cells mature by the stimulation to produce IL-17 and IL-22. In psoriasis skin formation, cytokines such as TNF-α, IL-23, and IL-17 work continuously. Since TNF-α inhibitors significantly suppressed IL-17A and IL-23p19 expression in psoriatic eruptions (Leonardi et al. 2012), by suppressing self-activation of Tip-DC by TNF-α, It can be seen that IL-23 and IL-17A production was suppressed. Anti-IL-17 and anti-IL-17RA antibodies suppress IL-17A and IL-17C, which are highly expressed in psoriatic eruptions. In particular, anti-IL-17RA antibody has been shown to normalize the expression of keratinocyte-related genes and IL-17C production two weeks after administration, followed by normalization of IL-17A production from leukocytes.

In mice, subcutaneous administration of IL-23 induced psoriatic eruption and IL-17A expression (K. A. et al. 2013), and IL-17C transgenic mice overexpressing IL-17C in keratinocytes showed psoriatic eruption. As shown in (8), the reaction of psoriasis-like eruption occurs in mice due to the chain of stimulation to T cells and epidermal cells starting from TLR.

Essentiality of the Key Events

Stressor, MIE and later events:MyD88 knock out(KO) mice

TLR7 (TLR7 / 8 in human) recognizes the imidazoquinoline derivative, binds to the adapter molecule MyD88, activates IRAKs (IL-1 receptor associated kinases), interacts with TRAF6 (TNF receptor associated factor 6) and IKK (Activates the IκB kinase complex). It phosphorylates IκB, induces its degradation, and transfers the transcription factor NF-κB to the nucleus. This pathway is called MyD88-dependent pathway and is essential for the production of inflammatory cytokines such as TNF-α (Akira S, Takeda K .: Nat Rev Immunol. Jul; 4: 499-511, 2004). When pDC is stimulated with a TLR7 / 8 ligand, the transcription factor IRF7 constitutively expressing pDC and MyD88 associate directly. IRF7 activity does not occur when pDCs of MyD88 KO mice are stimulated with TLR7 / 8 ligand. IRF7 is also activated by binding to TRAF6, leading to IFN-α production, which requires the Myd88 / TRAF6 / IRF7 complex. (Satoshi U, Shizuo A: Virus 54; 2: 145-152,2004)

Imiquimod 5% cream was applied to the left flank of female SKH-1 hairless mice (25 g body weight). The IFN-α and TNF-α concentrations in the skin after 1 and 2 hours of application increased these concentrations compared to the untreated skin.

In C57BL / 6 mice (8-12 weeks old) sensitized with 0.5% dinitrofluorobenzene (DNFB) as an antigen, imiquimod 5% cream was applied to the auricle once a day for 3 days. The application of imiquimod 5% cream promoted edema of the ears of mice (promoted DTH) compared to the base cream group. Imiquimod activates antigen-specific T cells by topical application to the skin. (Beserna Cream Interview Form Mochida Pharmaceutical Co., Ltd.)

KE-1 and later event:IL-17, IL-22 KO mice

In mice, psoriasis-like hyperplasia is induced by the application of IL-23, but this effect does not occur in IL-17A and IL-22 KO mice. IL-17A deficient mice show little epidermal hyperplasia after intradermal administration of IL-23. WT mice treated with anti-IL-17A Ab did not show IL-23-induced epidermal hyperplasia. IL-17 KO mice treated with IL-23 do not induce TNF-α mRNA and do not cause epidermal thickening. IL-22 did not increase in IL-17-/-mice after IL-23 administration, and IL-17 clearly increased in IL-22-/-mice. In IL-17-/-, IL-22-/-and WT mice treated with IL-23, immunohistochemically CD3 + T cells, CD11c (dendritic cells), F4 / 80 (macrophages), Gr-1 (Neutrophils) were analyzed. There was no difference in F4 / 80 and Gr-1 + cells in IL-17A-/-compared to WT mice, and CD3 + T cells decreased, but there was no obvious difference in IL-22-/-mice .

These data suggest that cytokines alone are not sufficient to mediate IL-23-induced epidermal changes, and that IL-17 and IL-22 are downstream mediators of mouse skin IL-23-induced changes. Therefore, Th17 cytokines are required for the generation of IL-23-mediated skin lesions.

KE-2 and later events: Mouse psoriasis-like dermatitis model

When TPA (12-O-tetradecanoy1phorbol-13-acetate) on the dorsal skin of K14 / mIL-1F6 gene-modified mice overexpress mouse IL-1F6 (IL-36a) selectively under the keratin 14 promoter was applied, skin pathological findings specific to psoriasis were observed, such as epidermal hyperplasia, epidermal exfoliation and micro-abscess formation, and wet inflammatory cells in the dermis. Quantitative RT-PCR measures mRNA expression levels of inflammatory chemokines and cytokines in skin tissues, and includes inflammatory chemokines: CCL3, CCL4, CXCL10, CXCL1, and cytokines: IL-23, IL-12, IL-1β, etc. These expressions were elevated. (Kyowa Hakko Kirin Co., Ltd.)

References

Appendix 1

List of MIEs in this AOP

Event: 1706: Stimulation, TLR7/8

Short Name: Stimulation of TLR7/8

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

TLR7 and TLR8 are conserved among humans and mice (Gupta et al. 2016). In addition, these molecules are also conserved among humans and rhesus monkeys.

Alignment of amino acid residues between human toll-like receptor 7 (NP_057646.1) and murine toll-like receptor 7 (NP_573474.1, NP_001277684.1, NP_001277685.1, NP_001277686.1, NP_001277687.1, XP_006528776.1, XP_011246087.1 and XP_011246088.1) was 80.74-80.76% identification. Murine TLR7 proteins have 1050 or 1053 amino acids. Human and rhesus toll-like receptor 7 (NP_001123898.1) was 98.00% identification. Rhesus TLR7 protein has 1049 amino acid residues.

In addition, alignment of amino acid residues between human toll-like receptor 8 (NP_619542.1) and murine toll-like receptor 8 (NP_001300689.1) was 70.97% identification. Likewise, human and rhesus toll-like receptor 8 (NP_001123899.1) was 96.73% identification. Murine and rhesus TLR8 referred here have 1029 and 1039 amino acids residues, respectively.

Studies of DC subsets isolated from humans and mice have revealed that TLRs have distinct expression patterns. TLR7 is expressed in freshly isolated human pDCs, whereas TLR8 is expressed in $CD11c^+$ human myeloid DCs (mDCs). In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas other reports showed that TLR7 was exclusively expressed in pDCs (Iwasaki and Medzhitov. 2004).

In mice, $CD4^+$ dendritic cells (DC), CD4/CD8 double negative DC and pDC express TLR7. All splenic DC subsets express TLR8. Moreover, mouse $CD8\alpha^+$ DCs lack TLR7 expression and fail to respond to TLR7 agonists. (Iwasaki and Medzhitov. 2004).

Key Event Description

Toll-like receptors (TLRs) are members of interleukin-1 (IL-1) receptor/TLR superfamily, as they share the intracellularToll-IL-1 receptor (TIR) domain with the IL-1 receptor.

Toll-like receptor (TLR) 7 and TLR8 is known to mediate the recognition of guanosine- and uridine-rich single-stranded RNA (ssRNA) derived from ssRNA viruses and synthetic antiviral imidazoquinoline components specifically that lead to activation of sequential signalling pathway (Akira et al. 2006, Blasius and Beutler. 2010). They also mediate the recognition of self RNA that is released from dead or dying cellsand activation of Myeloid differentiation primary response 88 (MyD88)-dependent signals can occur that leads to inflammation process as well as ssRNA derived from viruses.

TLR7 are exclusively expressed in plasmacytoid DCs (pDCs), which have the capacity to secrete vast amounts of type I interferon (IFN) in rapid response to viral infection (Gilliet et al. 2008, Reizis et al. 2011). TLR8 is express in various tissues, with its highest expression in monocytes. Myeloid DCs (mDCs) also express TLR8 in human (Iwasaki and Medzhitov. 2004). Thus, TLR8 ligands can directly activate mDCs via TLR8. TLR8 signalling activates mDCs to secrete TNF-α and IL-6 (Ganguly et al. 2009). TLR7 and TLR8 are localize in the endoplasmic reticulum of expressing cells (Lai et al. 2017).

Human TLR7 (hTLR7) and human TLR8 (hTLR8) contain 1049 and 1041 amino acid residues, respectively with molecular weight of 120.9 kDa and 119.8 kDa, respectively (Chuang and Ulvitch. 2000). The full-length hTLR7 protein includes a signal peptide of 26 amino acids (1–26 aa). The mature hTLR7 protein ectodomain, trans-membrane, and TIR domain are composite structure of 27–839, 840–860, and 889–1,036 amino acids, respectively (Gupta et al. 2016).

hTLR7 and hTLR8 form a subfamily of proteins that each contain an extracellular domain of >800 residues and share functional and structural features. hTLR7 and hTLR8 contains 27 and 26 leucine-rich repeats (LRRs), which is the largest number of LRRs among TLRs whose structures have been reported (Tanji et al. 2013).

As mentioned above, TLR7 and TLR8 are localize in the endoplasmic reticulum of expressing cells. They are deliver to the endosomes by interacting UNC93B1, which is a 12 membrane-spanning protein (Kawai and Akira. 2011, Itoh et al. 2011). After the trafficking, they initiate cellular responses upon their activation by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Lai et al. 2017).

Structural characterization was conducte with recombinant TLR7 from monkey (*Macaca mulatta*; 96.8% sequence identity with human TLR7) expressed in *Drosophila* S2 cells (Zhang et al. 2016). Rhesus TLR7 exists as a monomer in the absence of ligands. This TLR7 is activate by dimerization triggered by guanosine and uridine-containing ssRNA, which are degradation products of ssRNA, synergistically. Specifically, this TLR7 molecule has two ligand-binding sites. The first site conserved in TLR7 and TLR8 is use for small ligand-binding essential for its activation. The second site spatially distinct from that of TLR8 is use for ssRNA-binding that enhances the affinity of the first-site ligands. The first site preferentially recognize guanosine and the second site specifically bound to non-terminal uridine in ssRNA which have more than 3 bases. Rhesus TLR7 is also activate by dimerization induced by resiquimod alone, which is bound to only the first site (Zhang et al. 2016).

In contrast, hTLR8 exists as preformed dimer before ligand recognition. hTLR8 molecule has two ligand-binding sites as well as TLR7. The first site preferentially recognize uridine and second site recognize short-oligonucleotide. hTLR8 transforms into activated form upon binding of these two degradation products of ssRNA. hTLR8 is also activated by transformation induced by resiquimod alone, which is bound to only the first site (Tanji et al. 2015).

The key residues involved in TLR7 dimerization are LYS410, ASN503, SER504, GLY526, ASN527, SER530, THR532, ARG553, and TYR579 (Gupta et al. 2016).

Cellular signalling initiated by TLR7 activation with ssRNA in pDC is mediated in a Myeloid differentiation primary response 88 **(**MyD88)-dependent fashion, and activates NF-κB and IRF7, which results in induction of inflammatory cytokines and type I interferon, respectively (Kawai and Akira. 2011).

MyD88-dependent IRF7 activation in pDCs is mediate by activation of IRAK1, TRAF6, TRAF3, and IKKα and is facilitate by IFN-inducible Viperin expressed in the lipid body (Kawai and Akira. 2011).

IRF7, which is constitutively expresse by pDCs, binds MyD88 and forms a multiprotein signalling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (Kawai and Akira. 2008). In this complex, IRF7 becomes phosphorylate by IRAK1 and/or IKKα, dissociates from the complex and translocates into the nucleus to induce transcription of type I IFN by binding to its promoter proximal region (Kawai and Akira. 2008, Génin et al. 2009).

Signalling initiated by TLR8 engagement with ssRNAs in endosomes is also mediated the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

How it is Measured or Detected

In general, quantification of TLR7/8 activation can be done by:

- Reporter gene assay
- ELISA

Measurement of transcriptional activation of human TLR and NF-κB-luciferase co-transfected cells

HEK293 cells were transiently co-transfected with human TLR7 and a NF-κB-luciferase reporter. The cells were incubated for 48 hours following transfection and then stimulated with various concentrations of resiquimod or imiquimod. Luciferase activity was measured 48h post-stimulation and the results are reported as fold-increase in luciferase production relative to medium control (Gibson et al. 2002). Likewise, resiquimod (0.001-10 µg/mL) induced NF-κB activation in HEK293 cells transfected with human TLR7 or human TLR8 and a NF-κB-luciferase reporter is detected in the same manner (Jurk et al. 2002).

Measuring of cytokine levels in supernatants

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

TNF-α and IL-6 in cell-free supernatants collected after stimulation of mDCs by RNA-LL37 are measured by ELISA (Ganguly et al. 2009).

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List of Key Events in the AOP

Event: 1822: Maturation of TNF/iNOS-Producing Dendritic Cells

Short Name: Maturation, TNF/iNOS-Producing Dendritic Cells

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Tip-DCs are also observed in mice. Murine Tip-DCs are defined as splenic $CD11c^+$, $CD11b^+$, $MHC\text{-}II^+$, $CD40^+$, and $CD86^+$ cells producing iNOS and TNF. CD11b expression is observed in murine Tip-DC, however it is lacking on human cells (Lowes et al. 2005).

Key Event Description

Monocytes are formed in the bone marrow and continuously enter the blood circulation, where they constitute 10% of the total

leukocyte population in humans (Sprangers et al. 2016). They are recruited to inflammatory sites and differentiate into immature dendritic cells in situ (Tang-Huau and Segura. 2019). These immature dendritic cells, known as monocyte-derived dendritic cells (mo-DC) are distinguished from conventional or classical DCs which arise from a common DC precursor (Guilliams et al. 2014). They possess typical DC functions of antigen-presenting cells, including the ability to efficiently stimulate naive T cells and the capacity to express CCR7, and potentially enabling their migration to lymph nodes (Tang-Huau and Segura. 2019).

Mo-DC are HLA-DR^{+}CD11c^{+}CD14^{int}CD206^{+}CD1c^{+} cells. By contrast, they lack the macrophage markers CD16 and CD163. They also display a typical DC morphology: they are small size, possess dendrites and lack large cytoplasmic vacuoles (Tang-Huau and Segura. 2019). Human mo-DC are present in lungs, intestine and peritoneum in the steady-state. Peritoneal mo-DC secrete IL-6, TNF-α, IL-1β and IL-12p70 upon ex vivo re-stimulation. Mo-DC from bronchoalveolar lavage also secrete TNF-α upon re-stimulation (Tang-Huau and Segura. 2019).

Pathogen-derived components, such as Toll-like receptor ligands as well as inflammatory mediators induce maturation of mo-DC. These stimulants include LPS, ssRNA, IFN-α, TNF-α, IFN-γ or CD40L (León et al. 2005, Farkas and Kemény. 2011). TNF-α and inducible nitric oxide synthase (iNOS)-producing DCs (Tip-DCs) are abundant in inflamed tissue such as skin in patients of chronic inflammatory skin disease, and not present in the steady-state or normal skin tissue. These cells are derived from monocyte infiltrated during inflammation and contribute to innate immune response to pathogens including bacteria and parasites (Guilliams et al. 2014).

From the above, monocyte is considered to infiltrate into inflammatory site and differentiate to mo-DC and Tip-DC, sequentially in chronically inflamed tissue. This maturation process is induced and/or promoted by IFN-α, TNF-α and GM-CSF (Farkas and Kemény. 2011).

Tip-DCs express HLA-DR, CD40, CD86, as well as maturation markers DC-Lamp and CD83 but lack the CD207/Langerin and CD14 markers of Langerhans cells and monocytes. In addition, Tip-DCs found in psoriasis produce the inflammatory mediators IL-8, IL-1, STAT1, CCL 20, IL-20, IL-23p19, and IL-12/IL-23p40, which mediate Th1 and Th17 responses (Wilsmann-Theis et al. 2013).

How it is Measured or Detected

Detection of Tip-DC is considered to be done by:

- Flowcytometry
- RT-qPCR

Analysis of maturation marker expression on cell surface

Maturation markers such as CD80, CD86, CD40 and CD83 can be analyzed by flowcytometry (Wilsmann-Theis et al. 2013).

Quantification of mRNA expression of TNF-α and iNOS

Expression of TNF-α, iNOS, IL-12p35 and IL-23p19 mRNA in in vitro generated Tip-DC are quantified by RT-qPCR. (Wilsmann-Theis et al. 2013).

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Event: 1707: Increase, IL-23 from matured dendritic cells

Short Name: Increase of IL-23

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
dendritic cell

Organ term

Organ term
immune system

Domain of Applicability

Tip-DCs are also observed in mice. Murine Tip-DCs are defined as splenic CD11c$^+$, CD11b$^+$, MHC-II$^+$, CD40$^+$, and CD86$^+$ cells producing iNOS and TNF. CD11b expression is observed in murine Tip-DC, however it is lacking on human cells (Lowes et al. 2005).

Key Event Description

Increased IL-23 synthesis from TNF-α and inducible nitric oxide synthase (iNOS)-producing DCs (Tip-DCs) is a result of maturation from monocyte-derived dendritic cells (mo-DC) to Tip-DC which is HLA-DR$^+$CD40$^+$CD86$^+$DC-Lamp$^+$ and CD83$^+$ (Farkas and Kemény. 2011, Wilsmann-Theis et al. 2013).

Tip-DCs are abundant in inflamed tissue such as skin in patients of chronic inflammatory skin disease, and not present in the steady-state or normal skin tissue. These cells are derived from monocyte infiltrated during inflammation and contribute to innate immune response to pathogens including bacteria and parasites (Guilliams et al. 2014).

Increased production of cytokines including IL-12/IL-23p40 in Tip-DC stimulated with R848, an agonist of Toll-like receptor (TLR) 7/8 was reported (Hänsel et al. 2011). In addition, maturation-dependent cytokine production including IL-23 from 6 hours in-vitro matured Tip-DC were observed when stimulated with CD40L and TLR ligands, such as LPS, PGN, Pam3Cys and R848 (Hänsel et al. 2011).

IL-23 is a heterodimer, sharing a p40 subunit with IL-12 but having a distinct p19 subunit. IL-23 binds to IL-12Rβ1 but not IL-12Rβ2. The receptor for this cytokine is heterodimeric and uses a novel second subunit, IL-23R, which is a member of the hematopoietin receptor family (Lee et al. 2004).

How it is Measured or Detected

Measurement of IL23 protein

IL-23 in cell-free supernatants collected after R848 stimulation to moDCs is detected by ELISA (Schwarz et al. 2013).

Measurement of IL23 RNA levels

Expression of IL-23 mRNA in R848-stimulated moDCs is measured by qRT-PCR (Schwarz et al. 2013).

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Event: 1708: Th17 cell migration and inflammation induction

Short Name: Th17 cell migration and inflammation induction

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

dendritic cell

Organ term

Organ term

immune system

Domain of Applicability

Ras homolog gene family H (RhoH) is a membrane-bound adapter protein involved in proximal T cell receptor signaling, and spontaneously develops chronic dermatitis that closely resembles human psoriasis in RhoH gene-deficient mice. Ubiquitin protein ligase E3 component N recognition 5 (Ubr5) and nuclear receptor subfamily 2 group F member 6 (Nr2f6) expression levels are decreased at the lesion site, and protein levels and DNA binding activity of retinoic acid-related orphan receptors are increased is doing. As a result, T cells differentiated into Th17 cells due to increased production of IL-17 and IL-22. These results indicate that RhoH suppresses the differentiation of naive T cells into effector Th17 cells. RhoH is a gene expressed in blood cells, and when RhoH expression decreases in T cells, Th17 cells increase, IL-22 is produced in large quantities, and the epidermis thickens, leading to the formation of psoriasis pathology. Humans with low RhoH expression may become more severe if they suffer from psoriasis. Journal of Allergy and Clinical Immunology

The effect of the unique gut flora in psoriasis on the development and reactivity of inflammatory cells on the IL-23 / Th17 axis was analyzed in imiquimod-induced psoriasis model mice. Th17, γδ TCR-bearing lymphocytes in the spleen were measured from sterile (GF) mice, broad-spectrum antibiotic mixture-administered (ATB) mice, and conventional (CV) mice. GF mice and ATB-treated mice had fewer Th17 cells and γδTCR + cells than CV mice. This is thought to be due to the symbiotic bacteria that lack microbiota or changes due to ATB treatment reduce pro-inflammatory T cell response and regulate T cell development. In other words, it is proof that the interaction between the microorganisms of Clostridiales and Elysiperotricales and the host affects the reactivity of Th17 cells and is involved in the etiology of imiquimod-induced skin inflammation. The positive effect of antibiotic regulation of the gut flora on skin severity suggests the involvement of the gut and skin axes and is part of the management of psoriasis patients. (Zizana Z et al. 2016) * Wide-area antibiotic mixture (ATB): A mixture of metronidazole, colistin, streptomycin, and vancomycin.

Key Event Description

Psoriasis is known to play a major role in the etiology of T cell dysfunction, especially in over activation of the Th17 pathway, which Th17 cells were associated with Th1 and Th2 (Lisa C. et al. 2007) Th17 cell was identified as a cell population that produces different IL17. Abnormal activation of Toll-like receptors (TLR7, 8 and 9) is also involved in the initiation and maintenance of psoriasis. IMO-3100 (an antagonist of TLR7 and 9) and IMO-8400 (an antagonist of TLR7, 8 and 9) has been shown to reduce psoriasis-like skin lesions induced by intradermal administration of IL-23 on the back of mice (Mayte S-F et al. 2013). Immune cell infiltration in psoriasis lesions is composed of CD3 + Th1cell, Th17 cells and CD11c + dendritic cells (DC) (Chamian F et al 2005).

Cytokines such as TNF-α, IFN-γ, IL-17, IL-22, IL-23, IL-12, and IL-1β produced from these cells cause an inflammatory cascade. In particular, the IL-23 / Th17 axis plays an important role, and IL-23h is produce in DC, promotes the differentiation of naive CD4 + T cell progenitor cells into the Th17 phenotype, and stimulates the survival and expansion of the Th17 population (Harrington LE et al. 2005) (Veldhoen M et al. 2006). IL-17 produced from Th17 cells regulates the expression of defensin, S100 family protein and LL-37. These are innate immune responses in the skin and show higher expression of IL-23 in keratinocytes and dermal tissues of psoriatic lesions than in non-lesions (Liang SC et al. 2006).

Overproduction of Th1 and TH17 cytokines is a major cause of psoriasis, and glucocorticoid (GC) regulates epidermal differentiation and acts as a potent anti-inflammatory compound to suppress the pathology of psoriasis. Synthetic glucocorticoids are uses to suppress inflammatory disease including psoriasis, and induce the glucocorticoid-induced leucine zipper (GILZ), a protein that inhibits major immune cell signaling pathways. CILZ is deficient in lesioned skin of psoriasis patients and shows a negative correlation with the expression of pro-inflammatory cytokines IL-1, IL-23, IL-22, and STAT3. *Lisa et al.* was identified a T cell-specific role of CILZ that limits Th17 cell formation in vitro in response to the Th17-promoting cytokines IL-1β and IL-23 (Lisa M et al.2019). CILZ has the clinical significance of psoriasis as well as the non-redundant function of controlling pathogenic Th17 responses (Lisa M et al.2019).

One of the causes of psoriasis is an increase in pathogenic Th17 cells in people with a genetic predisposition stimulated by the production of Th17 polarized cytokines by bone marrow cells. The antibacterial peptide LL37, which forms a complex with nucleic acids released from cells, is an autoantigen that promotes the activation of cutaneous plasmacytoid dendritic cells and myeloid DCs, and Th17 cells are effector cytokines such as IL-17A. It activates keratinocytes directly through release. Activated keratinocytes proliferate abnormally and release inflammatory mediators and chemokines to amplify the inflammatory response (Boehncke WH et al.2015).

How it is Measured or Detected

IL17 + cell count measurement

Flow cytometric analysis of psoriasis skin biopsy showed increased IL-17 + and IL-22 + CD4 + T cells,

Measurement of IL17 protein levels (in skin and serum)

Increased frequency of IL-17 +, CCR6 +, and CCR4 + T cells. IL-22-producing cells (Th-22 cells) that do not produce IL-17 or IFNγ also increased (Benham et al. 2013).

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List of Adverse Outcomes in this AOP

Event: 1709: Psoriatic skin disease

Short Name: Skin disease

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:313 - Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	AdverseOutcome

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Mouse psoriasis-like dermatitis model: K14 / mIL-1F6 gene-modified mice overexpress mouse IL-1F6 (IL-36a) selectively under the keratin 14 promoter, and TPA: 12-O- tetradecanoy1phorbol-13-acetate(TPA) was applied, skin pathological featuresfindings specific to psoriasis-such as epidermal hyperplasia, epidemal exfoliation and micro-abscess formation, and wet inflammatory cells in the dermis-were observed. Quantitative RT-PCR. Measures mRNA expression levels of Inflammatory chemokines and cytokines in skin tissues, and includes inflammatory chemokines: CCL3, CCL4, CXCL10, CXCL1 and cytokines: IL-23, IL-12, IL-1β etc. Expression was observed. (Kyowa Hakko Kirin Co., Ltd.)

Epidermal keratinocyte expression genes that were elevated in psoriatic lesions of patients with psoriasis with stage-type skin eruption: mRNA expression level of keratin6a and 16, s100A7A, S100A12, DEFB4, IL-1F6, CCL20, IL-17C, etc. was rapidly reduced by 700 single intravenous dose of brodalumab and decreased to non-lesional skin level two weeks after administration.On the other hand, leukocyte expression genes with increased expression in psoriatic lesion skin: IL-17A, IL-17F, IL-23F, IL-12B, IL-22, IFN-γ and other mRNA expression levels decreased with brodalumab administration However, at 2 weeks after administration, the level did not decrease to the level of the non-lesional skin. Since the expression of pathophysiology-related genes is reduced prior to the decrease in the expression of leukocyte expression genes and the decrease in the PASI score, brodalumab expresses pathophysiology-related genes by blocking IL-17 signaling in the epidermal keratinocytes of psoriatic lesions It is possible to improve the skin eruption promptly. (Kyowa Hakko Kirin Co., Ltd. In-house materials)

The monoclonal antibody-mediated IL-17A (secukinumab / ixekizumab) and the receptor subunit IL-17RA (brodalumab) are effective approaches in the treatment of psoriasis. Blocking IL-17RA results in inhibition of the IL-17 family, including IL-17A, IL-17F, IL-17C, and IL-17E. Other drugs are under clinical development that target bimekizumab targeting IL-17A and IL-17F, IL-23 upstream of the IL-17 pathway, or signal transduction substances downstream. (Conrad C et al. 2018)

Key Event Description

Psoriasis is a complex inflammatory disease caused by activation of Th1 and TH17 cells. The epidermis is composed of keratinocytes that differentiate to form a permeable barrier. Abnormal balance between proliferation and differentiation of keratinocytes affects barrier function and causes inflammatory skin lesions. Psoriasis is a complex combination of genetic and environmental risk factors, and dysregulation of Th1 and Th17 lines is due to overproduction of cytokines including IFN-α, TNF-α, IL-23, IL-17, and IL-22. Causes overgrowth and skin immunity. (Harrington LE et al. 2005)

Histopathological features of psoriasis lesions include epidermal thickening, epidermal differentiation, and epidermal protrusions (reticular ridges), with intraepithelial neutrophil moistening (manlo-like abscess) and marked immune moistening consisting of T cells and dendritic cells. See an increase in. (Boehncke WH et al. 2018) Commonly used psoriasis model mice were produced by topical application of the TLR7 agonist imiquimod, which induces the IL-23-Th17 cell axis and histopathology of human psoriasis pathology. Strictly reproduce the target and molecular features. (Wagner EF et al.2010)

Psoriasis vulgaris shows overexpression of the S100 protein family soriacin (sorazine), cobunericin (kebuneridine), and epidermal antibacterial peptide (AMP). AMP is induced by IL-17 and itself functions as a chemotactic factor and cytokine. Mobilize CD4 + T cells and neutrophils that exacerbate inflammation. (Kanagawa Psoriasis Treatment Study Group)

Serum IL-17 levels in psoriasis patients are significantly higher than in healthy individuals, and brodalumab, a neutralizing antibody against the IL-17A receptor, has been shown to be effective in the treatment of psoriasis (Gilliet et al. 2004). In addition, antibody preparations against IL-17 ixekizumab (John K. et al. 2002) and Szeimies et al. 2004 were used to treat psoriasis, and with positive results, Th17-mediated pathways are important for the etiology of psoriasis. It is believed to play a role. Immunohistological examination of biopsies of skin areas with psoriatic plaques, including surrounding normal skin, shows an increase in the number of activated dendritic cells, especially CD1a-positive Langerhans cells in the epidermis of psoriatic lesions. In CD83-positive CD1a-negative Langerhans, -negative CD11c-positive dermal dendritic cells increased in the epidermis at the border of psoriatic plaques. In normal skin, there were significantly fewer CD3-positive T lymphocytes than lesions.

CD4 and CD8 T cells infiltrate both the epidermis and dermis and show increased expression of IL17A, IL22, and IFNG in epidermal CD4 and CD8 T cells near keratinocytes, but with lower dermal T cell upregulation. IL-22, produced primarily by lesion epidermal CD4 T cells, is associated with keratinocyte activation and the formation of epidermal thickening, a prominent morphological feature of psoriasis. Lesion epithelial CD8 T cells mainly produce IL-17A and promote the production of inflammatory cytokines and chemokines by keratinocytes. IL-17A is an important mediator of psoriatic inflammation through skin recruitment and activation of leukocytes. (Cheuk et al. 2014)

In patients with psoriasis, inflammatory keratin K6 and K16-positive keratinocytes were found, even in areas of normal-appearing skin that were not affected by psoriasis lesions. In addition, the transcription factor C / EBPβ, which is normally expressed only in the stratum granulosum of the healthy epidermis, was expressed throughout the epidermis, including the epidermis of the lesion. This suggests that early inflammatory changes have already occurred in areas that have not yet shown obvious skin lesions, and that these changes are caused by dendritic cells rather than lymphocytes. (Komine et al. 2007)

How it is Measured or Detected

Biopsy of the skin area and surrounding normal skin of patients with psoriasis vulgaris

The dendritic cell surface marker and lymphocyte surface marker of the section were used as the primary antibody.

In the vicinity of psoriasis lesions, an increased number of activated dendritic cells was observed, with CD1a-positive Langerhans cells in the epidermis and CD83-positive CD1a-negative Langerin-negative CD11c-positive dermal dendritic cells at the epidermal border.

There were significantly fewer CD3-positive T lymphocytes than lesions in normal skin.

Inflammatory keratin K6 and K16 positive keratinocytes were found in the normal part.

The transcription factor C / EBPβ, which is normally expressed only in the granular layer of the normal epidermis, was expressed in the entire epidermis in the same manner as the lesion. (Komine et al. 2007)

Serum amyloid A: SAA measurement ... measured in 35 psoriasis patients and healthy humans

DNA microarray analysis in lesions of psoriasis patients ... SAA levels are about 5 times higher than in normal skin. The average SAA of psoriasis patients was 19.1 ug / ml, and the average SAA after treatment was 6.9 ug / ml.

There is a correlation between SAA and psoriasis severity score (PASI).

Amyloid A deposition was observed in the skin-stained area of the psoriasis lesion skin area (Tanizaki H et al. 2013)

Regulatory Significance of the AO

Psoriasis model mice have been developed as a tool for understanding the etiology of this disease and as a preclinical model. Five representative models are based on K14-amphiregulin, K5-Stat3C, K5-Tie2, K5-TGF-β1, and imiquimod. There were statistically significant similarities between the gene expression patterns associated with epidermal development and keratinization in these

models and the gene expression patterns in human psoriasis. Direct high-level activation of keratinocytes via autoclean growth factor (amphiregulin) has the ability to induce cytokine-related genetic circuits that closely resemble human psoriasis. Through transgenes, inward mutations (CARD14), injury, and exposure to specific T cell-producing cytokines, activated keratinocytes induce and lead to a chronic inflammatory response that is highly consistent with psoriasis. However, there were frequent differences in the expression of immune-related genes between the models. (Cook PW et al. 1997)

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2017: Stimulation of TLR7/8 leads to Increase of IL-23

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Thirteen mammalian TLR members (10 in humans and 13 in mice) have been identified so far, of which TLR1, 2, 4, 5, and 6 are membrane bound and catalytic site for pathogenic structural components, whereas TLR3, 7, 8, and 9 expressed within the

endosomal compartment

are dedicated to nucleic acids. TLRs 1–9 are conserved among humans and mice, yet TLR10 is present only in humans and TLR11 strictly restricted to rodents (Gupta et al. 2016).

Mouse TLR10 is not functional because of a retrovirus insertion, and TLR11, TLR12 and TLR13 have been lost from the human genome (Kawai and Akira. 2010).

In addition, alignment of amino acid residues between human toll-like receptor 7 (AAF60188.1) and murine toll-like receptor 7 (AGX25544.1) was 80.74% identification. Both proteins have 1049 amino acid residues.

Structural characterization was conducted with recombinant TLR7 from monkey (*Macaca mulatta*; 96.8% sequence identify with human TLR7) expressed in *Drosophila* S2 cells (Zhang et al. 2016).

Studies of DC subsets isolated from humans and mice have revealed that TLRs have distinct expression patterns. Freshly isolated human pDCs express TLR7 and TLR9, whereas $CD11c^+$ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR8. In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas others found TLR7 was exclusively expressed in pDCs (Iwasaki and Medzhitov. 2004).

In mice, all splenic DC subsets express TLRs 1, 2, 4, 6, 8 and 9. However, mouse pDCs do not express TLR3. Moreover, mouse $CD8\alpha^+$ DCs lack TLR5 and TLR7 expression and fail to respond to TLR7 agonists. In short, $CD4^+$ DC, CD4CD8DN DC and pDC express TLR7 in mice (Iwasaki and Medzhitov. 2004).

Although unpublished, it has been reported that human slanDCs (Tip-DCs) lack the DNA-binding structureTLR9 but can express the endosomal RNA-binding receptorsTLR8 (slanDCs andCD1c$^+$ DCs) and TLR7 (slanDCs but not CD1c$^+$ DCs; Hänsel et al, unpublished data, June 2010) (Hänsel et al. 2011). There are not any other reports which mentioned TLR7 expression in Tip-DCs, so whether or not TLR7 exists in human Tip-DCs is still unknown.

IFN-α, but not TNF-α and IL-6 production by human pDCs after stimulation with self-RNA-LL37 complex was detected (Ganguly et al. 2009). However, in mice, IFN-α production from splenic pDCs was induced by IMQ treatment. The production of TNF-α and IL-23 was also induced by IMQ treatment. Although 4–8% of mPDCA-1$^-$ CD11c$^+$ DCs were contaminated in prepared mPDCA-1$^+$ pDC fraction, it was confirmed that splenic mPDCA-1$^-$ CD11c$^+$ DCs enriched to approximately 80% purity could not produce TNF-α and IL-23 by IMQ stimulation. In $Tlr7^{-/-}$ splenic pDCs, these cytokines (IFN-α, TNF-α and IL-23) were not induced by IMQ treatment, although stimulation by CpG, a TLR9 ligand, resulted in induction of these cytokines at the same level as was produced by wild-type splenic pDCs. These data indicate that, in mice, IMQ application can induce the production via TLR7 of IFN-α, TNF-α and IL-23 from pDCs existing in the skin in vivo (Ueyama et al. 2014).

When BMDCs were generated by 10-day culture with GM-CSF and IL-4 and characterized their phenotypes, CD11c+ BMDCs showed MHC II^{high}, CD11b^{high}, B220$^-$, CD86^{high}, Mac-3$^+$, and had the ability to produce high levels of TNF-α and NO/iNOS in response to LPS stimulation, which represents a similar phenotype to Tip-DCs (Xu et al. 2007, Ueyama et al. 2014).

In these BMDCs which represents a similar phenotype to Tip-DCs, IMQ weakly but significantly induced the production of IL-23. In addition, although IFN-α had no effect alone, co-stimulation with IFN-α and IMQ resulted in marked induction of IL-23 production. However, using BMDCs derived from $Tlr7^{-/-}$ mice, these effects of IMQ and IFN-α was not observed, verifying that it is also mediated via TLR7 (Ueyama et al. 2014).

In mice, purified bone marrow dendritic cells (BMDCs) derived from wild-type mice stimulated with IFN-α showed increase in Tlr7 mRNA expression (Ueyama et al. 2014). In addition, TLR7 expression was also observed in the inflamed skin of IMQ-treated mice (Ueyama et al. 2014). These data suggest that the synergistic effect of IMQ and IFN-α on BMDCs was caused by induction of TLR7 expression by IFN-α (Ueyama et al. 2014).

Taken together, in mice, IFN-α produced by IMQ-primed pDCs may enhance the effects of IMQ to activate Tip-DC, resulting in the release of a large amount of IL-23 in IMQ-induced psoriasis-like skin lesion (Ueyama et al. 2014).

Key Event Relationship Description

Toll-like receptors (TLRs) are members of interleukin-1 (IL-1) receptor/TLR superfamily, as they share the intracellularToll-IL-1 receptor (TIR) domain with the IL-1 receptor.

Toll-like receptor (TLR) 7 and TLR8 is known to mediate the recognition of guanosine- and uridine-rich single-stranded RNA (ssRNA) derived from ssRNA viruses and synthetic antiviral imidazoquinoline components (Akira et al. 2006; Blasius and Beutler. 2010). They also mediate the recognition of self RNA that is released from dead or dying cells.

Human TLR7 (hTLR7) and human TLR8 (hTLR8) contains 1049, 1041 amino acid residues with a calculated molecular weight of 120.9 kDa and 119.8 kDa respectively (Chuang and Ulvitch. 2000).

The full-length hTLR7 protein includes a signal peptide of 26 amino acids (1–26 aa). The mature hTLR7 protein ectodomain, trans-membrane, and TIR domain are composite structure of 27–839, 840–860, and 889–1,036 amino acids, respectively (Gupta et al. 2016).

hTLR7 and hTLR8 form a subfamily of proteins that each contain an extracellular domain of >800 residues and share functional and structural features. TLR8 contains 26 leucine-rich repeats (LRRs), which is the largest number of LRRs among TLRs whose structures have been reported (Tanji et al. 2013).

Monkey TLR7 exists as a monomer in the absence of ligands, and TLR7 dimerization is induced by R848 alone, but not by poly U or guanosine alone, although these two ligands synergistically triggered TLR7 dimerization (Zhang et al. 2016). In contrast, hTLR8 exists as preformed dimer before ligand recognition. TLR8 is activated by R848 alone, or both uridine and ssRNA synergistically (Tanji et al. 2013).

The key residues interacting two TLR7 molecules into dimer confirmation are LYS410, ASN503, SER504, GLY526, ASN527, SER530, THR532, ARG553, and TYR579 (Gupta et al. 2016).

TLR3, TLR7, TLR8, and TLR9 localize to the endoplasmic reticulum and are trafficked to the endosomal compartment where they initiate cellular responses upon their activation by PAMPs and DAMPs (Lai et al. 2017).

TLR7 are exclusively expressed in plasmacytoid DCs (pDCs), which have the capacity to secrete vast amounts of type I IFN rapidly in response to viral infection (Gilliet et al. 2008, Reizis et al. 2011).

TLR8 is expressed in various tissues, with its highest expression in monocytes. Myeloid DCs (mDCs) also express TLR8 in human (Iwasaki and Medzhitov. 2004). Thus, TLR8 ligands can directly activate mDCs via TLR8.

TLR7-mediated signaling in pDC is mediated in a MyD88-dependent fashion, which initiates an IRF7-mediated response, secreting vast amounts of IFN type 1 (Kawai and Akira. 2011).

MyD88-dependent IRF7 activation in pDCs is mediated by activation of IRAK1, TRAF6, TRAF3, and IKKα and is facilitated by IFN-inducible Viperin expressed in the lipid body (Kawai and Akira. 2011).

IRF7, which is constitutively expressed by pDCs, binds MyD88 and forms a multiprotein signaling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (Kawai and Akira. 2008). In this complex, IRF7 becomes phosphorylated by IRAK1 and/or IKKα, dissociates from the complex and translocates into the nucleus.

The interferons (IFNs) are a primary defense against pathogens because of the strong antiviral activities they induce. Three types of IFNs, types I, II and III, have been classified based on of their genetic, structural, and functional characteristics and their cell-surface receptors (Zhou et al. 2014). IFN-α belongs to the type I IFNs, the largest group which includes IFN-β, IFN-ε, IFN-ω, IFN-κ, IFN-δ, IFN-τ and IFN-ζ.

The IFN-α of type I IFN family in humans is composed of 12 subtypes encoded by 14 nonallelic genes including one pseudogene and two genes that encode the same protein. The various IFN-α subtypes have many common points. For example, all are clustered on chromosome 9 (Diaz et al. 1993). IFN-αs, which are composed of 165 to 166 aa, have 80% amino acid sequence identities (Li et al. 2018).

Upon engagement of ssRNAs in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

A distinct population of human blood DCs that are defined by the selective expression of the 6-sulfo LacNAc residue on the P-selectin glycoprotein ligand 1 membrane molecule was described previously. 6-Sulfo LacNAc DCs (slanDCs) stand out by a marked production of TNF-α, and they were recognized as the major source of IL-12p70 among blood leukocytes when stimulated with CD40 ligand or LPS of gramnegative bacteria (Hänsel et al. 2011).

According to the current concept, these inflammatory DCs are $CD1c^{-}$, $CD11c^{+}$ cells locally expressing TNF-α and iNOS. They were also referred to as TNF and inducible nitric oxide synthase–expressing DCs (Tip-DCs) (Lowes et al. 2005) or inflammatory dermal DCs (Zaba et al. 2009). In contrast, resident dermal DCs express CD1c and CD11c and were shown to lack inflammatory markers. The phenotype of slanDCs ($CD11c^{+}$ and $CD1c^{-}$) and their local production of IL-23p19, TNF-α, and iNOS identify slanDCs as being a population of inflammatory dermal DCs and so-called Tip-DCs in psoriasis (Hänsel et al. 2011).

Stimulation of blood DCs with self-RNA–LL37 complexes induced a robust TNF-α response (Hänsel et al. 2011). TNF-α affects Tip-DCs in an autocrine and/or paracrine manner (Zaba et al. 2007).

DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

R848 induces IL-23 production from activated human monocyte-derived DCs (moDCs) by enhanced transcriptional activity (Schwarz et al. 2013).

IL-23 is a heterodimer, sharing a p40 subunit with IL-12 but having a distinct p19 subunit. IL-23 binds to IL-12Rβ1 but not IL-12Rβ2. The receptor for this cytokine is heterodimeric and uses a novel second subunit, IL-23R, which is a member of the hematopoietin receptor family (Lee et al. 2004).

Evidence Supporting this KER

Biological Plausibility

The molecular structure and function of TLR7 and TLR8 are evident based on sufficient scientific findings as mentioned above. The known mechanisms for stimulation of TLR7/8 by each ligand are initiated by the formation of homodimer. TLR7-mediated signaling in pDC is mediated in a MyD88-dependent fashion, which initiates an IRF7, IRAK1, TRAF6, TRAF3, and IKKα-mediated response, secreting vast amounts of IFN type 1 (Kawai and Akira. 2011).

Similarly, upon engagement of ligands in endosomes, TLR8 initiate the MyD88-dependent pathway culminating in synthesis and release of proinflammatory mediators, such as TNF-α via NF-κB activation (Tanji et al. 2015).

DC activation is known to be enhanced by IFN-α in the presence of TNF-α (Luft et al. 1998).

R848 induces IL-23 production from activated human monocyte-derived DCs (moDCs) by enhanced transcriptional activity (Schwarz et al. 2013).

TNF and inducible nitric oxide synthase–expressing DCs also known as Tip-DCs or inflammatory dermal DCs differentiates from moDCs by inflammation (Hänsel et al. 2011).

As mentioned above, stimulation of TLR7 in pDCs, and TLR8 in moDCs and Tip-DCs leads to activation of Tip-DCs, which leads to the overproduction of IL-23 from matured Tip-DCs.

Empirical Evidence

Much experimental data is available that supports the stimulation of TLR7 in pDC induced by TLR7 agonist, which subsequently promote secretion of IFN-α in MyD88-dependent fashion. For example, three populations of cells were evaluated for type I IFN production following imidazoquinoline stimulation: human PBMC, pDC-depleted PBMC, and pDC-enriched cells. Human PBMC produce IFN-α following imiquimod (0.3–30 μM) or resiquimod (0.03–30 μM) treatment. Peak levels of IFN-α were reached with imiquimod and resiquimod at 3 μM. PBMC, depleted of pDC, did not produce detectable levels of IFN-α in response to imiquimod or resiquimod treatment.

The imidazoquinoline-treated pDC-enriched cultures produced 2–20 times more IFN-α than similarly treated PBMC as measured over the entire dose range. Peak levels of Resiquimod- and imiquimod-induced IFN-α production were reached with 0.3 μM and 30 μM, respectively (Gibson et al. 2002).

In addition, pDCs were stimulated with LL37 premixed with total human RNA extracted from U937 cells to confirm that LL37 can interact with self-RNA and convert it into a trigger of IFN-α production. U937-derived self-RNA induced dose-dependent IFN-α production when mixed with LL37, but not when given alone or mixed with the scrambled peptide GL37. Similar to self-DNA (Lande et al., 2007), pDCs activated by self-RNA mixed with LL37 produced high levels of IFN-α, but did not produce TNF-α or IL-6 or undergo maturation as assessed by measuring the expression of costimulatory molecules CD80 and CD86 (Ganguly et al. 2009).

Importantly, self-RNA isolated from a variety of cell types and tissue samples from various types of skin pathologies induced similar levels of IFN-α when mixed with LL37, indicating that cellular- or disease-dependent variations in RNA composition do not play a role in the responses to self-RNA. These data demonstrate that LL37 can convert otherwise nonstimulatory self-RNA into a trigger of pDC activation to produce IFN-α, and thus enable the RNA released during cell death to induce innate immune activation (Ganguly et al. 2009).

IFN-α induced in pDCs by self-RNA–LL37 complexes was inhibited in a dose-dependent manner by bafilomycin, which blocks endosomal acidification and TLR signaling. To specifically inhibit TLR7, we used the short oligonucleotide C661, which selectively blocks TLR7 (Barrat et al. 2005), as shown by the inhibition of IFN-α induction by the synthetic TLR7 agonist R837 but not the TLR9 agonist CpG2006. Pretreatment of pDCs with C661 specifically blocked the IFN-α induction by self-RNA–LL37 complexes, indicating that pDC activation by self-RNA–LL37 complexes occurs through TLR7 (Ganguly et al. 2009).

Self-RNA–LL37 complexes but not self-RNA alone activated mDCs to produce the proinflammatory cytokines TNF-α and IL-6, but not IFN-α (Ganguly et al. 2009). Self-RNA–LL37 complexes also activated mDCs to undergo maturation as shown by the up-regulation of CD80 and CD86 expression (Ganguly et al. 2009) . mDC activation by self-RNA–LL37 complexes was entirely dependent on self-RNA, given that these responses were abrogated by decreasing the amount of self-RNA in the complexes (unpublished data). In contrast to self-RNA–LL37 complexes, self-DNA–LL37 complexes were unable to activate mDCs (Ganguly et al. 2009) . In accordance with these findings, stimulation of mDCs with supernatants of apoptotic cells combined with LL37 induced the secretion of proinflammatory cytokines, and this secretion was entirely dependent on self-RNA because activity was abolished by depletion of self-RNA but not self-DNA (Ganguly et al. 2009).

Compared with stimulation with either supernatant of activated pDCs or self-RNA–LL37 alone, the combination of both significantly enhanced the activation of mDCs to secrete IL-6 and TNF-α and enhanced their differentiation into mature CD83+ DCs (Ganguly et al. 2009). This activity was completely blocked by antibodies against IFN-α, IFN-β and IFN-αβR (Ganguly et al. 2009). Thus, self-RNA–LL37 complexes can trigger mDC activation and maturation, and this process is enhanced by the concomitant activation of pDCs to produce IFN-α.

Self-RNA was also internalized by mDCs when complexed with LL37 but not when given alone. The cytokine production such as TNF-α and IL-6 of mDCs induced by self-RNA–LL37 complexes but not by the TLR4 agonist LPS was completely inhibited by bafilomycin in a dose-dependent manner, demonstrating that mDC activation by self-RNA–LL37 complexes involved endosomal TLR activation. Using 293T cells transfected with TLR8 and TLR3 expression vectors along with a NF-κB luciferase reporter plasmid, it was confirmed that self-RNA–LL37 complexes activated TLR8 but not TLR3. In support of this finding, synthetic short ssRNA sequences that activate TLR8 in human mDCs (Diebold et al. 2004, Heil et al. 2004) also activated mDCs when complexed

with LL37 but not when given alone (Ganguly et al. 2009).

Dose-dependent DC maturation was observed with increasing concentrations from 10 IU/ml up to 1000 IU/ml of IFN-α2a or IFN-α8 added to cultures containing GM-CSF, IL-4, and TNF-α. Both of the IFNs had a similar capacity to up-regulate HLA-A, B, C, CD80, and CD86 and to down-regulate CD1a and CD11b expression on the cell population (Luft et al. 1998).

DC cultured in GM-CSF, TNF-α, and IL-4-containing medium until day 14, and type I IFNs were added daily between days 14 and 17. Proportions of positive cells for each markers were analyzed by FACS on day 17 (Luft et al. 1998).

When GM-CSF, TNF-α, and IL-4-containing cultures were washed on day 14 and continued until day 17 in serum-free medium containing GM-CSF and IL-4, without or with TNF-α (20 ng/ml, standard conditions), IFN-a (1000 IU/ml), or both, IFN-α alone did not enhance DC maturation as compared with standard conditions. When both of TNF-α and IFN-α exist, optimal maturation was observed than either TNF-α or IFN-α alone. Thus, the enhancement of DC activation by IFN-α under serum-free conditions required the presence of TNF-α (Luft et al. 1998).

LL37 is highly expressed in the inflamed skin of psoriasis but is undetectable in inflamed skin of atopic dermatitis or in healthy skin (Lande et al. 2007). To determine whether extracellular self-RNA–LL37 complexes form in vivo, Staining skin sections with Ribogreen and DAPI revealed that numerous extracellular Ribogreen^{+}/ DAPI^{-} complexes in the dermal compartment of psoriatic skin lesions, but not in skin of atopic dermatitis or healthy skin (Ganguly et al. 2009). These tissue RNA complexes presented several features of self-RNA–LL37 complexes generated in vitro, including the size and bead-like branched structures resulting from the aggregation of smaller particles (Ganguly et al. 2009).

Skin sections of psoriatic tissues were stained with an anti-LL37 antibody and Ribogreen to determine whether the self-RNA complexes in the tissues contained LL37 and it was found that the majority of these complexes contained LL37 (Ganguly et al. 2009). Importantly, psoriatic skin also contained substantial numbers of particulate self-DNA–LL37 complexes.

Serial sections of lesional psoriatic skin were stained for RNA complexes and DC-LAMP, a lysosomal marker specific for mature mDCs to determine whether the presence of tissue self-RNA complexes is associated with the presence of activated DCs in psoriatic skin. Consistent with previous reports (Lowes et al. 2005), it was found that large clusters of DC-LAMP–positive mature mDCs (Ganguly et al. 2009). We also found tissue self-RNA–LL37 complexes within these clusters, and, occasionally, even inside the DCs as shown by the colocalization with endolysosomal compartments stained with DC-LAMP (Ganguly et al. 2009). The number of tissue self-RNA complexes significantly correlated with the numbers of DC-LAMP–positive mDCs in psoriatic skin (Ganguly et al. 2009). Together, these findings strongly support *in vitro* data that self-RNA complexes can activate mDCs and suggest that this pathway is operational in psoriasis.

When mRNA expression normalized to HARP for IL-23 subunits, such as p19 and p40 were quantified by RT-PCR in monocyte-derived DCs (moDCs) matured without and with etanercept, a dimeric human tumor necrosis factor recepter p75-Fc fusion protein made of 2 extra-cellular domains of the human 75kD TNFR linked by the constant Fc portion of human IgG1 (Haraoui and Bykerk. 2007), significant decrease in expression of IL-23 subunits p19 and p40 by etanercept were observed (Zaba et al. 2007). MoDCs cultured with etanercept decreased CD86 expression threefold and HLA-DR expression fivefold. In addition, moDCs cultured with etanercept were also an average of two to threefold less stimulatory than control DCs in a mixed leukocyte reaction. Gene array on control moDCs compared with those cultured with etanercept revealed that CD163, a macrophage scavenger receptor, was up-regulated 6.5-fold (Zaba et al. 2007).

In psoriatic dermis, mRNA expression normalized to HARP for multiple inflammatory products of Tip-DCs, including iNOS, TNF-α and IL23 p40 subunit, are reduced within 1–2 weeks after beginning etanercept, whereas the number of CD11c^{+} DCs in the tissue is minimally affected during this time, suggesting an initial blockade of cytokine production by these cells rather than cell reduction (Zaba et al. 2007). These facts suggest that TNF-α is an autocrine or paracrine inducer of IL-23 from Tip-DC (Zaba et al. 2007).

R848-treatment to moDCs, which were generated from monocytes isolated from buffy coats of healthy donors, resulted in concentration-dependent expression of IL-23. 2×10^{5} moDCs/ml were plated in DC medium and stimulated with 0-5 µg/ml R848. After 24 h of TLR stimulation, supernatants were harvested and cytokine expression was measured by ELISA. In addition, the combination of NOD1 and NOD2 agonists with R848 stimulated high levels of IL-23 secretion (Schwarz et al. 2013).

qRT-PCR for moDCs stimulated with TLR agonists in the absence or presence of NOD1 and NOD2 ligands at 8 h and 24 h post induction revealed that synergistic cytokine expression observed in NOD1/NOD2- and R848-stimulated cells is largely mediated by enhanced transcriptional activity (Schwarz et al. 2013).

In time kinetic studies, moDCs were stimulated with R848 in the absence or presence of MDP and iE-DAP which are ligands of NOD1/2, for 30 min, 2 h, 8 h or 24 h and mRNA levels of IL-23 as well as the cumulative cytokine release were measured by qRT-PCR and sandwich-ELISA, respectively. At the mRNA level, synergistic effects of both NOD ligands with R848 are already detectable after 8 h of stimulation. In agreement with IL-23 mRNA expression, synergistic effects are detectable by ELISA after 8 h; nevertheless, these effects are even more pronounced after 24 h of stimulation (Schwarz et al. 2013).

These findings show that dose responses and temporality of MIE and KE1 seem to be in sequence.

Uncertainties and Inconsistencies

Although unpublished, it has been reported that human slanDCs (Tip-DCs) lack the DNA-binding structureTLR9 but can express the endosomal RNA-binding receptorsTLR8 (slanDCs andCD1c^{+} DCs) and TLR7 (slanDCs but not CD1c^{+} DCs; Hänsel et al, unpublished data, June 2010) (Hänsel et al. 2011). There are not any other reports which mentioned TLR7 expression in Tip-DCs,

so whether or not TLR7 exists in human Tip-DCs is still unknown.

In addition, freshly isolated human pDCs have been reported to express TLR7 and TLR9, whereas $CD11c^+$ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR8. In some studies, TLR7 expression was detected on both pDCs and mDCs, whereas others found TLR7 was exclusively expressed in pDCs. Therefore, it is still unknown that whether or not TLR7 exists in human mDCs, and how much it does contribute recognition of R848 or LL37-RNA in these cells (Iwasaki and Medzhitov. 2004).

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

Much experimental data is available that supports the stimulation of TLR7 in pDC induced by TLR7 agonist, which subsequently promote secretion of IFN-α in MyD88-dependent fashion. For example, HEK293 cells were transiently co-transfected with human TLR7 and NF-κB-luciferase reporter. After 48 hours, the cells were stimulated with various concentrations of resiquimod or imiquimod. Luciferase activity was measured 48h post-stimulation and the results are reported as fold-increase relative to medium control. As a result, dose-dependent increase in NF-κB-dependent luciferase activity in HEK293 transfected with hTLR7 was observed with increasing concentrations from 0.01 μM up to 10 μM of resiquimod, and 0.1 μM up to 15 μM of imiquimod. Maximal NF-κB activation with resiquimod is achieved with 10-30 μM, which yields an 18-fold increase in luciferase production. Maximal NF-κB activation with imiquimod requires 10-15 μM compound and induces a 5-6-fold increase in luciferase production (Gibson et al. 2002).

In addition, three populations of cells were evaluated for type I IFN production following imidazoquinoline stimulation: human PBMC, pDC-depleted PBMC, and pDC-enriched cells. Human PBMC produce IFN-α following imiquimod (0.3–30 μM) or resiquimod (0.03–30 μM) treatment. Peak levels of IFN-α were reached with imiquimod and resiquimod at 3 μM. PBMC, depleted of pDC, did not produce detectable levels of IFN-α in response to imiquimod or resiquimod treatment.

The imidazoquinoline-treated pDC-enriched cultures produced 2–20 times more IFN-α than similarly treated PBMC as measured over the entire dose range. Peak levels of Resiquimod- and imiquimod-induced IFN-α production were reached with 0.3 μM and 30 μM, respectively (Gibson et al. 2002).

In different experiments, pDCs were stimulated with LL37 premixed with total human RNA extracted from U937 cells to confirm that LL37 can interact with self-RNA and convert it into a trigger of IFN-α production. U937-derived self-RNA induced dose-dependent IFN-α production when mixed with LL37, but not when given alone or mixed with the scrambled peptide GL37 (Ganguly et al. 2009).

R848 (0.001-10 μg/mL) induced NF-κB activation in HEK293 cells transfected with human TLR8 in a dose-dependent manner (Jurk et al. 2002). In addition, the production of TNF-α and IL-6, and the maturation

of mDCs induced by self-RNA–LL37 complexes but not by the TLR4 agonist LPS was completely inhibited by bafilomycin in a dose-dependent manner, demonstrating that mDC activation by self-RNA–LL37 complexes involved endosomal TLR activation (Ganguly et al. 2009).

Dose-dependent DC maturation was observed with increasing concentrations from 10 IU/ml up to 1000 IU/ml of IFN-α2a or IFN-α8 added to cultures containing GM-CSF, IL-4, and TNF-α. Both of the IFNs had a similar capacity to up-regulate HLA-A, B, C, CD80, and CD86 and to down-regulate CD1a and CD11b expression on the cell population (Luft et al. 1998).

DC cultured in GM-CSF, TNF-α, and IL-4-containing medium until day 14, and type I IFNs were added daily between days 14 and 17. Proportions of positive cells for each markers were analyzed by FACS on day 17 (Luft et al. 1998).

When GM-CSF, TNF-α, and IL-4-containing cultures were washed on day 14 and continued until day 17 in serum-free medium containing GM-CSF and IL-4, without or with TNF-α (20 ng/ml, standard conditions), IFN-a (1000 IU/ml), or both, IFN-α alone did not enhance DC maturation as compared with standard conditions. When both of TNF-α and IFN-α exist, optimal maturation was observed than either TNF-α or IFN-α alone. Thus, the enhancement of DC activation by IFN-α under serum-free conditions required the presence of TNF-α (Luft et al. 1998).

In accordance with these findings, compared with stimulation with either supernatant of activated pDCs or self-RNA–LL37 alone, the combination of both significantly enhanced the activation of mDCs to secrete IL-6 and TNF-α and enhanced their differentiation into mature CD83+ DCs (Ganguly et al. 2009). This activity was completely blocked by antibodies against IFN-α, IFN-β and IFN-αβR (Ganguly et al. 2009). Thus, self-RNA–LL37 complexes can trigger mDC activation and maturation, and this process is enhanced by the concomitant activation of pDCs to produce IFN-α.

KE 1

R848-treatment to moDCs, which were generated from monocytes isolated from buffy coats of healthy donors, resulted in concentration-dependent expression of IL-23. 2×10^5 moDCs/ml were plated in DC medium and stimulated with 0-5 μg/ml R848. After 24 h of TLR stimulation, supernatants were harvested and cytokine expression was measured by ELISA. In addition, the

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: Increase of IL-23 leads to Th17 cell migration and inflammation induction

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	adjacent	High	High

Evidence Supporting Applicability of this Relationship

In mice, application of IL-23 causes psoriatic-like epidermal hyperplasia, but this effect does not occur in IL-17A and IL-22KO mice. Therefore, it is thought that IL-17A and IL-22 play an important role downstream of IL-23 Rizzo HL. Et al. 2011 .

Recombinant mIL-23 (rmIL-23) injected into the ear of WT mice induced IL-17A and IL-22 expression, and showed ear swelling and epidermal hyperplasia. When rmIL-23 was injected into IL-22 KO mice, IL-22 was induced, but ear swelling and epidermal hyperplasia were less than in WT mice. When rmIL-23 was injected into IL-17A KO mice, IL-22 was induced, but ear swelling and epidermal hyperplasia hardly occurred. WT mice after administration of IL-22 or IL-17A inhibitor completely inhibited IL-23-induced epidermal hyperplasia. These results indicate that two cytokines, IL-22 and IL-17A, are downstream mediators of IL-23-induced changes in mouse skin and are required for the generation of IL-23-mediated skin lesions. (Hansel et al. 2011)

Key Event Relationship Description

IL-23 is important for differentiation and proliferation of Th17 cells. As a major source of IL-23, Tip-DC is present in the skin lesions of psoriatic patients and works to activate the Th17 pathway (Hansel et al. 2011).

Signaling through the heterodimeric IL-23 receptor (subunits of p19 and p40) of Th17 cells stimulates the production of proinflammatory keratinocyte cytokines that mediate the psoriatic response and induces the production of IL-17. Th17 cells are increased in the peripheral blood and lesion skin of psoriatic patients, and IL-17 and IL-22 produced from Th17 act on epidermal keratinocytes to cause inflammatory chemokines and hyperproliferation (Michelle A. et al. 2005).

IL-17A, which is highly expressed by Th17 cells, has a direct effect on the regulation of genes expressed by keratinocytes that are

involved in innate immune defense, including defensins,8, 9 S100 family proteins, lipocalin, and LL37/cathelicidin, as well as a range of CXCL chemokines that regulate neutrophil trafficking (Gilliet et al. 2004). IL-22, which is expressed by Th22 and Th17 cells, and related IL-20 family members promote keratinocyte hyperproliferation and abnormal differentiation (Krueger et al. 2012).

Evidence Supporting this KER

Biological Plausibility

IL-17A, which is highly expressed by TH17 cells, has a direct effect on the regulation of genes expressed by keratinocytes that are involved in innate immune defense, thorough expressions of defensins,8, 9, S100 family proteins, lipocalin and LL37/cathelicidin, as well as a range of CXCL chemokines that regulate neutrophil trafficking. IL-22, which is expressed by TH22 and TH17 cells, and related IL-20 family members promote keratinocyte hyperproliferation and abnormal differentiation Gilliet et al.2012 .

In vitro Reconstituted Human Epidermis (RHE) model stimulated for 48 hours with medium containing IL-17, IL-22 and TNFα mix (concentration 3 ng / mL) as psoriasis-specific cytokines. Controls were cultured in normal medium. After fixing RHE and embedding in paraffin, 4 µm sections were stained with hematoxylin-eosin or immunolabeled with anti-filaggrin, anti-S100A7, anti-hBD-2 mAb.

RHE stimulated with cytokine mix showed dramatic expression of these protein. In the RHE with normal medium, antibacterial peptide S100A7 was expressed locally, but BD-2 protein was not detected. This is due to the synergistic effect of IL-17 added to the IL-22 / TNFα combination. Filaggrin, S100A7 and BD-2 mRNA expression by RT-qPCR analysis increased 20-fold (S100A7) or -50-fold (BD-2) compared to controls. This is a downstream event that can be modeled using keratinocytes and cytokines and relies on upstream mechanisms of recruitment and activation of other innate adaptive immune cells. Bernard et al. 2012. .

Quantitative Understanding of the Linkage

Response-response relationship

KE1:

IL-23, which maintains Th17 cells, is released from TNF-a and inducible nitric oxide synthase (iNOS) -producing dendritic cells (TIP-DC). TIP-DC activates IL-17, IL-22, IL-23, and TNF-a mRNA expression in psoriatic skin. Cytokine staining analysis of peripheral blood mononuclear cell (PBMC) in patients with psoriasis showed a three-fold increase in Th17 cells compared to normal PBMC. Thl7 cells produce IL-22 and stimulate keratinocyte proliferation. IL-22 activates STAT3 and induces the production of cytokine (such as IL-8), chemokines and the synthesis of antimicrobial peptides (Zaba et al. 2005).

KE 2

The epidermis of psoriasis patients did not have many T cells, but the analysis was similar to peripheral blood and dermis. The proportion of Th17 cells in the dermis was significantly higher than that in normal skin, and TNF and IFN-g were produced from Th17 cells. Skin and peripheral blood contained a subset of Th17 cells producing IFN-g / TNF.

Keratin 16, IL-17, IFN-g, and IL-22 mRNA expression increased in psoriatic skin, but cyclosporine therapy returned these mRNA to normal levels. The average expression of IL-17 / human acidic ribosomal protein (hARP) in non-lesional skin was 0.4 compared to 10.8 in lesional skin, and cyclosporine administration returned to non-lesional levels. That IL-17 mRNA return to baseline, effective treatment supports that Th17 in psoriasis is a central pathogenic.(Lowes et al.2008)

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Relationship: 2019: Th17 cell migration and inflammation induction leads to Skin disease

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Stimulation of TLR7/8 in dendric cells leading to Psoriatic skin disease	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Initiation of plaque formation in the Aldara psoriasis mouse model is dependent on RORγt +, skin infiltrating γδ T cells, and innate lymphocyte cells (ILC). Vγ4 + γδT cells and innate lymphoid cells (ILC) are the dominant and important sources of IL-17A, IL-17F, and IL-22 in the formation of acute psoriatic lesions, rather than Th cells (Pantelyushin et al. 2012).

Amyloid A: SAA, an inflammatory marker, is high in the serum of patients with psoriasis. When C57B6 mice were given SAA protein subcutaneously on the back, epidermal thickening and inflammatory cell wetting were frequent on days 5-7. Skin inflammation was significantly suppressed when IL-12 / IL-23p40 protein, a target molecule of psoriasis biologics, was administered in advance. By SAA administration, a similar reaction to psoriatic eruption was formed in the immunological reaction, and a psoriatic eruption model mouse was established. (J Dermatolog Trest. 2013; 24 (6): 477-80)

Key Event Relationship Description

Th17 cells produce the cytokines IL-17 and IL-22. IL-17 is inflammatory, promotes the migration of neutrophils to psoriatic lesions, contributes to the formation of Munro's micro-abscess, and through DCL and memory T cells, including Th17 cells and CCR6, via CCL20 Incorporate into the affected area. IL-22 causes abnormal keratinocyte proliferation by down-regulating genes that control terminal differentiation, leading to altered differentiation and keratinization. Both IL-17 and IL-22 induce keratinocyte expression of the antibacterial S100A7 (psoriacin). Nograles et al. 2008

STAT3 is important for Th17 differentiation. Cytokine signaling SOCS3-deficient mice show increased IL-17 expression by increasing STAT3 activity in response to IL-23 binding to IL-17. Associated with increased activity of STAT3 in response to IL-23 capable of binding to IL-17 and IL-17F promoters. STAT3 overexpression promotes Th17 differentiation, whereas STAT3 deficiency inhibits Th17 differentiation. STAT3 signaling from IL-6, IL-21, IL-23 regulates the expression of lineage specific master transcription factors RORγt22, 63, 66 and RORα67. It has been found that patients with psoriasis with mutations in STAT3 cannot generate a Th17 response. Martinez et al. 2008

Evidence Supporting this KER

Biological Plausibility

The biological activity of the combination of cytokines was investigated. The combination of IL-17A and IFN-γ or IL-17A and TNF-α has a synergistic effect on CXCL8 production by keratinocytes. IL-17A and IL-22 exert a synergistic effect in upregulation of β-defensin 2: BD-2 and S100A9 production] IL-1α, IL-17, IL-22, Oncostatin M: OSM, and TNFα binding are associated with increased expression of inflammatory molecules such as soriacin / S100A7 or BD-2, IL-8 in vitro by NHEK Although very potent synergistic, removal of IL-22 from the cytokine mixture reduces CXCL8 and BD-2 expression by 30% and removal of IL-17 reduces it by 70%.

Ex vivo studies on human skin explants showed upregulation of BD-2, S100A7, and CXCL8 expression in response to the same combination of cytokines, and intradermal injection of cytokines into mice resulted in neutrophil infiltration and early epidermis CXCL1, CXCL2, CXCL3, S100A9, and BD-3 expression related to epidermal thickening was increased. (Bernard et aaal. 2012)

Empirical Evidence

Resident memory tissue T cells (TRM cells) confer both resistance and immunity depending on the local microenvironment, and CD8 TRM can be tracked by phenotypic markers CD49a and CD103. Circulating effector T cells infiltrate the site of skin inflammation and turn into long-lived epidermal TRM cells when the skin inflammation is resolved. Epidermal TRM cells are thought to form pathological site-specific disease memory at the site of recurrent psoriasis.(Cheuk et al. 2014)

Single cell suspensions of epidermis and dermis were analyzed by flow cytometry within 30 hours of sampling. In active psoriasis, CD8 T cells increased about 100-fold in the epidermis compared to normal skin, whereas CD4 T cells increased 10-fold in the dermis. In healthy skin, 20-30% of epidermal CD8 T cells co-expressed integrin CD103 and CD49a, which are phenotypic markers

of TRM cells. In active psoriasis, approximately half of epidermal CD8 T cells co-expressed these TRM phenotypic markers, a 100-fold increase compared to healthy skin. Cheuk et al. 2014

Uncertainties and Inconsistencies

Cytokines cannot be specified for genes associated with abnormalities in psoriatic skin. Many genes that are up-regulated in psoriatic lesions can be attributed to IFN-γ, including IL-17 and IL-22. Cytokines synthesized by Th1 / Th17 cells regulate different gene expression pathways in epidermal keratinocytes and other skin resident cells. The psoriatic transcriptome may result from activation of multiple independent pathways. Nograles et al. 2008

After daily topical application of Aldara containing imiquimod (IMQ) to humans, significant skin thickening, redness and scaling were observed 3 days later (doi: 10.1172 / JCI61862DS1). The clinical course of plaque formation on the ear and back skin and histopathology were similar. Aldara treatment resulted in impaired keratinocyte hyperproliferation and epidermal differentiation, as indicated by epidermal thickening and hyperkeratosis. There was a terminal neutrophil accumulation in the stratum corneum reminiscent of a Munro micro-abscess in psoriasis. Extensive leukocyte infiltration was observed in the dermis.(Pantelyushin et al. 2012)

Quantitative Understanding of the Linkage

Response-response relationship

KE2

High levels of Th17 cytokines were observed in psoriatic skin induced by CD4 + T cells. IL-23 p40 subunit or IL-22 significantly prevented the development of skin lesions.

IL-22-induced acantosis and inflammation were reduced in IL-22-deficient mice compared to WT mice. Blocking IL-22 increases IL-1α, IL-1β, IL-6, IL-17, IL-17F, and TNF-α. (K. A. et al. 2013)

AO

Anti-IL-17 antibody administration results in decreased keratinocyte proliferation and differentiation, leukocyte infiltration, and keratinocyte release of inflammatory cytokines. In psoriatic lesioned keratinocytes, changes in mRNA and protein expression of IL-17 regulatory products occurred. Within 2 weeks of antibody administration, the expression of LL37 (cathelicidin), β-defensin 2, S100A7, and S100A8 proteins was markedly decreased in keratinocytes, and the expression reached normal levels after 6 weeks. (Krueger et al. 2012)

Time-scale

Epidermal keratinocyte expression genes that were elevated in psoriatic lesions of patients with psoriasis with stage-type skin eruption: mRNA expression level of keratin6a and 16, s100A7A, S100A12, DEFB4, IL-1F6, CCL20, IL-17C, etc. was rapidly reduced by 700 single intravenous dose of brodalumab and decreased to non-lesional skin level 2 weeks after administration. On the other hand, leukocyte expression genes with increased expression in psoriatic lesion skin: IL-17A, IL-17F, IL-23F, IL-12B, IL-22, IFN-γ and other mRNA expression levels decreased with brodalumab administration. However, at 2 weeks after administration, the level did not decrease to the level of the non-lesional skin. Since the expression of pathophysiology-related genes is reduced prior to the decrease in the expression of leukocyte expression genes is reduced prior to the decrease in the expression of leukocyte expression genes and the decrease in the PASI score, Brodalumab is reduced expression of pathophysiology-related genes by blocking IL-17 signaling in the epidermal keratinocytes of psoriatic lesions It is possible to improve the skin eruption promptly. (Kyowa Hakko Kirin Co., Ltd.)

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AOP ID and Title:

AOP 314: Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)
Short Title: Binding to ER-α leading to exacerbation of SLE

Authors

Yasuharu Otsubo (1) Takao Ashikaga (1) Tomoki Fukuyama (1) Ken Goto (1) Shinko Hata (1) Shigeru Hisada (1) Shiho Ito (1) Hiroyuki Komatsu (1) Sumie Konishi (1) Tadashi Kosaka (1) Kiyoshi Kushima (1) Shogo Matsumura (1) Takumi Ohishi (1) Junichiro Sugimoto (1) Yasuhiro Yoshida (1)

(1) AOP Working Group, Testing Methodology Committee, The Japanese Society of Immunotoxicology

Corresponding author: Yasuharu Otsubo (otsubo-yasuharu@snbl.co.jp)

Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.73	Included in OECD Work Plan

Abstract

This AOP describes the linkage between the binding to estrogen receptor (ER) α in immune cells with the exacerbation of the autoimmune disease systemic lupus erythematosus (SLE).

Estrogen receptors (ERs), ERα and ERβ, are a group of proteins that are activated by the steroid hormone estrogen and are widely expressed in most tissue types, including most immune cells. ERα can be activated with exogenous and endogenous estrogens. Also, there are numerous xenoestrogens that exist in the environment and imitate estrogen. Bisphenol A (BPA) is an example of a xenoestrogen that is considered an endocrine disrupting (ED) compound. SLE is an autoimmune disease characterized by overproduction of a variety of anti-cell nuclear and other pathogenic autoantibodies. It is characterized by B-cell hyperactivity, polyclonal hypergammaglobulinemia, and immune complex deposition.

Binding to ERα in immune cells by a xenoestrogen or endogenous estrogen marks the molecular initiating event (MIE), which results in induction of GATA3 expression (KE1). One theory of immune regulation involves homeostasis between T-helper 1 (Th1) and T-helper2 (Th2) activity, however GATA3 expression induce increase of Th2 cells producing cytokine interleukin-4 (IL-4) (KE2), which results in increase of anti-DNA antibody from autoreactive B cell (KE3). This sequence of pathway means that the immune system skew from a Th1 to a Th2 profile, which results in the adverse outcome (AO) of exacerbated SLE.

We have identified a number of key events along this pathway and determined the key event relationships, based on which we have created an AOP for binding to ERα in immune cells leading to exacerbated SLE.

Background

It is well recognized that allergic diseases and autoimmune diseases are markedly increased the last several decades. About the same time, increasing scientific and social attention had been paid to environmentally dispersed chemicals that can enter the body by ingestion or adsorption and that mimic the actions of estrogens. These chemicals are termed endocrine disruptors (EDs) or environmental estrogens and are found in plastics (bisphenol-A, phthalates), pesticides (DDT, hexachlorobenzene, and dieldrin) and the like. Some of these estrogenic chemicals have also been shown to influence the immune system. Endocrine disruptors mimic hormones, block or alter hormone binding to receptors, or alter the metabolism of natural estrogens. It has been widely noted that females have stronger immune capabilities than males, as evidenced by their better immune responses to a variety of self-antigens and non-self-antigens, or vaccination. Paradoxically, the stronger immune response comes at a steep price, which is the high incidence of autoimmune diseases in females. This phenomenon of gender-based immune capability is largely attributed to the effects of sex hormones. Estrogens regulate the level of serum and uterine IgM, IgA, and IgG, and they augment antibody production to several nonself- antigens and self-antigens. It is possible that endocrine disruptors that mimic estrogenic activity may be involved in the increased incidence of autoimmune diseases such as SLE (Yurino H. 2004, Vaishali RM. 2018).

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	1710	Binding to estrogen receptor (ER)-α in immune cells	Binding to estrogen receptor (ER)-α

Sequence	Type	Event ID	Title	Short name
	KE	1711	Induction of GATA3 expression	Induction of GATA3 expression
	KE	1712	Increase of Th2 cells producing IL-4	Increase of Th2 cells producing IL-4
	KE	1713	Increase of anti-DNA antibody from autoreactive B cell	Increase of autoantibody production
	AO	1714	Exacerbation of systemic lupus erythematosus (SLE)	Exacerbation of SLE

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells	adjacent	Induction of GATA3 expression	Moderate	Moderate
Induction of GATA3 expression	adjacent	Increase of Th2 cells producing IL-4	Moderate	Moderate
Increase of Th2 cells producing IL-4	adjacent	Increase of anti-DNA antibody from autoreactive B cell	Moderate	Moderate
Increase of anti-DNA antibody from autoreactive B cell	adjacent	Exacerbation of systemic lupus erythematosus (SLE)	Moderate	Moderate

Stressors

Name	Evidence
Bisphenol A	Moderate
17beta-Estradiol	High

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI

Sex Applicability

Sex	Evidence
Mixed	High

It has long been appreciated that most autoimmune disorders are characterized by increased prevalence in females, suggesting a potential role for sex hormones (estrogen) in the etiology of autoimmunity. Females generally exhibit a stronger response to a variety of antigens including ERα ligands than males, which is perhaps one reason that they are more prone to develop autoimmune and allergic diseases such as SLE in greater severity than males. Therefore, this AOP is applicable to females and is dependent on the levels of estrogen, which means it varies with life stage, and age.

SLE frequently develop and progress in setting in which sympathoadrenomedullary and gonadal hormone levels are changing, e.g., during pregnancy, the postpartum period, or estrogen administration in menopause (Wilder RL. 1999). Women using oral contraceptives that contain estrogen or undergoing hormone replacement therapy are susceptible to major flare ups and exacerbation of the disease (Whitelaw DA. 2007).

The mechanisms described in this AOP are applicable to rodents and humans, and then the findings of this AOP are not found in any other species. However, Th2 dominant conditions induced by binding to ERα is considered likely to occur in a variety of mammalian species since ERα are expressed in all vertebrates (Eick GN. 2011).

Essentiality of the Key Events

Stressor , MIE and later events:

The NZB/W F1 mouse is the oldest classical model of lupus generated by the F1 hybrid between the NZB and NZW strains. The administration of the estrogen antagonist tamoxifen diminishes immune complex deposition in the kidneys and increases survival in NZB/W F1 strain. Renal disease was evaluated by the development of albuminuria and histological changes in the kidney (Wu WM. 2000). In females of the NZB/NZW F1 strain, disruption of ERα attenuated glomerulonephritis and increased survival and reduced anti-dsDNA antibodies (Bynote KK. 2008, Isenberg DA. 2007) and ovariectomy of NZB/W F1 mice not only delayed onset of the disease but also decreased autoantibody titer Meanwhile, restoration of estradiol in ovariectomized NZB/W F1 mice reestablished high numbers of autoantibody-producing (DNA-specific) B cells, and thereby suggests a pathogenic role of estrogen in lupus (Daniel P. 2011). Both NZB and NZW display limited autoimmunity, while NZB/W F1 hybrids develop severe lupus-like phenotypes comparable to that of lupus patients. In NZM female mice, ERα inactivation markedly prolonged life-span, lowered proteinuria, and ameliorated glomerulonephritis but resulted in higher serum anti-dsDNA antibody levels (Svenson JL. 2008).

KE1 and later events:

GATA3 mRNA expression has potential to induced IL-4 production in CD4+T cell (Lambert KC. 2005). The differentiation of activated CD4+T cells into the T helper type 1 (Th1) or Th2 fate is regulated by cytokines and the transcription factors T-bet and GATA-3. Early GATA-3 expression, required for Th2 differentiation, was induced by T cell factor 1 (TCF-1) and its cofactor β-catenin, mainly from the proximal Gata3 promoter upstream of exon 1b. TCF-1 blocked Th1 fate by negatively regulating interferon-γ (IFN-γ) expression independently of β-catenin. Thus, TCF-1 initiates Th2 differentiation of activated CD4+T cells by promoting GATA-3 expression and suppressing IFN-γ expression. Higher GATA-3 expression promotes IL-4 production and initiates Th2 differentiation (Qing Y. 2009). GATA-3 mRNA expression also increased in patients with SLE, compared with the healthy control groups (Zheng H. 2015, Sonia GR. 2012).

KE2 and later events:

Administration of mAb against IL-4 before the onset of lupus was effective in preventing the onset of lupus nephritis (Nakajima A. 1997).

KE3 and later events:

In a study to investigate a novel subpopulation of B-1 cells and its roles in murine lupus, anti-double-stranded DNA (anti-dsDNA) autoantibodies were preferentially secreted by a subpopulation of CD5+ B-1 cells that expressed programmed death ligand 2 (L2pB1 cells) (Xuemei Z. 2009). A substantial proportion of hybridoma clones generated from L2pB1 cells reacted to dsDNA. L2pB1 cells are potent antigen-presenting cells and a dramatic increase of circulating L2pB1 cells in lupus-prone BXSB mice correlates with elevated serum titers of anti-dsDNA antibodies (Xuemei Z. 2009).

Weight of Evidence Summary

Biological Plausibility

KER	KE_{up}-KE_{down}	Plausibility	Rationales supported by literatures
KER 1	Binding, Estrogen receptor α in immune cells - Induction, GATA3 expression	Weak	In immune cells, this event is confirmed indirectly; using artificial STAT6-ER fusion protein.
KER 2	Induction, GATA3 expression - Increase, Th2 cells producing IL-4	Strong	XXXX
KER 3	Increase, Th2 cells producing IL-4 - Increase, anti-DNA antibody production from autoreactive B cell	Weak	XXXX
KER 4	Increase, anti-DNA antibody production from autoreactive B cell -	Strong	XXXX

Empirical Support

KER	Empirical support of KERs
MIE=>KE 1 Binding, Estrogen receptor α in immune cells leads to Induction, GATA3 expression	Empirical support of the MIE => KE1 is weak. Rationale

	MIE: XXX KE XX: XXXX
KE 1=> KE 2: Induction, GATA3 expression leads to Increase, Th2 cells producing IL-4	Empirical support of the KE 1=> KE 2 is strong. Rationale KE XX: XXXX AO: XXXX
KE 2=> KE 3: Increase, Th2 cells producing IL-4 leads to Increase, anti-DNA antibody production from autoreactive B cell	Empirical support of the KE 2=> KE 3 is weak. Rationale KE XX: XXXX AO: XXXX
KE 3=>AO: Increase, antibody production from anti-DNA antibody production from autoreactive B cell leads to Exacerbation, systemic lupus erythematosus (SLE)	Empirical support of the KE 3 => AO is strong. Rationale KE XX: XXXX AO: XXXX

Quantitative Consideration

KER1

CD4^{+}T cell expressed GATA3 mRNA cultured with 10^{-9} M (272.4 pg/mL) concentrations of 17β-estradiol for 12-16 hr (Lambert KC. 2005).

BPA (0.1 mM) also indirectly induced GATA3 expression of Th cells, and this effect is mediated by dendritic cells exposed to BPA for 24 hr (Guo H. 2010). Naïve Th cells increased GATA3 expression cultured with dendritic cells exposure of BPA (0.1 mM) for 7 days.

KER2

Pre-stimulation 16 hr of 17β-estradiol (the concentration 10^{-9} M = 272.4 pg/mL) increased IL-4 secretion from CD4^{+}T cell (Lambert KC. 2005).

KER3

PBMCs or B cells were cultured for 7 days with 17β-estradiol (10^{-8} mol/L) and then, IgG and IgM production were increased up to about 150% (PBMC) and 200% (B cells) (Kanda N. 1999).

KER4

XXXX

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Appendix 1

List of MIEs in this AOP

Event: 1710: Binding to estrogen receptor (ER)-α in immune cells

Short Name: Binding to estrogen receptor (ER)-α

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	MolecularInitiatingEvent

Stressors

Name
Bisphenol A
17beta-Estradiol
Propylpyrazoletriol

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
immune system

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

E_2 activates ERα and ERβ with the same affinity although they share only 56% similarity in their ligand binding domains (Monroe DG. 2005, Papoutsi Z. 2009). Exposure E_2 induced thymic atrophy, and changing T-cell phenotype (decreasing double positive ($CD4^+CD8^+$) T cell and increasing double negative ($CD4^-CD8^-$) T cell) in thymus (Okasha SA. 2001).

BPA binds to both ERα and ERβ, and ERα binding affinity of BPA is lower than that of ERβ (Takayanagi S. 2006). While these bindings are less than 2000-fold affinity compared to the binding of estradiol to estrogen receptors (Krishnan AV. 1993).

Propylpyrazoletriol (PPT) is an ERα-selective agonist, which shows 410-fold selectivity for ERα as compared with ERβ (Kraichely DM. 2000, Li J. 2006). Li et al (2006) demonstrated that ovariectomized mice exposed PPT induced severe thymic atrophy, changing T-cell phenotype (CD4/CD8 phenotype profile) in thymus, and a reduction of mature B cell number in spleen. Since these effects by PPT were equal to or greater than E2, ERα plays the predominant role in the upregulation of immune responses.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	

Since ERα expresses in the cells of a vast variety of (vertebrate) species (Maria B. 2015) and there is common functionality in the immune systems of at least humans and mice, this AOP might be applicable to many mammal species, including humans and rodents. The estrogen receptors are composed of several domains important for hormone binding, DNA binding, dimer formation, and activation of transcription (Green S. 1986, Kumar V. 1986, Warnmark A. 2003). Interspecies sequence identities for the entire ERα are 88.5% (human-mouse), 87.5% (human-rat), and 97.5% (mouse-rat). For the ligand binding domain (ERα-LBD) alone, the interspecies sequence identities are 95.5% (human-mouse), 95.1% (human-rat), and 99.2% (mouse-rat) (White R. 1987).

ERα is widely expressed in most tissue types including most immune cells in males and females (Couse JF. 1997, Chelsea C. 2017). The ERs' expression patterns and functions vary in a receptor subtype, cell- and tissue-specific manner. In the adult human, large-scale sequencing approaches show that ERα mRNA is detected in numerous human tissues, with the highest levels in the uterus, liver, ovary, muscle, mammary gland, pituitary gland, adrenal gland, spleen and heart, and at lower levels in the prostate, testis, adipose tissue, thyroid gland, lymph nodes and spleen (Fagerberg L. 2014, Sayers EW. 2012) (www.ncbi.nlm.nih.gov/UniGene).

Estrogen level is higher in women than men. Ordinary estrogen levels in women are 20-30 pg/mL during diestrus, 100-200 pg/mL during estrus, and 5000-10000 pg/mL during pregnancy (Offner H. 2000). Therefore, the influence of ligand binding to ERα in immune cells is expressed more strong in women than men, especially high estrogen level period.

Key Event Description

ERα is expressed in all vertebrates (Eick GN. 2011). ERα was discovered in the late 1960s and was cloned and characterized in 1985 (Melissa C. 2011). ERα is expressed in a variety of immunocompetent cells, including thymocytes, CD4+ (Th1, Th2, Th17, and Tregs) and CD8+ cells and macrophages (Melissa C. 2011, Salem ML. 2004, Robinson DP. 2014). One study examined ERα expression in resting and activated PBMC subsets and found that ERα was expressed at higher levels in thymocytes, CD4+ T cells than B cells (Melissa C. 2011). ERα is a nuclear hormone transcription factor that classically binds with ligand (stressors), further stabilizing dimers that subsequently bind estrogen response elements (ERE) or non-ERE to transactivate or suppress specific target

genes (Parker MG. 1993, Goldstein RA. 1993, Sasson S. 1991, Brandt ME. 1997, Carolyn MK. 2001).

How it is Measured or Detected

The binding affinities of E_2 and BPA for ERα can be confirmed by radio receptor assay, and its dimer dissociation is measured using size exclusion chromatography (Brandt ME. 1997, Takayanagi S. 2006, OECD TG440 [*in vivo*] and TG455 [*in vitro*]). While the binding affinities of PPT for ERα was determined by competitive radiometric binding assays by chemiluminescence (Kraichely DM. 2000, Carlson KE. 1997).

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List of Key Events in the AOP

Event: 1711: Induction of GATA3 expression

Short Name: Induction of GATA3 expression

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	KeyEvent

Stressors

Name
17beta-Estradiol
Bisphenol A
4-Hydroxytamoxifen

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
immune system

Evidence for Perturbation by Stressor

17beta-Estradiol

Expression of GATA3 was induced in $CD4^+$T cells treated with E_2 at a concentration of 10^{-9} M (272.4 pg/mL) for 12-16 hours (Lambert KC. 2005). GATA3 expression has potential to induced IL-4 production in $CD4^+$T cell. In contrast, expression of T-bet was decreased, which means E_2 skew the immune system from a Th1 to a Th2 profile (Lambert KC. 2005).

Bisphenol A

GATA3 expression is induced in Th cells primed by dendritic cells exposed to BPA (Guo H. 2010). Purified naive T cells were cultured and expanded under Th1 culture conditions in the presence or absence of 0.3 µM 4-HT (Research Biochemicals Institute) for 2 weeks starting from days 1, 7, 14, or 21 (Kurata H. 1999).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence

All life stages

Life Stage	Evidence

Sex Applicability

Sex	Evidence
Mixed	

Involvement of GATA3 in Th2 cell development through ER is common in humans, rodents, and other mammalian species (Ho IC. 2009). protein sequence conservation between all six vertebrate members (mouse, human, dog, cow, armadillo, capuchin and opossum) identifies GATA3 as having the highest sequence similarity with both its GATA paralogs and orthologs, suggesting that it may be closest to the ancestral mammalian GATA factor (Tremblay M. 2018).

Key Event Description

Naïve CD4 T cells can differentiate into several different types of T helpers, and Th2 cells, capable of producing IL-4, IL-5 and IL-13, are involved in humoral immunity against extracellular pathogens and in the induction of asthma and other allergic diseases. It was reported that GATA-3 promotes Th2 responses through three different mechanisms (Zhu J. 2006). Cell fate determination in each lineage requires at least two types of transcription factors: the master regulators (GATA3) as well as the signal transducers and activator of transcription (STAT) proteins (Zhu J. 2010). A direct role in bridging distant regulatory elements has been demonstrated for GATA3 at Th2 cytokine loci (Spilianakis and Flavell, 2004). GATA3 is the Th2 master regulator (Zhu J 2010, Sung-Yun. 2004, Zhu J. 2004, Zheng W. 1997, Zhang DH. 1997), but it also plays important roles in multiple steps of CD4 T cell development (Ho IC. 2009). GATA3 can act as pioneer factors by initiating local chromatin opening and allowing the recruitment of other transcription factors to regulatory elements (Spilianakis and Flavell, 2004). Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells (Zhu J. 2004, Pai SY. 2004). GATA-3 mRNA expression also increased in patients with SLE, compared with the healthy control groups (Zheng H. 2015, Sonia GR. 2012).

How it is Measured or Detected

GATA3 mRNA in CD4 T cells can be detected by Real-time PCR (RT-PCR) (Lambert KC. 2005, Kurata H. 1999, Zhu J. 2001).

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Event: 1712: Increase of Th2 cells producing IL-4

Short Name: Increase of Th2 cells producing IL-4

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	KeyEvent

Stressors

Name
17beta-Estradiol
Bisphenol A

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T-helper 2 cell

Organ term

Organ term
immune system

Evidence for Perturbation by Stressor

17beta-Estradiol

In vitro, the addition of E2 significantly increased IL-4 secretion from ERα-replete CD4+T cells, while this effect was abrogated in ERα-deficient CD4+T cells. (Lambert KC. 2005).

Bisphenol A

Mouse lymphocytes stimulated with a massive amount of BPA (50 µM) were Th2 polarized, with prominent elevation of IL-4 as well as IL-10 (Lee J. 2010). Similarly, BPA enhanced IL-4 production in antigen-activated T cells by ELISA or RT-PCR, although the concentrations of BPA that they utilized (10-50 µM) were high (Lee MH. 2003). In this experiment, IL-4 level is confirmed baseline when treated with anti-CD4 mAb. Exposure to BPA in adulthood mice promoted antigen-stimulated levels of IL-4, IL-10, and IL-13, but not IFN-γ (Huimin Y. 2008).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Mixed	

Production of IL-4 from Th2 is common in humans, rodents, and other mammalian species.

Key Event Description

In naive CD4+ T cells, T cell expansion shifts toward a Th2 phenotype that produces Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13, thereby increasing antibody production from autoantibody-producing B cells. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, meanwhile Th1 cells produce IL-12, TNF-α, and IFN-γ. During Th2 polarization, IL-4 produced by Th2 cell. IL-12 plays a central role in promoting the differentiation of naive CD4+ T cells into mature Th1 effector cells. Secretion of IL-10 from Th2 has been suggested to downregulate the DC-derived IL-12 production and lead to a Th2 differentiation (Aste-Amezaga M. 1998). Th2 cells produce IL-4, which stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. The receptor for IL-4 is IL-4Rα, which expresses in B cells. IL-4 also plays an important role in the development of certain immune disorders, particularly allergies and some autoimmune diseases and especially when there is Th2 polarization. Th2 cells from GATA3 and STAT6 knockout animals showed reduction in IL-4 production (Zhu J. 2004, Pai SY. 2004).

How it is Measured or Detected

The levels of IL-4 in the cell supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), cytometric bead array (CBA) kits, or immunoblot analysis (Lee MH. 2003, Huimin Y. 2008, Lee J. 2010), and mRNA levels of IL-4 in the cells were assayed by reverse transcription–polymerase chain reaction (RT-PCR) (Lee MH. 2003, Lee J. 2010).

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Event: 1713: Increase of anti-DNA antibody from autoreactive B cell

Short Name: Increase of autoantibody production

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	KeyEvent

Stressors

Name
17beta-Estradiol
Bisphenol A
Diethylstilbestrol

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term

B cell

Organ term

Organ term

immune system

Evidence for Perturbation by Stressor

17beta-Estradiol

BPA as well as E_2 and diethylstilbestrol (DES) enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of ED was more prominent on aged BWF1 mice developing lupus nephritis. (Yurino H. 2004).

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 or B2 cells, B1 cells were prepared from peritoneal cells and B2 cells from spleen, B1 or B2 cells were cultured in the presence of endocrine disruptors (E_2: 100 nM, DES: 100 nM, BPA: 1 µM) for 4 days (Yurino H. 2004).

Direct exposure of PBMCs from SLE patients to E_2 induces secretion of anti-dsDNA antibodies and enhances the secretion of Igs, in particular IgG (Kanda N. 1999).

In both (NZB×NZW) F1 and MRL/lpr mice, estrogen treatment exacerbates the lupus disease, with augmented levels of autoantibodies against dsDNA and phospholipids as well as formation of circulating immune complexes (Grimaldi CM. 2002, Peeva E. 2000).

Hybridomas generated from E_2-treated mice express high-affinity, unmutated anti-DNA antibodies, indicating that naı¨ve B cells that are normally deleted or anergized are rescued from tolerance induction (Bynoe MS. 2000). E_2 treatment resulted in a rise in anti-DNA serum titers and in Ig deposition in renal glomeruli (Bynoe MS. 2000).

Bisphenol A

BPA as well as E_2 and diethylstilbestrol (DES) enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of ED was more prominent on aged BWF1 mice developing lupus nephritis. (Yurino H. 2004).

In a murine model of SLE, BPA increased the number of B cells producing autoantibodies, and IgM antibody secretion by B1 cells was augmented (Yurino H. 2004).

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 or B2 cells, B1 cells were prepared from peritoneal cells and B2 cells from spleen, B1 or B2 cells were cultured in the presence of endocrine disruptors (E_2: 100 nM, DES: 100 nM, BPA: 1 µM) for 4 days (Yurino H. 2004).

Diethylstilbestrol

BPA as well as E_2 and diethylstilbestrol (DES) enhanced anti-Br-RBC autoantibody production by B1 cells in vivo. IgM production by B1 cells in the presence of ED was more prominent on aged BWF1 mice developing lupus nephritis. (Yurino H. 2004).

To examine a direct effect of endocrine disruptors on IgM antibody production by B1 or B2 cells, B1 cells were prepared from peritoneal cells and B2 cells from spleen, B1 or B2 cells were cultured in the presence of endocrine disruptors (E_2: 100 nM, DES: 100 nM, BPA: 1 µM) for 4 days (Yurino H. 2004).

In both (NZB×NZW) F1 and MRL/lpr mice, estrogen treatment exacerbates the lupus disease, with augmented levels of autoantibodies against dsDNA and phospholipids as well as formation of circulating immune complexes (Grimaldi CM. 2002, Peeva E. 2000).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence

All life stages

Life Stage	Evidence

Sex Applicability

Sex	Evidence
Mixed	

Antibody production from B cells is common in humans, rodents, and other mammalian species. Since almost experiment are performed in female, it is considered that this event in SLE are noted more frequently in females.

Key Event Description

The receptor for IL-4 is IL-4Rα, which expresses in B cells. IL-4 produced by Th2 stimulates B-cells to proliferate, to switch immunoglobulin classes, and to differentiate into plasma and memory cells. Anti-DNA antibodies are produced from autoreactive B cell. In murine models, addition of estrogen or prolactin can lead to an autoimmune phenotype with an increase in mature high-affinity autoreactive B cells (Daniel P. 2011).

How it is Measured or Detected

[*in vivo* assay]

NZB/W F1 mice are used as model of SLE (Wu WM. 2000). BALB/c R4Ag-gamma 2b transgenic mice are used for evaluation of autoreactive B cells (Peeva E. 2005). These mice are administrated of the estrogen antagonist tamoxifen. Disruption of ERα (Bynote KK. 2008, Isenberg DA. 2007) and ovariectomy of NZB/W F1 mice are used as model of estrogen dysfunction (Daniel P. 2011). Survival and glomerulonephritis of these animals were evaluated.

Using female NZB/WF1 mice, silastic implants containing the powdered form of endocrine disruptors were placed subcutaneously on the back of ovariectomized mice. The implants were left in situ for 3 to 4 months and blood samples were collected periodically, and anti-DNA antibody was measured in ELISA using dsDNA (Yurino H. 2004).

[*in vitro* assay]

The amounts of anti-dsDNA, anti-glomerular antigens (GA), total IgG and IgM in the culture supernatants were measured by ELISA (Kanda N. 1999, Wu WM. 2000, Yurino H. 2004, Gabriela T. 2019, John LS. 2008, Wang Y.1996). Proliferative responses PBMCs or B cells were measured by [3H]-thymidine uptake, and the cell viability was assessed by a trypan blue exclusion test (Kanda N. 1999). Fluorescence activated cell sorting (FACScan) was used for the quantitated of total B cells and CD5+B cells expression in spleen and in peritoneal exudates or B cell subset analysis (Wu WM. 2000, Peeva E. 2005). Plaque forming cell (PFC) assay using autologous bromelain-treated erythrocytes (Br-RBC) was conducted to examine the effect of EDs on autoantibody production by B1 cells (Yurino H. 2004).

Enzyme-linked immunospot (ELISPOT) analysis confirmed a significant increase in the number of high-affinity anti-DNA antibody-secreting B cells in the spleens of E2-treated mice (Bynoe MS. 2000).

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List of Adverse Outcomes in this AOP

Event: 1714: Exacerbation of systemic lupus erythematosus (SLE)

Short Name: Exacerbation of SLE

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:314 - Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	AdverseOutcome

Stressors

Name
17beta-Estradiol
Bisphenol A

Biological Context

Level of Biological Organization
Individual

Evidence for Perturbation by Stressor

17beta-Estradiol

The NZB/W F1 mouse is the oldest classical model of lupus generated by the F1 hybrid between the NZB and NZW strains. In both NZB/W F1 and MRL/lpr mice, estrogen treatment exacerbates the lupus disease (Grimaldi CM. 2002, Peeva E. 2000). In postmenopausal women there was an increase in number of mild flares in women receiving estrogen supplementation suggesting that the addition of estrogen to a low estrogen state enhances flare rate (Buyon JP. 1998).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Mixed	

Exacerbation of SLE is common in humans and rodents, and is considered likely to occur in other animal species, as well. SLE is an autoimmune disease that occurs primarily in women (9:1 compared to men) (Rider V. 2001). SLE is an autoimmune disease that affects predominantly women during reproductive years, and its evolution is altered by hormonal events such as menses, menopause, and especially pregnancy (Luis JJ. 2014). The incidence of SLE is markedly increased in females of child-bearing age (Grainne M. 2013). Th1/Th2 shift is one of the most important immunologic changes during gestation. It is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses. For this reason, Th1-mediated diseases, such as rheumatoid arthritis, tend to improve, while Th2-mediated diseases, such as SLE tend to worsen during pregnancy (Doria A. 2006).

Female MRL/lpr mice that developed lymphadenopathy and a lupus-like disease also exhibited a 50% higher mortality rate than males at 5 months of age. In (NZB×NZW) F1 mice too, females develop signs of SLE several months before males, with severe autoimmune hemolytic anemia, glomerulonephritis, and autoantibodies to single-stranded DNA, doublestranded DNA, and histones (Carlsten H. 1992).

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Melissa, and Gary 2011). Low serum levels (60-100 pg/mL or 0.26-0.43 nM) of estradiol have been shown to increase Th1 T-cell development in vitro through an ERα mediated mechanism (Maret A. 2003). In contrast of SLE exacerbated by Th2, treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated autoimmune diseases (multiple sclerosis; MS, rheumatoid arthritis; RA, and experimental autoimmune encephalomyelitis; EAE, etc.) caused by Th1, while high doses (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset polarized T-cells to a Th2 phenotype in the EAE model (Bebo BF. 2001).

Key Event Description

SLE is an autoimmune disease characterized by overproduction of a variety of anti-cell nuclear and other pathogenic autoantibodies. It is characterized by B-cell hyperactivity, polyclonal hypergammaglobulinemia, and glomerulonephritis as immune complex deposition. Once SLE is suspected, the initial evaluation should include an antinuclear antibody (ANA) test. This is a highly sensitive test, with positive results in about 94% of patients with SLE. However, it also has low specificity, and may be positive in healthy patients. If ANA results show a 1:40 titer or higher, more specific tests should be performed, including measurement of anti–double-stranded DNA (anti-dsDNA), anti-Smith, anti-RNP, anticardiolipin, beta-2 glycoprotein antibodies and lupus anticoagulant; elevated levels of one or more of these biomarkers increase the likelihood of SLE (Nguyet-Cam VL. 2016). In the Systemic Lupus International Collaborating Clinics 2012 classification for SLE, biopsy-proven lupus nephritis plus positive ANA or anti-dsDNA is sufficient to fulfil SLE classification criteria (Bernard T. 2017). SLE is the prototypic multisystem autoimmune disorder with a broad spectrum of clinical presentations encompassing almost all organs and tissues including skin, kidney, heart, lungs, and joints. The pathogenesis of SLE includes both genetic and environmental components with female sex strongly influencing pathogenesis. These factors lead to an irreversible break in immunological tolerance manifested by immune responses against endogenous nuclear antigens (Daniel P. 2011).

It has been determined in a murine model of SLE that ERα is required for disease progression and that ERα deficiency impedes the course of the disease (Bynote KK. 2008). There is increased ERα mRNA expression in PBMCs of SLE patients (Inui A. 2007). It is considered that MIE affect later events and result in SLE.

How it is Measured or Detected

[*in vivo* assay]

Murine lupus models such as New Zealand Black (NZB)×New Zealand White (NZW) F1 (NZB/W F1), NZB.H-2bm12, NZB×SWR F1 (SNF1), MRL.lpr/lpr, and BXSB mice have led to a better understanding of the pathogenic mechanisms of lupus. All of these species of mice develop anti-dsDNA antibody, which is a characteristic of lupus, and die of uremia in early life. Among these murine lupus models, the natural course of NZB/W F1 mice is closer to human lupus than MRL.lpr/lpr and BXSB mice (Zhang DH. 1997, Pai SY. 2004, Daniel P. 2011).

For the disease onset, mice can monitor by proteinuria levels, body weights, blood urea nitrogen and appearance over time. (Gabriela T. 2019, John LS. 2008, Wang Y.1996). The major cause of death in the NZB/W F1 female is chronic glomerulonephritis with heavy mesangial deposits, tubular cast formation, proliferation of glomerular cells, prominent crescent formation, and a significant periglomerular and interstitial monocytic infiltrate. Extraglomerular renal deposits of IgG2a and C3 are present in the peritubular tissue and arterioles, and increase in frequency with age. Histological alterations in the kidney were assessed by Hematoxylin Eosin (H&E) and Periodic acid-Schiff (PAS) staining, expression of IgG and C3 was detected by immunohistochemistry (Gabriela T. 2019, Brian S. 1978).

To examine the relationship between oral contraceptive (OC) use and the development of SLE, analyzed data (1976 - 1990) from the Nurses' Health Study cohort. The questionnaire used to assemble biennially the group sought information on a variety of health conditions and exposures, such as use of OCs, use of post-menopausal hormones (PMH), current and past cigarette smoking habits and other health practices. Incidence of SLE was defined by; 1) strict American College of Rheumatology (ACR) classification criteria (> or = 4 ACR criteria), 2) > or = 4 ACR criteria and any physician's diagnosis, 3) > or = 4 ACR criteria and diagnosis by an ACR-certified rheumatologist, 4) > or = 3 ACR criteria, or 5) diagnosis by a physician even if the patient did not meet the ACR criteria. (Bertsias G. 2012, Sanchez-Guerrero J.1997).

Typical clinical symptoms include combinations of renal disease, swollen joints, skin rash, hematologic disorders, respiratory, and neurologic dysfunction.

Regulatory Significance of the AO

There are concerns about the increase in autoimmune diseases caused by estrogen-like substances, and its accurate in vitro toxicity assessment system is required in international regulations. The OECD has published a revised version of the guidance document on standardized test guidelines for evaluating ED (OECD. 2019).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2020: Binding to estrogen receptor (ER)-α leads to Induction of GATA3 expression

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

XXXX

Key Event Relationship Description

The hormone binding domain (HBD) of the ERα is required not only for binding ligand but also to form stable homodimers of the protein and mediate transcriptional activation by the receptor. There are two ligand-dependent signaling pathway. One is “classical” and the other is “tethered” pathway. A direct genomic interaction occurs between the ER ligand complex and specific sequences of DNA known as estrogen response elements (ERE) (Parker MG. 1993, Goldstein RA. 1993, Sasson S. 1991, Brandt ME. 1997). Transcriptional activation by ERα is mediated by two distinct activation functions: the constitutively active AF-1 domain, located in the N-terminal domain of the receptor protein, and the ligand-dependent AF-2 domain, located in the C-terminal domain of the receptor protein (Delaunay F. 2000). This is called “classical” signaling pathway. In addition to above classical mechanism, ligand-activated ERα interact with other transcription factor complexes and bind to non-EREs by attaching to other transcription factors and not with ERE directly. (Carolyn MK. 2001). This is also called “tethered” signaling pathway. The transcription factors GATA3 and STAT6 are essential for the establishment and/or maintenance of these interactions (Spilianakis and Flavell, 2004). In the tethered pathway, STAT6-ER fusion protein induce GATA-3 mRNA expression. Furthermore, in mammary gland but not in immune cells, GATA3 and ERα regulate each other and, along with FOXA1, can nucleate a remodeling complex at heterochromatic enhancer regions of ERα target genes, leading to the opening and epigenetic marking of sites for active transcription (Eeckhoute J. 2007, Kong SL. 2011). Alone, FOXA1 or ERα are not sufficient to fully open the chromatin, supporting a bona fide pioneer activity for GATA3 (Eeckhoute J. 2007, Kong SL. 2011).

Evidence Supporting this KER

Biological Plausibility

STAT6-ER fusion protein (STAT6:ER) induce expression of GATA-3 mRNAs in presence of 4-Hydroxytamoxifen (4-HT), estrogen analogue (Kurata H. 1999, Zhu J. 2001). Furthermore, A constitutively active form of Stat6 (STAT6VT) introduced GATA3 expression and resulted in both Th2 differentiation and enhanced cell expansion without IL-4 (Zhu J. 2001, Horiuchi S. 2011). CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under ThN conditions with added IL-4 (Zhu J. 2001). Therefore, it is considered that activated STAT6 after ligand-biniding to ERα induce GATA3 expression in immune cells.

Empirical Evidence

Expression of GATA3 was induced in T cells treated with E_2 at a concentration of 10^{-9} M (272.4 pg/mL) for 12-16 hours (Lambert KC. 2005). In contrast, expression of T-bet was decreased, which means E_2 skew the immune system from a Th1 to a Th2 profile. Stat6:ER Th1 cells expressed significant amounts of both GATA3 mRNAs in a 4-HT-dependent manner (Kurata H. 1999, Zhu J. 2001). Constitutively activated Stat6 (Stat6VT) is primed under null Th cell (ThN) conditions in the absence of human (h)IL-4. The expression level of Gata3 in this primed cells are checked by RT-PCR (Zhu J. 2001).

M12.4.1 cells, transfected with the luciferase reporter gene by inserting three copies of human STAT6 binding site oligonucleotide, are used nuclear extracts and electrophoretic mobility shift assay (EMSA) with 1 μM 4-HT. STAT6:ER DNA-binding activity is strongly and rapidly (within 1 hr) induced after addition of 4-HT to these cells. BA/F3 cells prepared as the same manner are stimulated with 1 μM 4-HT for 24 h at 37°C. The cells were harvested and assayed for luciferase activities using a Luciferase Assay Kit (Kamogawa Y. 1998).

Uncertainties and Inconsistencies

The “tethered” pathway is confirmed indirectly using artificial STAT6-ER fusion protein but not endogenous STAT6. It remains unknown whether the “classical” pathway is utilized after binding to ERα in immune cells.

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

XXXX

KE XX:

XXXX

Time-scale

XXXX

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during the menstrual cycle and gestation. Immune activity shifts across the menstrual cycle, with higher follicular-phase Th1 cell activity and higher luteal-phase Th2 cell activity (Tierney KL. 2015). This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria A. 2006). The effects of ERα signaling on T cells appear to be estrogen-dose dependent, i.e., low doses of estrogen stimulate a Th1 response, but higher doses promote a Th2 response (Priyanka HP. 2013).

Known Feedforward/Feedback loops influencing this KER

XXXX

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Relationship: 2021: Induction of GATA3 expression leads to Increase of Th2 cells producing IL-4

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

XXXX

Key Event Relationship Description

Intrachromosomal interactions in the Th2 cytokine locus may form the basis for the coordinated transcriptional regulation of cytokine-encoding genes by the Th2 locus control region (Spilianakis and Flavell, 2004). During Th2 cell differentiation, binding patterns of PcG and TrxG proteins are dynamically changed at the *Gata3* gene locus, and these epigenetic changes result in GATA3 protein upregulation, which consequently induces chromatin remodeling at the Th2 cytokine gene loci, including *Il4*, *Il5*, and *Il13* (Ansel KM. 2006, Horiuchi S. 2011).

Evidence Supporting this KER

Biological Plausibility

Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells. Th2 cells from both knockout animals showed reduction in IL-4 production. (Zhu J. 2004, Pai SY. 2004).

The GATA3 expression induced by TNF-α was enhanced in the presence of BPA. However, the T-bet expression did not change when tested at various culture conditions (Guo H. 2010, Uemura Y. 2008). IL-4 may serve multiple roles in the development of lupus: it may enhance autoantibody production via its direct B-cell effects (Ram RS. 2003).

Empirical Evidence

The proliferation of Stat6:ER Th2 cells was enhanced in a dose-dependent manner on days 10 and 31 after polarization by [^{3}H]thymidine incorporation (the effective concentration of 4-HT was between 0.08 and 2 μM, and the toxic concentration was greater than 5 μM) (Kurata H. 1999, Zhu J. 2001). Purified naive T cells were activated and infected with RV-Stat6:ER. The cells were cultured and expanded under Th culture conditions in the presence or absence of 0.3 μM 4-HT (Research Biochemicals Institute) for 2 weeks starting from days 1, 7, 14, or 21 and the cells were analyzed for cytokine (IL-4) expression by flow cytometer analysis of intracellular cytokine production or cytokine ELISA (Kurata H. 1999, Zhu J. 2001). CD4 T cells from Stat6-knockout mice are not able to drive Th2 differentiation and cell expansion under null Th cell (ThN) conditions with added with IL-4 (Zhu J. 2001).

Th2 differentiation is completely abolished both in vitro and in vivo when GATA3 is conditionally deleted in peripheral CD4 T cells from GATA-3-deficient (FF and FF cre) mice (Sung-Yun. 2004, Zhu J. 2004). Antigen-specific immune response is evaluated with lymphocyte from FF and FF cre mice injected with KLH, and cytokine production was measured by sandwich ELISA (Sung-Yun. 2004).

Quantitative Understanding of the Linkage

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham M. 2011). When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ.

Response-response relationship

MIE:

XXXX

KE XX:

XXXX

Time-scale

XXXX

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during gestation. This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria et al. 2006).

Known Feedforward/Feedback loops influencing this KER

XXXX

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Relationship: 2022: Increase of Th2 cells producing IL-4 leads to Increase of autoantibody production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	adjacent	Moderate	Moderate

Key Event Relationship Description

During process of B cell maturation, the autoreactive B cell which has high-affinity for DNA are normally silenced by anergy, and activated by stimulation with antigen independent CD40 ligand (CD154) or IL-4 from Th2 cells. The receptor for IL-4 is IL-4Rα, which expresses in B cells. In the development of T-cell dependent antibody producing cells, the interaction between IL-4 and its receptor stimulates B-cells to mature (proliferate, switch immunoglobulin classes). As a result, production of anti-DNA antibody from activated autoreactive B cells in increased.

Evidence Supporting this KER

Biological Plausibility

Lack of ERα, in either male or female mice, did not increase B cell precursors (Smithson G. 1998). Restoration of estradiol in ovariectomized NZB/W F1 mice reestablished high numbers of autoantibody-producing (DNA-specific) B cells, and thereby suggests a pathogenic role of estrogen in lupus (Daniel P. 2011).

Anergic B cells, dsDNA-specific models, can be stimulated by IL-4 specific antibody in vitro, suggesting that they are capable of responding to T-cell-derived signals (Acevedo-Suarez CA. 2005, Noorchashm H. 1999, Mandik-Nayak L. 2000, Eris JM. 1994).

Transfer of either IL-4-stimulated splenocytes from 5-mo-old NZB/W F1 mice into NZB/W F1 mice of the same age enhanced the production of IgG anti-dsDNA Ab. Consistently, administration of mAb against IL-4 before the onset of lupus was effective in preventing the onset of lupus nephritis (Nakajima A. 1997).

Empirical Evidence

The administration of the estrogen antagonist tamoxifen diminishes anti-DNA antibody levels by ELISA as well as decreases percentages of total B cells and CD5+ B cells by FCM (Wu WM. 2000). Tamoxifen blocks estrogen-induced B cell maturation but not survival (Peeva E. 2005). ERα deficiency in (NZB×NZW) F1 female mice downregulated levels of anti-dsDNA IgG antibodies,

and the absence of ERα In (NZB×NZW) F1 males resulted in decreased anti-dsDNA antibodies (Bynote KK. 2008).

Uncertainties and Inconsistencies

Estrogen upregulates CD40L (CD154) on T cells from SLE patients (Desai-Mehta A. 1996, Li X. 2006). Anti-CD40L antibodies downregulate CD86 expression on normal and SLE B lymphocytes, blockade of CD86 only diminishes anti-DNA antibody production by SLE B cells (Nagafuchi H. 2003). Moreover, mice overexpressing CD40L develop a lupus-like disease with high levels of antibodies to nuclear antigens, DNA, and histones, as well as glomerulonephritis (Higuchi T. 2002). Activation of autoreactive B cell may be involved in stimulation not only IL-4, but also CD40 ligand (CD154) of Th2 cell as well as the other immune cells.

B1 cells from aged mice exhibited increased expression of ERα mRNA compared to young mice (Yurino H. 2004). Since the ER of B cell is also expressed, there may be a direct route that does not go through Th2.

Quantitative Understanding of the Linkage

Response-response relationship

MIE:

XXXX

KE XX:

XXXX

Time-scale

XXXX

Known modulating factors

XXXX

Known Feedforward/Feedback loops influencing this KER

XXXX

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Relationship: 2023: Increase of autoantibody production leads to Exacerbation of SLE

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to estrogen receptor (ER)-α in immune cells leading to exacerbation of systemic lupus erythematosus (SLE)	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

XXXX

Key Event Relationship Description

The presence of many autoantibodies is a hallmark of SLE. In particular, autoantibodies directed to double-stranded DNA (dsDNA) are characteristic (Isenberg DA. 2007). SLE patients appear to produce significant amounts of the anti-dsDNA autoantibodies that cause the disease. Anti-dsDNA antibody exists even in healthy people, but in SLE patients, an increase in anti-dsDNA antibody has been observed and is also used for definitive diagnosis of SLE. Activation of autoantibody-producing B cells only serves to exacerbate that condition.

Evidence Supporting this KER

Biological Plausibility

SLE has been seen to flare up during pregnancy (Petri M. 1991). The aberrant T cell dysfunction in SLE is also associated with high levels of autoantibodies (Crispin JC. 2010).

Premenopausal women receiving low estrogen containing birth control pills did not have an increased flare rate compared to women receiving placebo suggesting that adding estrogen to an already high estrogen state had no effect on disease (Buyon JP. 1996).

Empirical Evidence

In a study to investigate a novel subpopulation of B-1 cells and its roles in murine lupus, anti-double-stranded DNA (anti-dsDNA) autoantibodies were preferentially secreted by a subpopulation of CD5+ B-1 cells that expressed programmed death ligand 2 (L2pB1 cells) (Xuemei Z. 2009). A substantial proportion of hybridoma clones generated from L2pB1 cells reacted to dsDNA. L2pB1 cells are potent antigen-presenting cells and a dramatic increase of circulating L2pB1 cells in lupus-prone BXSB mice correlates with elevated serum titers of anti-dsDNA antibodies (Xuemei Z. 2009).

Uncertainties and Inconsistencies

Stat6-deficient New Zealand Mixed (NZM) 2328 mice display a significant reduction in incidence of kidney disease, with a dramatic increase in survival, despite the presence of high levels of anti-dsDNA Abs same like the wild-type NZM 2328 animals (Chaim O. 2003). In NZM 2410 mice, STAT6 deficiency or anti-IL-4 Ab treatment decreases type 2 cytokine responses and ameliorates kidney disease, particularly glomerulosclerosis, despite the presence of high levels of IgG anti-dsDNA Abs same like the wild-type littermates or PBS-treated controls (Ram RS. 2003). Anti-dsDNA antibodies are not what we think they are, as they may be antibodies operational in quite different biological contexts, although they bind dsDNA by chance. This may not mean that these antibodies are not pathogenic but they do not inform how they are so (Ole PR. 2019). In other words, might be that the high levels of anti-dsDNA Abs does not always exacerbate SLE.

Quantitative Understanding of the Linkage

Response-response relationship

The effects of estrogen receptor signaling on T cells also appear to be dose dependent (Cunningham M. 2011). When estrogen levels are low, T cell expansion shift toward a Th1 phenotype that produces IL-12, TNF-α, and IFN-γ. This response results in cellular immunity inducing inflammation and exacerbating cellular type autoimmune diseases (multiple sclerosis; MS, rheumatoid arthritis; RA, and experimental autoimmune encephalomyelitis; EAE, etc.) caused by Th1 rather than SLE. Treatment with low serum levels (60-100 pg/mL or 0.26-0.43 nM) of estradiol increased Th1 T-cell development in vitro by acting through an ERα mediated mechanism (Maret A. 2003). Treatment with low doses of estrogen (25 pg/ml or 0.1 nM) ameliorated autoimmune diseases caused by Th1, while high dose levels (>1000 pg/ml or 4.3 nM), which mimic pregnancy levels, prevented EAE onset and polarized T-cells to a Th2 phenotype in the EAE. (Bebo BF. 2001, Korn-Lubetzki I. 1984).

Time-scale

XXXX

Known modulating factors

The Th1/Th2 shift is one of the most important immunologic changes during the menstrual cycle and gestation. Immune activity shifts across the menstrual cycle, with higher follicular-phase Th1 cell activity and higher luteal-phase Th2 cell activity (Tierney KL. 2015). This is due to the progressive increase of estrogens, which reach peak level in the third trimester of pregnancy. At these high levels, estrogens suppress the Th1-mediated responses and stimulate Th2-mediated immunologic responses (Doria A. 2006). Incidence of flare in patients with SLE is increased during pregnancy and within the 3-months postpartum (Amanda E. 2018).

Known Feedforward/Feedback loops influencing this KER

XXXX

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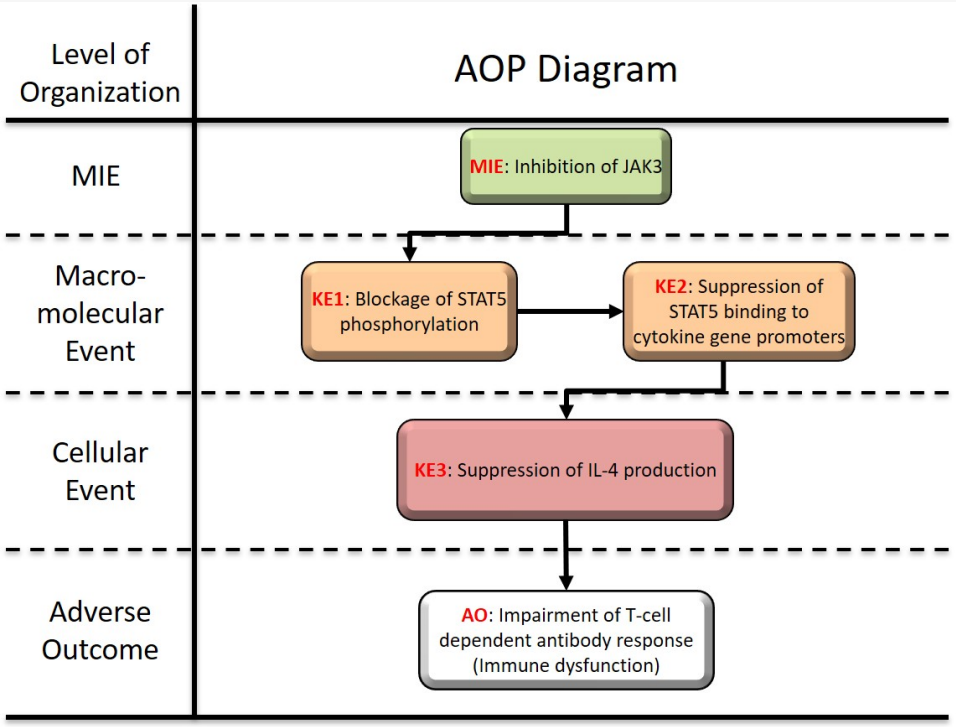
AOP315

AOP ID and Title:

AOP 315: Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response
Short Title: Immune dysfunction induced by JAK3 inhibition

Graphical Representation



Authors

Yasuhiro Yoshida (1) Takao Ashikaga (1) Tomoki Fukuyama (1) Ken Goto (1) Shinko Hata (1) Shigeru Hisada (1) Shiho Ito (1) Hiroyuki Komatsu (1) Sumie Konishi (1) Tadashi Kosaka (1) Kiyoshi Kushima (1) Shogo Matsumura (1) Takumi Ohishi (1) Yasuharu Otsubo (1) Junichiro Sugimoto (1)

(1) AOP Working Group, Testing Methodology Committee, The Japanese Society of Immunotoxicology

Corresponding author: Yasuhiro Yoshida (freude@med.uoeh-u.ac.jp)

Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.74	Included in OECD Work Plan

Abstract

Signal transduction between immune-related cells depends in many cases on cytokines. The transduction involves cell surface cytokine receptors as well as direct cell-to-cell interaction. Cytokines influence the movement, proliferation, differentiation, and activation of lymphocytes and other leukocytes in a variety of ways. Some cytokine receptors require an activation step through a Janus-kinase (JAK)/signal transducer and activator of transcription (STAT) system. When cytokines bind to specific cytokine receptors, the receptors form dimers, which more closely resemble JAK molecules. JAK is activated and phosphorylates adjacent cytokine receptors. STATs bind to the phosphorylated receptor sites and are in turn phosphorylated by the activated JAK. The phosphorylated STAT is dimerized and translocated into the nucleus. There it binds to the promoter regions of cytokine genes, which initiates the transcription of these genes in the nucleus.

In mammals, four JAK families of enzymes (JAK1, JAK2, JAK3, and TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) are utilized by more than 50 cytokines and growth factors to mediate intracellular signaling. In particular, pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6, IL-13, IL-21, and IL-23 have been implicated in inflammatory diseases that utilize the JAK pathway. In addition, TH2 derived cytokines, including IL-31 and thymic stromal lymphopoietin (TSLP), are ligands for murine and human sensory nerves. These cytokines have critical roles in evoking itchiness. Because these cytokines also interact with JAK, several JAK inhibitors have received a lot of attention recently as therapeutic agents for major inflammatory diseases and pruritic diseases.

This proposed AOP consists of JAK3 inhibition as a MIE, blockade of STAT5 phosphorylation as the first key event (KE1), suppression of STAT5 binding to the promoter regions of cytokine genes as KE2, suppression of IL-4 production as KE3, and suppression of T cell

dependent antibody response (TDAR) as an AO. This AOP especially focuses on the inhibition of JAK3, which is required for signal transduction by cytokines through the common γ chain of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In the proposed AOP, JAK3 selective inhibitors that include PF-06651600 (CAS No: 1792180-81-4) and the 4-aminopiperidine-based compound RB1 are stressors. STAT5 that is phosphorylated by JAK3 forms a homo-dimer that translocate to the nucleus and induces expressions of genes, such as IL-4. Therefore, JAK3 inhibition leads to the suppressed binding of STAT5 to the promoter regions of cytokine genes and the subsequent suppression of IL-4 production. Thus, JAK/STAT regulation plays an important role in the TDAR. TDAR is frequently affected by immunosuppressive conditions and is a major endpoint in many preclinical immunotoxicity studies.

Background

Although many stressors inhibit JAK3 activity, this AOP is based on immunosuppression caused by the recently developed and highly selective JAK3 inhibitors PF-06651600 and RB1. A significant body of scientific literature has been published concerning these two inhibitors. We look forward to future amendments to this AOP with up-to-date information on other stressors, which will clarify the link between inhibition of JAK activity and impairment of TDAR.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	1715	Inhibition of JAK3	Inhibition of JAK3
	KE	1716	Blockade of STAT5 phosphorylation	STAT5 inhibition
	KE	1717	Suppression of STAT5 binding to cytokine gene promoters	Suppression of STAT5 binding to cytokine gene promoters
	KE	1718	Suppression of IL-4 production	Suppression of IL-4 production
	AO	1719	Impairment of T-cell dependent antibody response	Impairment, TDAR

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition of JAK3	adjacent	Blockade of STAT5 phosphorylation	High	High
Blockade of STAT5 phosphorylation	adjacent	Suppression of STAT5 binding to cytokine gene promoters	High	High
Suppression of STAT5 binding to cytokine gene promoters	adjacent	Suppression of IL-4 production	High	High
Suppression of IL-4 production	adjacent	Impairment of T-cell dependent antibody response	High	High

Stressors

Name	Evidence
PF-06651600 (CAS No 1792180-81-4),	High
RB1	High

Overall Assessment of the AOP

JAKs are a family of nonreceptor tyrosine kinases and consist of four members: JAK1, JAK2, JAK3, and Tyk2 (Johnston, et al. 1994). All four mediate signals initiated by cytokines through interactions with receptors for IL-2, IL-5, IL-7, IL-9, and IL-15 via the common γ chain (Witthuhn, et al. 1994). Different studies have shown that JAK3 is widely expressed in different organs (Witthuhn, et al. 1994). Previous studies with IL-2Rγ-null mice showed that JAK3 is related to the development of spontaneous inflammatory bowel disease symptoms (Miyazaki, et al. 1994). Moreover, abnormal activation of JAK3 was associated with human hematology (Ihle, et al. 1997), indicating that a tight balance of its activity is essential for normal hematopoietic development.

Although JAK1, JAK2, and Tyk2 are widely expressed, JAK3 is predominantly expressed in hematopoietic cells and is associated only with th common γ chain of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (Nosaka, et al. 1995). IL-4 is a very well-known cytokine that is crucial in the

polarization of naïve T cells to type 2 helper T cells. IL-4 plays a major role in the growth and proliferation of many immune cells, such as natural killer (NK) cells and T cells (Dhupkar and Gordon 2017). Homozygous mutant mice harboring a disrupted JAK3 gene display profound reductions in thymocytes and severe B cell and T cell lymphopenia, similar to severe combined immunodeficiency disease (SCID), and functionally deficient residual T cells and B cells. Thus, JAK3 plays a critical role in γ chain signaling and lymphoid development.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

The proposed AOP involves inhibition of JAK activity, which leads to suppression of TDAR independent of life stage, sex, or age. Since JAK3 inhibitors (PF-06651600, RB1) are currently under phase 2 clinical evaluation for the treatment of rheumatoid arthritis, the AOP appears to be applicable to all life stages. JAK3 inhibitor-induced outcomes in humans are mimicked by similar responses in a variety of animal models, including non-human primates and rodents. Thus, immunosuppression induced by inhibition of JAK3 activity is considered to occur across a variety of mammalian species. For example, PF-06651600 was reported to reduce paw swelling with an unbound EC50 of 169 nM in rat adjuvant-induced arthritis. Similarly, PF-06651600 significantly reduced disease severity in an experimental autoimmune encephalomyelitis mouse model at 30 or 100 mg/kg or prophylactically at 20 and 60 mg/kg. PF-06651600 will be evaluated in clinical trials (Telliez, et al. 2016).

Essentiality of the Key Events

MIE: Inhibition of JAK3

JAK3 was initially identified (Johnston, et al. 1994, Witthuhn, et al. 1994) in studies designed to identify the JAK family member involved in the signaling of a group of cytokines with shared utilization of the γ chain first identified in the IL-2 receptor complex. It was subsequently demonstrated that JAK3 physically associates with the γ chain and is activated in a receptor complex that also contains JAK1, which associates with the ligand-specific α or β chain of the receptors (Miyazaki, et al. 1994). JAK3 is somewhat unique within the JAK family in that it is predominantly expressed in hematopoietic cells and is only activated in response to cytokines that use the γ chain (Ihle, et al. 1997). The phenotype of the JAK3 deletion mice is striking, with a range of deficiencies that collectively constitute SCID (Nosaka, et al. 1995, Thomis, et al. 1995). At the same time, two groups identified individuals that lacked JAK3 and exhibited somatically acquired SCID (Macchi, et al. 1995, Russell, et al. 1995). One of the most striking components of the phenotype are the dramatic reductions in both the T and B-cell lineages. Comparable reductions are seen in mice that lack IL-7 (von Freeden-Jeffry, et al. 1995), the IL-7 receptor α chain (Peschon, et al. 1994), or the γ chain. Despite the reduced numbers, the cells that do develop are phenotypically normal. These results are consistent with the hypothesis that activation of JAK3 is critical in the expansion, but not differentiation, of early lymphoid lineage-committed cells. In addition to the reduced numbers, the differentiated lymphoid cells that are generated fail to respond to the spectrum of cytokines that utilize the γ chain and activate JAK3 normally. In addition, there are other examples of JAK3 mutant mice. Primary immunodeficiencies (PIDs) are inborn errors that cause developmental and/or functional defects in the immune system (Picard, et al. 2015). PIDs are usually rare and monogenic. They present clinically with a broad array of phenotypes, including increased susceptibility to infection. One of the most deadly categories of PID is SCID. SCID is invariably caused by severe developmental and/or functional defects of T lymphocytes. However, SCID may also present with variable defects of B and/or NK cells. The B6.Cg-Nr1d1tm1Ven/LazJ mouse line harbors a spontaneous mutation in JAK3, which generates the SCID phenotype (Robinette, et al. 2018). STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells (Rani and Murphy 2016, Wittig and Groner 2005). The activation of STAT5 is transient and tightly regulated in normal cells (Quezada Urban, et al. 2018). The transcription factor STAT5 is expressed in all lymphocytes and plays a key role in multiple aspects of lymphocyte development and function (Owen and Farrar 2017). STAT5 was initially identified as a transcription factor activated by prolactin in mammary gland epithelial cells (Schmitt-Ney, et al. 1992, Wakao, et al. 1992). Subsequent studies identified STAT5 binding activity in T cells (Beadling, et al. 1994). Other authors described that the expression of STAT5 in multiple cell types and its' activation by a number of cytokines, including the common γ-chain-dependent cytokines IL-2, IL-4, IL-7, IL-13, and IL-15 (Lin, et al. 1995).

KE	description
KE1: Blockade of STAT5 phosphorylation	STAT5 refer to two proteins that share 94% structural homology and are transcribed from separate genes, STAT5A and STAT5B. Binding of these extracellular ligands to their target receptors induces the activation of receptor-associated JAK kinases that phosphorylate key tyrosine residues within the receptor, providing docking sites for the SRC homology 2 (SH2) domains of the inactive cytoplasmic STAT5 monomers. STAT5 is then phosphorylated at specific tyrosine residues, either Y694 (STAT5A) or Y699 (STAT5B) of the C-

	terminus. Subsequently, STAT5 undergoes a conformational change and phosphorylated STAT5 monomers form either homo- or hetero-STAT5X-STATX dimers through reciprocal phosphotyrosine–SH2 interactions (Cumaraswamy, et al. 2014, Tothova, et al. 2021). This means that STAT5 will never be activated without this phosphorylation step.
KE2: Suppression of STAT5 binding to cytokine gene promoters	Activated STAT5 dimers translocate to the nucleus, where they bind to STAT5 DNA response elements inducing transcription of genes involved in proliferation, cell differentiation, inflammation (cytokines) and cell survival. Since the STAT5 monomer does not bind directly to the DNA element, inhibiting the STAT5 phosphorylation step suppresses STAT5 activity.
KE3: Suppression of IL-4 production	The observation that STAT5 is activated by multiple cytokines in T cells suggests that it might play a critical role in the development and/or function of these cells. Disruption of the Stat5a gene or Stat5b gene reportedly resulted in relatively modest phenotypes. For example, Stat5a-/- mice displayed defects in mammary gland development and lactation, while Stat5b-/- mice displayed defects in response to growth hormone in male mice and NK cell proliferation (Imada, et al. 1998, Liu, et al. 1997). To determine whether combined deletion of Stat5a and Stat5b might result in more profound immunodeficiencies, subsequent studies deleted the first coding exons of both Stat5a and Stat5b. This intervention resulted in the production of truncated forms of STAT5a and STAT5b, which acted as functional hypomorphs. These mice had surprisingly mild defects in lymphocyte development, although T cells were grossly dysfunctional as they could no longer proliferate in response to IL-2 (Moriggl, et al. 1999, Teglund, et al. 1998). Finally, complete deletion of Stat5a and Stat5b using Cre-LoxP approaches demonstrated that STAT5a and STAT5b are absolutely required for lymphocyte development, as Stat5a/b-/- mice had profound blocks in lymphocyte development, which mimicked that observed in Il7r-/- mice (Cui, et al. 2004, Yao, et al. 2006). These studies definitively demonstrated the retention of appreciable STAT5 function in STAT5 hypomorph mice. Thus, T cell damage due to STAT5 deficiency or inactivation leads to suppression of the production of cytokines such as IL-4.

AOP: Impairment of T cell dependent antibody response (Immune dysfunction)

Weight of Evidence Summary

T cell development is mainly regulated by the JAK-STAT system. JAK3 deficiency in T cells induces multiple types of immunosuppression, including TDAR.

JAK3-deficient mice reportedly displayed profound reductions in thymocytes and severe B cell and T cell lymphopenia, similar to SCID disease. The residual T cells and B cells were functionally deficient (Peschon, et al. 1994).

Mice lacking JAK3 also showed a severe block in B cell development at the pre-B stage in the bone marrow. In contrast, although the thymuses of these mice were small, T cell maturation progressed relatively normally. In response to mitogenic signals, peripheral T cells in JAK3-deficient mice did not proliferate and secreted small amounts of IL-4. These data demonstrate that JAK3 is critical for the progression of B cell development in the bone marrow and for the functional competence of mature T cells (Nosaka, et al. 1995).

Furthermore, the abnormal architecture of lymphoid organs suggested the involvement of JAK3 in epithelial cells. T cells that developed in the mutant mice did not respond to IL-2, IL-4, or IL-7 (Ito, et al. 2017).

PF-06651600 and RB1 specifically inhibit JAK3 with over 100-fold preference over JAK2, JAK1, and TYK2 in kinase assays. Reduced inflammation and associated pathology have been described in collagen-induced arthritis mice. Importantly, the administration of PF-06651600 or RB1 results in decreased pro-inflammatory cytokines and JAK3 and STAT phosphorylation in mice. The findings suggest that the inhibition of JAK3/STAT signaling is closely correlated with the induction of multiple types of immunosuppression, including TDAR.

Quantitative Consideration

KER1 (MIE => KE1)

Treatment with the highly selective JAK3 inhibitor PF-06651600 or RB1 suppresses the complex formation of STAT5 in the nucleus. IL-2 stimulates STAT5 and induces tyrosine phosphorylation of STAT5 (Wakao, et al. 1995). RB1 inhibits the phosphorylation of STAT5 elicited by IL-2, as evidenced by an IC50 value of 31 nM in the peripheral blood mononuclear cells (PBMCs) of humans. PBMCs isolated from the buffy coats of healthy volunteers by density gradient centrifugation on Lymphoprep were cultured in complete RPMI 1640 medium (containing 10% fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin) plus 10 µg/mL lectin phytohemagglutinin (PHA) for 3 days. The cells were then treated with recombinant human IL-6 (400 ng/mL), recombinant human IL-2 (100 ng/mL), or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL) at 37°C for 20 min. To terminate the stimulation, the cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 min. The cells were incubated with anti-pSTAT3 and anti-CD4 antibodies, or anti-pSTAT5 and anti-CD4 antibodies at 4°C overnight, washed twice with PBS, and analyzed with by flow cytometry (Ju, et al. 2011).

The fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes was observed to increase upon incubation of peripheral blood with IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM fo two rats). Additionally, the effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T cells was evaluated. Parallel with the results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 127 nM in human lymphocytes (Ito, et al. 2017).

KER2 (KE1 => KE2)

STAT5 can be activated and phosphorylated by cytokines, such as IL-2 and IL-15. Tyrosine phosphorylation of STAT5 is important for the dimerization of STAT5 (Wakao, et al. 1995). The STAT5 dimer has an identical DNA binding specificity and immunoreactivity.

KER3 (KE2 => KE3)

STAT5 is phosphorylated by JAK kinases, allowing its dimerization and translocation into the nucleus where it can bind to its specific DNA binding sites. Electrophoretic mobility shift assay (EMSA) data revealed that IL-2 activation induced STAT5 dimerization and DNA binding to the gamma interferon activated site (GAS) motif in the IL-4 receptor alpha promoter region (John, et al. 1999). Other EMSA data showed that dexamethasone (10^{-6} M) inhibited STAT5 DNA binding in mononuclear cells in a dose-dependent fashion at dexamethasone concentrations of 10^{-8} to 10^{-7} M (Bianchi, et al. 2000). Dexamethasone could inhibit tyrosine phosphorylation, and nuclear translocation of STAT5 in primary T cells. The mechanism of inhibition involved suppression of IL-2 receptor and JAK3 expression.

KER4 (KE3 => AO)

Binding of IL-4 to the T cell receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

In co-cultured human T and B cells stimulated with anti-CD3 monoclonal antibody, the calcineurin inhibitors (CNIs) FK506 and cyclosporin A (CsA) lowered the levels of T cell cytokines, including IL-2 and IL-4, and inhibited IgM and IgG production in a dose-dependent manner (Heidt, et al. 2010).

The collective results demonstrate the quantitative relationships between the inhibition of IL-4 by specific antibodies or CNI and suppression of antibody production.

1. Support for Biological Plausibility of KER	
MIE => KE1: Inhibition of JAK3 to blockade of STAT5 phosphorylation	Biological Plausibility of the MIE => KE1 is Strong. Rationale: Administration of PF-06651600 or RB1 results in decreased pro-inflammatory cytokines and JAK3 and STAT phosphorylation in mice.
KE1 => KE2: Blockade of STAT5 phosphorylation to suppression of STAT5 binding to cytokine gene promoters	Biological Plausibility of the KE1 => KE2 is Strong. Rationale: STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells. STAT5 is activated by phosphorylation of a single constituent tyrosine residue (Y694) and is negatively regulated by dephosphorylation. A wide variety of growth factors and cytokines can activate STAT5 through the JAK-STAT pathway. The activation of STAT5 is transient and tightly regulated in normal cells.
KE2 => KE3: Suppression of STAT5 binding to cytokine gene promoters to impairment of T cell dependent antibody response (Immune dysfunction)	Biological Plausibility of the KE2 => KE3 is strong. Rationale: In response to mitogenic signals, peripheral T cells in JAK3-deficient mice did not proliferate and secreted small amounts of IL-4. These data demonstrate that JAK3 is critical for the progression of B cell development in the bone marrow and for the functional competence of mature T cells.
KE3 => AO: Suppression, IL-4 production to impairment, T cell dependent Antibody response	Biological Plausibility of the KE3 => KE4 is strong. Rationale: In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells provide help for B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to the impairment of TDAR.
2. Support for Essentiality of AOP	Rationale for Essentiality of KEs in the AOP is strong:
3. Empirical Support for KERs	
MIE => KE1: Inhibition of JAK3 to blockade of STAT5 phosphorylation	Empirical Support of the MIE => KE1 is strong. Rationale: Treatment with the highly selective JAK3 inhibitor PF-06651600 or RB1 suppresses the complex formation of STAT5 in the nucleus. IL-2 stimulates STAT5 and induces tyrosine phosphorylation of STAT5. RB1 inhibits the

	phosphorylation of STAT5 elicited by IL-2, as evidenced by an IC50 value of 31 nM in the peripheral blood mononuclear cells (PBMCs) of humans. PBMCs isolated from the buffy coats of healthy volunteers by density gradient centrifugation on Lymphoprep were cultured in complete RPMI 1640 medium (containing 10% fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin) plus 10 µg/mL lectin phytohemagglutinin (PHA) for 3 days. The cells were then treated with recombinant human IL-6 (400 ng/mL), recombinant human IL-2 (100 ng/mL), or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL) at 37°C for 20 min. To terminate the stimulation, the cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 min. The cells were incubated with anti-pSTAT3 and anti-CD4 antibodies, or anti-pSTAT5 and anti-CD4 antibodies at 4°C overnight, washed twice with PBS, and analyzed with by flow cytometry. The fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes was observed to increase upon incubation of peripheral blood with IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM for two rats). Additionally, the effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T cells was evaluated. Parallel with the results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 127 nM in human lymphocytes.
KE1 => KE2: Blockade of STAT5 phosphorylation to suppression of STAT5 binding to cytokine gene promoters	Empirical Support of the KE1 => KE2 is strong. Rationale: STAT5 can be activated and phosphorylated by cytokines, such as IL-2 and IL-15. Tyrosine phosphorylation of STAT5 is important for the dimerization of STAT5. The STAT5 dimer has an identical DNA binding specificity and immunoreactivity.
KE2 => KE3: Suppression of STAT5 binding to cytokine gene promoters to impairment of T cell dependent antibody response (Immune dysfunction)	Empirical Support of the KE2 => KE3 is strong. Rationale: STAT5 is phosphorylated by JAK kinases, allowing its dimerization and translocation into the nucleus where it can bind to its specific DNA binding sites. Electrophoretic mobility shift assay (EMSA) data revealed that IL-2 activation induced STAT5 dimerization and DNA binding to the gamma interferon activated site (GAS) motif in the IL-4 receptor alpha promoter region. Other EMSA data showed that dexamethasone (10^{-6} M) inhibited STAT5 DNA binding in mononuclear cells in a dose-dependent fashion at dexamethasone concentrations of 10^{-8} to 10^{-7} M. Dexamethasone could inhibit tyrosine phosphorylation, and nuclear translocation of STAT5 in primary T cells. The mechanism of inhibition involved suppression of IL-2 receptor and JAK3 expression.
KE3 => AO: Suppression, IL-4 production to impairment, T cell dependent Antibody response	Empirical Support of the KE3 => KE4 is strong. Rationale: Binding of IL-4 to the T cell receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR. In co-cultured human T and B cells stimulated with anti-CD3 monoclonal antibody, the calcineurin inhibitors (CNIs) FK506 and cyclosporin A (CsA) lowered the levels of T cell cytokines, including IL-2 and IL-4, and inhibited IgM and IgG production in a dose-dependent manner. The collective results demonstrate the quantitative relationships between the inhibition of IL-4 by specific antibodies or CNI and suppression of antibody production.

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Appendix 1

List of MIEs in this AOP

Event: 1715: Inhibition of JAK3

Short Name: Inhibition of JAK3

Key Event Component

Process	Object	Action
regulation of binding	tyrosine-protein kinase JAK3	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	MolecularInitiatingEvent

Stressors

Name
PF-06651600 (CAS No 1792180-81-4), RB1

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus rattus	Rattus rattus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

JAKs are a family of nonreceptor protein tyrosine kinases that are critical for cytokine-receptor-binding-triggered signal transduction through STAT to the nuclei of cells. In mammals, the JAK1, JAK2, and TYK2 kinases are ubiquitously expressed. In contrast, the expression of JAK3 is more restricted. It is predominantly expressed in hematopoietic cells and is highly regulated by cell development and activation (Gaffen, et al. 1995, Xu, et al. 1996). JAK3 is solely activated by type I cytokine receptors, featuring a common γ-chain subunit that is activated by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-7 (Peschon, et al. 1994). Mutations in either the γ chain or JAK3 have been identified as a cause of SCID in humans, which manifests as a depletion of T, B, and NK cells with no other defects (Darnell 1997, Decker, et al. 1997).

Loss-of-function mutations in JAK3 cause autosomal recessive SCID. Defects in this form of SCID are restricted to the immune system, which leads to the development of immunosuppressive JAK inhibitors.

Key Event Description

Janus tyrosine kinase (JAK) 3 is a member of the JAK family that is constitutively associated with the Box-1 region of the cytokine receptor intracellular domain. JAK3 is activated upon ligand-induced receptor dimerization (Stahl, et al. 1994).

The PF-06651600 selective JAK3 inhibitor is undergoing phase 2 clinical evaluation for use in treating rheumatoid arthritis. This compound inhibits JAK3 kinase activity with an IC50 of 33.1 nM (IC50 > 10000 nM). It lacks activity against JAK1, JAK2, or TYK2 (Telliez, et al. 2016, Thorarensen, et al. 2017). The RB1 novel and highly selective JAK3 inhibitor blocks JAK3 kinase in vitro and abrogates functional activity in various cell types (Pei, et al. 2018). When orally administered to mice, RB1 mediate the JAK-STAT pathway and reduces the clinical and microscopic manifestations of paw damage in collagen-induced arthritis mice.

How it is Measured or Detected

Enzymatic activities against JAK1, JAK2, JAK3, and TYK2 were examined using a Caliper Mobility Shift Assay. In the presence of an ATP concentration at Km for ATP for each JAK isoform, RB1 inhibited JAK3 kinase activity with an IC50 value of 40 nM without inhibiting JAK1, JAK2, or TYK2 (IC50 > 5000 nM) (Gianti and Zauhar 2015). The PF-06651600 JAK3 inhibitor displays potent inhibitory activity with an IC50 of 33.1 nM (IC50>10 000 nM), with no activity against JAK1, JAK2, and TYK2. PF-06651600 inhibits the phosphorylation of STAT5 elicited by IL-2, IL-4, IL-7, and IL-15 with an IC50 of 244, 340, 407, and 266 nM, respectively (Telliez, et al. 2016).

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List of Key Events in the AOP

Event: 1716: Blockade of STAT5 phosphorylation

Short Name: STAT5 inhibition

Key Event Component

Process	Object	Action
protein dephosphorylation	signal transducer and transcription activator STAT	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide Pimozide

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

STAT5 is expressed in hematopoietic cells, including T cells and B cells from humans, rodents, and other mammalian species (Thibault, et al. 2016).

Key Event Description

The STAT family of proteins regulate gene transcription upon activation. The proteins rely on cytokine signaling and a number of growth factors through the JAK/STAT pathway (Kisseleva, et al. 2002). STAT activation is regulated by phosphorylation of protein monomers at conserved tyrosine residues, followed by binding to phospho-peptide pockets and subsequent dimerization (Gianti and Zauhar 2015). STAT5 has been implicated in cell growth and differentiation. STAT5 was originally purified and cloned from mammary epithelial cells in sheep and identified as a signal transducer that confers the specific biological responses of prolactin (Wakao, et al. 1992, Xu, et al. 1996).Thus, STAT5 proteins function as signal transduction molecules in the cytoplasm and as transcription factors upon translocation to the nucleus.

How it is Measured or Detected

Phosphorylation of STAT5 tyrosine can be detected by specific antibodies using several detection systems, including flow cytometry. In one study, phosphorylated STAT5 expression was measured in T lymphocytes, and MFIs were reported for each subset (Osinalde, et al. 2017). A cell-permeable non-peptidic nicotinoyl hydrazone compound selectively targets the SH2 domain of STAT5 (IC50 = 47 µM against STAT5b SH2 domain EPO peptide binding activity), with markedly less recognition of the SH2 domain of STAT1, STAT3, or Lck (IC50 >500 µM). The compound was reported to block STAT5/STAT5 DNA binding activity in K562 nuclear extract and inhibit IFN-α-stimulated STAT5 tyrosine phosphorylation in Daudi cells, with no effect on STAT1 or STAT3 (Muller, et al. 2008).

Tyrosine phosphorylation of STAT5 induced by IL-2 has been analyzed using an anti-STAT5 antibody. In the study, this antibody immunoprecipitated STAT5 (p94 kDa). Peripheral blood lymphocytes were untreated (control) or treated with IL-2, IL-4, or IL-15 for 15 min. The extracts were incubated with biotinylated oligonucleotide bound to streptavidin-coated agarose. The agarose beads were washed and the eluted protein was immunoblotted with an antibody to STAT5 (Stahl, et al. 1994).

Other authors described the inhibition of JAK3 kinase activity by PF-06651600, followed by inhibition of the phosphorylation of STAT5 elicited by IL-2, IL-4, IL-7, and IL-15 with IC50 values of 244, 340, 407, and 266 nM, respectively (Telliez, et al. 2016).

Pimozide is a specific inhibitor of STAT5 phosphorylation. Pimozide decreased the survival of chronic myelogenous leukemia cells resistant to kinase inhibitors (Nelson, et al. 2011). IL-2 markedly stimulated STAT5 phosphorylation in PBMCs from patients with chronic kidney disease (CKD). Pretreatment with pimozide (3 µM) dramatically suppressed IL-2-induced STAT5 phosphorylation, indicating that it is a potent blocker of IL-2-stimulated STAT5 phosphorylation in PBMCs from CKD patients.

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Event: 1717: Suppression of STAT5 binding to cytokine gene promoters

Short Name: Suppression of STAT5 binding to cytokine gene promoters

Key Event Component

Process	Object	Action
negative regulation of DNA binding	protein-DNA complex	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculoides	Mus musculoides	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

STAT5 is expressed in hematopoietic cells, such as T and B cells from humans, rodents, and other mammalian species (Gilmour, et al. 1995).

Key Event Description

IL-2 and other cytokines rapidly activate JAK1 and JAK3 (Beadling, et al. 1994) in peripheral blood lymphocytes. The activation of JAK kinases and STAT proteins by IL-2 and IFN-α does not include the T cell antigen receptor in human T lymphocytes (Beadling, et al. 1994). After activation of JAKs, latent STAT transcription factors induce dimeric STAT proteins (Gaffen, et al. 1995). These proteins then translocate to the nucleus, where they bind to and regulate the transcriptional activation of the promoters of target genes. Dimeric STAT proteins can bind to the palindromic gamma interferon-activated (GAS) sequence TTCNmGAA, where m is 3 for all the STATs, except STAT6. The latter can additionally bind to GAS motifs. The m for STAT6 denotes 4 (Darnell 1997, Decker, et al. 1997, Ihle 1996, Leonard and O'Shea 1998).

How it is Measured or Detected

EMSA using nuclear extracts and specific oligonucleotides, including transcription factor binding sites, such as cytokine-inducible SH2-containing protein (CIS) gene promoters, are useful to evaluate DNA binding activity (Johnston, et al. 1995). Activated STAT5 binds to specific DNA-probes in splenocytes (Liu, et al. 2010). A cell-permeable non-peptidic nicotinoyl hydrazone compound selectively targets the SH2 domain of STAT5 (IC50 = 47 μM against STAT5b SH2 domain EPO peptide binding activity), with markedly less recognition of the SH2

domain of STAT1, STAT3, or Lck (IC50 > 500 µM). This compound inhibited STAT5/STAT5 DNA binding activity in K562 nuclear extract and inhibited IFN-α-stimulated STAT5 tyrosine phosphorylation in Daudi cells, but not STAT1 or STAT3 (Muller, et al. 2008).

Nuclear extracts were prepared from untreated YT cells or cells treated with recombinant IL-2 (2 nM) for 30 min at 37°C. EMSA was performed using glycerol-containing 5% polyacrylamide gels (29:1) containing 0.5× Tris-borate-EDTA buffer. For supershift assays, nuclear extracts were preincubated for 10 min with antibodies against STAT5. Oligonucleotide sequences from PRRIFV have been used as probes (Maeshima, et al. 2012). Other authors described a supershift ESMA that involved preincubating whole-cell extract with 3 µL of pan-STAT5 antiserum that recognizes both STAT5a and STAT5b. Electrophoresis was carried out at room temperature using 5% or 6% polyacrylamide gels (Heidt, et al. 2010).

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Event: 1718: Suppression of IL-4 production

Short Name: Suppression of IL-4 production

Key Event Component

Process	Object	Action
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tofacitinib (CP690,550)

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

T cell

Organ term

Organ term

immune system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In one study, only 1% of CD4 T cells from STAT5a-/- mice primed with soluble anti-CD3 and anti-CD28 with IL-2 produced IL-4, whereas 10.5% of control C57BL/6 CD4 T cells produced IL-4 (Cote-Sierra, et al. 2004).

Cells from STAT5A-deficient mice or cells treated with phospho-STAT5 peptide are defective in Th2 differentiation. STAT5A single-deficient mice showed impaired Th2 differentiation. Reconstituting STAT5A by retroviral infection restored the capacity of cells to induce IL-4 (Kagami, et al. 2001).

IL-2 directly activates STAT5A and STAT5B. T cells from mice deficient in either STAT5A or STAT5B did not show a dramatic change in T cell proliferation, but cells from mice in which both had been knocked out proliferated poorly in response to IL-4 (Moriggl, et al. 1999).

Key Event Description

IL-4 is a mammalian protein found in *Homo sapiens*. IL-4 is pivotal in shaping the nature of immune responses. Upon activation, naïve periph CD4+ T cells begin to synthesize and secrete cytokines. Type 2 helper cells (Th2 cells) produce IL-4, IL-5, IL-6, and IL-13. IL-4 is a 15-kD polypeptide with pleiotropic effects on many cell types. In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th cells. Th2 cells assist B cells in promoting class switching from IgM to IgG1 and IgE (Choi and Reiser 1998).

STAT5 phosphorylation facilitates the dimerization of STAT5, transport to the nucleus, and gene regulation (Levy and Darnell 2002). DNaseI hypersensitivity sites II (HSII) and III (HSIII) in intron 2 have been identified in several regions of the Il4/Il13 locus. STAT5A binding to sites nea HSII and HSIII could provide a mechanism through which STAT5A mediates IL-4 gene accessibility and participates in the induction of IL-4 production (Zhu, et al. 2003). The CD3 antibody-induced phosphorylation of STAT5 can be downregulated by tofacitinib, suggesting that JAK inhibition by tofacitinib can downregulate STAT5-dependent cytokine signaling. Tofacitinib was shown to abrogate anti-CD3-induced STAT5 activation in CD4+ T cells and inhibit IL-4 production from CD4+ T cells (Migita, et al. 2011).

How it is Measured or Detected

In one study, CD4+ T cells were stimulated with CD3 monoclonal antibodies in the presence or absence of tofacitinib (CP-690550) for 48 h. Supernatants were collected and the levels of IL-4 production were measured by ELISA (Migita, et al. 2011). The authors also extract total R after 8 h or 24 h of stimulation and measured IL-4 mRNA expression was measured by real-time PCR (Migita, et al. 2011).

In another study, flow cytometry analysis involving intracellular staining was used to measure cytosolic IL-4 content in stimulated cells (Zhu, e 2001). Relative gene expression levels were determined by quantitative RT-PCR using Taqman Gene Expression primer probe sets and ABI PRISM 7700 or 7900 Taqman systems (Applied Biosystems). The comparative threshold cycle method and internal controls (cyclophillin or β were used to normalize the expression of target gene (IL-4) (Ghoreschi, et al. 2011).

Cytokine content was quantified in appropriately diluted samples in duplicate using ELISA kits to test matched antibody pairs with biotin-

horseradish peroxidase-streptavidin detection and 3,3',5,5'-tetramethylbenzidine substrate. ELISA plates were scanned using the UVmax plate reader (Molecular Devices) using SOFT max software (Dumont, et al. 1998).

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List of Adverse Outcomes in this AOP

Event: 1719: Impairment of T-cell dependent antibody response

Short Name: Impairment, TDAR

Key Event Component

Process	Object	Action
T cell activation involved in immune response		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	AdverseOutcome

Stressors

Name
Cyclosporin, FK506, Basiliximab, PFOA (perfluorooctanoic acid)
Tacrolimus (also FK506)

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNI-induced impairment of TDAR has been demonstrated in rodent studies. In one study, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino, et al. 1987). Likewise, oral administration of FK506 to rats over a 4-week period reduced the production of both anti-KLH-IgG and IgM after subcutaneous immunization with KLH (Ulrich, et al. 2004). Other authors described that treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkeys resulted in reduction of serum SRBC-specific IgM and IgG (Gaida, et al. 2015). As for humans, in vitro experiments showed that treatment with FK506 or CsA of PBMCs from blood bank donors suppressed the production of IgM and IgG specific to T cell dependent antigens (Heidt, et al. 2010). In SKW6.4 cells (IL-6 dependent, IgM-secreting, human B cell line) cultures, FK506 or CsA suppressed the production of IgM in the presence of T cell activation (Sakuma, et al. 2001). Considering that FK506 and CsA reduce T cell derived IL-2, these findings strongly suggest that impairment of TDAR following reduced production of IL-2 occurs at least in common among humans, monkeys, and rodents.

Yang et al. (2002b) exposed male C57BL/6 mice to a single concentration (0.02%) of PFOA in the diet for 16 days. TDAR was measured after inoculating PFOA-treated mice with horse red blood cells intravenously on day 10; serum levels of horse red blood cell-specific IgM and IgG in response to the immunization were significantly decreased (Yang, et al. 2002).

The suppression of TDAR in adult C57BL/6 female mice has been observed in several studies. NOEL of 1.88 mg/kg/d and LOEL of 3.75 mg/kg/d were identified for PFOA administered in drinking water for over 15 days (Dewitt, et al. 2008).

The suppression of TDAR in adrenalectomized or sham-operated C57BL/6N female mice was observed when PFOA was provided in drinking water for 10 days at doses of 0, 3.75, 7.5, or 15 mg/kg/d. TDAR was determined as the primary antibody response to the T cell dependent antigen in SRBCs. The day after exposure ended, SRBCs were introduced intravenously and SRBC-specific IgM was measured 5 days later (DeWitt, et al. 2009).

Key Event Description

The production of antibodies to T cell-dependent antigens is a coordinated process involving B cells, antigen-presenting cells, and T cell derived cytokines. The B cells are stimulated to proliferate and differentiate. The TDAR might be altered if any of these cell populations are affected.

IL-2 and IL-4 are produced and secreted by helper T cells. Both are important in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation. Both events induce and enhance TDAR. IL-2 promotes differentiation of B cells, which stimulates differentiation of activated T cells to Th2 cells. The suppressed production of IL-2 and IL-4 impairs TDAR (Justiz Vaillant and Qurie 2020).

A mutant form of human IL-4, in which the tyrosine residue at position 124 is replaced by aspartic acid (hIL-4Y124D), was reported to specifically block IL-4 and IL-13-induced proliferation of B cells. In addition, hIL-4Y124D also strongly inhibited both IL-4- or IL-13-induced IgG4 and IgE synthesis in cultures of PBMCs, or highly purified sIgD+ B cells cultured in the presence of anti-CD40 monoclonal antibodies. IL-4 may be necessary to produce antibodies and to proliferate in B cells. The mutation of IL-4 may impair TDAR (Aversa, et al. 1993).

IL-4 stimulates B cells to proliferate, switch immunoglobulin classes, and differentiate to plasma and memory cells. Suppressing the production of these B cell related cytokines appears to impair TDAR, as evident from the results of FK506 treatment (Heidt, et al. 2010).

STAT5 is able to inhibit peroxisome proliferator activated receptor (PPAR)-regulated gene transcription. Conversely, ligand-activated PPAR can inhibit STAT5-regulated transcription. As a peroxisome proliferator, perfluorooctanoic acid (PFOA) induces PPARs. The suppression of TDAR has been observed with a no observable effect level (NOEL) of 1.88 mg/kg/d and lowest observed adverse effect level (LOEL) of 3.75 mg/kg/d for PFOA administered in drinking water over 15 days (Dewitt, et al. 2008). The increase in PPAR expression induced by PFOA may inhibit STAT5-regulated transcription, which is important for IL-4 production in TDAR.

How it is Measured or Detected

TDAR can be examined in vivo and in vitro. In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with ELISA (Onda, et al. 2014) or with a plaque-forming cell (PFC) assay.

To assess keyhole limpet hemocyanin (KLH) antigen-specific T cell proliferation, 1×10^5 CD4+ T cells were co-cultured with 2×10^5

autologous PBMCs in 96-well plates in the presence of KLH. Cells were cultured for 5 or 7 days before being pulsed with 0.5 µCi ^{3}[H]-thymidine (PerkinElmer) for 18 h. The cells were harvested using a 96-well cell FilterMate harvester. ^{3}[H]-thymidine incorporation in CD4+ T cell response to biopharmaceuticals was measured by liquid scintillation counting using a TopCount NXT (Schultz, et al. 2017).

In another in vivo study, rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH. Rat serum was examined for T cell dependent, antigen-specific IgM and IgG levels by ELISA (Ulrich, et al. 2004).

Other authors repeatedly administered CNIs, including FK506 and CsA, to mice orally for 4 days and immunized with sheep red blood cells (SRBCs). Spleen cells were examined using a PFC assay (Kino, et al. 1987). Antigen-specific plaque-forming splenocytes were reduced at doses of 3.2, 10, 32, and 100 mg/kg of FK506 or 32 and 100 mg/kg CsA.

In another study, cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBCs. Serum was examined for anti-SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Gaida, et al. 2015).

In the final in vivo study cited here, mice were exposed to a single pharyngeal aspiration of 1,2:5,6-Dibenzanthracene, after which the supernatants of splenocytes were cultured for 24 h in the presence of lipopolysaccharide and assayed using a mouse IgM or IgG matched pairs antibody kit (Smith, et al. 2010).

For in vitro studies, total IgM and IgG levels in culture supernatants are often measured after polyclonal T cell activation rather than after antigen stimulation in immune cell cultures.

In one study, T and B cells isolated from human PBMCs were co-cultured with CNIs for 9 days in the presence of polyclonal T cell stimulation. The supernatants were examined for IgM and IgG levels by ELISA. Treatment with FK506 or CsA reduced the levels of IgM and IgG at concentrations of 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) or 50 and 100 ng/mL (41.6 and 83.2 nM), respectively (Heidt, et al. 2010).

In another study, SKW6.4 IL-6-dependent IgM-secreting human B cells were cultured for 4 days with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. IgM produced in the culture supernatants was measured by ELISA. FK506 or CsA reduced the levels of IgM at concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma, et al. 2001).

Regulatory Significance of the AO

TDAR is considered to be the most important endpoint of immunotoxicity, because T cells, B cells, and antigen-presenting cells, such as dendritic cells, are involved in inducing and developing TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

The ICH S8 immunotoxicity testing guideline on pharmaceuticals recommends that TDAR can be evaluated whenever the target cells of immunotoxicity are not clear based on pharmacology and findings in standard toxicity studies. For the assessment of pesticides, the United States Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC.

Finally, the draft Food and Drug Administration guidance of nonclinical safety evaluation for immunotoxicology recommends the TDAR assay.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2024: Inhibition of JAK3 leads to STAT5 inhibition

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Nelson et al. reported that a membrane proximal region of the interleukin-2 receptor gamma c chain sufficient for Jak kinase activation and induction of proliferation in T cells (Nelson, et al. 1996). Furthermore, Kirken RA et al. demonstrated that activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and STAT5 recruitment by a COOH-terminal region of the IL-2 receptor beta-chain (Kirken, et al. 1995). Therefore, STAT activation is regulated by JAK via phosphorylation. Thus, JAK inhibitors commonly interfere with STAT activation.

Evidence Supporting this KER

STAT5 refer to two proteins that share 94% structural homology and are transcribed from separate genes, STAT5A and STAT5B. Binding of these extracellular ligands to their target receptors induces the activation of receptor-associated JAK kinases that phosphorylate key tyrosine residues within the receptor, providing docking sites for the SRC homology 2 (SH2) domains of the inactive cytoplasmic STAT5 monomers. STAT5 is then phosphorylated at specific tyrosine residues, either Y694 (STAT5A) or Y699 (STAT5B) of the C-terminus. Subsequently, STAT5 undergoes a conformational change and phosphorylated STAT5 monomers form either homo- or hetero- STAT5X-STATX dimers through reciprocal phosphotyrosine–SH2 interactions (Cumaraswamy, et al. 2014, Tothova, et al. 2021). This means that STAT5 will never be activated without this phosphorylation step by JAK3.

Biological Plausibility

STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis, of hematopoietic and immune cells (Wakao, et al. 1992). STAT5 is activated by JAK3 phosphorylation of a single tyrosine residue (Y694).

Empirical Evidence

GM-CSF-induced phosphorylation of STAT5 is inhibited by the RB1 selective JAK3 inhibitor. This suggests that JAK3 inhibition downregulates STAT5-dependent cytokine signaling (Al-Shami, et al. 1998).

Quantitative Understanding of the Linkage

The fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes was reportedly increased upon incubation of peripheral blood with IL-2. Peficitinib is a pan-JAK family inhibitor that can inhibit STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM for two rats). The effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T cells has been evaluated. In parallel with results obtained from rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after the addition of IL-2, but peficitinib inhibited STAT5 phosphorylation in a dose-dependent manner with a mean IC50 of 127 nM in human lymphocytes (Gianti and Zauhar 2015).

Response-response relationship

MIE:

Dose-response analysis of the effects of RB1 on JAK3 kinase activity showed that RB1 inhibits JAK3 kinase activity in a dose-dependent manner with an IC50 value of 40 nM, without inhibiting JAK1, JAK2, or TYK2 (Pei, et al. 2018).

Normal rats were administered peficitinib at 10 and 20 mg/kg. Thirteen hours later, the animals were bled and STAT5 phosphorylation was assessed. IL-2-induced STAT5 phosphorylation of CD3-positive lymphocytes in peripheral blood from the peficitinib-treated rats was suppressed by 37% at a dose of 10 mg/kg and 78% at 20 mg/kg (Gianti and Zauhar 2015).

Time-scale

The enzymatic activities against JAK1, JAK2, JAK3, and TYK2 were immediately tested in CTLL-2 cells using a Caliper Mobility Shift Assay with an ATP concentration at Km (Pei, et al. 2018). CTLL-2 cells were treated with 10 µM adenosine (plus coformycin) for 15 min at 37°C and then stimulated with IL-2 (10 U/mL) for different lengths of time (5 min-12 h). Adenosine dramatically decreased dose-dependent STAT5A/B tyrosine phosphorylation in response to IL-2 over the entire 12 h time course (Zhang, et al. 2004).

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Relationship: 2025: STAT5 inhibition leads to Suppression of STAT5 binding to cytokine gene promoters

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding

Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High	Quantitative Understanding
AOP Name	**Adjacency**		**Weight of Evidence**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Key Event Relationship Description

STAT proteins bind with their SH2 domains (which are located between amino acids 600 and 700) to phosphorylated tyrosine residues of transmembrane receptors (Heim, et al. 1995, Stahl, et al. 1995). Once STATs are bound to the receptors, the receptor-associated Jak kinases phosphorylate them on a single tyrosine residue located carboxy terminal of the SH2 domain. Changing this tyrosine to phenylalanine results in STATs that are no longer functional (Shuai, et al. 1993). Two STATs dimerize through specific reciprocal SH2–phosphotyrosine interaction and translocate to the nucleus. After translocation into the nucleus, STATs bind DNA response elements in promoters of target genes. The putative DNA-binding domain lies between amino acids 400 and 500. After DNA binding STATs interact directly or indirectly with the RNA polymerase II complex. The DNA sequence elements in the promoters of genes that bind STAT proteins can be classified in two groups. The prototype of the first class is the interferon-stimulated response element (ISRE).

The second class comprises the GAS-like response elements. STAT5 homodimers have been shown to bind to at least one of the GAS-like elements (Heim 1996).

Evidence Supporting this KER

The observation that STAT5a/STAT5b/double KO mice are defective in IL-2-induced IL-2Rα expression, suggested that STAT5 is essential for this expression (Kim, et al. 2001, Moriggl, et al. 1999).

In another study, CD25 associated with the intermediate affinity IL-2Rβγ subunits to form the high-affinity heterotrimeric IL-2Rαβγ. In response to ligation with IL-2, signaling of the complex through the IL-2Rβγ chains resulted in the phosphorylation of STAT5 (Waldmann 2006).

STAT5a/b mutant peripheral T cells in mice are profoundly deficient in proliferation and fail to undergo cell cycle progression or to express genes controlling cell cycle progression. STAT5 proteins are essential mediators of IL-2 signaling in T cells (Willerford, et al. 1995).

IL-2 binding to CD25 triggers the grouping with IL-2Rβ and γ chains, leading to signal transduction through STAT5, mitogen-activated protein kinase, and phosphoinositide 3-kinases (PI3Ks) (Fujii, et al. 1995, Ravichandran and Burakoff 1994, Remillard, et al. 1991). Within all T cell populations, IL-2 signaling appears to be primarily mediated through phosphorylation of STAT5 (Hirakawa, et al. 2016).

Biological Plausibility

Upon T cell receptor stimulation, IL-2/STAT5 signaling promotes T cell differentiation. This is the first key step in generating effector T cells that can target pathogens (Liao, et al. 2013).

Increasing the concentrations of IL-2 to superphysiological levels (1000 units/mL), which would eliminate the required upregulation of the IL-2 receptor α chain, also failed to induce a proliferative response in cells from Stat5a/b mutant mice (Willerford, et al. 1995).

Splenic lymphocytes from STAT5a/b, but not STAT5a or STAT5b, mutant mice failed to significantly respond to increasing concentrations of IL-2 in the presence of anti-CD3 (Moriggl, et al. 1999).

Empirical Evidence

Reversible protein phosphorylation plays a key role in IL-2 receptor-mediated activation of JAK3 and STAT5 in lymphocytes (Ross, et al. 2010).

In another study, adenosine was shown to act through A2 receptors and associated cAMP/protein kinase A-dependent signaling pathways to activate Src homology region 2 domain-containing phosphatase-2 (SHP-2) and cause STAT5 dephosphorylation. The dephosphorylation resulted in reduced IL-2R signaling in T cells (Zhang, et al. 2004).

Quantitative Understanding of the Linkage

CD2 signaling of human PBMCs results in activation of the -3.6-kb IFN-γ promoter. In contrast, mutation of the -3.6-kb STAT5 site attenuates promoter activity. Functional activation is accompanied by STAT5A, but scant STAT5B nucleoprotein binds to the STAT5 binding site on the IFN-γ promoter, as determined by competition and supershift assays. Western and fluorescence-activated cell sorting analyses revealed increased phospho-STAT5 following CD2 signaling (Gonsky, et al. 2004).

Response-response relationship

Inhibition of phosphatase activity by calyculin A treatment of YT cells resulted in a significant induction of serine phosphorylation of JAK3 and STAT5, and serine/threonine phosphorylation of IL-2Rβ. Moreover, inhibition of protein phosphatase 2 (PP2A) diminished IL-2-induced tyrosine phosphorylation of IL-2Rβ, JAK3, and STAT5, and abolished STAT5 DNA binding activity (Ross, et al. 2010).

Known modulating factors

As a property of STAT, it is known that DNA binding ability is acquired by forming a dimer, and it is considered that a modifying factor does not intervene in that respect.

Known Feedforward/Feedback loops influencing this KER

IL-2 acts on the same cell that secretes the cytokine. For instance, IL-2 produced by T cells operates on the same T cells that produce this cytokine, or on neighboring cells. With the highest levels in secondary lymphoid organs, IL-2 is believed to act in an autocrine or paracrine manner to support effector and memory CD8 T cell differentiation (Kalia and Sarkar 2018).

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DOI: 10.4049/jimmunol.173.2.932.

Relationship: 2026: Suppression of STAT5 binding to cytokine gene promoters leads to Suppression of IL-4 production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

A STAT5 binding site (TTCATGGAA) has been identified in intron 2 of the Il4 gene, near HSII (Hural, et al. 2000). Another potential STAT5 binding site (TTCTAAGAA) is conserved between mice and humans, and is located near HSIII. STAT5A binds to the sites near HSII and HSIII, which could provide a mechanism through which STAT5A mediates Il4 gene accessibility and participates in the induction of IL-4 production. Enhanced STAT5 signaling results in a larger proportion of cells producing IL-4. A consensus STAT site that preferentially associates with STAT5 contributes to its enhancer activity in mast cells. The intron element plays a role in acquiring and/or maintaining the IL-4 gene locus in a demethylated state in IL-4-producing cells.

Constitutively active STAT5A (STAT5A1*6) restores the capacity to produce IL-4 in cells primed under Th2 conditions in the absence of IL-2, suggesting that STAT5 activation plays a critical role in Th2 differentiation (Zhu, et al. 2003, Zhu, et al. 2004). Additionally, IL-2 critically regulates Th2 differentiation in a STAT5-dependent manner, acting early at the locus encoding IL-4Ra to induce expression of this receptor (IL-4Rα) (Liao, et al. 2008) and later to open chromatin accessibility at the Th2 locus, which encodes IL-4 and IL-13 (Cote-Sierra, et al. 2004).

The development of Th2 cells was reportedly impaired in STAT5a-/-CD4+ T cells, even in the presence of IL-4. Retrovirus-mediated expression of STAT5A restored Th2 cell differentiation in STAT5a-/-CD4+ T cells. Th2 cell-mediated immune responses were diminished in STAT5a-/- mice. When stimulated with anti-CD3 mAb, CD4+ T cells that produced IL-4, but not IFN-γ (Th2 cells), were significantly decreased in STAT5a-/- mice compared with those in wild-type mice, suggesting that STAT5A plays a regulatory role in T helper cell differentiation (Kagami, et al. 2001).

Evidence Supporting this KER

IL-2 stabilizes the accessibility of the Il4 gene. STAT5, a key transducer of IL-2 function, binds to sites in the second intron of the Il4 gene (Cote-Sierra, et al. 2004).

5C.C7 cells infected with a retrovirus expressing a constitutively active form of STAT5A (STAT5A1*6) were shown to be primed for IL-4 production.

STAT5a/b mutant peripheral T cells in mice are profoundly deficient in proliferation and fail to undergo cell cycle progression or to express genes controlling cell cycle progression. STAT5 proteins are essential mediators of IL-2 signaling in T cells (Willerford, et al. 1995).

IL-2 is one of the earliest cytokines produced by activated T cells and mediates its actions primarily through the activation of STAT5 proteins. A STAT5-chromatin immunoprecipitation assay (ChIP) was performed using chromatin from freshly isolated CD4 T cells to identify in vivo IL-2-activated STAT5 gene targets. The immunoprecipitated chromatin yielded a number of distinct clones based on sequencing. One clone mapped to chromosome 16 152,916 to 153,096 upstream of the C-MAF gene, and contained a consensus GAS motif (Rani, et al. 2011).

Heat map analysis of expression profiles of IL-2 regulated genes (sorted by superenhancer binding scores for STAT5, from strongest to

weakest) revealed that STAT5-bound superenhancer-containing genes were highly induced by IL-2 (Li, et al. 2018).

Cells primed under Th2, but not Th1, conditions showed an association of STAT5A with HSII and HSIII. In addition, cells infected with the STAT5A1*6 retrovirus acquired IL-4-producing capacity, and STAT5 was associated with DNA elements near HSII and HSIII (Zhu, et al. 2003).

CD4+ T cell-mediated allergic inflammation was reportedly diminished in STAT5A-deficient (STAT5a-/-) mice. Furthermore, Th2 cell differentiation was also impaired in STAT5a-/- mice, even when purified CD4+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of IL-4 (Kagami, et al. 2001).

Biological Plausibility

Th2 cell differentiation from antigen-stimulated splenocytes was significantly decreased in STAT5a-/- mice as compared with that in wild-type mice. The intrinsic expression of STAT5a in CD4+ T cells is required for Th2 cell differentiation and STAT5a is involved in the development of CD4+CD25+ immunoregulatory T cells that modulate T helper cell differentiation toward Th2 cells (Kagami, et al. 2001).

IL-4 production was reportedly induced by STAT5 phosphorylation. STAT5 phosphorylation facilitates STAT5 dimerization, transport to the nucleus, and gene regulation (56-Levy-2002). PPARs are members of the nuclear hormone receptor superfamily. STAT5 is able to inhibit PPAR-regulated gene transcription. Conversely, ligand-activated PPAR can inhibit STAT5-regulated transcription. STAT5 and PPAR disparate pathways are subject to mutually inhibitory crosstalk. The extent of the inhibitory crosstalk was dependent on the relative expression levels of each transcription factor (Shipley and Waxman 2004).

Empirical Evidence

When stimulated with anti-CD3 mAb, CD4 T cells that produced IL-4, but not IFN-γ (Th2 cells), were significantly decreased in STAT5a-/- mice as compared with those in wild-type mice. In contrast, CD4 T cells that produced IFN-γ, but not IL-4 (Th1 cells), were significantly increased in STAT5a-/- mice, and T helper cell differentiation was biased toward Th1 cells in STAT5a-/- mice (Kagami, et al. 2001).

In another study, BALB/c mice were exposed to PFNA (0, 1, 3, or 5 mg/kg/day) for 14 days. Exposure to PFNA led to a decrease in the weight of lymphoid organs. Cell cycle arrest and apoptosis were observed in the spleen and thymus following PFNA exposure. PFNA reduced the production of IL-4 by splenic lymphocytes and was associated with increases in messenger RNA (mRNA) of PPAR (Fang, et al. 2008). In a related study using male Sprague-Dawley rats given the same PFNA doses for the same duration, similar effects were observed on body and thymus weights and mRNA of PPARα.

Other authors described that cells infected with STAT5A retrovirus acquired the capacity to produce IL-4 when cultured in the presence of anti-IL-4; the strength of STAT5 signaling correlated with the percentage of IL-4 producers observed in the primed cell population (Zhu, et al. 2003).

STAT5 interacts with transcriptional regulatory regions and regulates T cell differentiation by enhancing key genes (Adamson, et al. 2009). Th2 differentiation in both mouse and human CD4 T cells is critically dependent on IL-2 (Ben-Sasson, et al. 1990, McDyer, et al. 2002).

Uncertainties and Inconsistencies

GAS is a STAT3-target gene, therefore STAT3 could regulate IL-4 production (Campia, et al. 2015). Additionally, Lederer et al. demonstrated that STAT6 binds to a sequence in the IL-4 promoter (Lederer, et al. 1996).

Quantitative Understanding of the Linkage

$CD4^+$ T cell blasts from BALB/c mice were cultured in the presence or absence of the antioxidant N-acetylcysteine (NAC). T cells preferentially followed a Th2 differentiation pathway. Treatment of $CD4^+$ T cell blasts with 10 mM NAC increased Th1 cytokine production and decreased IL-4 production as compared to untreated controls. T cells treated with NAC also showed decreased levels of phosphorylated STAT5 (Shatynski, et al. 2012).

Mycophenolic acid (MPA) treatment dramatically reduced STAT5 phosphorylation, without affecting the expression of CD25 and the levels of IL-2 (He, et al. 2011). Significantly lower concentrations of IL-4 were detected in the supernatants of MPA (5 μM)-treated T cells (Liu, et al. 2013).

Response-response relationship

Once STATs are recruited to the activated JAK/receptor complex and are tyrosine phosphorylated within the SH2 domain by JAKs, they form dimers and/or tetramers, translocate to the nucleus, and associate with promoter regions, such as gamma activated sequence (GAS) elements. STAT dimers can bind to GAS DNA sequences (TTCN3GAA) to induce transcription. The STAT5 dimers can also form tetramers through interactions between residues (I28, F81, and L82) in their N-terminal regions. These STAT5 tetramers bind to pairs of GAS motifs separated by a linker of 6–22 nucleotides (Lin, et al. 2012). Mutational studies have demonstrated that STAT5 is important for IL-2-induced gene expression. The interaction of STATs with gene promoters can enhance the expression of its target genes (Able, et al. 2017).

It was reported that while the wild-type construct displayed 4.6-fold IL-2 inducibility in YT cells, selective mutation of GAScI (M1), GASn (M2), and GAScII (M3) motifs modestly lowered IL-2 inducibility (M1 1.7-fold, M2 2.9-fold, M3 1.6-fold, respectively). Double mutation of GAScI and GASn (M4) or GASn and GAScII (M5) more potently decreased IL-2 inducibility, and simultaneous mutation of GAScI and GAScII (M6) or of all the GAS motifs (M7) abrogated IL-2 inducibility (M4 1.2-fold, M5 1.4-fold, M6 1.0-fold, M7 1.0-fold, respectively). These results suggest that all the GAS motifs are required for maximal IL-2 inducibility, including IL-4 induction (Kim, et al. 2001).

Time-scale

A STAT5 binding site (TTCATGGAA) has been identified in intron 2 of the Il4 gene. HS V (also known as CNS2) is a 3' enhancer in the Il4

locus. HS V is essential for IL-4 production by Tfh cells. Mice lacking HS V display marked defects in Th2 humoral immune responses, as evidenced by abrogated IgE and sharply reduced IgG1 production in vivo. HS V-deficient (ΔV) mice displayed complete abrogation of IgE production despite only mild reduction in Th2 responses. HS V-deficiency affected Il4 transcription in T cells naïve T cells lacking the HS V (CNS2) region were completely unable to produce Il4 transcripts following ex vivo stimulation with anti-CD3 and anti-CD28 antibodies for 180 min. In a similar time course assay (240 min), in vitro differentiated Th2 cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin showed only a 50% reduction in Il4 transcription (Vijayanand, et al. 2012).

Phosphorylation of STAT5 was reportedly decreased by nearly two-fold in NOX2-deficient T cells as compared to that in wild-type controls by intracellular staining 12 and 24 h after activation with immobilized anti-CD3 and soluble anti-CD28. PCR analysis also revealed decreases in Il4 and Il4rα mRNA expression in NOX2-deficient T cells (Shatynski, et al. 2012).

Known modulating factors

Adenosine can inhibit IL-2-dependent proliferation of CTLL-2 T cells. This inhibition was reportedly associated with a reduction in tyrosine phosphorylation of STAT5A and STAT5B, which was mediated by the activation of a protein tyrosine phosphatase (PTP). The PTP Src homology region 2 domain-containing phosphatase-2 (SHP-2) was implicated in STAT5A/B dephosphorylation because adenosine strongly increased tyrosine phosphorylation of SHP-2 and the formation of complexes consisting of SHP-2 and STAT5 in IL-2- stimulated CTLL-2 T cells. In contrast, adenosine did not affect the phosphorylation status of the upstream kinases JAK1 or JAK3. The inhibitory effect of adenosine on STAT5A/B phosphorylation was mediated through cell surface A_{2a} and A_{2b} receptors, and involved associated cAMP/protein kinase A (PKA)-dependent signaling pathways (Zhang, et al. 2004).

Known Feedforward/Feedback loops influencing this KER

STAT5 can upregulate a number of molecules, including cytokine-inducible SH2 proteins (CIS family, also referred to as the SOCS or SSI family) (Yasukawa, et al. 2000). Some CIS family proteins might be involved in the cross-regulation of cytokine networks and may regulate Th1 and Th2 cell differentiation (Dickensheets, et al. 1999, Losman, et al. 1999). CIS1, a prototype of CIS family proteins, is induced by STAT5 and inhibits STAT5 activation by blocking the interaction between STAT5 and cytokine receptors (Yasukawa, et al. 2000). Thus, CIS1 seems to function in classical negative feedback of STAT5 signaling.

IL-2 acts on the same cell that secretes the cytokine. For instance, IL-2 produced by T cells operates on the same T cells that make this cytokine or on nearby cells. With the highest levels in secondary lymphoid organs, IL-2 is believed to act in an autocrine or paracrine manner to support effector and memory CD8 T cell differentiation (Kalia and Sarkar 2018). IL-2Rα expression is triggered by antigens, mitogen lectins, or antibodies to the TCR through STAT5. These signals also result in the secretion of IL-2, which in turn can increase and prolong IL-2Rα expression, thus acting as a positive feedback regulator of its own high-affinity receptor (Waldmann 1989). Therefore, STAT5 deficiency disrupted T cell function.

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Relationship: 2027: Suppression of IL-4 production leads to Impairment, TDAR

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

The effects of FK506 on serum concentrations of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over 4 weeks and immunized with KLH (Ulrich, et al. 2004). The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt, et al. 2010, Sakuma, et al. 2001). In thymectomized mice, the development of KLH-specific effector CD4 T cells was reportedly reduced and these cells were suppressed in their production of IL-4 (Bradley, et al. 1991). The effects of FK506 and CsA on the production of IL-2 have been demonstrated using mice and human cells. These facts suggest that there are no species differences between humans and rodents in the inhibition of IL-4 production and TDAR induction.

Key Event Relationship Description

IL-2 induces T cell proliferation Therefore, the suppression of IL-2 production leads to the impairment of TDAR. The IL-2-JAK3-STAT5 axis regulates Th1 cell differentiation, suggesting that IL-2 mediated JAK3-STAT5 signaling may generically operate in the production of Th1-related cytokines (Shi, et al. 2008).

IL-2 is produced and secreted by helper T cells. IL-2 has important roles in the development of TDAR. IL-2 promotes differentiation of B cells by stimulating differentiation of activated T cells to Th2 T cells. Therefore, suppressed production of IL-2 impairs T cell dependent antibody production.

In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

T cells, B cells, and antigen-presenting cells, such as dendritic cells, are involved in the induction and development of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

After treatment with FK506 or CsA, production of IL-2, IL-4, and other cytokines decreases in T cells (Dumont, et al. 1998, Dumont, et al. 1998). This reduces stimulation of B cells as well as proliferation, activation, and class switching, leading to impairment of TDAR. Therefore, FK506 and CsA are potent inhibitors of T cell dependent antibody production. Suppression of the production of these B cell related cytokines appears to be the main factor in the impairment of TDAR (Heidt, et al. 2010).

Evidence Supporting this KER

In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

Biological Plausibility

FK506 and rapamycin suppress the mRNA expression levels of IL-2 and IL-4 in T cells, which stimulate the proliferation of B cells (Heidt, et al. 2010).

Several in vivo studies in rodents have shown decreased TDAR following treatment with FK506 (Kino, et al. 1987, Ulrich, et al. 2004). In vitro tests examined antibody production in blood samples obtained from blood bank donors and PBMCs treated with FK506 and CsA. The suppressed production of immunoglobulin (Ig) M and G antibodies to T cell dependent antigens was demonstrated (Heidt, et al. 2010).

T cells, B cells, and antigen-presenting cells, such as dendritic cells, are involved in the induction and development of TDAR. Thus, changes in any of these immune cell populations can influence TDAR. However, concerning the suppression of humoral immunity induced by the inhibition of CN phosphatase activity, CNIs do not affect B cells directly. Rather, the effect is indirect via T cells. FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B-cell response by FK506 and CsA appears solely due to inhibition of T helper cells (Heidt, et al. 2010).

Therefore, it is concluded that decreased amounts of IL-4, in addition to IL-2, secreted from helper T cells, is the main factor in the suppression of TDAR.

Empirical Evidence

Empirical support for the suppression of IL-4 production leads to impairment, and the T cell dependent antibody response is strong.

Rationale

In CD3/PMA activated human T cells, FK506 suppressed the production of IL-2, IL-4, and IFN-γ at concentrations of 1.2 to 12.5 nM and

inhibited the expression of IL-2, IL-4, and IFN-γ mRNA at concentrations of 10 nM (Dumont, et al. 1998).

After 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced. The FK506 levels were 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) and the CsA levels were 50 and 100 ng/mL (41 and 83 nM) (Heidt, et al. 2010).

After a 4-day culture of SKW6.4 IL-6-dependent IgM-secreting human B cells and anti-CD3/CD28 stimulation of the PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 and 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma, et al. 2001).

Rats were treated with FK506 for over 4 weeks and immunized with KLH. The serum concentrations of anti-KLH IgM and IgG were reduced at a dose of 3 mg/kg/day (Ulrich, et al. 2004).

In vitro suppression of T cell derived cytokines and T cell dependent antibody production or antibody production after polyclonal T cell stimulation showed similar dose responses to CNIs. Time gaps were found between these two KEs, which showed earlier onset of cytokine production and delayed onset of antibody production.

Uncertainties and Inconsistencies

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Additional suppression of other immune functions may also be possible.

Quantitative Understanding of the Linkage

CsA treatment achieved 100% maximal inhibition of the ex vivo IL-2 response on Days 0, 9, and 16. CsA treatment achieved 82 [± 10]%, 68 [± 25]%, and 82 [± 9]% maximal inhibition of the ex vivo IL-4 response on Days 0, 9, and 16, respectively.

Response-response relationship

In a rat T cell proliferation assay, IL-2-induced T cell proliferation was inhibited by peficitinib in a concentration-dependent manner with an IC50 of 10 nM and by tofacitinib with a similar IC50 of 24 nM (Gianti and Zauhar 2015). In addition, cynomolgus monkeys treated with CsA showed suppression of IL-2 and TDAR using SRBCs in a dose-dependent manner (Gaida, et al. 2015).\

In the human T-B-cell co-culture stimulated with anti-CD3 monoclonal antibody, CNIs of FK506 and CsA lowered the mRNA levels of T cell cytokines at 8 h post-stimulation including IL-2 and IL-4 at 1.0 ng/mL (1.24 nM) FK506 or 100 ng/mL (90.7 nM) CsA, and inhibited IgM and IgG productions after 9 days at 0.3 and 1.0 ng/mL FK506 and 50 and 100 ng/mL CsA (Heidt, et al. 2010).

Time-scale

In human T cell culture, suplatast tosilate (an inhibitor of the production of cytokines by Th2 cells) inhibited IL-4 production after 3 days and antigen-specific IgE production after 10 days (Taiho 2013).

Other authors described that in human T-B-cell co-cultures, FK506 and CsA lowered the mRNA levels of IL-2 and IL-4 at 8 h post-stimulation and inhibited IgM and IgG production after 9 days (Heidt, et al. 2010).

Treatment with CsA (50 mg/kg) twice daily in cynomolgus monkeys resulted in reduction of IL-4 cytokine production from PMA/ionocycin stimulation of whole blood starting on day 0 and continuing through the end of the study on day 16. CsA treatment achieved 82 [±10]%, 68 [± 25]%, and 82 [± 9]% 100% maximal inhibition of ex vivo IL-4 response on days 0, 9, and 16. SRBC-specific IgM and IgG were significantly lower in animals dosed with CsA than in animals dosed with the vehicle control on days 9, 12, and 16 post-immunization. There was ≥80% or greater reduction in SRBC-specific IgM on days 9–16. SRBC-specific IgG was decreased by ≥95% on days 9–16 (Gaida, et al. 2015). This was similar to the degree of inhibition observed in rats using an KLH immunization model (Smith, et al. 2003).

Known modulating factors

Treatment with CsA (cyclosporin A) at 50 mg/kg BID (bis in die) resulted in reduction of IL-2, IL-4 cytokine production from PMA/ionomycin stimulation of whole blood in synomolgus monkey starting on Day 0 and continuing through the end of study on Day 16. In addition, Tacrolimus concentration was 1.0 ng/ml. Tacrolimus inhibited IL-2 and IL-4 mRNA levels. Glycosylation-inhibiting factor (GIF) secreted from CD4 cells suppressed IL-4 mRNA levels of the same cells during the initial 24 h of CD3/CD28 stimulation.

Known Feedforward/Feedback loops influencing this KER

B cells are required for the generation and / or maintenance of Th2 responses. Germinal center B cells regulate Th2 development through an IL-4 dependent process. Type 2 immunity and allergic responses are initiated by T cells and DCs, this response may be sustained and potentially amplified by an IL-4-driven feedback loop between Ag-specific T and B cells (Harris, et al. 2005).

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