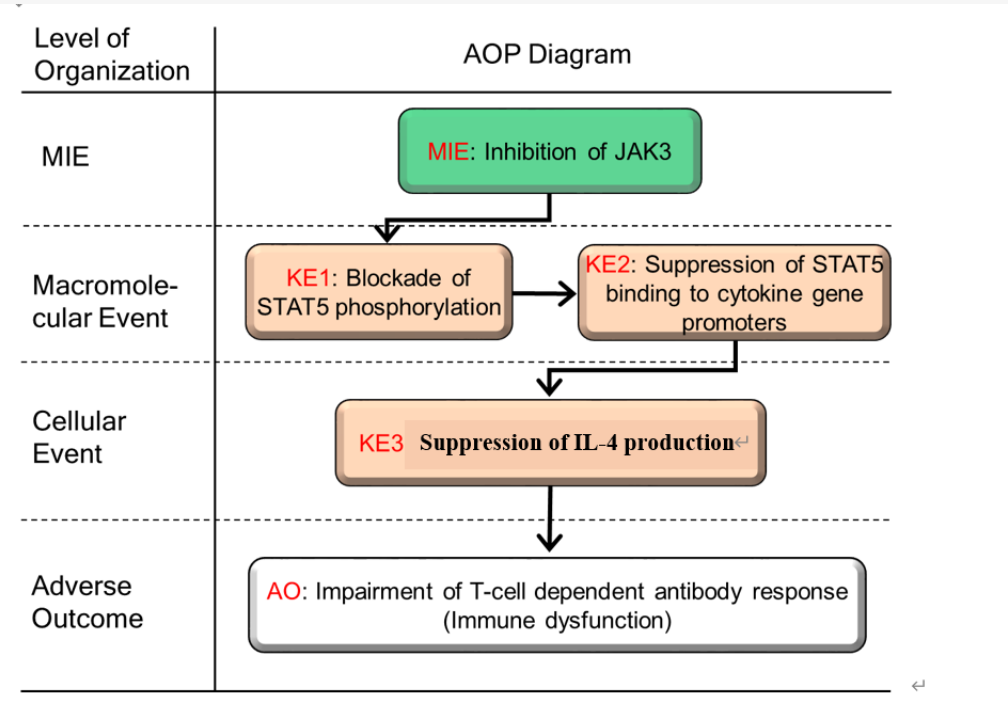
AOP ID and Title:

AOP 315: Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response
Short Title: Immune dysfunction induced by JAK3 inhibition

Graphical Representation



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Status

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Abstract

Signal transduction between immune-related cells depends in many cases on cytokines and takes place via cell surface cytokine receptors as well as direct cell-to-cell interaction. Cytokines influence the movement, proliferation, differentiation, and activation of lymphocytes and other leukocytes in a variety of ways.

Some receptors for cytokines require and activation step through a Janus-kinase (JAK)/Signal Transducers and Activator of Transcription (STAT) system. When cytokine binds to its specific cytokine receptors, the cytokine receptors form dimers, which more closely resemble the JAK molecules. The JAK then activates to phosphorylate adjacent cytokine receptors. STATs bind to the phosphorylated sites of the receptors and are then phosphorylated by the activated JAK. The phosphorylated STAT is dimerized to be translocated into nucleus and bind to promoter regions of cytokine genes, which starts transcription of cytokine genes in the nucleus.

In mammals, four JAK families of enzymes (JAK1, JAK2, JAK3, TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) are utilized by more than 50 cytokines and growth factors to mediate intracellular signaling. In particular, pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6, IL-13, IL-21 and IL-23 have been implicated in inflammatory diseases that utilize the JAK pathway. In addition, TH2 derived cytokines, including IL-31 and thymic stromal lymphopoietin (TSLP), are ligands for murine and human sensory nerves and have a critical function that evokes itchiness. Because these cytokines also interact with JAK, several JAK-inhibitors have received a lot of attention recently as a therapeutic agent for

major inflammatory diseases and pruritic diseases.

This proposed AOP consists of JAK3 inhibition as a MIE, blockade of STAT5 phosphorylation as a KE1, suppression of STAT5 binding to the promoter regions of cytokine genes as a KE2, suppression of IL-4 production as a 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 gamma (γ) chain of the interleukin receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In this proposed AOP, JAK3 selective inhibitors (e.g. PF-06651600 (CAS No 1792180-81-4), RB1) are stressors. Upon the phosphorylation of STAT5 by JAK3, it makes a homo-dimer to translocate to nucleus and induce gene expression such as IL-4. Therefore, JAK3 inhibition leads to the suppression of STAT5 binding to the promoter regions of cytokine genes and the subsequent suppression of IL-4 production. In this way, JAK/STAT regulation plays an important role in TDAR. TDAR is frequently affected under immunosuppressive conditions and is a major endpoint in many preclinical immunotoxicity studies.

Background

Although there are numerous stressors that inhibit JAK3 activity, this AOP is based on immunosuppression caused by recently developed, highly selective JAK3 inhibitors PF-06651600 and RB1, about which a significant body of scientific literature has been published.

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

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	1715	Inhibition of JAK3	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
	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 kinase and consists of four members: JAK1, JAK2, JAK3, and Tyk2 (**1**-Johnston-1994). All

four members mediate signals initiated by cytokines through interactions with receptors for IL-2, IL-5, IL-7, IL-9, and IL-15 via the common γ chain (**2**-Witthuhn-1994). Different studies have shown that JAK3 is widely expressed in different organs (**2**-Witthuhn-1994). Previous studies with IL-2Rγ-null mice showed that JAK3 is related to the development of spontaneous inflammatory bowel disease (IBD) symptoms (**3**-Miyazaki-1994). Moreover, abnormal activation of JAK3 was associated with human hematological (**4**-Ihle-1997), indicating that a tight balance of its activity was essential for normal hematopoietic development.

Although JAK1, JAK2, and Tyk2 are each widely expressed, JAK3 is predominantly expressed in hematopoietic cells and is known to associate only with the common γ (γc) chain of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (**5**-Nosaka-1995). IL-4 is a very well-known cytokine that plays a crucial role in the polarization of naïve T cells to type 2 helper T cells. It plays a major role in the growth and proliferation of many immune cells such NK and T cells (**6**-Dhupkar-2017). Homozygous mutant mice in which the JAK3 gene had been disrupted were generated by gene targeting. JAK3-deficient mice had profound reductions in thymocytes and severe B cell and T cell lymphopenia similar to severe combined immunodeficiency disease (SCID), and the residual T cells and B cells were functionally deficient. Thus, JAK3 plays a critical role in γc signaling and lymphoid development.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	High

This proposed AOP involves inhibition of JAK activity leading to suppression of TDAR and is not dependent on life stage, sex, or age. Since JAK3 inhibitors (PF-06651600, RB1) are currently under a phase 2 clinical evaluation to treat rheumatoid arthritis, the AOP appears to be applicable to all life stages. Since JAK3 inhibitor-induced outcomes in humans are mimicked by similar responses in a variety of animal models, including non-human primates and rodents, immunosuppression induced by inhibition of JAK3 activity is considered to occur across a variety of mammalian species. For example, PF-06651600 reduces paw swelling with an unbound EC50 of 169 nM in the rat adjuvant-induced arthritis. Similarly, PF-06651600 significantly reduces disease severity in the experimental autoimmune encephalomyelitis (EAE) mouse model at 30 or 100 mg/kg or prophylactically at 20 and 60 mg/kg. Then, PF-06651600 is going to clinical trials (**7**-Telliez-2016).

Essentiality of the Key Events

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

JAK3 was initially identified (1-Johnston-1994,2-Witthuhn-1994) in studies to identify the JAK family member that was involved in the signaling of a group of cytokines that shared in common the utilization of the γc chain first identified in the interleukin 2 (IL-2) receptor complex. It was subsequently demonstrated that JAK3 physically associates with the γc chain and is activated in a receptor complex that also contains JAK1, which associates with the ligand specific alpha or beta chain of the receptors (3-Miyazaki-1994).JAK3 is somewhat unique within the JAK family in that it is predominantly expressed in hematopoietic cells and is only activated in the responses to cytokines that use the γc chain (4-Ihle-1997). The phenotype of the JAK3 deletion mice was quite striking and consisted of a range of deficiencies which collectively constituted SCID (5-Nosaka-1995,8-Thomis-1995). At the same time, two groups identified individuals that lacked JAK3 and exhibited somatically acquired SCID (9-Macchi-1995,10-Russell-1995). One of the most striking components of the phenotype is the dramatic reduction seen in both the T and B cell lineages. Comparable reductions are seen in mice that lack IL-7 (11-von Freeden-Jeffry-1995), the IL-7 receptor alpha chain (12-Peschon-1994), or the γc chain. In spite of the reduced numbers, the cells that do develop are phenotypically normal. These results are consistent with the hypothesis that activation of JAK3 give it a critical role in the expansion but not the differentiation of early lymphoid lineage-committed cells. In addition to the reduced numbers, the differentiated lymphoid cells that are generated fail to respond to the spectrum of cytokines that utilize the γc chain and activate JAK3 normally.

B6.Cg-Nr1d1tm1Ven/LazJ mouse

Primary immunodeficiencies (PIDs) are inborn errors that cause developmental and/or functional defects in the immune system (13-Picard-2015). Most frequently rare and monogenic, PIDs present clinically with a broad array of phenotypes including increased susceptibility to infection. One of the most deadly categories of PID is SCID, which is invariably caused by severe developmental and/or functional defects of T lymphocytes, but may also present with variable defects of B and/or Natural Killer (NK) cells. The B6.Cg-Nr1d1tm1Ven/LazJ mouse line harbors a spontaneous mutation in JAK3, which generates an SCID phenotype (14-Robinette-2018).

KE1: STAT5-KO mice

STAT5 plays a major role in regulating vital cellular functions such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells (15-Rani-2016,16-Wittig-2005). STAT5 is activated by phosphorylation of a single tyrosine residue (Y694 in STAT5) and negatively regulated by dephosphorylation. A wide variety of growth factors and cytokines can activate STAT5 through the JAK-STAT pathway. The activation of STAT5 is transient and tightly regulated in normal cells (17-Quezada Urban-2018).

The following phenotypes are observed in STAT5-KO mice:

The transcription factor STAT5 is expressed in all lymphocytes and plays a key role in multiple aspects of lymphocyte development and function (18-Owen-2017). STAT5 was initially identified as a transcription factor activated by prolactin in mammary gland epithelial cells (19-Schmitt-Ney-1992,20-Wakao-1992). Subsequent studies identified STAT5 binding activity in T cells (21-Beadling-1994), and it was later established that STAT5 was expressed in multiple cell types and activated by a number of cytokines, including the common γc-dependent cytokines interleukin 2 (IL2), IL4, IL7, IL13, and IL15 (22-Lin-1995).

STAT5 in T-cell development

The observation that STAT5 is activated by multiple cytokines in T cells suggested that it might play a critical role in the development or function (or both) of these cells. Disruption of Stat5a or Stat5b genes alone resulted in relatively modest phenotypes; for example, Stat5a-/- mice had defects in mammary gland development and lactation while Stat5b-/- mice had defects in response to growth hormone in male mice and natural killer cell proliferation (23-Imada-1998,24-Liu-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 that acted as functional hypomorphs. These mice too had surprisingly mild defects in lymphocyte development, although T cells were grossly dysfunctional, as they could no longer proliferate in response to IL-2 (25-Moriggl-1999,26-Teglund-1998). Finally, complete deletion of Stat5a and Stat5b using

Weight of Evidence Summary

Biological Plausibility

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

JAK3-deficient mice had profound reductions in thymocytes and severe B cell and T cell lymphopenia similar to SCID disease, and the residual T cells and B cells were functionally deficient (**12**-Peschon-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 (**5**-Nosaka-1995).

Furthermore, the abnormal architecture of lymphoid organs suggested the involvement of JAK3 in the function of epithelial cells. T cells developed in the mutant mice did not respond to either IL-2, IL-4, or IL-7 (**29**-Ito-2017).

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

Quantitative Consideration

KER1 (MIE=>KE 1)

Treatment of highly selective JAK3 inhibitors (PF-06651600 or RB1) clearly suppresses the complex formation of STAT5 in the nucleus. IL-2 have been demonstrated to stimulate STAT5 and induce tyrosine phosphorylation of STAT5 (**35**-Wakao-1995). Highly-selective JAK3 inhibitor RB1 inhibited the phosphorylation of STAT5 elicited by IL-2 at IC50 value of 31 nM in the raw peripheral blood mononuclear cells (PBMCs) of humans. PBMCs were isolated from the buffy coats of healthy volunteers using density gradient centrifugation on Lymphoprep. Cells were cultured in complete RPMI 1640 medium (containing 10% foetal bovine serum, 100 mg/ml streptomycin and 100 U/ml penicillin) plus 10 µg/ml lectin phytohemagglutinin (PHA) for 3 days and then treated with either recombinant human IL-6 (400 ng/ml), recombinant human IL-2 (100 ng/ml), or recombinant human GM-CSF (50 ng/ml) at 37 °C for 20 min. To terminate the stimulation, cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 minutes; cells were incubated with anti-pSTAT3 and anti-CD4 Abs, or anti-pSTAT5 and anti-CD4 Abs at 4 °C overnight, washed twice with PBS, and analysed with an flow cytometer (**36**-Ju-2011).

Fluorescence intensity for phospho-STAT5 in CD3-positive lymphocytes increased upon incubation of peripheral blood with IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM for two rats). Additionally, the effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T-cells was evaluated. Paralleling results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2, but peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 127 nM in human lymphocytes (**29**-Ito-2017).

KER2 (KE1 =>KE 2)

STAT5 could be activated and phosphorylated by cytokines such as IL-2 and IL-15. Tyrosine phosphorylation of STAT5 is important for dimerization of STAT5 (**35**-Wakao-1995). Dimer of STAT5 has an identical DNA binding specificity and immunoreactivity.

KER3 (KE2 =>KE 3)

STAT5 is phosphorylated by the 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) showed that IL-2 activation induced STAT5 dimerization and DNA binding to gamma interferon-activated site (GAS) motif in IL-4 receptor alpha promoter region (**37**-John-1999). Furthermore, mononuclear cells cultured with dex (dexamethazone) (10^{-6}M) inhibited STAT5 DNA binding. EMSAs showed that Dex inhibited STAT5 DNA binding in dose-dependent fashion, including concentrations of dex (10^{-8}M 10^{-7}M) (**38**-Bianchi-2000).

KER4 (KE3 =>AO)

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.

In the human T-B cell co-culture stimulated with anti-CD3 monoclonal antibody, calcineurin inhibitors (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 (**39**-Heidt-2010).

These results show 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

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

non

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
Mixed	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. The JAK family consists of four members: JAK1, JAK2, JAK3 and TYK. In mammals JAK1, JAK2 and TYK2 are ubiquitously expressed. In contrast, the expression of JAK3 is more restricted: it is predominantly expressed in haematopoietic cells and is highly regulated with cell development and activation (**43**-Xu-1996,**44**-Gaffen-1995). JAK3 is solely activated by type I cytokine receptors featuring a common γ-chain (γc) subunit that are activated by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-7 (**12**-Peschon-1994). Mutations in either the γ-chain or JAK3 have been identified as a cause of severe combined immunodeficiency disease (SCID) in humans, which manifests as a depletion of T, B, and natural killer (NK) cells with no other defects (**45**-Darnell-1997,**46**-Decker-1997).

Loss-of-function mutations in the tyrosine kinase JAK3 cause autosomal recessive SCID. Defects in this form of SCID are restricted to the immune system, which led to the development of the immunosuppressive JAK inhibitors.

Key Event Description

Janus tyrosine kinase (JAK) 3 is a member of the JAK family, is constitutively associated with the Box-1 region of the cytokine receptor intracellular domain, and becomes activated upon ligand-induced receptor dimerization (**40**-Stahl-1994).

To date, PF-06651600 is the only single isoform-selective JAK3 inhibitor to undergo a phase 2 clinical evaluation to treat rheumatoid arthritis. This compound inhibits JAK3 kinase activity with an IC50 of 33.1 nM but without activity (IC50 > 10000 nM) against JAK1, JAK2, or Tyrosine kinase (TYK)2 (**7**-Telliez-2016,**41**-Thorarensen-2017). RB1 has been also identified as a novel and highly selective JAK3 inhibitor, which blocks in vitro JAK3 kinase and functional activity in various cell types. When administered to mice orally, RB1 has been proven to mediate the JAK-Signal Transducer and Activator of Transcription (STAT) (JAK-STAT) pathway and reduce the clinical and microscopic manifestations of paw damage in collagen induced arthritis mice.

How it is Measured or Detected

The enzymatic activities against JAK1, JAK2, JAK3, and TYK2 are tested by 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) (**42**-Gianti-2015). PF-06651600 is also a potent JAK3-selective inhibitor which can inhibit the JAK3 kinase activity with an IC50 of 33.1 nM but without activity (IC50>10 000 nM) against JAK1, JAK2, and TYK2. PF-06651600 inhibits 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 (**7**-Telliez-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

Process	Object	Action
protein dephosphorylation	signal transducer and activator of 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 (**50**-Thibault-2016).

Key Event Description

Signal transducer and activator of transcription (STAT) is a family of proteins that regulate gene transcription upon activation, operated by cytokine signaling and a number of growth factors through the Janus-Associated-Kinase (JAK)/STAT pathway (**47**-Kisseleva-2002). The STAT5 is a member of the STAT family of proteins, implicated in cell growth and differentiation. STAT activation is regulated by phosphorylation of protein monomers at conserved tyrosine residues, followed by binding to phospho-peptide pockets and subsequent dimerization (**42**-Gianti-2015). 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 (**20**-Wakao-1992,**43**-Xu-1996).Thus, STAT5 proteins play a double role as signal transduction molecules in the cytoplasm and as transcription factors

upon translocation to the nuclear compartment.

How it is Measured or Detected

Phosphorylation of tyrosine of STAT5 was detected by specific antibodies using several detection systems, including a flow cytometer. Phosphprylated-STAT5 expression was measured in T lymphocytes, and MFIs were reported for each subset (**48**-Osinalde-2017). A cell-permeable nonpeptidic nicotinoyl hydrazone compound that selectively targets the SH2 domain of STAT5 (IC50 = 47 μM against STAT5b SH2 domain EPO peptide binding activity), while exhibiting much less effect towards the SH2 domain of STAT1, STAT3, or Lck (IC50 >500 μM). Shown to block STAT5/STAT5 DNA binding activity in K562 nuclear extract and inhibit IFN-α-stimulated STAT5, but not STAT1 or STAT3, tyrosine phosphorylation in Daudi cells (**49**-Muller-2008).

Tyrosine phosphorylation of STAT5 induced by IL-2 can be analyzed with an anti-STAT5 antibody. This antibody immunoprecipitates STAT5 (p94 kDa). Peripheral blood lymphocytes were untreated (control) or treated with IL-2, IL-4, or IL-15 for 15 min, and extracts were incubated with biotinylated GRR oligonucleotide bound to streptavidin-coated agarose. The agarose beads were then washed, and the eluted protein was immunoblotted with antibody to STAT5 (**40**-Stahl-1994).

JAK3 selective inhibitor PF-06651600 can also inhibit the JAK3 kinase activity 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 (**7**-Telliez-2016).

IL-2 remarkably stimulated STAT5 phosphorylation in peripheral blood mononuclear cells (PBMCs) from Chronic kidney disease (CKD) patients. Pimozide is a specific inhibitor of STAT5 phosphorylation.

Pimozide (3 μM) pretreatment 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

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, B cells from humans, rodents and other mammalian species (**55**-Gilmour-1995).

Key Event Description

Interleukin-2 (IL-2) and other cytokines rapidly activate the Janus-Associated-Kinase (JAK)1 and JAK3 (**21**-Beadling-1994) in peripheral blood lymphocytes (PBLs). The activation of JAK kinases and signal transducer and activator of transcription (STAT) proteins by IL-2 and interferon (IFN)-α does not include the T cell antigen receptor in human T lymphocytes. (EMBO J. 13:5606–5615). After activation of JAKs, latent STAT transcription factors make dimeric STAT proteins (**44**-Gaffen-1995). The STAT 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, which can additionally bind to GAS motifs where m is 4 (**45**-Darnell-1997,**46**-Decker-1997,**51**-Ihle-1996,**52**-Leonard-1998).

How it is Measured or Detected

Electrophoretic mobility shift assays (EMSAs) using nuclear extracts and specific oligo including transcription factor binding sites such as cytokine-inducible SH2-containing proteins (CIS) gene promoters is useful for evaluation of DNA binding activity (**30**-Johnston-1995). Yoshida et al. demonstrated that activated Stat5 binds specific DNA-probe in splenocytes (**53**-Liu-2010). A cell-permeable nonpeptidic nicotinoyl hydrazone compound that selectively targets the SH2 domain of STAT5 (IC50 = 47 µM against STAT5b SH2 domain EPO peptide binding activity), while exhibiting much less effect towards the SH2 domain of STAT1, STAT3, or Lck (IC50 >500 µM). It shows blockage STAT5/STAT5 DNA binding activity in K562 nuclear extract and inhibit IFN-α-stimulated STAT5, but not STAT1 or STAT3, tyrosine phosphorylation in Daudi cells (**49**-Muller-2008).

Nuclear extracts were prepared from untreated YT cells or cells that had been treated with recombinant IL-2 (2 nM) for 30 min at 37°C. EMSAs were performed by using glycerol-containing 5% polyacrylamide gels (29:1) containing 0.5X Tris-borate-EDTA buffer. For supershifting assays, nuclear extracts were preincubated for 10 min with antibodies to STAT5. Oligonucleotide sequences from PRRIFV were used as probes (**54**-Maeshima-2012).

Supershifting was performed by preincubating the whole-cell extract with 3 µl of a pan-STAT5 antiserum that recognizes both STAT5a and STAT5b. Electrophoresis was carried out at room temperature using 5% or 6% polyacrylamide gels (**39**-Heidt-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

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 (**62**-Cote-Sierra-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, and reconstituting STAT5A by retroviral infection restored the capacity of cells to make IL-4 (**63**-Kagami-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 have been knocked out proliferate poorly in response to IL-4 (**25**-Moriggl-1999).

Key Event Description

IL-4 is mammalian protein found in Homo sapiens. IL-4 plays a pivotal role in shaping the nature of immune responses. Upon activation, naive peripheral 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 Th2 cells. Th2 cells provide help for B cells to promote class switching from IgM to IgG1 and IgE (**56**-Choi-1998).

STAT5 phosphorylation facilitates STAT5 dimerization, transport to the nucleus and gene regulation (**57**-Levy-2002). DNaseI hypersensitivity sites II (HSII) and III (HSIII) in intron 2 were included in several regions of the Il4/Il13 locus. STAT5A binds to the sites near HSII and HSIII, which could provide a mechanism through which STAT5A mediates IL-4 gene accessibility and participates in the induction of IL-4 production (**32**-Zhu-2003). The CD3 antibody-induced phosphorylation of STAT5 was down regulated by Tofacitinib, suggesting that JAK3 inhibition by Tofacitinib down regulated STAT5-dependent cytokine signaling. Tofacitinib abrogated anti-CD3-induced STAT5 activation in CD4+ T cells and inhibited IL-4 production from CD4+ T cells (**58**-Migita-2011).

How it is Measured or Detected

CD4+ T cells were stimulated with CD3 monoclonal antibodies in the presence or absence of tofacitinib (CP690,550) for 48 hr. Supernatants were collected and the levels of IL-4 production was measured by ELISA (**58**-Migita-2011).

CD4+ T cells were stimulated with CD3 monoclonal antibodies in the presence or absence of tofacitinib. After 8-hr or 24-hr stimulation, total RNA was extracted and IL-4 mRNA expression was measured by real-time PCR (**58**-Migita-2011).

Flow cytometry analysis (intracellular staining) were used for measurement of cytosolic IL-4 content in stimulated cells (**59**-Zhu-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-actin) were used to normalize expression of target gene (IL-4) (**60**-Ghoreschi-2011).

Quantitation of cytokine content was done on appropriately diluted samples, run in duplicate, using Sandwich Enzyme-Linked Immuno Sorbent 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) (**61**-Dumont-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

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

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 (**70**-Kino-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 (**69**-Ulrich-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 (**71**-Gaida-2015). 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 (**73**-Sakuma-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, monkey, 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 (HRBC) intravenously on day 10; serum levels of HRBC-specific IgM and IgG in response to the immunization were significantly decreased (**74**-Yang-2002).

The suppression of TDAR in adult C57BL/6J or C57BL/6N female mice has been observed in several studies and a NOEL of 1.88 mg/kg/d and LOEL of 3.75 mg/kg/d identified for PFOA administered in drinking water over 15 days (**66**-Dewitt-2008).

The suppression of TDAR in adrenalectomised (adx) or sham-operated C57BL/6N female mice has been observed for PFOA administration (0, 3.75, 7.5, or 15 mg/kg/d in drinking water for 10 days). Immune tests: TDAR, ie. primary antibody response to T-cell dependent antigen (SRBC). Day after exposure ended, i.v. SRBC with SRBC-specific IgM measurement 5d later (**75**-DeWitt-2009).

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.

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 TDAR. IL-2 promotes differentiation of B cells through IL-2 stimulates differentiation of the activated T cell into Th2 cell. Therefore, suppressed production of IL-2 and IL-4 impairs TDAR (**64**-Justiz Vaillant-2020).

A mutant form of human IL-4, in which the tyrosine residue at position 124 is replaced by aspartic acid (hIL-4Y124D), specifically blocks 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 peripheral blood mononuclear cells, or highly purified sIgD + B cells cultured in the presence of anti-CD40 mAbs. It was suggested that IL-4 is necessary to product antibodies for B cells to proliferate B cells, and the mutation of IL-4 may cause the impairment of TDAR (**65**-Aversa-1993).

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 impair TDAR, as seen in the result of FK506 treatment (**39**-Heidt-2010).

STAT5 is able to inhibit PPAR (peroxisome proliferator activated receptors)-regulated gene transcription and conversely, ligand-activated PPAR able to inhibit STAT5-regulated transcription. As a peroxisome proliferator, PFOA is able to induce PPARs. The suppression of TDAR has been observed in a NOEL of 1.88 mg/kg/d and LOEL of 3.75 mg/kg/d identified for PFOA administered in drinking water over 15 days (**66**-Dewitt-2008). It was suggested that the increase of PPAR expression induced by PFOA inhibited STAT5-regulated transcription which is important for IL-4 production in TDAR.

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) (**67**-Onda-2014) or with a plaque-forming cell (PFC) assay.

To assess keyhole limpet hemocyanin (KLH) antigen-specific T cell proliferation, $1x10^5$ CD4+ T cells were co-cultured with $2x10^5$ autologous peripheral blood mononuclear cell (PBMCs) in 96-well plates in the presence of KLH. Cells were cultured for five or seven days before being pulsed with 0.5µCi 3[H]-thymidine (Perkin Elmer, Groningen; Netherlands) for 18 hours. The cells were harvested using a 96-well cell FilterMate harvester (PerkinElmer, Warrenville road IL, USA). 3[H]-thymidine incorporation was measured by liquid scintillation counting using a TopCount NXT (Perkin Elmer, Warrenville road CD4+ T cell response to biopharmaceuticals (**68**-Schultz-2017).

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 (**69**-Ulrich-2004).

Mice were repeatedly administered calcineurin inhibitors (CNIs) including FK506 and cyclosporin A (CsA) orally for 4 days and immunized with SRBC, after which spleen cells were examined using a PFC assay (**70**-Kino-1987). 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.

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 (**71**-Gaida-2015).

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) (**72**-Smith-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 (0.37 and 1.24 nM) or 50 and 100 ng/mL (41.6 and 83.2 nM) respectively (**39**-Heidt-2010).

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 (**73**-Sakuma-2001).

Regulatory Significance of the AO

Regulatory Significance of the Adverse Outcome

TDAR is considered to be the most important endpoint of immunotoxicity, because T cells, B cells, and antigen-presenting cells such as dendritic cells are involved in inducing and developing of TDAR. Thus, changes in any of these immune cell populations can influence TDAR.

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

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

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 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
Mixed	High

non

Key Event Relationship Description

Signal transducer and activator of transcription (STAT) activation is regulated by JAK through phosphorylation. Thus, JAK inhibitors commonly interfere with STAT activation.

Evidence Supporting this KER

non

Biological Plausibility

STAT5 plays a major role in regulating vital cellular functions such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells (**20**-Wakao-1992). STAT5 is activated by phosphorylation of a single tyrosine residue (Y694 in STAT5). Janus kinase (JAK)3 phosphorylates tyrosine Y694 of STAT5.

Empirical Evidence

GM-CSF-induced phosphorylation of STAT5 was inhibited by RB1, which is a highly selective JAK3 inhibitor. This suggests that JAK3 inhibition may downregulate STAT5-dependent cytokine signaling (**76**-Al-Shami-1998).

Uncertainties and Inconsistencies

Non

Quantitative Understanding of the Linkage

Fluorescence intensity for phospho-STAT5 in CD3-positive lymphocytes increased upon incubation of peripheral blood with IL-2 treatment. Peficitinib (pan-JAK family inhibitor) 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 were evaluated. Paralleling results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2, but peficitinib inhibited STAT5 phosphorylation in a in a dose-dependent manner with a mean IC50 of 127 nM in human lymphocytes (**42**-Gianti-2015).

Response-response relationship

MIE:

Dose-response analysis of the effects of JAK3 highly selective inhibitor RB1 on JAK3 kinase activity showed that RB1 inhibits JAK3 kinase activity in a dose-dependent manner with an IC50 value of 40nM without inhibiting JAK1, JAK2 or Tyrosine kinase 2 (TYK2) (**77**-Pei-2018).

Normal rats were administered with peficitinib at 10 and 20 mg/kg, bled the animals after 13 h of dosing, and then assessed STAT5 phosphorylation. IL-2-induced STAT5 phosphorylation of CD3-positive lymphocytes in peripheral blood from peficitinib-treated rats was suppressed by 37% at a dose of 10 mg/kg and 78% at 20 mg/kg (**42**-Gianti-2015).

Time-scale

The enzymatic activities against JAK1, JAK2, JAK3, and TYK2 were immediately tested by a Caliper Mobility Shift Assay with an ATP concentration at Km (**77**-Pei-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 (**78**-Zhang-2004).

Known modulating factors

non

Known Feedforward/Feedback loops influencing this KER

non

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[Relationship: 2025: STAT5 inhibition leads to Suppression of STAT5 binding]

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

Non

Key Event Relationship Description

Signal transducer and activator of transcription (STAT)5 phosphorylation induced Interleukin (IL)-2 receptor expression. Therefore the suppression of STAT5 phosphorylation interfere with IL-2 production after STAT5 DNA binding to promoter region.

Evidence Supporting this KER

STAT5a/ STAT5b/double knockout mice defects in IL-2-induced IL-2Rα expression suggesting that STAT5 is essential for that expression (**25**-Moriggl-1999,**79**-Kim-2001).

CD25 associates with the intermediate affinity IL-2Rβγ subunits to form the high affinity heterotrimeric IL-2Rαβγ. In response to ligation with IL-2, this complex signals through the IL-2Rβγ chains, resulting in phosphorylation of STAT5 (**80**-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. The STAT5 proteins are essential mediators of IL-2 signaling in T cells (**31**-Willerford-1995).

IL-2 binding to CD25 triggers the grouping with IL-2Rβ and γ-chains, thus leading to signal transduction through STAT5, mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinases (PI3K) (**81**-Remillard-1991,**82**-Ravichandran-1994,**83**-Fujii-1995). Within all T cell populations, IL-2 signaling appears to be primarily mediated through phosphorylation of STAT5 (**84**-Hirakawa-2016).

Biological Plausibility

Upon T cell receptor (TCR) stimulation, IL-2/STAT5 signaling promotes T cell differentiation, which is the first key step to generating effector T cells that can target pathogens (**85**-Liao-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 with cells from Stat5a/b mutant mice (**31**-Willerford-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 (**25**-Moriggl-1999).

Empirical Evidence

Reversible protein phosphorylation plays a key role in IL-2 receptor-mediated activation of Janus tyrosine kinase 3 (JAK3) and STAT5 in lymphocytes (**86**-Ross-2010).

Adenosine acts 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 that results in reduced IL-2R

signaling in T cells (**78**-Zhang-2004).

Uncertainties and Inconsistencies

Non

Quantitative Understanding of the Linkage

CD2 signaling of human peripheral blood mononuclear cells results in activation of the -3.6-kb Interferon-γ (IFN-γ) promoter, whereas mutation of the -3.6-kb STAT5 site attenuates promoter activity. Functional activation is accompanied by STAT5A but little STAT5B nucleoprotein binding to the IFN-γ STAT5 site, as determined by competition and supershift assays. Western and FACS analysis shows increased phospho-STAT5 following CD2 signaling (**87**-Gonsky-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 (**86**-Ross-2010).

Time-scale

Non

Known modulating factors

At present, no evidence is found.

Known Feedforward/Feedback loops influencing this KER

IL-2 acts either on the same cell that secretes the cytokine, for instance, IL-2 produced by T cells operates on the same T cells that made it or on a nearby cell. With 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 (**88**-Kalia-2018).

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Relationship: 2026: Suppression of STAT5 binding 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
Mixed	High

non

Key Event Relationship Description

A STAT5 binding site (TTCATGGAA) has been identified in intron 2 of the Il4 gene, near HSII (**89**-Hural-2000). Another potential STAT5 binding site (TTCTAAGAA), conserved between mouse and human, and was found 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.

Constitutive 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 (**32**-Zhu-2003,**33**-Zhu-2004). Additionally, IL-2 critically regulate TH2 differentiation in a STAT5-dependent manner, acting early at the locus encoding the IL-4Ra to induce expression of this receptor (IL-4Rα) (**34**-Liao-2008) and later to open chromatin accessibility at the TH2 locus, which encodes IL-4, IL-13 (**62**-Cote-Sierra-2004).

The development of Th2 cells was impaired in STAT5a-/-CD4+ T cells even in the presence of IL-4 and that the retrovirus-mediated expression of STAT5a restored Th2 cell differentiation in STAT5a-/-CD4+ T cells. Th2 cell–mediated immune responses are 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 as compared with those in wild-type mice, suggesting that STAT5a plays a regulatory role in T helper cell differentiation (**63**-Kagami-2001).

Evidence Supporting this KER

IL-2 stabilizes the accessibility of the Il4 gene, and STAT5, a key transducer of IL-2 function, binds to sites in the second intron of the Il4 gene (**62**-Cote-Sierra-2004).

5C.C7 cells infected with a retrovirus expressing a constitutively active form of STAT5A (STAT5A1*6) were 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. The STAT5 proteins are essential mediators of IL-2 signaling in T cells (**31**-Willerford-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 at –152,916 to –153,096 upstream of the C-MAF gene, and contained a consensus interferon gamma activated sequence (GAS) motif (**90**-Rani-2011).

Heat map of expression profiles of IL-2–regulated genes (sorted by superenhancer binding scores for STAT5, from strongest to weakest) reveals STAT5-bound superenhancer-containing genes were highly induced by IL-2 (**91**-Li-2018).

Cells primed under Th2 but not Th1 conditions show association of STAT5A with HSII and HSIII. We have also shown that cells infected with the STAT5A1*6 retrovirus acquire IL-4-producing capacity and that STAT5 is associated with DNA elements near HSII and HSIII (**32**-Zhu-2003).

CD4+ T cell–mediated allergic inflammation is diminished in STAT5a-deficient (STAT5a-/-) mice. Further, Th2 cell differentiation was also impaired in STAT5a-/- mice even when purified CD4+ T cells were stimulated with anti-CD3 plus anti-CD28 antibodies in the presence of interleukin-4 (**63**-Kagami-2001).

Biological Plausibility

Th2 cell differentiation from antigen-stimulated splenocytes was significantly decreased in STAT5a-/- mice as compared with that in wild-type mice. Intrinsic expression of STAT5a in CD4+ T cells is required for Th2 cell differentiation and that STAT5a is involved in the development of CD4+CD25+ immunoregulatory T cells that modulate T helper cell differentiation toward Th2 cells (**63**-Kagami-2001).

IL-4 production was induced by STAT5 phosphorylation. STAT5 phosphorylation facilitates STAT5 dimerization, transport to the nucleus and gene regulation (56-Levy-2002). STAT5 is able to inhibit PPAR-regulated gene transcription and conversely, ligand-activated PPAR able to inhibit STAT5-regulated transcription. PPARs (peroxisome proliferator activated receptors) are members of the nuclear hormone receptor superfamily. STAT5 and PPAR disparate pathways are subject to mutually inhibitory crosstalk. The extent of the inhibitory crosstalk was dependent on the relative levels of expression of each transcription factor (**92**-Shipley-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 that T helper cell differentiation is biased toward Th1 cells in STAT5a-/- mice (**63**-Kagami-2001).

BALB/c mice were exposed to the PFNA (0, 1, 3, or 5 mg/kg/day) for fourteen days. Exposure to PFNA led to a decrease in the weight of the lymphoid organs. Cell cycle arrest and apoptosis were observed in the spleen and thymus following PFNA exposure. PFNA reduced production of IL-4 by splenic lymphocytes and were associated with increases in messenger RNA (mRNA) of PPAR (**93**-Fang-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 PPARa.

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 (**32**-Zhu-2003).

STAT5 interact with transcriptional regulatory regions and are known to regulate T cell differentiation by enhancing key genes (**94**-Adamson-2009). Th2 differentiation in both mouse and human CD4T cells is critically dependent on IL-2 (**95**-Ben-Sasson-1990,**96**-McDyer-2002).

Uncertainties and Inconsistencies

At present, no evidence is found.

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). BALB/c T cells preferentially follow a Th2 differentiation pathway, and treatment of BALB/c $CD4^+$ T cell blasts with 10 mM NAC increased Th1 cytokine production and decreased IL-4 production as compared to untreated controls. BALB/c T cells treated with NAC also showed decreased phosphorylated STAT5 (**97**-Shatynski-2012).

MPA (Mycophenolic Acid) treatment dramatically reduced STAT5 phosphorylation, without affecting the expression of CD25 and the levels of IL-2 (**98**-He-2011). Significantly lower concentrations of IL-4 were detected in the supernatants of MPA (5 μM) treated T cells (**99**-Liu-2013).

Response-response relationship

Once STATs are recruited to the activated JAK/receptor complex and 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 gamma interferon–activated sequence (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 (**100**-Lin-2012). Mutational studies demonstrate 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 (**101**-Able-2017).

While the wild-type construct showed 4.6-fold IL-2 inducibility in YT cells, selective mutation of the 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 of 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 of the GAS motifs are required for maximal IL-2 inducibility including IL-4 induction(**79**-Kim-2001).

Time-scale

A STAT5 binding site (TTCATGGAA) has been identified in intron 2 of the Il4 gene. Hypersensitivity site (HS) V (also known as CNS2), a 3' enhancer in the *Il4* locus, 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 were the complete abrogation of IgE production despite only mild reduction in Th2 responses. HS V-deficiency affected Il4 transcription in T cells. Naive T cells lacking the HS V (CNS2) region were completely unable to produce Il4 transcripts following ex-vivo stimulated 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 PMA and ionomycin showed only a 50% reduction in Il4 transcription (**102**-Vijayanand-2012).

Phosphorylation of STAT5 was found to be decreased nearly two-fold in NOX2-deficient T cells as compared to wild type controls by intracellular staining at 12 and 24 hours after activation with immobilized anti-CD3 and soluble anti-CD28. PCR analysis also found decreases in Il4 and Il4rα mRNA expression in NOX2-deficient T cells (**97**-Shatynski-2012).

Known modulating factors

Adenosine inhibited IL-2-dependent proliferation of the CTLL-2 T cell line. The adenosine inhibitory effect was associated with a reduction in tyrosine phosphorylation of STAT5a and STAT5b that 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 (**78**-Zhang-2004).

Known Feedforward/Feedback loops influencing this KER

STAT5 has been shown to up-regulate a number of molecules, including cytokine-inducible SH2 proteins (CIS family, also referred to as the SOCS or SSI family) (**103**-Yasukawa-2000). Some CIS family proteins might be involved in the cross-regulation of cytokine network and may regulate Th1 cell and Th2 cell differentiation (**104**-Losman-1999,**105**-Dickensheets-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 (**103**-Yasukawa-2000). Thus, CIS1 seems to function in a classical negative feedback of STAT5 signaling.

IL-2 acts either on the same cell that secretes the cytokine, for instance, IL-2 produced by T cells operates on the same T cells that made it or on a nearby cell. With 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 (**88**-Kalia-2018). IL-2Rα expression is triggered by antigen, mitogen lectins, or antibodies to the TCR through STAT5. These signals also result in 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 (**106**-Waldmann-1989). Therefore, STAT5 deficiency induced disrupted T cell functions

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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 concentration of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over four weeks and immunized with KLH (**69**-Ulrich-2004).

The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (**39**-Heidt-2010,**73**-Sakuma-2001).

In thymectomized mice, development of KLH-specific effector CD4 T cells is reduced and these cells were suppressed in production of IL-4 (**111**-Bradley-1991). The effects of FK506 and CsA on 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 inhibitions of IL-4 production and TDAR induction.

Key Event Relationship Description

Interleukin (IL)-2 induced T cell proliferation. Therefore, the suppression of IL-2 production leads to the impairment of T cell dependent antibody responses (TDAR). IL-2-JAK3-STAT5 axis has been demonstrated to regulate Th1 cell differentiation, suggesting that IL-2-mediated JAK3-STAT5 signaling may generically operate in the production of Th1-related cytokines (**107**-Shi-2008)

IL-2 are produced and secreted by helper T cells and play important roles in the development of TDAR. 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 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 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.

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.

After treatment with FK506 or cyclosporin A (CsA), production of IL-2, IL-4, and other cytokines decreases in T cells (**61**-Dumont-1998,**108**-Dumont-1998), reducing stimulation of B cells as well as proliferation, activation, and class switching, and leading to impairment of TDAR. Therefore, FK506 and CsA are potent inhibitors of T-cell–dependent-antibody production and suppressing the production of these B-cell-related cytokines appears to be the main factor in impairment of TDAR (**39**-Heidt-2010).

Evidence Supporting this KER

non

Biological Plausibility

FK506 and Rapamycin suppress mRNA expression levels of IL-2 and IL-4 in T cells that stimulate proliferation of B cells (**39**-Heidt-2010).

Several in vivo studies in rodents showed decreased TDAR by the treatment of FK506 (**69**-Ulrich-2004,**70**-Kino-1987). In in vitro tests examining antibody production in blood samples obtained from blood-bank donors, peripheral blood mononuclear cell (PBMC) treated with FK506 and CsA suppressed the production of immunoglobulin (Ig) M and G antibodies to T-cell dependent antigens (**39**-Heidt-2010).

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 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 (**39**-Heidt-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 for suppression of TDAR.

Empirical Evidence

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

Rationale

- In CD3/phorbol 12-myristate 13-acetate (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 as well as inhibited expression of IL-2, IL-4, and IFN-γ mRNA at the concentrations of 10 nM (**61**-Dumont-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 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 (**39**-Heidt-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 (**73**-Sakuma-2001).
- Rats were treated with FK506 for over four weeks and immunized with KLH, after which serum concentration of anti-KLH IgM and IgG reduced at the dose levels of 3 mg/kg/day (**69**-Ulrich-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, 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. There remains some possibility of additional suppression of other immune functions.

Quantitative Understanding of the Linkage

non

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 (**42**-Gianti-2015).

In addition, cynomolgus monkeys treated wth CsA showed suppression of IL-2 and TDAR using sheep red blood cells with a dose dependent manner (**71**-Gaida-2015).

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 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 (**39**-Heidt-2010).

Time-scale

In human T cell culture, suplatast tosilate (an inhibitor of the production of cytokines on Th2 cell) inhibits IL-4 production after 3 days and antigen specific IgE production after 10 days (**109**-Taiho-2013).

In the human T-B cell co-culture, FK506 and CsA lowered the m-RNA levels of IL-2 and IL-4 at 8 h post-stimulation and inhibited IgM and IgG productions after 9 days (**39**-Heidt-2010).

Treatment with CsA at 50 mg/kg twice daily in cynomolgus monkey 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 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 compared to animals dosed with the vehicle control on Days 9, 12, and 16 post-immunization. There was an 80% or greater reduction in SRBC-specific IgM on Days9-16. SRBC-specific IgG was decreased by ≥95% on Days 9-16 (**71**-Gaida-2015). This similar to the degree of inhibition observed in rats using a KLH immunization model (**110**-Smith-2003).

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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