STAT4: A critical regulator of inflammation *in vivo*

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Running title: STAT4 *in vivo*

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Abstract

STAT4 is a central mediator in generating inflammation during protective immune responses and immune-mediated diseases. In the eight years since their first description, STAT4-deficient mice have defined the role of STAT4 in a variety of *in vivo* model systems. Despite the extensive study and use of these mice, the exact role of STAT4 *in vivo* is still unclear. In this review, I will focus on describing the phenotypes of STAT4-deficient immune responses to pathogens and in diseases. Comparing the effects of STAT4-deficiency among numerous model systems will further enhance the development of a systemic model of STAT4 function *in vivo*.

Key words: STAT4, inflammation, IFN-γ, IL-12, IL-23, pathogenesis, disease models

Signal Transducer and Activator of Transcription 4 (STAT4) is a critical mediator of pro-inflammatory immune responses. STAT proteins are a family of factors implicated in a variety of biological processes (1,2). Latent STAT4 molecules are activated by cytokine stimulation to form homodimers. The dimers then move to the nucleus, bind DNA and modulate gene transcription. This mechanism provides a direct link between cell surface cytokine/growth factor stimulation and gene activation in the nucleus. IL-12, the hallmark activator of STAT4, is a pleiotropic cytokine expressed by macrophages and dendritic cells and is implicated in many inflammatory diseases (3). IL-12 is a two-chain cytokine comprised of disulfide linked p35 and p40 chains. The cytokine binds specifically to two non-covalently linked receptor subunits expressed on NK, activated T and B cells. The subunits are termed IL-12Rβ1 and IL-12Rβ2 and respectively associate with the tyrosine kinases Tyk2 and Jak2 (4,5). The biological effects of IL-12 include induction of IFN-γ expression in NK and activated T cells, increasing cytotoxic responses in both T and NK cells, inducing proliferation of activated T cells and stimulating the development of fully functional Th1 cells.

STAT4 was first cloned by cross hybridization with other cloned STAT proteins (6,7). It is the only STAT that shows tissue-restricted expression, with mRNA found mainly in lymphoid and myeloid tissues. Initial studies of STAT4-deficient mice demonstrated that STAT4 is required for most IL-12 biological responses including IFN-γ production. It is also required for the normal differentiation of Th1 cells and the expression of Th1 specific genes such as IFN-γ, LTα, IL-18Rα, IL-12Rβ2, ERM and functional Selectin ligands (8-14). The normal development and viability of STAT4-deficient mice has facilitated their use in a large number of models of inflammatory disease, infection and basic immune responses. In the eight years since the first reports on the STAT4-deficient mice, over 40 reports have described *in vivo* pathologies that are STAT4-dependent or –

independent. The focus of this review will be to highlight common themes and note differences in the results of these various models to generate a better understanding of the function of STAT4 *in vivo*.

Protein antigen immunization

Immunization of STAT4-deficient mice results in a typical Th2-like immune response. In several studies STAT4-deficient mice have been immunized with Ovalbumin, Hen egg-white lysozyme and keyhole limpet hemocyanin (15-18). There have not been dramatic differences in proliferative responses between wild-type and STAT4-deficient immunized mice suggesting that any defects or alterations in immune responses are not a product of failure of antigen-induced T cell expansion (15-18). However, T cells from immunized STAT4-deficient mice produce low amounts of IFN-γ and increased amounts of Th2 cytokines such as IL-4, IL-5 and IL-10 (15-19). Because of the Th1-to-Th2 shift in the immune response, antigen-specific antibody production is also shifted from an IgG2a to an IgG1 response (17,18). The shift also decreases inflammation in delayed-type hypersensitivity (15). These results create a paradigm for immune responses in the STAT4-deficient model wherein a more Th2-like environment is generated. This paradigm is supported by evidence from numerous disease systems described in the following sections.

Autoimmune diseases

As STAT4 is required for the development of fully functional Th1 cells, STAT4-deficient mice are protected from the effects of T cell mediated autoimmune diseases. In models of Experimental Autoimmune Encephalomyelis (myelin oligodendrocyte glycoprotein peptide induced), arthritis (proteoglycan-induced), colitis (transplantation induced), myocarditis (cardiac myosin

induced) and diabetes (NOD mouse and virally induced) STAT4-deficient mice display less disease and decreased parameters of inflammation compared to wild-type mice (20-25). Decreased IFN-γ secretion is common to all of these models. As a result a switch from IFN-γ-induced antigen-specific IgG2a antibodies to IgG1 was observed in the arthritis model but surprisingly not in the EAE model (20,21). TNFα secretion was also reduced in the arthritis and myocarditis models, though it was not examined in other models (21,23). The Th1 to Th2 shift is modest in autoimmune disease models wherein STAT4-deficient T cells had increased IL-5 in the EAE model and modestly increased IL-10 in the NOD model but no changes in other Th2 cytokine levels (20,24). Interestingly, while IFN-γ-deficiency does mimic STAT4-deficiency in the arthritis model (21), IFN-γ-deficient mice are not protected from EAE, myocarditis or colitis (22,23,26). Thus, while IFN-γ is an important STAT4-induced immune mediator, STAT4 must regulate other genes that are critical for the development of inflammatory disease.

In contrast, STAT4-deficient mice are not protected from autoimmune diseases that are predominantly antibody mediated. In models of Myasthenia Gravis and Graves' disease STAT4-deficient mice have levels of disease very similar to wild-type mice (27,28). However, there are characteristics of the STAT4-deficient immune response in these systems including decreased IFN-γ and increased IL-4 production, and a modest shift from antigen specific IgG2a to IgG1 antibodies.

Different scenarios are observed in models of Systemic Lupus Erythematosus using two different lines of New Zealand Mixed mice. In these models STAT4-deficient mice actually develop worse inflammatory disease than seen in control mice including increases in kidney inflammation and proteinuria although there were paradoxical decreases in autoantibody levels and antibody and

complement deposition in the kidney (29,30). These phenotypes are accompanied by the classic Stat4-deficient immune response including decreased IFN-γ, increased IL-4 and a switch in autoantibody isotypes from IgG2a to IgG1. The STAT4-dependent mechanisms causing the disease phenotype are still unclear.

Atopy and airway hypersensitivity

Since STAT4-deficiency often results in a Th2 skewed immune response *in vivo*, it would be expected that STAT4-deficient mice would develop worse airway hypersensitivity than control mice. This is indeed observed in examination of airway resistance following infection of C57BL/6 background mice with Respiratory Syncytial Virus. Compared to control mice, STAT4-deficient mice have increased airway resistance with increased eosinophil infiltration in the lung and goblet cell hypertrophy (31). However, in a cockroach allergen induced model of airway hyperreactivity, STAT4-deficient mice showed the opposite phenotype of decreased airway resistance compared to control mice (32). There was correspondingly no increase in Th2 cytokines and no changes in serum IgE levels. However, there were significant decreases in chemokine production in the lung that correlates with decreased lung infiltration. This latter model suggests that even in a prototypic Th2 immune response, there may be a role for STAT4-dependent Th1 immunity in the establishment of pathology.

Transplantation, Tumor Immunity and Tolerance

Dogma would predict that transplant rejection and tumor immunity, being inflammatory responses, would require STAT4-dependent immunity. However, experimental results would suggest that STAT4 has only a limited role in these immune responses. STAT4-deficient mice rejected

cardiac allografts at the same rate as wild-type mice with similar levels of graft infiltration, despite a decrease in IFN-γ production (16,33). Since there are aspects of Th1 immunity that are STAT4-independent, including a low level of IFN-γ production, it is possible that even diminished Th1 immunity is sufficient for acute rejection responses. STAT4-deficient mice were also more easily tolerized by blockade of CD40-CD40L signaling compared to similarly treated wild-type mice, which may be a result of the Th2 cytokine environment (16).

In a model of chronic cardiac allograft rejection, STAT4 did contribute to the level of infiltration and post-transplant vasculopathy (34). Additionally, STAT4-deficient spleen and bone marrow cells were less efficient at mediating graft-versus-host disease (GVHD) resulting in extended survival of recipient mice (35). STAT4-deficient cells displayed a shift from Th1 to Th2 profiles with decreased IFN-γ and IL-2 and increased IL-4 and IL-10. There were also large increases in serum IgG1 and IgE. Some recipients of STAT4-deficient grafts demonstrated unusual clinical phenotypes including a lack of post-transplant diarrhea and increased incidence of severe skin GVHD. Mice receiving STAT4-deficient grafts had liver infiltration comparable to recipients of wild-type transplants but milder intestinal infiltrates and colitis than recipients of wild-type cells. Thus, there may also be distinct requirements for STAT4 in acute versus chronic rejection responses.

The role of STAT4 in ischemia-reperfusion injury (IRI) is less clear due to conflicting reports. One report suggested that, although STAT4 is found activated following hepatic IRI, IL-12-dependent tissue damage was independent of STAT4 (36). Several parameters of hepatic injury, including increased TNFα, myeloperoxidase (MPO) content and serum alanine aminotransferase (ALT) were indistinguishable between wild-type and STAT4-deficient mice. A separate report

clearly observed a role for STAT4 in hepatic IRI with STAT4-deficient mice having decreased serum ALT, MPO content and Th1 cytokines (TNFα, IL-2, IFN-γ) (37). This was correlated with the expression of heme-oxygenase-1 in STAT4-deficient, but not wild-type, livers. Treatment of STAT4-deficient mice with an inhibitor of heme oxygenase abrogated the protective effect of STAT4-deficiency (37). There was a modest protective effect of STAT4-deficiency in a similar model of renal IRI when serum creatinine was measured, although no significant changes were observed between wild-type and STAT4-deficient mice when tissue damage, infiltration, MPO content and inflammatory cytokine production were examined (38). Although the explanation for the differences in these models is unclear, reasons may include slight differences in time analyzed and experimental procedures.

Rejection of synegenic tumors is similar to transplantation in that tumor antigens may function as histocompatibility antigens. However, rejection of a syngeneic mammary carcinoma tumor was nearly identical in wild-type and STAT4-deficient mice (39). In contrast, STAT4-deficient mice had an increased incidence of thymic lymphomas following chemical-induction (40). However, it is not clear if this result is due to decreased immune surveillance or an inherent predisposition of STAT4-deficient thymic cells to transformation.

Some models of tolerance to nominal antigens are thought to function through immune deviation; the shift of an immune response from a Th1 to a Th2 response. However, there has also been no requirement for STAT4 demonstrated in two models of tolerance induction. Analysis of immune cell responses following oral or neonatal tolerance demonstrated equivalent levels of non-responsiveness in wild-type and STAT4-deficient mice. Oral tolerance was accompanied by a

decrease in both Th1 and Th2 cytokines and in both antigen-specific IgG1 and IgG2a (17). Neonatal tolerance was accompanied by a modest shift towards Th2 immune responses but decreases in both antigen-specific IgG1 and IgG2a antibodies (18). Since there is no evidence of spontaneous inflammation or autoimmune disease in STAT4-deficient mice, there is also no evidence of any defect in thymic education or peripheral tolerance, supporting the lack of involvement of STAT4 in these immunoregulatory mechanisms.

Parasite infections

Protozoan parasites are intracellular pathogens that require Th1 immunity for clearance. As such, STAT4-deficient mice do not develop effective immunity against this class of parasites. STAT4-deficient mice develop significantly higher parasite burdens following infections with *Leishmania major*, *Leishmania mexicana*, *Trypanosoma cruzi* and *Toxoplasma gondii* (41-44). While increased burden is correlated with decreased IFN-γ following *L. Major* infection, there is no significant increase in IL-4 production or shift in antibody isotypes, which are nearly identical to wild-type mice (43). Similarly, STAT4-deficiency confers susceptibility to a resistant STAT6-deficient genetic background, which lack Th2 responses (45). The increased *T. cruzi* burden results in lethality for STAT4-deficient mice (44). Decreased lesion IFN-γ is accompanied by decreased TNFα, anti-*T. cruzi* IgG2a and an increase in anti- *T. cruzi* IgG1 (44). Increased parasite burden in *T. gondii* infected STAT4-deficient mice also results in lethality (41,46). This is accompanied by decreased IFN-γ production, decreased iNOS in lesions as well as increased Th2 cytokine expression. While STAT4-deficient lymphocytes proliferated well *in vitro*, ex vivo experiments suggested that STAT4-deficient cells *in vivo* were not fully activated and had defects in expansion. Lethality following infection could be delayed modestly by injection of IFN-γ and more significantly by a

combination of IL-2 and IL-18, the latter combination also increasing IFN-γ *in vivo* (44). Thus, STAT4-dependent immunity is required for effective immunity to protozoans.

Helminthic parasites are extracellular pathogens that are generally thought to require Th2 immunity for resistance. However, there is evidence that Th1 immunity may also be important in some of these infections. Following infection with *Schistosoma mansoni*, STAT4-deficeint mice generated decreased pulmonary granuloma size but similar hepatic granulomas to wild-type littermates, despite decreased granuloma eosinophil content (47). In this model, STAT4-deficient mice have decreased IFN-γ production but no increase in Th2 cytokines. Anti-*S. mansoni* egg antigen isotypes mirror this with decreased IgG2a but no changes in IgG1. There was also a marked decrease in antigen specific IgG3, though the mechanism of this is still unclear. Infection with *Taenia crassiceps* resulted in a typical STAT4-deficient response including decreased IFN-γ and antigen specific IgG2a with increased Th2 cytokines, antigen specific IgG1 and total IgE compared to control mice (48). This response resulted in a dramatic increase in the parasite burden of STAT4-deficient mice with increased eosinophils and only modestly decreased lymphocyte numbers recruited to the site of infection, compared to control mice. In the absence of STAT4, there was a decreased ability of the cells to proliferate *in vitro*. There was also a decreased ability of macrophages from STAT4-deficient infected mice to secrete TNFα, IL-1β, IL-12, and nitrite, compared to control mice and this correlated with peritoneal macrophages of a distinct phenotype in wild-type and STAT4-deficient infected mice (48). While it is difficult to draw conclusions with only a limited number of infectious models, it also seems likely that STAT4-dependent immune responses may be required for optimal immunity to some helminthic parasites.

Hematopoiesis

It was initially observed that STAT4-deficient mice had no defects in hematopoiesis with normal development of lymphoid and myeloid cells (9,11). Subsequent analysis of backcrossed mice demonstrated that STAT4-deficient mice have decreased hematopoietic progenitor numbers (CFU-GM, BFU-E and CFU-GEMM) and decreased levels of cycling of the progenitors that are present (49). This defect is a result of STAT4 function in T cells and suggests that Th1 cells regulate hematopoietic progenitor cell homeostasis. Oncostatin M was secreted in decreased levels in STAT4-deficient cultures and injection of Oncostatin M *in vivo* normalized hematopoietic progenitor numbers and cycling in STAT4-deficient mice (49). Further analysis demonstrated that there was no significant difference in the ability of wild-type or STAT4-deficient progenitors to respond to GM-CSF, Flt3 ligand or Steel factor (50). However, STAT4-deficient progenitor cells are refractory to the myelosuppresive effects of chemokines (50). Despite these progenitor defects, there are no significant decreases in the numbers of mature myeloid cells or PMNs in STAT4-deficient mice suggesting that other mechanisms compensate for progenitor cell defects.

Viral infections

CD8+ T cells have less dependence upon STAT4 for IFN-γ production. Following anti-CD3 stimulation, CD8+ STAT4-deficient T cells produce wild-type levels of IFN-γ, suggesting that, unlike CD4+ T cells, the IFN-γ primed differentiated state in CD8 T cells is independent of STAT4 (51). However, STAT4 is still required if CD8 cells are stimulated with IL-12. STAT4 is required for a burst of IFN-γ following infection with LCMV (52). However, antigen primed STAT4-deficient mice are capable of clearing an LCMV infection with efficiency equal to that of wild-type mice (25). LCMV-specific cytotoxicity was also similar between wild-type and STAT4-deficient

mice. STAT4-deficient mice are also able to clear influenza infection, producing slight changes in cytokine production and similar antibody titers, despite a modest switch from IgG2a to IgG1 (53). There were also no significant differences in the ability of STAT4-deficient mice to respond to a Vesicular Stomatitis Virus infection (54). Thus, while STAT4-deficient immune responses are altered during viral infections there is sufficient generation of cytotoxic T cells and neutralizing antibodies to limit viral infections, at least in the models studied thus far.

Bacterial infections

STAT4-deficient mice have altered responses and pathology to sepsis caused by cecal ligation and puncture (CLP) and are protected from CLP induced mortality (55,56). STAT4-deficient mice had higher aerobic and anaerobic bacterial burdens at early time points (56), but had levels similar to wild-type by 24 hours after CLP (55). There were no significant differences in organ neutrophil infiltration assessed by counting and MPO content at early time points in the liver, lung, kidney and peritoneum, though levels were lower in STAT4-deficient liver than in wild-type liver by 24 hours (55,56). However, STAT4-deficient mice did have significantly decreased levels of serum aspartate transaminase, ALT, blood urea nitrogen and creatinine than wild-type mice, indicating decreased kidney and liver injury. STAT4-deficient mice also had decreased MIP-2 and KC levels in several organs with increased IL-10 and IL-13 in the liver (55).

Responses are somewhat different when STAT4-deficient mice are injected with LPS directly. In response to 3 mg/kg *Escherichia coli* 0111:B4 LPS, STAT4-deficient mice were more sensitive to lethal endotoxemia (57). Sensitivity was dependent on IL-12, since anti-IL-12 increased survival, suggesting that STAT4-independent IL-12 signaling increases lethality. Despite increased

sensitivity to LPS, there were no differences between wild-type and STAT4-deficient mice in TNFα, IL-6, MIP-2, MIP-1α, MCP-1 production, serum ALT levels or levels of cellular infiltration (57). In contrast, STAT4-deficient mice were somewhat protected from endotoxemia following 30-50 mg/kg injections of *Salmonella typhimurium* LPS, compared to wild-type mice (58). STAT4-deficient mice had decreased serum IFN-γ levels, but normal levels of TNFα and IL-12 (58). STAT4-deficient macrophages had normal activation of NF-κB, ERK, p38 and production of IFNβ. Thus, the mechanism of increased or decreased sensitivity to these bacterial lipopolysaccharides is unclear.

STAT4 is also involved in the formation of sepsis-induced adhesion formations and abscesses. STAT4-deficient mice had lower levels of postsurgical adhesion formation following cecal abrasion surgery; a response found to depend upon IL-17 (59). STAT4-deficient mice also have decreased abscess formation following infection with *Bacteroides fragilis* or *Streptococcus pneumoniae* type I capsules (60). Abscess formation was also dependent on IL-17 and as there was decreased IL-17 production in STAT4-deficient peritoneal cavities, STAT4 seems to be a component of the IL-17 pro-inflammatory pathway.

Similar to results with intracellular parasites, STAT4-deficient mice are susceptible to infection with intracellular bacteria. Following infection with *Mycobacterium tuberculosis* there were increased bacterial burdens in the lungs and spleens of STAT4-deficient mice as well as decreased expression of iNOS and IFNα in infected lungs, compared to those observed in control mice (61). Electron microscopy revealed that STAT4-deficient macrophages phagocytosed bacteria but that bacteria were not as effectively killed as they were in wild-type macrophages. Similarly, I have also observed that STAT4-deficient mice have increased susceptibility to *Listeria*

monocytogenes infection (unpublished observation) and recurrent infections with *Mycobacterium avium* occur in patients with defects in STAT4 nuclear localization (62).

Other STAT4 activating cytokines

We have focused much of the discussion on IL-12 as a central activator of STAT4. Indeed, IL-12 is the most potent activator of STAT4. However, IL-23 and IFN-α can activate STAT4 and it is becoming clear that these cytokines may require STAT4 for certain aspects of their function as well (52,63,64). STAT4-deficient and IFN-αR-deficient mice lack a burst of IFN-γ production that is normal in IL-12 p35-deficient mice (52) suggesting that this STAT4 function is IFN-α-dependent but IL-12-independent. IL-23, which shares a p40 chain with IL-12 and whose receptor shares the IL-12Rβ1 chain with the IL-12R, plays a critical role in inflammatory disease. In some disease models, it may be IL-23, not IL-12, that is required for pathogenesis. For example, both the IL-23 p19-deficient, p40-deficient and STAT4-deficient mice are protected from EAE (20,65). By contrast, mice deficient in IL-12 p35, the IL-12 specific chain, are susceptible to disease. Thus, the phenotype in the STAT4-deficient mice, in all models described, may actually be a composite effect of defects in IL-12, IL-23 and IFN-α signaling, and possibly other cytokine signaling pathways as well.

Summary

STAT4 functions in multiple cell types and at multiple time points throughout an immune response. Early in a response, STAT4 aids in innate immunity allowing NK cells and antigen presenting cells (macrophages and dendritic cells) to produce IFN-γ, cytokines, chemokines and inflammatory mediators that are critical in the initial stages of an inflammatory response. It is also likely that STAT4-dependent tissue destruction occurs through these early acting cells. STAT4 is

then critical in the differentiation and effector function of Th1 cells. In the absence of STAT4, initial inflammation is markedly decreased and T cells develop into a more Th2-like phenotype altering the cells that are recruited during an inflammatory response and the antibody isotypes produced by the humoral response. This shift in components of the innate and adaptive immune systems severely limits the capacity of STAT4-deficient mice to eliminate some infections, but leaves them relatively protected from the effects of localized inflammatory responses. Continuing use of STAT4-deficient mice in disease models and the future identification of STAT4 target genes will facilitate the development of pharmaceuticals targeting STAT4 or its downstream mediators to treat many human diseases.

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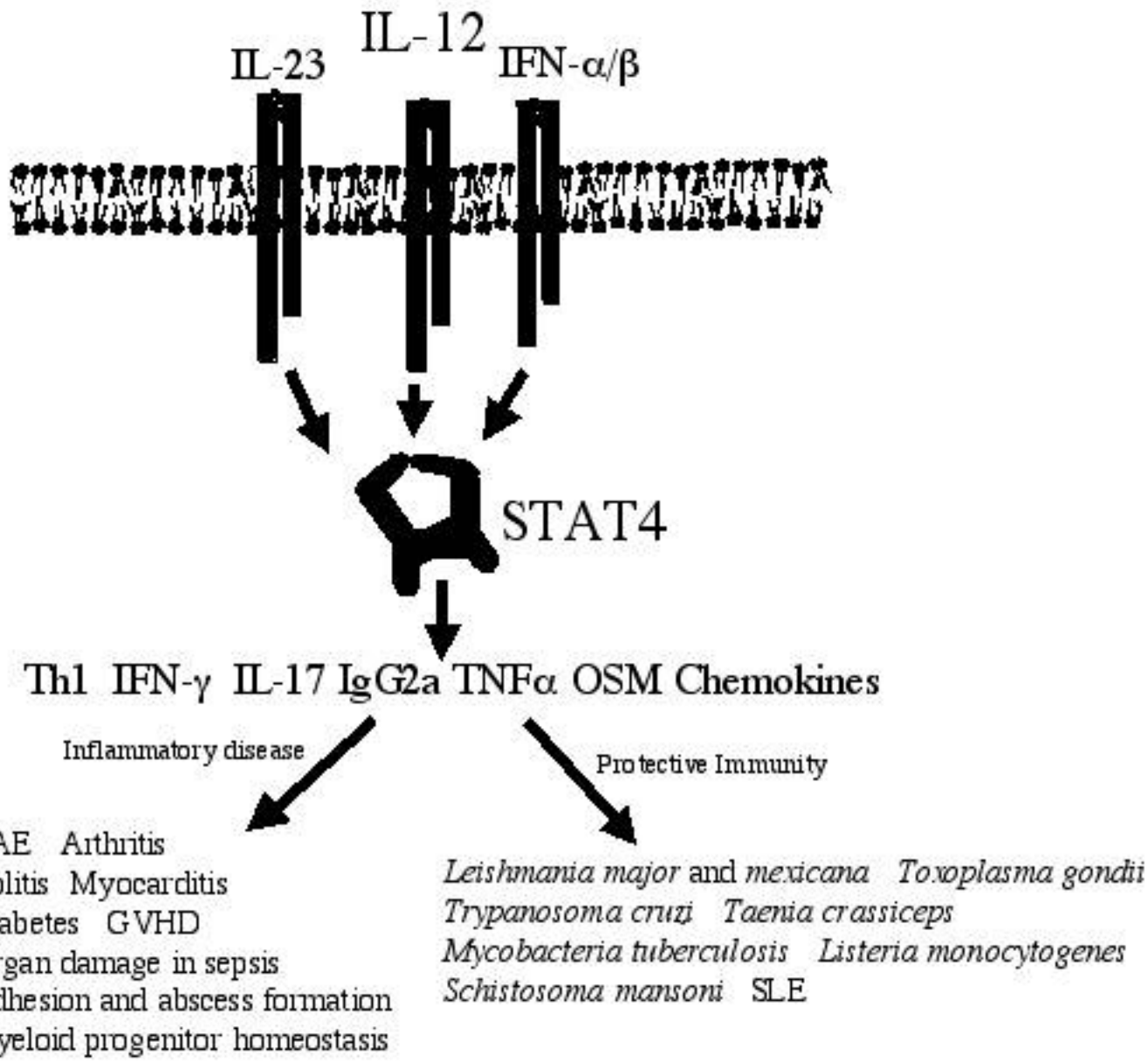
Figure Legend

Figure 1. Summary of STAT4 function in vivo. Cytokines which activate STAT4 result in biological responses which can be protective in response to various pathogens but when uncontrolled result in tissue destruction and organ damage.


IL-12
IL-23
IFN-α/β
STAT4
Th1 IFN-γ IL-17 IgG2a TNFα OSM Chemokines
Inflammatory disease
Protective Immunity
EAE Arthritis
Colitis Myocarditis
Diabetes GVHD
Organ damage in sepsis
Adhesion and abscess formation
Myeloid progenitor homeostasis
Leishmania major and *mexicana* *Toxoplasma gondii*
Trypanosoma cruzi *Taenia crassiceps*
Mycobacteria tuberculosis *Listeria monocytogenes*
Schistosoma mansoni SLE