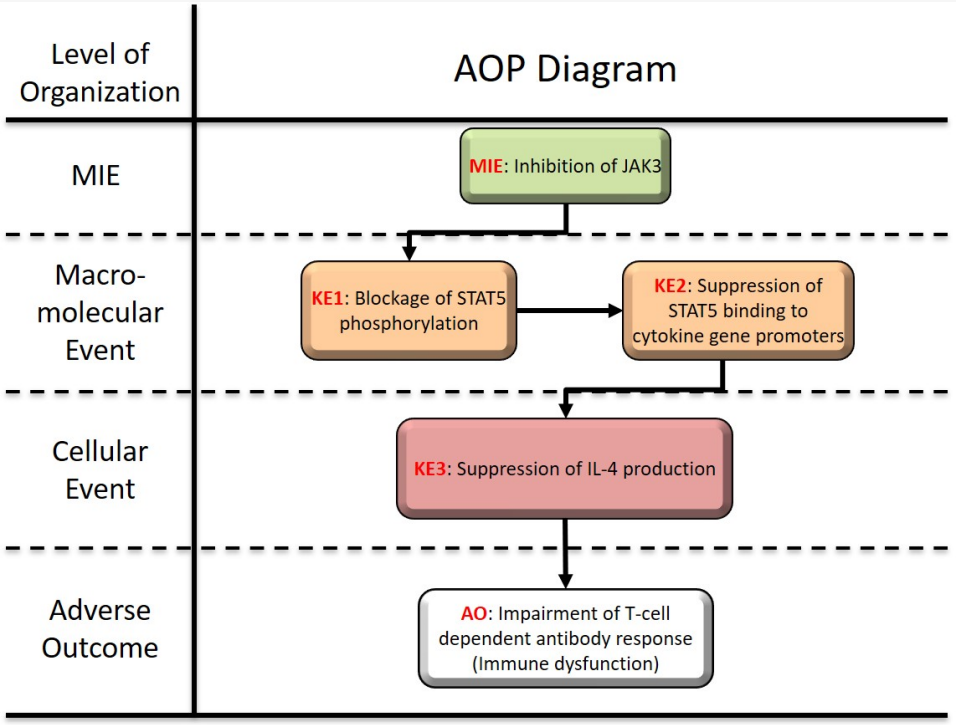
AOP ID and Title:

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

Graphical Representation



Authors

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

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

Corresponding author: Yasuhiro Yoshida (freude@med.uoeh-u.ac.jp)

Status

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 Do not cite	Under Development	1.74	Included in OECD Work Plan

Abstract

Signal transduction between immune-related cells depends in many cases oncytokines. 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 the 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 muta 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-0665160(was reported to reduce paw swelling with an unbound EC50 of 169 nM in rat adjuvant-induced arthritis. Similarly, PF-0665160(significantly reduced disease severity in an experimental autoimmune encephalomyelitis mouse model at 30 or 100 mg/kg or prophylactically at 20 and 60 mg/kg. PF-06651600 will be evaluated in clinical trials (Telliez, et al. 2016).

Essentiality of the Key Events

MIE: Inhibition of JAK3

JAK3 was initially identified (Johnston, et al. 1994, Witthuhn, et al. 1994) in studies designed to identify the JAK family member involved in the signaling of a group of cytokines with shared utilization of the γ chain first identified in the IL-2 receptor complex. It was subsequently demonstrated that JAK3 physically associates with the γ chain and is activated in a receptor complex that also contains JAK1, which associates with the ligand-specific α or β chain of the receptors (Miyazaki, et al. 1994). JAK3 is somewhat unique within the JAK family in that it is predominantly expressed in hematopoietic cells and is only activated in response to cytokines that use the γ chain (Ihle, et al. 1997). The phenotype of the JAK3 deletion mice is striking, with a range of deficiencies that collectively constitute SCID (Nosaka, et al. 1995, Thomis, et al. 1995). At the same time, two groups identified individuals that lacked JAK3 and exhibited somatically acquired SCID (Macchi, et al. 1995, Russell, et al. 1995). One of the most striking components of the phenotype are the dramatic reductions in both the T and B-cell lineages. Comparable reductions are seen in mice that lack IL-7 (von Freeden-Jeffry, et al. 1995), the IL-7 receptor α chain (Peschon, et al. 1994), or the γ chain. Despite the reduced numbers, the cells that do develop are phenotypically normal. These results are consistent with the hypothesis that activation of JAK3 is critical in the expansion, but not differentiation, of early lymphoid lineage-committed cells. In addition to the reduced numbers, the differentiated lymphoid cells that are generated fail to respond to the spectrum of cytokines that utilize the γ chain and activate JAK3 normally. In addition, there are other examples of JAK3 mutant mice. Primary immunodeficiencies (PIDs) are inborn errors that cause developmental and/or functional defects in the immune system (Picard, et al. 2015). PIDs are usually rare and monogenic. They present clinically with a broad array of phenotypes, including increased susceptibility to infection. One of the most deadly categories of PID is SCID. SCID is invariably caused by severe developmental and/or functional defects of T lymphocytes. However, SCID may also present with variable defects of B and/or NK cells. The B6.Cg-Nr1d1tm1Ven/LazJ mouse line harbors a spontaneous mutation ir JAK3, which generates the SCID phenotype (Robinette, et al. 2018). STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells (Rani and Murphy 2016, Wittig and Groner 2005). The activation of STAT5 is transient and tightly regulated in normal cells (Quezada Urban, et al. 2018). The transcription factor STAT5 is expressed in all lymphocytes and plays a key role in multiple aspects of lymphocyte development and function (Owen and Farrar 2017). STAT5 was initially identified as a transcription factor activated by prolactin in mammary gland epithelial cells (Schmitt-Ney, et al. 1992, Wakao, et al. 1992). Subsequent studies identified STAT5 binding activity in T cells (Beadling, et al. 1994). Other authors described that the expression of STAT5 in multiple cell types and its' activation by a number of cytokines, including the common γ-chain-dependent cytokines IL-2, IL-4, IL-7, IL-13, and IL-15 (Lin, et al. 1995).

KE	description

KE1: Blockade of STAT5 phosphorylation	STAT5 refer to two proteins that share 94% structural homology and are transcribed from separate genes, STAT5A and STAT5B. Binding of these extracellular ligands to their target receptors induces the activation of receptor-associated JAK kinases that phosphorylate key tyrosine residues within the receptor, providing docking sites for the SRC homology 2 (SH2) domains of the inactive cytoplasmic STAT5 monomers. STAT5 is then phosphorylated at specific tyrosine residues, either Y694 (STAT5A) or Y699 (STAT5B) of the C-terminus. Subsequently, STAT5 undergoes a conformational change and phosphorylated STAT5 monomers form either homo- or hetero- STAT5X-STATX dimers through reciprocal phosphotyrosine–SH2 interactions (Cumaraswamy, et al. 2014, Tothova, et al. 2021). This means that STAT5 will never be activated without this phosphorylation step.
KE2: Suppression of STAT5 binding to cytokine gene promoters	Activated STAT5 dimers translocate to the nucleus, where they bind to STAT5 DNA response elements inducing transcription of genes involved in proliferation, cell differentiation, inflammation (cytokines) and cell survival. Since the STAT5 monomer does not bind directly to the DNA element, inhibiting the STAT5 phosphorylation step suppresses STAT5 activity.
KE3: Suppression of IL-4 production	The observation that STAT5 is activated by multiple cytokines in T cells suggests that it might play a critical role in the development and/or function of these cells. Disruption of the Stat5a gene or Stat5b gene reportedly resulted in relatively modest phenotypes. For example, Stat5a-/- mice displayed defects in mammary gland development and lactation, while Stat5b-/- mice displayed defects in response to growth hormone in male mice and NK cell proliferation (Imada, et al. 1998, Liu, et al. 1997). To determine whether combined deletion of Stat5a and Stat5b might result in more profound immunodeficiencies, subsequent studies deleted the first coding exons of both Stat5a and Stat5b. This intervention resulted in the production of truncated forms of STAT5a and STAT5b, which acted as functional hypomorphs. These mice had surprisingly mild defects in lymphocyte development, although T cells were grossly dysfunctional as they could no longer proliferate in response to IL-2 (Moriggl, et al. 1999, Teglund, et al. 1998). Finally, complete deletion of Stat5a and Stat5b using Cre-LoxP approaches demonstrated that STAT5a and STAT5b are absolutely required for lymphocyte development, as Stat5a/b-/- mice had profound blocks in lymphocyte development, which mimicked that observed in Il7r-/- mice (Cui, et al. 2004, Yao, et al. 2006). These studies definitively demonstrated the retention of appreciable STAT5 function in STAT5 hypomorph mice. Thus, T cell damage due to STAT5 deficiency or inactivation leads to suppression of the production of cytokines such as IL-4.

AOP: Impairment of T cell dependent antibody response (Immune dysfunction)

Weight of Evidence Summary

T cell development is mainly regulated by the JAK-STAT system. JAK3 deficiency in T cells induces multiple types of immunosuppression, including TDAR.

JAK3-deficient mice reportedly displayed profound reductions in thymocytes and severe B cell and T cell lymphopenia, similar to SCID disease. The residual T cells and B cells were functionally deficient (Peschon, et al. 1994).

Mice lacking JAK3 also showed a severe block in B cell development at the pre-B stage in the bone marrow. In contrast, although the thymuses of these mice were small, T cell maturation progressed relatively normally. In response to mitogenic signals, peripheral T cells in JAK3-deficient mice did not proliferate and secreted small amounts of IL-4. These data demonstrate that JAK3 is critical for the progression of B cell development in the bone marrow and for the functional competence of mature T cells (Nosaka, et al. 1995).

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

PF-06651600 and RB1 specifically inhibit JAK3 with over 100-fold preference over JAK2, JAK1, and TYK2 in kinase assays. Reduced inflammation and associated pathology have been described in collagen-induced arthritis mice. Importantly, the administration of PF-06651600 or RB1 results in decreased pro-inflammatory cytokines and JAK3 and STAT phosphorylation in mice. The findings suggest that the inhibition of JAK3/STAT signaling is closely correlated with the induction of multiple

types of immunosuppression, including TDAR.

Quantitative Consideration

KER1 (MIE => KE1)

Treatment with the highly selective JAK3 inhibitor PF-06651600 or RB1 suppresses the complex formation of STAT5 in the nucleus. IL-2 stimulates STAT5 and induces tyrosine phosphorylation of STAT5 (Wakao, et al. 1995). RB1 inhibits the phosphorylation of STAT5 elicited by IL-2, as evidenced by an IC50 value of 31 nM in the peripheral blood mononuclear cells (PBMCs) of humans. PBMCs isolated from the buffy coats of healthy volunteers by density gradient centrifugation on Lymphoprep were cultured in complete RPMI 1640 medium (containing 10% fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin) plus 10 µg/mL lectin phytohemagglutinin (PHA) for 3 days. The cells were then treated with recombinant human IL-6 (400 ng/mL), recombinant human IL-2 (100 ng/mL), or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL) at 37°C for 20 min. To terminate the stimulation, the cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 min. The cells were incubated with anti-pSTAT3 and anti-CD4 antibodies or anti-pSTAT5 and anti-CD4 antibodies at 4°C overnight, washed twice with PBS, and analyzed with by flow cytometry (Ju, et al. 2011).

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

KER2 (KE1 => KE2)

STAT5 can be activated and phosphorylated by cytokines, such as IL-2 and IL-15. Tyrosine phosphorylation of STAT5 is important for the dimerization of STAT5 (Wakao, et al. 1995). The STAT5 dimer has an identical DNA binding specificity and immunoreactivity.

KER3 (KE2 => KE3)

STAT5 is phosphorylated by JAK kinases, allowing its dimerization and translocation into the nucleus where it can bind to its specific DNA binding sites. Electrophoretic mobility shift assay (EMSA) data revealed that IL-2 activation induced STAT5 dimerization and DNA binding to the gamma interferon activated site (GAS) motif in the IL-4 receptor alpha promoter region (John, et al. 1999). Other EMSA data showed that dexamethasone (10^{-6} M) inhibited STAT5 DNA binding in mononuclear cells in a dose-dependent fashion at dexamethasone concentrations of 10^{-8} to 10^{-7} M (Bianchi, et al. 2000). Dexamethasone could inhibit tyrosine phosphorylation, and nuclear translocation of STAT5 in primary T cells. The mechanism of inhibition involved suppression of IL-2 receptor and JAK3 expression.

KER4 (KE3 => AO)

Binding of IL-4 to the T cell receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

In co-cultured human T and B cells stimulated with anti-CD3 monoclonal antibody, the calcineurin inhibitors (CNIs) FK506 and cyclosporin A (CsA) lowered the levels of T cell cytokines, including IL-2 and IL-4, and inhibited IgM and IgG production in a dose-dependent manner (Heidt, et al. 2010).

The collective results demonstrate the quantitative relationships between the inhibition of IL-4 by specific antibodies or CNI and suppression of antibody production.

1. Support for Biological Plausibility of KER	
MIE => KE1: Inhibition of JAK3 to blockade of STAT5 phosphorylation	Biological Plausibility of the MIE => KE1 is Strong. Rationale: Administration of PF-06651600 or RB1 results in decreased pro-inflammatory cytokines and JAK3 and STAT phosphorylation in mice.
KE1 => KE2: Blockade of STAT5 phosphorylation to suppression of STAT5 binding to cytokine gene promoters	Biological Plausibility of the KE1 => KE2 is Strong. Rationale: STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis of hematopoietic and immune cells. STAT5 is activated by phosphorylation of a single constituent tyrosine residue (Y694) and is negatively regulated by dephosphorylation. A wide variety of growth factors and cytokines can activate STAT5 through the JAK-STAT pathway. The activation of STAT5 is transient and tightly regulated in normal cells.

KE2 => KE3: Suppression of STAT5 binding to cytokine gene promoters to impairment of T cell dependent antibody response (Immune dysfunction)	Biological Plausibility of the KE2 => KE3 is strong. Rationale: In response to mitogenic signals, peripheral T cells in JAK3-deficient mice did not proliferate and secreted small amounts of IL-4. These data demonstrate that JAK3 is critical for the progression of B cell development in the bone marrow and for the functional competence of mature T cells.
KE3 => AO: Suppression, IL-4 production to impairment, T cell dependent Antibody response	Biological Plausibility of the KE3 => KE4 is strong. Rationale: In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells provide help for B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to the impairment of TDAR.
2. Support for Essentiality of AOP	Rationale for Essentiality of KEs in the AOP is strong:
3. Empirical Support for KERs	
MIE => KE1: Inhibition of JAK3 to blockade of STAT5 phosphorylation	Empirical Support of the MIE => KE1 is strong. Rationale: Treatment with the highly selective JAK3 inhibitor PF-06651600 or RB1 suppresses the complex formation of STAT5 in the nucleus. IL-2 stimulates STAT5 and induces tyrosine phosphorylation of STAT5. RB1 inhibits the phosphorylation of STAT5 elicited by IL-2, as evidenced by an IC50 value of 31 nM in the peripheral blood mononuclear cells (PBMCs) of humans. PBMCs isolated from the buffy coats of healthy volunteers by density gradient centrifugation on Lymphoprep were cultured in complete RPMI 1640 medium (containing 10% fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin) plus 10 µg/mL lectin phytohemagglutinin (PHA) for 3 days. The cells were then treated with recombinant human IL-6 (400 ng/mL), recombinant human IL-2 (100 ng/mL), or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL) at 37°C for 20 min. To terminate the stimulation, the cells were fixed with Lyse/Fix Buffer and then incubated with 100% methanol for 30 min. The cells were incubated with anti-pSTAT3 and anti-CD4 antibodies, or anti-pSTAT5 and anti-CD4 antibodies at 4°C overnight, washed twice with PBS, and analyzed with by flow cytometry. The fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes was observed to increase upon incubation of peripheral blood with IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM for two rats). Additionally, the effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T cells was evaluated. Parallel with the results in rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after adding IL-2. Peficitinib inhibited STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 127 nM in human lymphocytes.
KE1 => KE2: Blockade of STAT5 phosphorylation to suppression of STAT5 binding to cytokine gene promoters	Empirical Support of the KE1 => KE2 is strong. Rationale: STAT5 can be activated and phosphorylated by cytokines, such as IL-2 and IL-15. Tyrosine phosphorylation of STAT5 is important for the dimerization of STAT5. The STAT5 dimer has an identical DNA binding specificity and immunoreactivity.

KE2 => KE3: Suppression of STAT5 binding to cytokine gene promoters to impairment of T cell dependent antibody response (Immune dysfunction)	Empirical Support of the KE2 => KE3 is strong. Rationale: STAT5 is phosphorylated by JAK kinases, allowing its dimerization and translocation into the nucleus where it can bind to its specific DNA binding sites. Electrophoretic mobility shift assay (EMSA) data revealed that IL-2 activation induced STAT5 dimerization and DNA binding to the gamma interferon activated site (GAS) motif in the IL-4 receptor alpha promoter region. Other EMSA data showed that dexamethasone (10^{-6} M) inhibited STAT5 DNA binding in mononuclear cells in a dose-dependent fashion at dexamethasone concentrations of 10^{-8} to 10^{-7} M. Dexamethasone could inhibit tyrosine phosphorylation, and nuclear translocation of STAT5 in primary T cells. The mechanism of inhibition involved suppression of IL-2 receptor and JAK3 expression.
KE3 => AO: Suppression, IL-4 production to impairment, T cell dependent Antibody response	Empirical Support of the KE3 => KE4 is strong. Rationale: Binding of IL-4 to the T cell receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR. In co-cultured human T and B cells stimulated with anti-CD3 monoclonal antibody, the calcineurin inhibitors (CNIs) FK506 and cyclosporin A (CsA) lowered the levels of T cell cytokines, including IL-2 and IL-4, and inhibited IgM and IgG production in a dose-dependent manner. The collective results demonstrate the quantitative relationships between the inhibition of IL-4 by specific antibodies or CNI and suppression of antibody production.

References

Beadling C, Guschin D, Witthuhn BA, Ziemiecki A, Ihle JN, Kerr IM, Cantrell DA. 1994. Activation of JAK kinases and STAT proteins by interleukin-2 and interferon alpha, but not the T cell antigen receptor, in human T lymphocytes. EMBO J 13:5605-5615.

Bianchi M, Meng C, Ivashkiv LB. 2000. Inhibition of IL-2-induced Jak-STAT signaling by glucocorticoids. Proc Natl Acad Sci U S A 97:9573-9578. DOI: 10.1073/pnas.160099797.

Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng CX, Robinson GW, Hennighausen L. 2004. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol Cell Biol 24:8037-8047. DOI: 10.1128/MCB.24.18.8037-8047.2004.

Cumaraswamy AA, Lewis AM, Geletu M, Todic A, Diaz DB, Cheng XR, Brown CE, Laister RC, Muench D, Kerman K, Grimes HL, Minden MD, Gunning PT. 2014. Nanomolar-Potency Small Molecule Inhibitor of STAT5 Protein. ACS Med Chem Lett 5:1202-1206. DOI: 10.1021/ml500165r.

Dhupkar P, Gordon N. 2017. Interleukin-2: Old and New Approaches to Enhance Immune-Therapeutic Efficacy. Adv Exp Med Biol 995:33-51. DOI: 10.1007/978-3-319-53156-4_2.

Heidt S, Roelen DL, Eijsink C, Eikmans M, van Kooten C, Claas FH, Mulder A. 2010. Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. Clin Exp Immunol 159:199-207. DOI: 10.1111/j.1365-2249.2009.04051.x.

Ihle JN, Nosaka T, Thierfelder W, Quelle FW, Shimoda K. 1997. Jaks and Stats in cytokine signaling. Stem Cells 15 Suppl 1:105-111; discussion 112. DOI: 10.1002/stem.5530150814.

Imada K, Bloom ET, Nakajima H, Horvath-Arcidiacono JA, Udy GB, Davey HW, Leonard WJ. 1998. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. J Exp Med 188:2067-2074.

Ito M, Yamazaki S, Yamagami K, Kuno M, Morita Y, Okuma K, Nakamura K, Chida N, Inami M, Inoue T, Shirakami S, Higashi Y. 2017. A novel JAK inhibitor, peficitinib, demonstrates potent efficacy in a rat adjuvant-induced arthritis model. J Pharmacol Sci 133:25-33. DOI: 10.1016/j.jphs.2016.12.001.

John S, Vinkemeier U, Soldaini E, Darnell JE, Jr., Leonard WJ. 1999. The significance of tetramerization in promoter recruitment by Stat5. Mol Cell Biol 19:1910-1918.

Johnston JA, Bacon CM, Finbloom DS, Rees RC, Kaplan D, Shibuya K, Ortaldo JR, Gupta S, Chen YQ, Giri JD, et al. 1995. Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15. Proc Natl Acad Sci U S A 92:8705-8709.

Johnston JA, Kawamura M, Kirken RA, Chen YQ, Blake TB, Shibuya K, Ortaldo JR, McVicar DW, O'Shea JJ. 1994.

Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. Nature 370:151-153. DOI: 10.1038/370151a0.

Ju W, Zhang M, Jiang JK, Thomas CJ, Oh U, Bryant BR, Chen J, Sato N, Tagaya Y, Morris JC, Janik JE, Jacobson S, Waldmann TA. 2011. CP-690,550, a therapeutic agent, inhibits cytokine-mediated Jak3 activation and proliferation of T cells from patients with ATL and HAM/TSP. Blood 117:1938-1946. DOI: 10.1182/blood-2010-09-305425.

Liao W, Schones DE, Oh J, Cui Y, Cui K, Roh TY, Zhao K, Leonard WJ. 2008. Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. Nat Immunol 9:1288-1296. DOI: 10.1038/ni.1656.

Lin JX, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, Yamauchi A, Bloom ET, Mietz J, John S, et al. 1995. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity 2:331-339.

Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L. 1997. Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev 11:179-186.

Macchi P, Villa A, Giliani S, Sacco MG, Frattini A, Porta F, Ugazio AG, Johnston JA, Candotti F, O'Shea JJ, et al. 1995. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). Nature 377:65-68. DOI: 10.1038/377065a0.

Miyazaki T, Kawahara A, Fujii H, Nakagawa Y, Minami Y, Liu ZJ, Oishi I, Silvennoinen O, Witthuhn BA, Ihle JN, et al. 1994. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. Science 266:1045-1047.

Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249-259.

Nosaka T, van Deursen JM, Tripp RA, Thierfelder WE, Witthuhn BA, McMickle AP, Doherty PC, Grosveld GC, Ihle JN. 1995. Defective lymphoid development in mice lacking Jak3. Science 270:800-802.

Owen DL, Farrar MA. 2017. STAT5 and CD4 (+) T Cell Immunity. F1000Res 6:32. DOI: 10.12688/f1000research.9838.1.

Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, Park LS, Ziegler SF, Williams DE, Ware CB, Meyer JD, Davison BL. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med 180:1955-1960.

Picard C, Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, Etzioni A, Holland SM, Klein C, Nonoyama S, Ochs HD, Oksenhendler E, Puck JM, Sullivan KE, Tang ML, Franco JL, Gaspar HB. 2015. Primary Immunodeficiency Diseases: an Update on the Classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015. J Clin Immunol 35:696-726. DOI: 10.1007/s10875-015-0201-1.

Quezada Urban R, Diaz Velasquez CE, Gitler R, Rojo Castillo MP, Sirota Toporek M, Figueroa Morales A, Moreno Garcia O, Garcia Esquivel L, Torres Mejia G, Dean M, Delgado Enciso I, Ochoa Diaz Lopez H, Rodriguez Leon F, Jan V, Garzon Barrientos VH, Ruiz Flores P, Espino Silva PK, Haro Santa Cruz J, Martinez Gregorio H, Rojas Jimenez EA, Romero Cruz LE, Mendez Catala CF, Alvarez Gomez RM, Fragoso Ontiveros V, Herrera LA, Romieu I, Terrazas LI, Chirino YI, Frecha C, Oliver J, Perdomo S, Vaca Paniagua F. 2018. Comprehensive Analysis of Germline Variants in Mexican Patients with Hereditary Breast and Ovarian Cancer Susceptibility. Cancers (Basel) 10. DOI: 10.3390/cancers10100361.

Rani A, Murphy JJ. 2016. STAT5 in Cancer and Immunity. J Interferon Cytokine Res 36:226-237. DOI: 10.1089/jir.2015.0054.

Robinette ML, Cella M, Telliez JB, Ulland TK, Barrow AD, Capuder K, Gilfillan S, Lin LL, Notarangelo LD, Colonna M. 2018. Jak3 deficiency blocks innate lymphoid cell development. Mucosal Immunol 11:50-60. DOI: 10.1038/mi.2017.38.

Russell SM, Tayebi N, Nakajima H, Riedy MC, Roberts JL, Aman MJ, Migone TS, Noguchi M, Markert ML, Buckley RH, O'Shea JJ, Leonard WJ. 1995. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. Science 270:797-800.

Schmitt-Ney M, Happ B, Hofer P, Hynes NE, Groner B. 1992. Mammary gland-specific nuclear factor activity is positively regulated by lactogenic hormones and negatively by milk stasis. Mol Endocrinol 6:1988-1997. DOI: 10.1210/mend.6.12.1491685.

Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN. 1998. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841-850.

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.

Thomis DC, Gurniak CB, Tivol E, Sharpe AH, Berg LJ. 1995. Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. Science 270:794-797.

Tothova Z, Tomc J, Debeljak N, Solar P. 2021. STAT5 as a Key Protein of Erythropoietin Signalization. Int J Mol Sci 22. DOI: 7109 [pii]10.3390/ijms22137109ijms22137109 [pii].

von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 181:1519-1526.

Wakao H, Harada N, Kitamura T, Mui AL, Miyajima A. 1995. Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways. EMBO J 14:2527-2535.

Wakao H, Schmitt-Ney M, Groner B. 1992. Mammary gland-specific nuclear factor is present in lactating rodent and bovine mammary tissue and composed of a single polypeptide of 89 kDa. J Biol Chem 267:16365-16370.

Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW. 1995. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity 3:521-530.

Witthuhn BA, Silvennoinen O, Miura O, Lai KS, Cwik C, Liu ET, Ihle JN. 1994. Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. Nature 370:153-157. DOI: 10.1038/370153a0.

Wittig I, Groner B. 2005. Signal transducer and activator of transcription 5 (STAT5), a crucial regulator of immune and cancer cells. Curr Drug Targets Immune Endocr Metabol Disord 5:449-463.

Yao Z, Cui Y, Watford WT, Bream JH, Yamaoka K, Hissong BD, Li D, Durum SK, Jiang Q, Bhandoola A, Hennighausen L, O'Shea JJ. 2006. Stat5a/b are essential for normal lymphoid development and differentiation. Proc Natl Acad Sci U S A 103:1000-1005. DOI: 10.1073/pnas.0507350103.

Zhu J, Cote-Sierra J, Guo L, Paul WE. 2003. Stat5 activation plays a critical role in Th2 differentiation. Immunity 19:739-748. DOI: 10.1016/s1074-7613(03)00292-9.

Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, Killeen N, Urban JF, Jr., Guo L, Paul WE. 2004. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat Immunol 5:1157-1165. DOI: 10.1038/ni1128.

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).

References

Darnell JE, Jr. 1997. STATs and gene regulation. Science 277:1630-1635.

Decker T, Kovarik P, Meinke A. 1997. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. J Interferon Cytokine Res 17:121-134. DOI: 10.1089/jir.1997.17.121.

Gaffen SL, Lai SY, Xu W, Gouilleux F, Groner B, Goldsmith MA, Greene WC. 1995. Signaling through the interleukin 2 receptor beta chain activates a STAT-5-like DNA-binding activity. Proc Natl Acad Sci U S A 92:7192-7196.

Gianti E, Zauhar RJ. 2015. An SH2 domain model of STAT5 in complex with phospho-peptides define "STAT5 Binding Signatures". J Comput Aided Mol Des 29:451-470. DOI: 10.1007/s10822-015-9835-6.

Pei H, He L, Shao M, Yang Z, Ran Y, Li D, Zhou Y, Tang M, Wang T, Gong Y, Chen X, Yang S, Xiang M, Chen L. 2018. Discovery of a highly selective JAK3 inhibitor for the treatment of rheumatoid arthritis. Sci Rep 8:5273. DOI: 10.1038/s41598-018-23569-y.

Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, Park LS, Ziegler SF, Williams DE, Ware CB, Meyer JD, Davison BL. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med 180:1955-1960.

Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, et al. 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science 263:92-95.

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.

Thorarensen A, Dowty ME, Banker ME, Juba B, Jussif J, Lin T, Vincent F, Czerwinski RM, Casimiro-Garcia A, Unwalla R, Trujillo JI, Liang S, Balbo P, Che Y, Gilbert AM, Brown MF, Hayward M, Montgomery J, Leung L, Yang X, Soucy S, Hegen M, Coe J, Langille J, Vajdos F, Chrencik J, Telliez JB. 2017. Design of a Janus Kinase 3 (JAK3) Specific Inhibitor 1-((2S,5R)-5-((7H-Pyrrolo[2,3-d]pyrimidin-4-yl)amino)-2-methylpiperidin-1-yl)prop -2-en-1-one (PF-06651600) Allowing for the Interrogation of JAK3 Signaling in Humans. J Med Chem 60:1971-1993. DOI: 10.1021/acs.jmedchem.6b01694.

Xu BC, Wang X, Darus CJ, Kopchick JJ. 1996. Growth hormone promotes the association of transcription factor STAT5 with the growth hormone receptor. J Biol Chem 271:19768-19773.

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 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.

References

Gianti E, Zauhar RJ. 2015. An SH2 domain model of STAT5 in complex with phospho-peptides define "STAT5 Binding Signatures". J Comput Aided Mol Des 29:451-470. DOI: 10.1007/s10822-015-9835-6.

Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene 285:1-24.

Muller J, Sperl B, Reindl W, Kiessling A, Berg T. 2008. Discovery of chromone-based inhibitors of the transcription factor STAT5. Chembiochem 9:723-727. DOI: 10.1002/cbic.200700701.

Nelson EA, Walker SR, Weisberg E, Bar-Natan M, Barrett R, Gashin LB, Terrell S, Klitgaard JL, Santo L, Addorio MR, Ebert BL, Griffin JD, Frank DA. 2011. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. Blood 117:3421-3429. DOI: 10.1182/blood-2009-11-255232

blood-2009-11-255232 [pii].

Osinalde N, Sanchez-Quiles V, Blagoev B, Kratchmarova I. 2017. Data on interleukin (IL)-2- and IL-15-dependent changes in IL-2Rbeta and IL-2Rgamma complexes. Data Brief 11:499-506. DOI: 10.1016/j.dib.2017.02.030.

Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, et al. 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science 263:92-95.

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.

Thibault G, Paintaud G, Legendre C, Merville P, Coulon M, Chasseuil E, Ternant D, Rostaing L, Durrbach A, Di Giambattista F, Buchler M, Lebranchu Y. 2016. CD25 blockade in kidney transplant patients randomized to standard-dose or high-dose basiliximab with cyclosporine, or high-dose basiliximab in a calcineurin inhibitor-free regimen. Transpl Int 29:184-195. DOI: 10.1111/tri.12688.

Wakao H, Schmitt-Ney M, Groner B. 1992. Mammary gland-specific nuclear factor is present in lactating rodent and bovine mammary tissue and composed of a single polypeptide of 89 kDa. J Biol Chem 267:16365-16370.

Xu BC, Wang X, Darus CJ, Kopchick JJ. 1996. Growth hormone promotes the association of transcription factor STAT5 with the growth hormone receptor. J Biol Chem 271:19768-19773.

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 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).

References

Beadling C, Guschin D, Witthuhn BA, Ziemiecki A, Ihle JN, Kerr IM, Cantrell DA. 1994. Activation of JAK kinases and STAT proteins by interleukin-2 and interferon alpha, but not the T cell antigen receptor, in human T lymphocytes. EMBO J 13:5605-5615.

Darnell JE, Jr. 1997. STATs and gene regulation. Science 277:1630-1635.

Decker T, Kovarik P, Meinke A. 1997. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. J Interferon Cytokine Res 17:121-134. DOI: 10.1089/jir.1997.17.121.

Gaffen SL, Lai SY, Xu W, Gouilleux F, Groner B, Goldsmith MA, Greene WC. 1995. Signaling through the interleukin 2 receptor beta chain activates a STAT-5-like DNA-binding activity. Proc Natl Acad Sci U S A 92:7192-7196.

Gilmour KC, Pine R, Reich NC. 1995. Interleukin 2 activates STAT5 transcription factor (mammary gland factor) and specific gene expression in T lymphocytes. Proc Natl Acad Sci U S A 92:10772-10776. DOI: 10.1073/pnas.92.23.10772.

Heidt S, Roelen DL, Eijsink C, Eikmans M, van Kooten C, Claas FH, Mulder A. 2010. Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. Clin Exp Immunol 159:199-207. DOI: 10.1111/j.1365-2249.2009.04051.x.

Ihle JN. 1996. STATs: signal transducers and activators of transcription. Cell 84:331-334.

Johnston JA, Bacon CM, Finbloom DS, Rees RC, Kaplan D, Shibuya K, Ortaldo JR, Gupta S, Chen YQ, Giri JD, et al. 1995. Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15. Proc Natl Acad Sci U S A 92:8705-8709.

Leonard WJ, O'Shea JJ. 1998. Jaks and STATs: biological implications. Annu Rev Immunol 16:293-322. DOI: 10.1146/annurev.immunol.16.1.293.

Liu J, Yoshida Y, Kunugita N, Noguchi J, Sugiura T, Ding N, Arashidani K, Fujimaki H, Yamashita U. 2010. Thymocytes are activated by toluene inhalation through the transcription factors NF-kappaB, STAT5 and NF-AT. J Appl Toxicol 30:656-660. DOI: 10.1002/jat.1536.

Maeshima K, Yamaoka K, Kubo S, Nakano K, Iwata S, Saito K, Ohishi M, Miyahara H, Tanaka S, Ishii K, Yoshimatsu H, Tanaka Y. 2012. The JAK inhibitor tofacitinib regulates synovitis through inhibition of interferon-gamma and interleukin-17 production by human CD4+ T cells. Arthritis Rheum 64:1790-1798. DOI: 10.1002/art.34329.

Muller J, Sperl B, Reindl W, Kiessling A, Berg T. 2008. Discovery of chromone-based inhibitors of the transcription factor STAT5. Chembiochem 9:723-727. DOI: 10.1002/cbic.200700701.

Event: 1718: Suppression of IL-4 production

Short Name: Suppression of IL-4 production

Key Event Component

Process	Object	Action
interleukin-4 production	interleukin-4	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:315 - Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	KeyEvent

Stressors

Name
Tofacitinib (CP690,550)

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
T cell

Organ term

Organ term
immune system

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

In one study, only 1% of CD4 T cells from STAT5a-/- mice primed with soluble anti-CD3 and anti-CD28 with IL-2 produced IL-4, whereas 10.5% of control C57BL/6 CD4 T cells produced IL-4 (Cote-Sierra, et al. 2004).

Cells from STAT5A-deficient mice or cells treated with phospho-STAT5 peptide are defective in Th2 differentiation. STAT5A single-deficient mice showed impaired Th2 differentiation. Reconstituting STAT5A by retroviral infection restored the capacity of cells to induce IL-4 (Kagami, et al. 2001).

IL-2 directly activates STAT5A and STAT5B. T cells from mice deficient in either STAT5A or STAT5B did not show a dramatic change in T cell proliferation, but cells from mice in which both had been knocked out proliferated poorly in response to IL-4 (Moriggl, et al. 1999).

Key Event Description

IL-4 is a mammalian protein found in *Homo sapiens*. IL-4 is pivotal in shaping the nature of immune responses. Upon activation naïve 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 induc proliferation and differentiation into Th2 cells. Th2 cells assist B cells in promoting class switching from IgM to IgG1 and IgE (Cl 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 near HSII and HSIII could provide a mechanism through which STAT5A mediates IL-4 gene accessibility 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 JAK3 inhibition by tofacitinib can downregulate STAT5-dependent cytokine signa 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-6905 for 48 h. Supernatants were collected and the levels of IL-4 production were measured by ELISA (Migita, et al. 2011). In additio total RNA was extracted after 8 h or 24 h of stimulation, and IL-4 mRNA expression was measured by real-time PCR. (Migita, et 2011).

In another study, flow cytometry analysis involving intracellular staining was used to measure cytosolic IL-4 content in stimula cells (Zhu, et al. 2001). Relative gene expression levels were determined by quantitative RT-PCR using Taqman Gene Expressi primer probe sets and ABI PRISM 7700 or 7900 Taqman systems (Applied Biosystems). The comparative threshold cycle metho internal controls (cyclophillin or β-actin) 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).

References

Choi P, Reiser H. 1998. IL-4: role in disease and regulation of production. Clin Exp Immunol 113:317-319. DOI: 10.1046/j.1365-2249.1998.00690.x.

Cote-Sierra J, Foucras G, Guo L, Chiodetti L, Young HA, Hu-Li J, Zhu J, Paul WE. 2004. Interleukin 2 plays a central role in Th2 differentiation. Proc Natl Acad Sci U S A 101:3880-3885. DOI: 10.1073/pnas.0400339101.

Dumont FJ, Staruch MJ, Fischer P, DaSilva C, Camacho R. 1998. Inhibition of T cell activation by pharmacologic disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. J Immunol 160:2579-2589.

Ghoreschi K, Jesson MI, Li X, Lee JL, Ghosh S, Alsup JW, Warner JD, Tanaka M, Steward-Tharp SM, Gadina M, Thomas CJ, Minnerly JC, Storer CE, LaBranche TP, Radi ZA, Dowty ME, Head RD, Meyer DM, Kishore N, O'Shea JJ. 2011. Modulation of innate and adaptive immune responses by tofacitinib (CP-690,550). J Immunol 186:4234-4243. DOI: 10.4049/jimmunol.1003668.

Kagami S, Nakajima H, Suto A, Hirose K, Suzuki K, Morita S, Kato I, Saito Y, Kitamura T, Iwamoto I. 2001. Stat5a regulates T helper cell differentiation by several distinct mechanisms. Blood 97:2358-2365. DOI: 10.1182/blood.v97.8.2358.

Levy DE, Darnell JE, Jr. 2002. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 3:651-662. DOI: 10.1038/nrm909.

Migita K, Miyashita T, Izumi Y, Koga T, Komori A, Maeda Y, Jiuchi Y, Aiba Y, Yamasaki S, Kawakami A, Nakamura M, Ishibashi H. 2011. Inhibitory effects of the JAK inhibitor CP690,550 on human CD4(+) T lymphocyte cytokine production. BMC Immunol 12:51. DOI: 10.1186/1471-2172-12-51.

Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249-259.

Zhu J, Cote-Sierra J, Guo L, Paul WE. 2003. Stat5 activation plays a critical role in Th2 differentiation. Immunity 19:739-748. DOI: 10.1016/s1074-7613(03)00292-9.

Zhu J, Guo L, Watson CJ, Hu-Li J, Paul WE. 2001. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. J Immunol 166:7276-7281. DOI: 10.4049/jimmunol.166.12.7276.

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

Name
Cyclosporin, FK506, Basilixim...
PFOA (perfluorooctanoic acid)
Tacrolimus (also FK506)

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CNI-induced impairment of TDAR has been demonstrated in rodent studies. In one study, oral administration of FK506 or CsA to mice for 4 days impaired the response of PFC in splenocytes after intravenous immunization with sheep erythrocytes (Kino, et al. 1987). Likewise, oral administration of FK506 to rats over a 4-week period reduced the production of both anti-KLH-IgG and IgM after subcutaneous immunization with KLH (Ulrich, et al. 2004). Other authors described that treatment with CsA at 50 mg/kg BID via oral gavage in cynomolgus monkeys resulted in reduction of serum SRBC-specific IgM and IgG (Gaida, et al. 2015). As for humans, in vitro experiments showed that treatment with FK506 or CsA of PBMCs from blood bank donors suppressed the production of IgM and IgG specific to T cell dependent antigens (Heidt, et al. 2010). In SKW6.4 cells (IL-6 dependent, IgM-secreting, human B cell line) cultures, FK506 or CsA suppressed the production of IgM in the presence of T cell activation (Sakuma, et al. 2001). Considering that FK506 and CsA reduce T cell derived IL-2, these findings strongly suggest that impairment of TDAR following reduced production of IL-2 occurs at least in common among humans, monkeys, and rodents.

Yang et al. (2002b) exposed male C57BL/6 mice to a single concentration (0.02%) of PFOA in the diet for 16 days. TDAR was measured after inoculating PFOA-treated mice with horse red blood cells intravenously on day 10; serum levels of horse red blood cell-specific IgM and IgG in response to the immunization were significantly decreased (Yang, et al. 2002).

The suppression of TDAR in adult C57BL/6 female mice has been observed in several studies. NOEL of 1.88 mg/kg/d and LOEL of 3.75 mg/kg/d were identified for PFOA administered in drinking water for over 15 days (Dewitt, et al. 2008).

The suppression of TDAR in adrenalectomized or sham-operated C57BL/6N female mice was observed when PFOA was provided in drinking water for 10 days at doses of 0, 3.75, 7.5, or 15 mg/kg/d. TDAR was determined as the primary antibody response to the T cell dependent antigen in SRBCs. The day after exposure ended, SRBCs were introduced intravenously and SRBC-specific IgM was measured 5 days later (DeWitt, et al. 2009).

Key Event Description

The production of antibodies to T cell-dependent antigens is a coordinated process involving B cells, antigen-presenting cells, and T cell derived cytokines. The B cells are stimulated to proliferate and differentiate. The TDAR might be altered if any of these cell populations are affected.

IL-2 and IL-4 are produced and secreted by helper T cells. Both are important in the development of TDAR. IL-4 affects maturation and class switching of B cells as well as proliferation. Both events induce and enhance TDAR. IL-2 promotes differentiation of B cells, which stimulates differentiation of activated T cells to Th2 cells. The suppressed production of IL-2 and IL-4 impairs TDAR (Justiz Vaillant and Qurie 2020).

A mutant form of human IL-4, in which the tyrosine residue at position 124 is replaced by aspartic acid (hIL-4Y124D), was reported to specifically block IL-4 and IL-13-induced proliferation of B cells. In addition, hIL-4Y124D also strongly inhibited both IL-4- or IL-13-induced IgG4 and IgE synthesis in cultures of PBMCs, or highly purified sIgD+ B cells cultured in the presence of anti-CD40 monoclonal antibodies. IL-4 may be necessary to produce antibodies and to proliferate in B cells. The mutation of IL 4 may impair TDAR (Aversa, et al. 1993).

IL-4 stimulates B cells to proliferate, switch immunoglobulin classes, and differentiate to plasma and memory cells. Suppressing the production of these B cell related cytokines appears to impair TDAR, as evident from the results of FK506

treatment (Heidt, et al. 2010).

STAT5 is able to inhibit peroxisome proliferator activated receptor (PPAR)-regulated gene transcription. Conversely, ligand-activated PPAR can inhibit STAT5-regulated transcription. As a peroxisome proliferator, perfluorooctanoic acid (PFOA) induces PPARs. The suppression of TDAR has been observed with a no observable effect level (NOEL) of 1.88 mg/kg/d and lowest observed adverse effect level (LOEL) of 3.75 mg/kg/d for PFOA administered in drinking water over 15 days (Dewitt, et al. 2008). The increase in PPAR expression induced by PFOA may inhibit STAT5-regulated transcription, which is important for IL-4 production in TDAR.

How it is Measured or Detected

TDAR can be examined in vivo and in vitro. In vivo studies of antigen-specific antibodies are usually performed by measuring serum antibody levels with ELISA (Onda, et al. 2014) or with a plaque-forming cell (PFC) assay.

To assess keyhole limpet hemocyanin (KLH) antigen-specific T cell proliferation, 1×10^5 CD4+ T cells were co-cultured with 2×10^5 autologous PBMCs in 96-well plates in the presence of KLH. Cells were cultured for 5 or 7 days before being pulsed with 0.5 µCi ^{3}[H]-thymidine (PerkinElmer) for 18 h. The cells were harvested using a 96-well cell FilterMate harvester. ^{3}[H]-thymidine incorporation in CD4+ T cell response to biopharmaceuticals was measured by liquid scintillation counting using a TopCount NXT (Schultz, et al. 2017).

In another in vivo study, rats were repeatedly administered FK506 orally for 4 weeks and immunized with KLH. Rat serum was examined for T cell dependent, antigen-specific IgM and IgG levels by ELISA (Ulrich, et al. 2004).

Other authors repeatedly administered CNIs, including FK506 and CsA, to mice orally for 4 days and immunized with sheep red blood cells (SRBCs). Spleen cells were examined using a PFC assay (Kino, et al. 1987). Antigen-specific plaque-forming splenocytes were reduced at doses of 3.2, 10, 32, and 100 mg/kg of FK506 or 32 and 100 mg/kg CsA.

In another study, cynomolgus monkeys received 50 mg/kg CsA twice a day via oral gavage (10 h apart) for 23 days and were immunized with SRBCs. Serum was examined for anti-SRBC IgM and IgG levels using an ELISA specific for SRBC antigen (Gaida, et al. 2015).

In the final in vivo study cited here, mice were exposed to a single pharyngeal aspiration of 1,2:5,6-Dibenzanthracene, after which the supernatants of splenocytes were cultured for 24 h in the presence of lipopolysaccharide and assayed using a mouse IgM or IgG matched pairs antibody kit (Smith, et al. 2010).

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

In one study, T and B cells isolated from human PBMCs were co-cultured with CNIs for 9 days in the presence of polyclonal T cell stimulation. The supernatants were examined for IgM and IgG levels by ELISA. Treatment with FK506 or CsA reduced the levels of IgM and IgG at concentrations of 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) or 50 and 100 ng/mL (41.6 and 83.2 nM), respectively (Heidt, et al. 2010).

In another study, SKW6.4 IL-6-dependent IgM-secreting human B cells were cultured for 4 days with anti-CD3/CD28 antibody-stimulated PBMC culture supernatant. IgM produced in the culture supernatants was measured by ELISA. FK506 or CsA reduced the levels of IgM at concentrations of 0.01 to 100 ng/mL or 0.1 to 1000 ng/mL (Sakuma, et al. 2001).

Regulatory Significance of the AO

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

The ICH S8 immunotoxicity testing guideline on pharmaceuticals recommends that TDAR can be evaluated whenever the target cells of immunotoxicity are not clear based on pharmacology and findings in standard toxicity studies. For the assessment of pesticides, the United States Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances 870.7800 immunotoxicity testing guideline recommends TDAR using SRBC.

Finally, the draft Food and Drug Administration guidance of nonclinical safety evaluation for immunotoxicology recommends the TDAR assay.

References

Aversa G, Punnonen J, Cocks BG, de Waal Malefyt R, Vega F, Jr., Zurawski SM, Zurawski G, de Vries JE. 1993. An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: support for a common component shared by IL-4 and IL-13 receptors. J Exp Med 178:2213-2218. DOI: 10.1084/jem.178.6.2213.

Dewitt JC, Copeland CB, Strynar MJ, Luebke RW. 2008. Perfluorooctanoic acid-induced immunomodulation in adult C57BL/6J or C57BL/6N female mice. Environ Health Perspect 116:644-650. DOI: 10.1289/ehp.10896.

DeWitt JC, Shnyra A, Badr MZ, Loveless SE, Hoban D, Frame SR, Cunard R, Anderson SE, Meade BJ, Peden-Adams MM, Luebke RW, Luster MI. 2009. Immunotoxicity of perfluorooctanoic acid and perfluorooctane sulfonate and the role of peroxisome proliferator-activated receptor alpha. Crit Rev Toxicol 39:76-94. DOI: 10.1080/10408440802209804.

Gaida K, Salimi-Moosavi H, Subramanian R, Almon V, Knize A, Zhang M, Lin FF, Nguyen HQ, Zhou L, Sullivan JK, Wong M,

McBride HJ. 2015. Inhibition of CRAC with a human anti-ORAI1 monoclonal antibody inhibits T-cell-derived cytokine production but fails to inhibit a T-cell-dependent antibody response in the cynomolgus monkey. J Immunotoxicol 12:164-173. DOI: 10.3109/1547691X.2014.915897.

Heidt S, Roelen DL, Eijsink C, Eikmans M, van Kooten C, Claas FH, Mulder A. 2010. Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. Clin Exp Immunol 159:199-207. DOI: 10.1111/j.1365-2249.2009.04051.x.

Justiz Vaillant AA, Qurie A. 2020. Interleukin. In *StatPearls*: Treasure Island (FL)

Kino T, Hatanaka H, Hashimoto M, Nishiyama M, Goto T, Okuhara M, Kohsaka M, Aoki H, Imanaka H. 1987. FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. J Antibiot (Tokyo) 40:1249-1255. DOI: 10.7164/antibiotics.40.1249.

Onda M, Ghoreschi K, Steward-Tharp S, Thomas C, O'Shea JJ, Pastan IH, FitzGerald DJ. 2014. Tofacitinib suppresses antibody responses to protein therapeutics in murine hosts. J Immunol 193:48-55. DOI: 10.4049/jimmunol.1400063.

Sakuma S, Kato Y, Nishigaki F, Magari K, Miyata S, Ohkubo Y, Goto T. 2001. Effects of FK506 and other immunosuppressive anti-rheumatic agents on T cell activation mediated IL-6 and IgM production in vitro. Int Immunopharmacol 1:749-757.

Schultz HS, Reedtz-Runge SL, Backstrom BT, Lamberth K, Pedersen CR, Kvarnhammar AM, consortium A. 2017. Quantitative analysis of the CD4+ T cell response to therapeutic antibodies in healthy donors using a novel T cell:PBMC assay. PLoS One 12:e0178544. DOI: 10.1371/journal.pone.0178544.

Smith DC, Smith MJ, White KL. 2010. Systemic immunosuppression following a single pharyngeal aspiration of 1,2:5,6-dibenzanthracene in female B6C3F1 mice. J Immunotoxicol 7:219-231. DOI: 10.3109/1547691X.2010.487193.

Ulrich P, Paul G, Perentes E, Mahl A, Roman D. 2004. Validation of immune function testing during a 4-week oral toxicity study with FK506. Toxicol Lett 149:123-131. DOI: 10.1016/j.toxlet.2003.12.069.

Yang Q, Abedi-Valugerdi M, Xie Y, Zhao XY, Moller G, Nelson BD, DePierre JW. 2002. Potent suppression of the adaptive immune response in mice upon dietary exposure to the potent peroxisome proliferator, perfluorooctanoic acid. Int Immunopharmacol 2:389-397. DOI: 10.1016/s1567-5769(01)00164-3.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2024: Inhibition of JAK3 leads to STAT5 inhibition

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Nelson et al. reported that a membrane proximal region of the interleukin-2 receptor gamma c chain sufficient for Jak kinase

activation and induction of proliferation in T cells (Nelson, et al. 1996). Furthermore, Kirken RA et al. demonstrated that activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and STAT5 recruitment by a COOH-terminal region of the IL-2 receptor beta-chain (Kirken, et al. 1995). Therefore, STAT activation is regulated by JAK via phosphorylation. Thus, JAK inhibitors commonly interfere with STAT activation.

Evidence Supporting this KER

STAT5 refer to two proteins that share 94% structural homology and are transcribed from separate genes, STAT5A and STAT5B. Binding of these extracellular ligands to their target receptors induces the activation of receptor-associated JAK kinases that phosphorylate key tyrosine residues within the receptor, providing docking sites for the SRC homology 2 (SH2) domains of the inactive cytoplasmic STAT5 monomers. STAT5 is then phosphorylated at specific tyrosine residues, either Y694 (STAT5A) or Y699 (STAT5B) of the C-terminus. Subsequently, STAT5 undergoes a conformational change and phosphorylated STAT5 monomers form either homo- or hetero- STAT5X-STATX dimers through reciprocal phosphotyrosine–SH2 interactions (Cumaraswamy, et al. 2014, Tothova, et al. 2021) This means that STAT5 will never be activated without this phosphorylation step by JAK3.

Biological Plausibility

STAT5 plays a major role in regulating vital cellular functions, such as proliferation, differentiation, and apoptosis, of hematopoietic and immune cells (Wakao, et al. 1992). STAT5 is activated by JAK3 phosphorylation of a single tyrosine residue (Y694).

Empirical Evidence

GM-CSF-induced phosphorylation of STAT5 is inhibited by the RB1 selective JAK3 inhibitor. This suggests that JAK3 inhibition downregulates STAT5-dependent cytokine signaling (Al-Shami, et al. 1998).

Quantitative Understanding of the Linkage

The fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes was reportedly increased upon incubation of peripheral blood with IL-2. Peficitinib is a pan-JAK family inhibitor that can inhibit STAT5 phosphorylation in a concentration-dependent manner with a mean IC50 of 124 nM (101 and 147 nM for two rats). The effect of peficitinib on IL-2 stimulated STAT5 phosphorylation in human peripheral T cells has been evaluated. In parallel with results obtained from rats, the fluorescence intensity of phospho-STAT5 in CD3-positive lymphocytes increased in human peripheral blood after the addition of IL-2, but peficitinib inhibited STAT5 phosphorylation in a dose-dependent manner with a mean IC50 of 127 nM in human lymphocytes (Gianti and Zauhar 2015).

Response-response relationship

MIE:

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

Normal rats were administered peficitinib at 10 and 20 mg/kg. Thirteen hours later, the animals were bled and STAT5 phosphorylation was assessed. IL-2-induced STAT5 phosphorylation of CD3-positive lymphocytes in peripheral blood from the peficitinib-treated rats was suppressed by 37% at a dose of 10 mg/kg and 78% at 20 mg/kg (Gianti and Zauhar 2015).

Time-scale

The enzymatic activities against JAK1, JAK2, JAK3, and TYK2 were immediately tested in CTLL-2 cells using a Caliper Mobility Shift Assay with an ATP concentration at Km (Pei, et al. 2018). CTLL-2 cells were treated with 10 μM adenosine (plus coformycin) for 15 min at 37°C and then stimulated with IL-2 (10 U/mL) for different lengths of time (5 min-12 h). Adenosine dramatically decreased dose-dependent STAT5A/B tyrosine phosphorylation in response to IL-2 over the entire 12 h time course (Zhang, et al. 2004).

References

Al-Shami A, Mahanna W, Naccache PH. 1998. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Selective activation of Jak2, Stat3, and Stat5b. J Biol Chem 273:1058-1063. DOI: 10.1074/jbc.273.2.1058.

Cumaraswamy AA, Lewis AM, Geletu M, Todic A, Diaz DB, Cheng XR, Brown CE, Laister RC, Muench D, Kerman K, Grimes HL, Minden MD, Gunning PT. 2014. Nanomolar-Potency Small Molecule Inhibitor of STAT5 Protein. ACS Med Chem Lett 5:1202-1206. DOI: 10.1021/ml500165r.

Gianti E, Zauhar RJ. 2015. An SH2 domain model of STAT5 in complex with phospho-peptides define "STAT5 Binding Signatures". J Comput Aided Mol Des 29:451-470. DOI: 10.1007/s10822-015-9835-6.

Kirken RA, Rui H, Malabarba MG, Howard OM, Kawamura M, O'Shea JJ, Farrar WL. 1995. Activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and STAT5 recruitment by a COOH-terminal region of the IL-2 receptor beta-chain. Cytokine 7:689-700. DOI: S1043466685700816 [pii]

10.1006/cyto.1995.0081.

Nelson BH, Lord JD, Greenberg PD. 1996. A membrane-proximal region of the interleukin-2 receptor gamma c chain sufficient for Jak kinase activation and induction of proliferation in T cells. Mol Cell Biol 16:309-317. DOI: 10.1128/MCB.16.1.309.

Pei H, He L, Shao M, Yang Z, Ran Y, Li D, Zhou Y, Tang M, Wang T, Gong Y, Chen X, Yang S, Xiang M, Chen L. 2018. Discovery of a highly selective JAK3 inhibitor for the treatment of rheumatoid arthritis. Sci Rep 8:5273. DOI: 10.1038/s41598-018-23569-y.

Tothova Z, Tomc J, Debeljak N, Solar P. 2021. STAT5 as a Key Protein of Erythropoietin Signalization. Int J Mol Sci 22. DOI: 7109 [pii]10.3390/ijms22137109ijms22137109 [pii].

Wakao H, Schmitt-Ney M, Groner B. 1992. Mammary gland-specific nuclear factor is present in lactating rodent and bovine mammary tissue and composed of a single polypeptide of 89 kDa. J Biol Chem 267:16365-16370.

Zhang H, Conrad DM, Butler JJ, Zhao C, Blay J, Hoskin DW. 2004. Adenosine acts through A2 receptors to inhibit IL-2-induced tyrosine phosphorylation of STAT5 in T lymphocytes: role of cyclic adenosine 3',5'-monophosphate and phosphatases. J Immunol 173:932-944. DOI: 10.4049/jimmunol.173.2.932.

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

STAT proteins bind with their SH2 domains (which are located between amino acids 600 and 700) to phosphorylated tyrosine residues of transmembrane receptors (Heim, et al. 1995, Stahl, et al. 1995). Once STATs are bound to the receptors, the receptor-associated Jak kinases phosphorylate them on a single tyrosine residue located carboxy terminal of the SH2 domain. Changing this tyrosine to phenylalanine results in STATs that are no longer functional (Shuai, et al. 1993). Two STATs dimerize through specific reciprocal SH2–phosphotyrosine interaction and translocate to the nucleus. After translocation into the nucleus, STATs bind DNA response elements in promoters of target genes. The putative DNA-binding domain lies between amino acids 400 and 500. After DNA binding STATs interact directly or indirectly with the RNA polymerase II complex. The DNA sequence elements in the promoters of genes that bind STAT proteins can be classified in two groups. The prototype of the first class is the interferon-stimulated response element (ISRE).

The second class comprises the GAS-like response elements. STAT5 homodimers have been shown to bind to at least one of the GAS-like elements (Heim 1996).

Evidence Supporting this KER

The observation that STAT5a/STAT5b/double KO mice are defective in IL-2-induced IL-2Rα expression, suggested that STAT5 is essential for this expression (Kim, et al. 2001, Moriggl, et al. 1999).

In another study, CD25 associated with the intermediate affinity IL-2Rβγ subunits to form the high-affinity heterotrimeric IL-2Rαβγ. In response to ligation with IL-2, signaling of the complex through the IL-2Rβγ chains resulted in the phosphorylation of STAT5 (Waldmann 2006).

STAT5a/b mutant peripheral T cells in mice are profoundly deficient in proliferation and fail to undergo cell cycle progression or to express genes controlling cell cycle progression. STAT5 proteins are essential mediators of IL-2 signaling in T cells (Willerford, et al. 1995).

IL-2 binding to CD25 triggers the grouping with IL-2Rβ andγ chains, leading to signal transduction through STAT5, mitogen-activated protein kinase, and phosphoinositide 3-kinases (PI3Ks) (Fujii, et al. 1995, Ravichandran and Burakoff 1994, Remillard, et al. 1991). Within all T cell populations, IL-2 signaling appears to be primarily mediated through phosphorylation of STAT5 (Hirakawa, et al. 2016).

Biological Plausibility

Upon T cell receptor stimulation, IL-2/STAT5 signaling promotes T cell differentiation. This is the first key step in generating effector T cells that can target pathogens (Liao, et al. 2013).

Increasing the concentrations of IL-2 to superphysiological levels (1000 units/mL), which would eliminate the required upregulation of the IL-2 receptor α chain, also failed to induce a proliferative response in cells from Stat5a/b mutant mice (Willerford, et al. 1995).

Splenic lymphocytes from STAT5a/b, but not STAT5a or STAT5b, mutant mice failed to significantly respond to increasing concentrations of IL-2 in the presence of anti-CD3 (Moriggl, et al. 1999).

Empirical Evidence

Reversible protein phosphorylation plays a key role in IL-2 receptor-mediated activation of JAK3 and STAT5 in lymphocytes (Ross, et al. 2010).

In another study, adenosine was shown to act through A2 receptors and associated cAMP/protein kinase A-dependent signaling pathways to activate Src homology region 2 domain-containing phosphatase-2 (SHP-2) and cause STAT5 dephosphorylation. The dephosphorylation resulted in reduced IL-2R signaling in T cells (Zhang, et al. 2004).

Quantitative Understanding of the Linkage

CD2 signaling of human PBMCs results in activation of the -3.6-kb IFN-γ promoter. In contrast, mutation of the -3.6-kb STAT5 site attenuates promoter activity. Functional activation is accompanied by STAT5A, but scant STAT5B nucleoprotein binds to the STAT5 binding site on the IFN-γ promoter, as determined by competition and supershift assays. Western and fluorescence-activated cell sorting analyses revealed increased phospho-STAT5 following CD2 signaling (Gonsky, et al. 2004).

Response-response relationship

Inhibition of phosphatase activity by calyculin A treatment of YT cells resulted in a significant induction of serine phosphorylation of JAK3 and STAT5, and serine/threonine phosphorylation of IL-2Rβ. Moreover, inhibition of protein phosphatase 2 (PP2A) diminished IL-2-induced tyrosine phosphorylation of IL-2Rβ, JAK3, and STAT5, and abolished STAT5 DNA binding activity (Ross, et al. 2010).

Known modulating factors

As a property of STAT, it is known that DNA binding ability is acquired by forming a dimer, and it is considered that a modifying factor does not intervene in that respect.

Known Feedforward/Feedback loops influencing this KER

IL-2 acts on the same cell that secretes the cytokine. For instance, IL-2 produced by T cells operates on the same T cells that produce this cytokine, or on neighboring cells. With the highest levels in secondary lymphoid organs, IL-2 is believed to act in an autocrine or paracrine manner to support effector and memory CD8 T cell differentiation (Kalia and Sarkar 2018).

References

Fujii H, Nakagawa Y, Schindler U, Kawahara A, Mori H, Gouilleux F, Groner B, Ihle JN, Minami Y, Miyazaki T, et al. 1995. Activation of Stat5 by interleukin 2 requires a carboxyl-terminal region of the interleukin 2 receptor beta chain but is not essential for the proliferative signal transmission. Proc Natl Acad Sci U S A 92:5482-5486. DOI: 10.1073/pnas.92.12.5482.

Gonsky R, Deem RL, Bream J, Young HA, Targan SR. 2004. Enhancer role of STAT5 in CD2 activation of IFN-gamma gene expression. J Immunol 173:6241-6247. DOI: 10.4049/jimmunol.173.10.6241.

Heim MH. 1996. The Jak-STAT pathway: specific signal transduction from the cell membrane to the nucleus. Eur J Clin Invest 26:1-12. DOI: 10.1046/j.1365-2362.1996.103248.x.

Heim MH, Kerr IM, Stark GR, Darnell JE, Jr. 1995. Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. Science 267:1347-1349. DOI: 10.1126/science.7871432.

Hirakawa M, Matos TR, Liu H, Koreth J, Kim HT, Paul NE, Murase K, Whangbo J, Alho AC, Nikiforow S, Cutler C, Ho VT, Armand P, Alyea EP, Antin JH, Blazar BR, Lacerda JF, Soiffer RJ, Ritz J. 2016. Low-dose IL-2 selectively activates subsets of CD4(+) Tregs and NK cells. JCI Insight 1:e89278. DOI: 10.1172/jci.insight.89278.

Kalia V, Sarkar S. 2018. Regulation of Effector and Memory CD8 T Cell Differentiation by IL-2-A Balancing Act. Front Immunol 9:2987. DOI: 10.3389/fimmu.2018.02987.

Kim HP, Kelly J, Leonard WJ. 2001. The basis for IL-2-induced IL-2 receptor alpha chain gene regulation: importance of two widely separated IL-2 response elements. Immunity 15:159-172.

Liao W, Lin JX, Leonard WJ. 2013. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity 38:13-25. DOI: 10.1016/j.immuni.2013.01.004.

Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249-259.

Ravichandran KS, Burakoff SJ. 1994. The adapter protein Shc interacts with the interleukin-2 (IL-2) receptor upon IL-2 stimulation. J Biol Chem 269:1599-1602.

Remillard B, Petrillo R, Maslinski W, Tsudo M, Strom TB, Cantley L, Varticovski L. 1991. Interleukin-2 receptor regulates activation of phosphatidylinositol 3-kinase. J Biol Chem 266:14167-14170.

Ross JA, Cheng H, Nagy ZS, Frost JA, Kirken RA. 2010. Protein phosphatase 2A regulates interleukin-2 receptor complex formation and JAK3/STAT5 activation. J Biol Chem 285:3582-3591. DOI: 10.1074/jbc.M109.053843.

Shuai K, Stark GR, Kerr IM, Darnell JE, Jr. 1993. A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. Science 261:1744-1746. DOI: 10.1126/science.7690989.

Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell JE, Jr., Yancopoulos GD. 1995. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. Science 267:1349-1353. DOI: 10.1126/science.7871433.

Waldmann TA. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol 6:595-601. DOI: 10.1038/nri1901.

Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW. 1995. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity 3:521-530.

Zhang H, Conrad DM, Butler JJ, Zhao C, Blay J, Hoskin DW. 2004. Adenosine acts through A2 receptors to inhibit IL-2-induced tyrosine phosphorylation of STAT5 in T lymphocytes: role of cyclic adenosine 3',5'-monophosphate and phosphatases. J Immunol 173:932-944. DOI: 10.4049/jimmunol.173.2.932.

Relationship: 2026: Suppression of STAT5 binding to cytokine gene promoters leads to Suppression of IL-4 production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

A STAT5 binding site (TTCATGGAA) has been identified in intron 2 of the Il4 gene, near HSII (Hural, et al. 2000). Another potential STAT5 binding site (TTCTAAGAA) is conserved between mice and humans, and is located near HSIII. STAT5A binds to the sites near HSII and HSIII, which could provide a mechanism through which STAT5A mediates Il4 gene accessibility and participates in the induction of IL-4 production. Enhanced STAT5 signaling results in a larger proportion of cells producing IL-4. A consensus STAT site that preferentially associates with STAT5 contributes to its enhancer activity in mast cells. The intron element plays a role in acquiring and/or maintaining the IL-4 gene locus in a demethylated state in IL-4-producing cells.

Constitutively active STAT5A (STAT5A1*6) restores the capacity to produce IL-4 in cells primed under Th2 conditions in the absence of IL-2, suggesting that STAT5 activation plays a critical role in Th2 differentiation (Zhu, et al. 2003, Zhu, et al. 2004). Additionally, IL-2 critically regulates Th2 differentiation in a STAT5-dependent manner, acting early at the locus encoding IL-4Ra to induce expression of this receptor (IL-4Rα) (Liao, et al. 2008) and later to open chromatin accessibility at the Th2 locus, which encodes IL-4 and IL-13 (Cote-Sierra, et al. 2004).

The development of Th2 cells was reportedly impaired in STAT5a-/-CD4+ T cells, even in the presence of IL-4. Retrovirus-mediated expression of STAT5A restored Th2 cell differentiation in STAT5a-/-CD4+ T cells. Th2 cell-mediated immune responses were diminished in STAT5a-/- mice. When stimulated with anti-CD3 mAb, CD4+ T cells that produced IL-4, but not IFN-γ (Th2 cells), were significantly decreased in STAT5a-/- mice compared with those in wild-type mice, suggesting that STAT5A plays a regulatory role in T helper cell differentiation (Kagami, et al. 2001).

Evidence Supporting this KER

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

5C.C7 cells infected with a retrovirus expressing a constitutively active form of STAT5A (STAT5A1*6) were shown to be primed for IL-4 production.

STAT5a/b mutant peripheral T cells in mice are profoundly deficient in proliferation and fail to undergo cell cycle progression or to express genes controlling cell cycle progression. STAT5 proteins are essential mediators of IL-2 signaling in T cells (Willerford, et al. 1995).

IL-2 is one of the earliest cytokines produced by activated T cells and mediates its actions primarily through the activation of STAT5 proteins. A STAT5-chromatin immunoprecipitation assay (ChIP) was performed using chromatin from freshly isolated CD4 T cells to identify in vivo IL-2-activated STAT5 gene targets. The immunoprecipitated chromatin yielded a number of distinct clones based on sequencing. One clone mapped to chromosome 16 152,916 to 153,096 upstream of the C-MAF gene, and contained a consensus GAS motif (Rani, et al. 2011).

Heat map analysis of expression profiles of IL-2 regulated genes (sorted by superenhancer binding scores for STAT5, from strongest to weakest) revealed that STAT5-bound superenhancer-containing genes were highly induced by IL-2 (Li, et al. 2018).

Cells primed under Th2, but not Th1, conditions showed an association of STAT5A with HSII and HSIII. In addition, cells infected with the STAT5A1*6 retrovirus acquired IL-4-producing capacity, and STAT5 was associated with DNA elements near HSII and HSIII (Zhu, et al. 2003).

CD4+ T cell-mediated allergic inflammation was reportedly diminished in STAT5A-deficient (STAT5a-/-) mice. Furthermore, Th2 cell differentiation was also impaired in STAT5a-/- mice, even when purified CD4+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of IL-4 (Kagami, et al. 2001).

Biological Plausibility

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

IL-4 production was reportedly induced by STAT5 phosphorylation. STAT5 phosphorylation facilitates STAT5 dimerization, transport to the nucleus, and gene regulation (56-Levy-2002). PPARs are members of the nuclear hormone receptor superfamily. STAT5 is able to inhibit PPAR-regulated gene transcription. Conversely, ligand-activated PPAR can inhibit STAT5-regulated transcription. STAT5 and PPAR disparate pathways are subject to mutually inhibitory crosstalk. The extent of the inhibitory crosstalk was dependent on the relative expression levels of each transcription factor (Shipley and Waxman 2004).

Empirical Evidence

When stimulated with anti-CD3 mAb, CD4 T cells that produced IL-4, but not IFN-γ (Th2 cells), were significantly decreased in STAT5a-/- mice as compared with those in wild-type mice. In contrast, CD4 T cells that produced IFN-γ, but not IL-4 (Th1 cells), were significantly increased in STAT5a-/- mice, and T helper cell differentiation was biased toward Th1 cells in STAT5a-/- mice (Kagami, et al. 2001).

In another study, BALB/c mice were exposed to PFNA (0, 1, 3, or 5 mg/kg/day) for 14 days. Exposure to PFNA led to a decrease in the weight of lymphoid organs. Cell cycle arrest and apoptosis were observed in the spleen and thymus following PFNA exposure. PFNA reduced the production of IL-4 by splenic lymphocytes and was associated with increases in messenger RNA (mRNA) of PPAR (Fang, et al. 2008). In a related study using male Sprague-Dawley rats given the same PFNA doses for the same duration, similar effects were observed on body and thymus weights and mRNA of PPARα.

Other authors described that cells infected with STAT5A retrovirus acquired the capacity to produce IL-4 when cultured in the presence of anti-IL-4; the strength of STAT5 signaling correlated with the percentage of IL-4 producers observed in the primed cell population (Zhu, et al. 2003).

STAT5 interacts with transcriptional regulatory regions and regulates T cell differentiation by enhancing key genes (Adamson, et al. 2009). Th2 differentiation in both mouse and human CD4 T cells is critically dependent on IL-2 (Ben-Sasson, et al. 1990, McDyer, et al. 2002).

Uncertainties and Inconsistencies

GAS is a STAT3-target gene, therefore STAT3 could regulate IL-4 production (Campia, et al. 2015). Additionally, Lederer et al. demonstrated that STAT6 binds to a sequence in the IL-4 promoter (Lederer, et al. 1996).

Quantitative Understanding of the Linkage

$CD4^+$ T cell blasts from BALB/c mice were cultured in the presence or absence of the antioxidant N-acetylcysteine (NAC). T

cells preferentially followed a Th2 differentiation pathway. Treatment of $CD4^+$ T cell blasts with 10 mM NAC increased Th1 cytokine production and decreased IL-4 production as compared to untreated controls. T cells treated with NAC also showed decreased levels of phosphorylated STAT5 (Shatynski, et al. 2012).

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

Response-response relationship

Once STATs are recruited to the activated JAK/receptor complex and are tyrosine phosphorylated within the SH2 domain by JAKs, they form dimers and/or tetramers, translocate to the nucleus, and associate with promoter regions, such as gamma activated sequence (GAS) elements. STAT dimers can bind to GAS DNA sequences (TTCN3GAA) to induce transcription. The STAT5 dimers can also form tetramers through interactions between residues (I28, F81, and L82) in their N-terminal regions. These STAT5 tetramers bind to pairs of GAS motifs separated by a linker of 6–22 nucleotides (Lin, et al. 2012). Mutational studies have demonstrated that STAT5 is important for IL-2-induced gene expression. The interaction of STATs with gene promoters can enhance the expression of its target genes (Able, et al. 2017).

It was reported that while the wild-type construct displayed 4.6-fold IL-2 inducibility in YT cells, selective mutation of GAScI (M1), GASn (M2), and GAScII (M3) motifs modestly lowered IL-2 inducibility (M1 1.7-fold, M2 2.9-fold, M3 1.6-fold, respectively). Double mutation of GAScI and GASn (M4) or GASn and GAScII (M5) more potently decreased IL-2 inducibility, and simultaneous mutation of GAScI and GAScII (M6) or of all the GAS motifs (M7) abrogated IL-2 inducibility (M4 1.2-fold, M5 1.4-fold, M6 1.0-fold, M7 1.0-fold, respectively). These results suggest that all the GAS motifs are required for maximal IL-2 inducibility, including IL-4 induction (Kim, et al. 2001).

Time-scale

A STAT5 binding site (TTCATGGAA) has been identified in intron 2 of the Il4 gene. HS V (also known as CNS2) is a 3’ enhancer in the Il4 locus. HS V is essential for IL-4 production by Tfh cells. Mice lacking HS V display marked defects in Th2 humoral immune responses, as evidenced by abrogated IgE and sharply reduced IgG1 production in vivo. HS V-deficient (ΔV) mice displayed complete abrogation of IgE production despite only mild reduction in Th2 responses. HS V-deficiency affected Il4 transcription in T cells naïve T cells lacking the HS V (CNS2) region were completely unable to produce Il4 transcripts following ex vivo stimulation with anti-CD3 and anti-CD28 antibodies for 180 min. In a similar time course assay (240 min), in vitro differentiated Th2 cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin showed only a 50% reduction in Il4 transcription (Vijayanand, et al. 2012).

Phosphorylation of STAT5 was reportedly decreased by nearly two-fold in NOX2-deficient T cells as compared to that in wild-type controls by intracellular staining 12 and 24 h after activation with immobilized anti-CD3 and soluble anti-CD28. PCR analysis also revealed decreases in Il4 and Il4rα mRNA expression in NOX2-deficient T cells (Shatynski, et al. 2012).

Known modulating factors

Adenosine can inhibit IL-2-dependent proliferation of CTLL-2 T cells. This inhibition was reportedly associated with a reduction in tyrosine phosphorylation of STAT5A and STAT5B, which was mediated by the activation of a protein tyrosine phosphatase (PTP). The PTP Src homology region 2 domain-containing phosphatase-2 (SHP-2) was implicated in STAT5A/B dephosphorylation because adenosine strongly increased tyrosine phosphorylation of SHP-2 and the formation of complexes consisting of SHP-2 and STAT5 in IL-2- stimulated CTLL-2 T cells. In contrast, adenosine did not affect the phosphorylation status of the upstream kinases JAK1 or JAK3. The inhibitory effect of adenosine on STAT5A/B phosphorylation was mediated through cell surface A_{2a} and A_{2b} receptors, and involved associated cAMP/protein kinase A (PKA)-dependent signaling pathways (Zhang, et al. 2004).

Known Feedforward/Feedback loops influencing this KER

STAT5 can upregulate a number of molecules, including cytokine-inducible SH2 proteins (CIS family, also referred to as the SOCS or SSI family) (Yasukawa, et al. 2000). Some CIS family proteins might be involved in the cross-regulation of cytokine networks and may regulate Th1 and Th2 cell differentiation (Dickensheets, et al. 1999, Losman, et al. 1999). CIS1, a prototype of CIS family proteins, is induced by STAT5 and inhibits STAT5 activation by blocking the interaction between STAT5 and cytokine receptors (Yasukawa, et al. 2000). Thus, CIS1 seems to function in classical negative feedback of STAT5 signaling.

IL-2 acts on the same cell that secretes the cytokine. For instance, IL-2 produced by T cells operates on the same T cells that make this cytokine or on nearby cells. With the highest levels in secondary lymphoid organs, IL-2 is believed to act in an autocrine or paracrine manner to support effector and memory CD8 T cell differentiation (Kalia and Sarkar 2018). IL-2Rα expression is triggered by antigens, mitogen lectins, or antibodies to the TCR through STAT5. These signals also result in the secretion of IL-2, which in turn can increase and prolong IL-2Rα expression, thus acting as a positive feedback regulator of its own high-affinity receptor (Waldmann 1989). Therefore, STAT5 deficiency disrupted T cell function.

References

Able AA, Burrell JA, Stephens JM. 2017. STAT5-Interacting Proteins: A Synopsis of Proteins that Regulate STAT5 Activity. Biology (Basel) 6. DOI: 10.3390/biology6010020.

Adamson AS, Collins K, Laurence A, O'Shea JJ. 2009. The Current STATus of lymphocyte signaling: new roles for old players. Curr Opin Immunol 21:161-166. DOI: 10.1016/j.coi.2009.03.013.

Ben-Sasson SZ, Le Gros G, Conrad DH, Finkelman FD, Paul WE. 1990. IL-4 production by T cells from naive donors. IL-2 is required for IL-4 production. J Immunol 145:1127-1136.

Campia I, Buondonno I, Castella B, Rolando B, Kopecka J, Gazzano E, Ghigo D, Riganti C. 2015. An Autocrine Cytokine/JAK/STAT-Signaling Induces Kynurenine Synthesis in Multidrug Resistant Human Cancer Cells. PLoS One 10:e0126159. DOI: 10.1371/journal.pone.0126159

PONE-D-14-48346 [pii].

Cote-Sierra J, Foucras G, Guo L, Chiodetti L, Young HA, Hu-Li J, Zhu J, Paul WE. 2004. Interleukin 2 plays a central role in Th2 differentiation. Proc Natl Acad Sci U S A 101:3880-3885. DOI: 10.1073/pnas.0400339101.

Dickensheets HL, Venkataraman C, Schindler U, Donnelly RP. 1999. Interferons inhibit activation of STAT6 by interleukin 4 in human monocytes by inducing SOCS-1 gene expression. Proc Natl Acad Sci U S A 96:10800-10805. DOI: 10.1073/pnas.96.19.10800.

Fang X, Zhang L, Feng Y, Zhao Y, Dai J. 2008. Immunotoxic effects of perfluorononanoic acid on BALB/c mice. Toxicol Sci 105:312-321. DOI: 10.1093/toxsci/kfn127.

He X, Smeets RL, Koenen HJ, Vink PM, Wagenaars J, Boots AM, Joosten I. 2011. Mycophenolic acid-mediated suppression of human CD4+ T cells: more than mere guanine nucleotide deprivation. Am J Transplant 11:439-449. DOI: 10.1111/j.1600-6143.2010.03413.x.

Hural JA, Kwan M, Henkel G, Hock MB, Brown MA. 2000. An intron transcriptional enhancer element regulates IL-4 gene locus accessibility in mast cells. J Immunol 165:3239-3249. DOI: 10.4049/jimmunol.165.6.3239.

Kagami S, Nakajima H, Suto A, Hirose K, Suzuki K, Morita S, Kato I, Saito Y, Kitamura T, Iwamoto I. 2001. Stat5a regulates T helper cell differentiation by several distinct mechanisms. Blood 97:2358-2365. DOI: 10.1182/blood.v97.8.2358.

Kalia V, Sarkar S. 2018. Regulation of Effector and Memory CD8 T Cell Differentiation by IL-2-A Balancing Act. Front Immunol 9:2987. DOI: 10.3389/fimmu.2018.02987.

Kim HP, Kelly J, Leonard WJ. 2001. The basis for IL-2-induced IL-2 receptor alpha chain gene regulation: importance of two widely separated IL-2 response elements. Immunity 15:159-172. DOI: 10.1016/s1074-7613(01)00167-4.

Lederer JA, Perez VL, DesRoches L, Kim SM, Abbas AK, Lichtman AH. 1996. Cytokine transcriptional events during helper T cell subset differentiation. J Exp Med 184:397-406. DOI: 10.1084/jem.184.2.397.

Li Y, Liu X, Wang W, Wang S, Zhang J, Jiang S, Wang Y, Li L, Li J, Zhang Y, Huang H. 2018. Low-dose IL-2 expands CD4(+) regulatory T cells with a suppressive function in vitro via the STAT5-dependent pathway in patients with chronic kidney diseases. Ren Fail 40:280-288. DOI: 10.1080/0886022X.2018.1456462.

Liao W, Schones DE, Oh J, Cui Y, Cui K, Roh TY, Zhao K, Leonard WJ. 2008. Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. Nat Immunol 9:1288-1296. DOI: 10.1038/ni.1656.

Lin JX, Li P, Liu D, Jin HT, He J, Ata Ur Rasheed M, Rochman Y, Wang L, Cui K, Liu C, Kelsall BL, Ahmed R, Leonard WJ. 2012. Critical Role of STAT5 transcription factor tetramerization for cytokine responses and normal immune function. Immunity 36:586-599. DOI: 10.1016/j.immuni.2012.02.017.

Liu Y, Yang T, Li H, Li MH, Liu J, Wang YT, Yang SX, Zheng J, Luo XY, Lai Y, Yang P, Li LM, Zou Q. 2013. BD750, a benzothiazole derivative, inhibits T cell proliferation by affecting the JAK3/STAT5 signalling pathway. Br J Pharmacol 168:632-643. DOI: 10.1111/j.1476-5381.2012.02172.x.

Losman JA, Chen XP, Hilton D, Rothman P. 1999. Cutting edge: SOCS-1 is a potent inhibitor of IL-4 signal transduction. J Immunol 162:3770-3774.

McDyer JF, Li Z, John S, Yu X, Wu CY, Ragheb JA. 2002. IL-2 receptor blockade inhibits late, but not early, IFN-gamma and CD40 ligand expression in human T cells: disruption of both IL-12-dependent and -independent pathways of IFN-gamma production. J Immunol 169:2736-2746. DOI: 10.4049/jimmunol.169.5.2736.

Rani A, Afzali B, Kelly A, Tewolde-Berhan L, Hackett M, Kanhere AS, Pedroza-Pacheco I, Bowen H, Jurcevic S, Jenner RG, Cousins DJ, Ragheb JA, Lavender P, John S. 2011. IL-2 regulates expression of C-MAF in human CD4 T cells. J Immunol 187:3721-3729. DOI: 10.4049/jimmunol.1002354.

Shatynski KE, Chen H, Kwon J, Williams MS. 2012. Decreased STAT5 phosphorylation and GATA-3 expression in NOX2-deficient T cells: role in T helper development. Eur J Immunol 42:3202-3211. DOI: 10.1002/eji.201242659.

Shipley JM, Waxman DJ. 2004. Simultaneous, bidirectional inhibitory crosstalk between PPAR and STAT5b. Toxicol Appl Pharmacol 199:275-284. DOI: 10.1016/j.taap.2003.12.020.

Vijayanand P, Seumois G, Simpson LJ, Abdul-Wajid S, Baumjohann D, Panduro M, Huang X, Interlandi J, Djuretic IM, Brown DR, Sharpe AH, Rao A, Ansel KM. 2012. Interleukin-4 production by follicular helper T cells requires the conserved Il4 enhancer hypersensitivity site V. Immunity 36:175-187. DOI: 10.1016/j.immuni.2011.12.014.

Waldmann TA. 1989. The multi-subunit interleukin-2 receptor. Annu Rev Biochem 58:875-911. DOI: 10.1146/annurev.bi.58.070189.004303.

Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW. 1995. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity 3:521-530.

Yasukawa H, Sasaki A, Yoshimura A. 2000. Negative regulation of cytokine signaling pathways. Annu Rev Immunol 18:143-164. DOI: 10.1146/annurev.immunol.18.1.143.

Zhang H, Conrad DM, Butler JJ, Zhao C, Blay J, Hoskin DW. 2004. Adenosine acts through A2 receptors to inhibit IL-2-induced tyrosine phosphorylation of STAT5 in T lymphocytes: role of cyclic adenosine 3',5'-monophosphate and phosphatases. J Immunol 173:932-944. DOI: 10.4049/jimmunol.173.2.932.

Zhu J, Cote-Sierra J, Guo L, Paul WE. 2003. Stat5 activation plays a critical role in Th2 differentiation. Immunity 19:739-748. DOI: 10.1016/s1074-7613(03)00292-9.

Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, Killeen N, Urban JF, Jr., Guo L, Paul WE. 2004. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat Immunol 5:1157-1165. DOI: 10.1038/ni1128.

Relationship: 2027: Suppression of IL-4 production leads to Impairment, TDAR

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

The effects of FK506 on serum concentrations of anti-KLH antibodies IgM and IgG have been demonstrated in rats treated with FK506 for over 4 weeks and immunized with KLH (Ulrich, et al. 2004). The effects of FK506 and CsA on the levels of IgM and IgG in the culture supernatant have been demonstrated in human cells (Heidt, et al. 2010, Sakuma, et al. 2001). In thymectomized mice, the development of KLH-specific effector CD4 T cells was reportedly reduced and these cells were suppressed in their production of IL-4 (Bradley, et al. 1991). The effects of FK506 and CsA on the production of IL-2 have been demonstrated using mice and human cells. These facts suggest that there are no species differences between humans and rodents in the inhibition of IL-4 production and TDAR induction.

Key Event Relationship Description

IL-2 induces T cell proliferation Therefore, the suppression of IL-2 production leads to the impairment of TDAR. The IL-2-JAK3-STAT5 axis regulates Th1 cell differentiation, suggesting that IL-2 mediated JAK3-STAT5 signaling may generically operate in the production of Th1-related cytokines (Shi, et al. 2008).

IL-2 is produced and secreted by helper T cells. IL-2 has important roles in the development of TDAR. IL-2 promotes differentiation of B cells by stimulating differentiation of activated T cells to Th2 T cells. Therefore, suppressed production of IL-2 impairs T cell dependent antibody production.

In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

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

After treatment with FK506 or CsA, production of IL-2, IL-4, and other cytokines decreases in T cells (Dumont, et al. 1998, Dumont, et al. 1998). This reduces stimulation of B cells as well as proliferation, activation, and class switching, leading to impairment of TDAR. Therefore, FK506 and CsA are potent inhibitors of T cell dependent antibody production. Suppression of the production of these B cell related cytokines appears to be the main factor in the impairment of TDAR (Heidt, et al. 2010).

Evidence Supporting this KER

In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Th2 cells assist B cells and

promote class switching from IgM to IgG1 and IgE. Therefore, the suppression of IL-4 production leads to impairment of TDAR.

Biological Plausibility

FK506 and rapamycin suppress the mRNA expression levels of IL-2 and IL-4 in T cells, which stimulate the proliferation of B cells (Heidt, et al. 2010).

Several in vivo studies in rodents have shown decreased TDAR following treatment with FK506(Kino, et al. 1987, Ulrich, et al. 2004). In vitro tests examined antibody production in blood samples obtained from blood bank donors and PBMCs treated with FK506 and CsA. The suppressed production of immunoglobulin (Ig) M and G antibodies to T cell dependent antigens was demonstrated (Heidt, et al. 2010).

T cells, B cells, and antigen-presenting cells, such as dendritic cells, are involved in the induction and development of TDAR. Thus, changes in any of these immune cell populations can influence TDAR. However, concerning the suppression of humoral immunity induced by the inhibition of CN phosphatase activity, CNIs do not affect B cells directly. Rather, the effect is indirect via T cells. FK506 and CsA are capable of inhibiting immunoglobulin production when B cells are cultured with non-pre-activated T cells, but FK506 and CsA fail to inhibit immunoglobulin levels when pre-activated T cells are used to stimulate B cells. Hence, the inhibition of B-cell response by FK506 and CsA appears solely due to inhibition of T helper cells (Heidt, et al. 2010).

Therefore, it is concluded that decreased amounts of IL-4, in addition to IL-2, secreted from helper T cells, is the main factor in the suppression of TDAR.

Empirical Evidence

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

Rationale

In CD3/PMA activated human T cells, FK506 suppressed the production of IL-2, IL-4, and IFN-γ at concentrations of 1.2 to 12.5 nM and inhibited the expression of IL-2, IL-4, and IFN-γ mRNA at concentrations of 10 nM(Dumont, et al. 1998).

After 9-day culture of B cells and non-pre-activated T cell stimulation with FK506 or CsA, the levels of IgM and IgG in the culture supernatant were reduced. The FK506 levels were 0.3 and 1.0 ng/mL (0.37 and 1.24 nM) and the CsA levels were 50 and 100 ng/mL (41 and 83 nM) (Heidt, et al. 2010).

After a 4-day culture of SKW6.4 IL-6-dependent IgM-secreting human B cells and anti-CD3/CD28 stimulation of the PBMC culture supernatant with FK506 or CsA, the level of IgM in the culture supernatant was reduced at concentrations of 0.01 to 100 ng/mL (0.01 to 124 nM) of FK506 and 0.1 to 1000 ng/mL (0.08 to 832 nM) of CsA (Sakuma, et al. 2001).

Rats were treated with FK506 for over 4 weeks and immunized with KLH. The serum concentrations of anti-KLH IgM and IgG were reduced at a dose of 3 mg/kg/day (Ulrich, et al. 2004).

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

Uncertainties and Inconsistencies

IL-2 affects multiple populations of immune cells expressing IL-2 receptors, while IL-4 mainly acts on B cells. Additional suppression of other immune functions may also be possible.

Quantitative Understanding of the Linkage

CsA treatment achieved 100% maximal inhibition of the ex vivo IL-2 response on Days 0, 9, and 16. CsA treatment achieved 82 [± 10]%, 68 [± 25]%, and 82 [± 9]% maximal inhibition of the ex vivo IL-4 response on Days 0, 9, and 16, respectively.

Response-response relationship

In a rat T cell proliferation assay, IL-2-induced T cell proliferation was inhibited by peficitinib in a concentration-dependent manner with an IC50 of 10 nM and by tofacitinib with a similar IC50 of 24 nM (Gianti and Zauhar 2015). In addition, cynomolgus monkeys treated with CsA showed suppression of IL-2 and TDAR using SRBCs in a dose-dependent manner (Gaida, et al. 2015).\

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

Time-scale

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

Other authors described that in human T-B-cell co-cultures, FK506 and CsA lowered the mRNA levels of IL-2 and IL-4 at 8 h post-stimulation and inhibited IgM and IgG production after 9 days (Heidt, et al. 2010).

Treatment with CsA (50 mg/kg) twice daily in cynomolgus monkeys resulted in reduction of IL-4 cytokine production from

PMA/ionocycin stimulation of whole blood starting on day 0 and continuing through the end of the study on day 16. CsA treatment achieved 82 [±10]%, 68 [± 25]%, and 82 [± 9]% 100% maximal inhibition of ex vivo IL-4 response on days 0, 9, and 16. SRBC-specific IgM and IgG were significantly lower in animals dosed with CsA than in animals dosed with the vehicle control on days 9, 12, and 16 post-immunization. There was ≥80% or greater reduction in SRBC-specific IgM on days 9–16. SRBC-specific IgG was decreased by ≥95% on days 9–16 (Gaida, et al. 2015). This was similar to the degree of inhibition observed in rats using an KLH immunization model (Smith, et al. 2003).

Known modulating factors

Treatment with CsA (cyclosporin A) at 50 mg/kg BID (bis in die) resulted in reduction of IL-2, IL-4 cytokine production from PMA/ionomycin stimulation of whole blood in synomolgus monkey starting on Day 0 and continuing through the end of study on Day 16. In addition, Tacrolimus concentration was 1.0 ng/ml. Tacrolimus inhibited IL-2 and IL-4 mRNA levels. Glycosylation-inhibiting factor (GIF) secreted from CD4 cells suppressed IL-4 mRNA levels of the same cells during the initial 24 h of CD3/CD28 stimulation.

Known Feedforward/Feedback loops influencing this KER

B cells are required for the generation and / or maintenance of Th2 responses. Germinal center B cells regulate Th2 development through an IL-4 dependent process. Type 2 immunity and allergic responses are initiated by T cells and DCs, this response may be sustained and potentially amplified by an IL-4-driven feedback loop between Ag-specific T and B cells (Harris, et al. 2005).

References

Bradley LM, Duncan DD, Tonkonogy S, Swain SL. 1991. Characterization of antigen-specific CD4+ effector T cells in vivo: immunization results in a transient population of MEL-14-, CD45RB- helper cells that secretes interleukin 2 (IL-2), IL-3, IL-4, and interferon gamma. J Exp Med 174:547-559. DOI: 10.1084/jem.174.3.547.

Dumont FJ, Koprak S, Staruch MJ, Talento A, Koo G, DaSilva C, Sinclair PJ, Wong F, Woods J, Barker J, Pivnichny J, Singer I, Sigal NH, Williamson AR, Parsons WH, Wyvratt M. 1998. A tacrolimus-related immunosuppressant with reduced toxicity. Transplantation 65:18-26. DOI: 10.1097/00007890-199801150-00005.

Dumont FJ, Staruch MJ, Fischer P, DaSilva C, Camacho R. 1998. Inhibition of T cell activation by pharmacologic disruption of the MEK1/ERK MAP kinase or calcineurin signaling pathways results in differential modulation of cytokine production. J Immunol 160:2579-2589.

Gaida K, Salimi-Moosavi H, Subramanian R, Almon V, Knize A, Zhang M, Lin FF, Nguyen HQ, Zhou L, Sullivan JK, Wong M, McBride HJ. 2015. Inhibition of CRAC with a human anti-ORAI1 monoclonal antibody inhibits T-cell-derived cytokine production but fails to inhibit a T-cell-dependent antibody response in the cynomolgus monkey. J Immunotoxicol 12:164-173. DOI: 10.3109/1547691X.2014.915897.

Gianti E, Zauhar RJ. 2015. An SH2 domain model of STAT5 in complex with phospho-peptides define "STAT5 Binding Signatures". J Comput Aided Mol Des 29:451-470. DOI: 10.1007/s10822-015-9835-6.

Harris DP, Goodrich S, Mohrs K, Mohrs M, Lund FE. 2005. Cutting edge: the development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor alpha, and Th2 cells. J Immunol 175:7103-7107. DOI: 175/11/7103 [pii]10.4049/jimmunol.175.11.7103.

Heidt S, Roelen DL, Eijsink C, Eikmans M, van Kooten C, Claas FH, Mulder A. 2010. Calcineurin inhibitors affect B cell antibody responses indirectly by interfering with T cell help. Clin Exp Immunol 159:199-207. DOI: 10.1111/j.1365-2249.2009.04051.x.

Kino T, Hatanaka H, Hashimoto M, Nishiyama M, Goto T, Okuhara M, Kohsaka M, Aoki H, Imanaka H. 1987. FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. J Antibiot (Tokyo) 40:1249-1255. DOI: 10.7164/antibiotics.40.1249.

Sakuma S, Kato Y, Nishigaki F, Magari K, Miyata S, Ohkubo Y, Goto T. 2001. Effects of FK506 and other immunosuppressive anti-rheumatic agents on T cell activation mediated IL-6 and IgM production in vitro. Int Immunopharmacol 1:749-757.

Shi M, Lin TH, Appell KC, Berg LJ. 2008. Janus-kinase-3-dependent signals induce chromatin remodeling at the Ifng locus during T helper 1 cell differentiation. Immunity 28:763-773. DOI: 10.1016/j.immuni.2008.04.016.

Smith HW, Winstead CJ, Stank KK, Halstead BW, Wierda D. 2003. A predictive F344 rat immunotoxicology model: cellular parameters combined with humoral response to NP-CgammaG and KLH. Toxicology 194:129-145. DOI: 10.1016/j.tox.2003.07.002.

Taiho PC, Ltd. 2013. Drug interview form IPD capsule 50 and 100. . Revised 5th edition.

Ulrich P, Paul G, Perentes E, Mahl A, Roman D. 2004. Validation of immune function testing during a 4-week oral toxicity study with FK506. Toxicol Lett 149:123-131. DOI: 10.1016/j.toxlet.2003.12.069