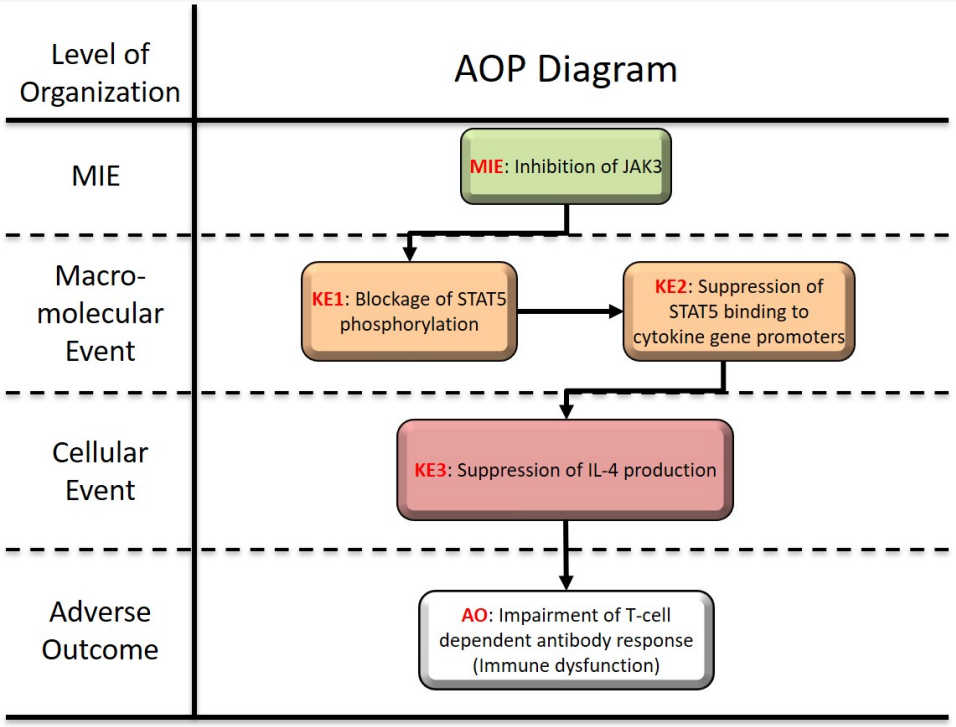
AOP ID and Title:

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

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.74	Included in OECD Work Plan

Abstract

Signal transduction between immune-related cells depends in many cases on cytokines. The transduction involves cell surface cytokine receptors as well as direct cell-to-cell interaction. Cytokines influence the movement, proliferation, differentiation, and activation of lymphocytes and other leukocytes in a variety of ways. Some cytokine receptors require an activation step through a Janus-kinase (JAK)/signal transducer and activator of transcription (STAT) system. When cytokines bind to specific cytokine receptors, the receptors form dimers, which more closely resemble JAK molecules. JAK is activated and phosphorylates adjacent cytokine receptors. STATs bind to the phosphorylated receptor sites and are in turn phosphorylated by the activated JAK. The phosphorylated STAT is dimerized and translocated into the nucleus. There it binds to the promoter regions of cytokine genes, which initiates the transcription of these genes in the nucleus.

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

This proposed AOP consists of JAK3 inhibition as a MIE, blockade of STAT5 phosphorylation as the first key event (KE1), suppression of STAT5 binding to the promoter regions of cytokine genes as KE2, suppression of IL-4 production as KE3, and suppression of T cell

dependent antibody response (TDAR) as an AO. This AOP especially focuses on the inhibition of JAK3, which is required for signal transduction by cytokines through the common γ chain of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In the proposed AOP, JAK3 selective inhibitors that include PF-06651600 (CAS No: 1792180-81-4) and the 4-aminopiperidine-based compound RB1 are stressors. STAT5 that is phosphorylated by JAK3 forms a homo-dimer that translocate to the nucleus and induces expressions of genes, such as IL-4. Therefore, JAK3 inhibition leads to the suppressed binding of STAT5 to the promoter regions of cytokine genes and the subsequent suppression of IL-4 production. Thus, JAK/STAT regulation plays an important role in the TDAR. TDAR is frequently affected by immunosuppressive conditions and is a major endpoint in many preclinical immunotoxicity studies.

Background

Although many stressors inhibit JAK3 activity, this AOP is based on immunosuppression caused by the recently developed and highly selective JAK3 inhibitors PF-06651600 and RB1. A significant body of scientific literature has been published concerning these two inhibitors. We look forward to future amendments to this AOP with up-to-date information on other stressors, which will clarify the link between inhibition of JAK activity and impairment of TDAR.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	1715	Inhibition of JAK3	Inhibition of JAK3
	KE	1716	Blockade of STAT5 phosphorylation	STAT5 inhibition
	KE	1717	Suppression of STAT5 binding to cytokine gene promoters	Suppression of STAT5 binding to cytokine gene promoters
	KE	1718	Suppression of IL-4 production	Suppression of IL-4 production
	AO	1719	Impairment of T-cell dependent antibody response	Impairment, TDAR

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition of JAK3	adjacent	Blockade of STAT5 phosphorylation	High	High
Blockade of STAT5 phosphorylation	adjacent	Suppression of STAT5 binding to cytokine gene promoters	High	High
Suppression of STAT5 binding to cytokine gene promoters	adjacent	Suppression of IL-4 production	High	High
Suppression of IL-4 production	adjacent	Impairment of T-cell dependent antibody response	High	High

Stressors

Name	Evidence
PF-06651600 (CAS No 1792180-81-4),	High
RB1	High

Overall Assessment of the AOP

JAKs are a family of nonreceptor tyrosine kinases and consist of four members: JAK1, JAK2, JAK3, and Tyk2 (Johnston, et al. 1994). All four mediate signals initiated by cytokines through interactions with receptors for IL-2, IL-5, IL-7, IL-9, and IL-15 via the common γ chain (Witthuhn, et al. 1994). Different studies have shown that JAK3 is widely expressed in different organs (Witthuhn, et al. 1994). Previous studies with IL-2Rγ-null mice showed that JAK3 is related to the development of spontaneous inflammatory bowel disease symptoms (Miyazaki, et al. 1994). Moreover, abnormal activation of JAK3 was associated with human hematology (Ihle, et al. 1997), indicating that a tight balance of its activity is essential for normal hematopoietic development.

Although JAK1, JAK2, and Tyk2 are widely expressed, JAK3 is predominantly expressed in hematopoietic cells and is associated only with th common γ chain of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (Nosaka, et al. 1995). IL-4 is a very well-known cytokine that is crucial in the

polarization of naïve T cells to type 2 helper T cells. IL-4 plays a major role in the growth and proliferation of many immune cells, such as natural killer (NK) cells and T cells (Dhupkar and Gordon 2017). Homozygous mutant mice harboring a disrupted JAK3 gene display profound reductions in thymocytes and severe B cell and T cell lymphopenia, similar to severe combined immunodeficiency disease (SCID), and functionally deficient residual T cells and B cells. Thus, JAK3 plays a critical role in γ chain signaling and lymphoid development.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	High

The proposed AOP involves inhibition of JAK activity, which leads to suppression of TDAR independent of life stage, sex, or age. Since JAK3 inhibitors (PF-06651600, RB1) are currently under phase 2 clinical evaluation for the treatment of rheumatoid arthritis, the AOP appears to be applicable to all life stages. JAK3 inhibitor-induced outcomes in humans are mimicked by similar responses in a variety of animal models, including non-human primates and rodents. Thus, immunosuppression induced by inhibition of JAK3 activity is considered to occur across a variety of mammalian species. For example, PF-06651600 was reported to reduce paw swelling with an unbound EC50 of 169 nM in rat adjuvant-induced arthritis. Similarly, PF-06651600 significantly reduced disease severity in an experimental autoimmune encephalomyelitis mouse model at 30 or 100 mg/kg or prophylactically at 20 and 60 mg/kg. PF-06651600 will be evaluated in clinical trials (Telliez, et al. 2016).

Essentiality of the Key Events

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

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.

B6.Cg-Nr1d1tm1Ven/LazJ mouse

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).

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 (Rani and Murphy 2016, Wittig and Groner 2005). 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 (Quezada Urban, et al. 2018).

Phenotypes 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 (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).

STAT5 in T cell development

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.

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).

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.

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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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Telliez JB, Dowty ME, Wang L, Jussif J, Lin T, Li L, Moy E, Balbo P, Li W, Zhao Y, Crouse K, Dickinson C, Symanowicz P, Hegen M, Banker ME, Vincent F, Unwalla R, Liang S, Gilbert AM, Brown MF, Hayward M, Montgomery J, Yang X, Bauman J, Trujillo JI, Casimiro-Garcia A, Vajdos FF, Leung L, Geoghegan KF, Quazi A, Xuan D, Jones L, Hett E, Wright K, Clark JD, Thorarensen A. 2016. Discovery of a JAK3-Selective Inhibitor: Functional Differentiation of JAK3-Selective Inhibition over pan-JAK or JAK1-Selective Inhibition. ACS Chem Biol 11:3442-3451. DOI: 10.1021/acschembio.6b00677.

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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

Organ term

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

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 **Sex** High **Evidence**

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

STAT activation is regulated by JAK via phosphorylation. Thus, JAK inhibitors commonly interfere with STAT activation.

Evidence Supporting this KER

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

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

STAT5 phosphorylation can induce IL-2 receptor expression. Therefore, the suppression of STAT5 phosphorylation interferes with IL-2 production after STAT5 DNA binding to the promoter region.

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

At present, there is no evidence of factors.

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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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

At present, no evidence is available.

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

Term	Scientific Term	Evidence	Links
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

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

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

At present, no evidence exists.

Known Feedforward/Feedback loops influencing this KER

At present, no evidence is exists.

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