Modulation of Regulatory T Cells by Cytokines

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The incidence of immunopathology is increasing; and treatments usually involve systemic immunosuppression, with significant side-effects. Regulatory T cells (Tregs) inhibit immune responses in a targeted manner, and are being studied as potential therapeutic agents. Interleukin 2 (IL-2) is among the few well-characterized modulators of Tregs. IL-2 stimulation increases Treg function, but inhibits suppression in co-cultures with T helper (Th) cells, due to dominant effects on Th cells. Characterizing cytokine modulation of Tregs is important because Treg-targeted therapy would be used primarily to treat diseases that induce cytokine production. Recent work has implicated IL-6 in the regulation of Tregs in the lung and in the pathogenesis of several diseases. We investigated the influence of IL-2 and IL-6 on Tregs, and found that they increased Treg suppressive function, proliferation, and expression of FoxP3 and CTLA4. Interestingly, both cytokines are regulated by suppressor of cytokine signaling 3 (SOCS3), and we found that unstimulated Tregs lacked SOCS3, whereas naïve Th cells expressed it abundantly. SOCS3 over-expression in Tregs inhibited proliferation, FoxP3 and CTLA4 expression, and suppressive function. Whereas IL-2 and SOCS3 seemed to act during homeostatic conditions, IL-4 is active in disease states. IL-4 is required for experimental asthma induction in mice due to its critical role in the development of Th2 cells, which protect against helminth infections. Consequently, we analyzed regulation of Tregs by IL-4 *in vivo* during allergic airway inflammation. Pulmonary Treg numbers were similar in tolerized mice compared

to those with inflammation, and adoptive transfer of Tregs inhibited inflammation, implying that cytokines might have inhibited Treg function. However, IL-4Rα-/- Tregs, which lack the IL-4 receptor alpha chain, were less effective in reducing inflammation. I*n vitro*, IL-4 increased Treg proliferation and maintenance of FoxP3 expression, did not alter Treg suppressive function, and increased Th cell resistance to suppression. Also, IL-4-mediated effects on Tregs and Th cells required signal transducer and activator of transcription 6 (STAT6). Therefore, IL-4 increased Treg function *in vivo* by enhancing Treg proliferation through a STAT6-dependent mechanism. In summary, SOCS3 may serve as a future therapeutic agent, whereas the net effect of IL-4 blockade therapy remains unclear.

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1 INTRODUCTION

1.1 BASIC IMMUNOLOGY

The immune system protects an organism from microbial, viral, and parasitic colonization. However, this function is confounded by an environment containing many chemicals, allergens, and harmless microbes that can potentially activate the immune system. Therefore, the immune system must maintain a balance between tolerance of harmless antigens and immunity against pathogens.

Cells of the innate immune system provide rapid recognition and clearance of most microorganisms before they are able to cause disease or induce an adaptive response. The innate immune system is comprised of many cell types, including macrophages, neutrophils, mast cells, dendritic cells (DCs), eosinophils, natural killer (NK) cells, and basophils. Many of these cells are able to either phagocytose small pathogens, such as macrophage uptake of viruses and bacteria, or they secrete toxic compounds and proteolytic enzymes to inhibit larger infectious agents, as in the case of eosinophil degranulation during helminth infection. In most cases, these cells use genetically encoded pattern recognition receptors such as toll-like receptors (TLRs) that bind to carbohydrate, protein, lipid, and nucleic acid structures that are released by damaged cells, or are components of pathogens that are not produced by the host. As a result, necrotic cells or pathogen-specific products serve as “danger signals”, which cause rapid activation of the

immune system and promote initiation of adaptive immunity (1, 2). In addition, innate immune function can be significantly enhanced by adaptive immune responses, which produce antibodies and cytokines to enhance targeting and activation of innate immune cells.

In contrast to the rapidly induced general responses that are provided by the innate immune system, adaptive immune cells are highly antigen specific, and as a result, require a longer period of time to provide a protective response. The adaptive immune response is capable of responding to infectious agents never encountered previously, while remaining unresponsive to self antigens and "harmless" allergens. In order to recognize pathogens not present during the evolution of the immune system, the adaptive immune response uses a system of random recombination to create receptors that have the potential to bind almost any protein (1, 2). The adaptive immune system is comprised of several cell types including: B cells, CD8+ T cells, CD4+ T helper (Th) cells, γδ TCR+ T cells, CD8+ suppressor cells, CD4+ regulatory T cells (Tregs), and NKT cells (1, 2). This document will focus primarily on Th cells and Tregs.

Since receptor specificity of the adaptive immune system is not coded in the genome, cells must undergo negative selection during development to prevent autoimmune responses, which involves deletion of cells with high affinity for MHC Class II:self antigen complexes (1, 2). However, harmless foreign antigens such as those in food, allergens, or commensal organisms, that are not present in the thymus still do not generally provoke an immune response (1, 2). In this setting of constant stimulation by harmless antigens interrupted by brief periods of infection, a multifaceted system of immune regulation is critical to inhibit responses by Th cells that escaped negative selection in the thymus, inappropriate activation by commensal flora, or bystander responses to self antigens during infection.

Of all the adaptive immune cells, Th cells are possibly the most important in the initiation and modulation of adaptive immune responses. This document will discuss several layers of regulation involving Th cells, including immune receptor selection and lymphocyte activation, and will focus on Tregs, with respect to cytokine signaling and modulation of cytokine signal transduction. An overview is depicted in Figure 1 and is described briefly in the legend. A detailed description will be provided in subsequent sections.

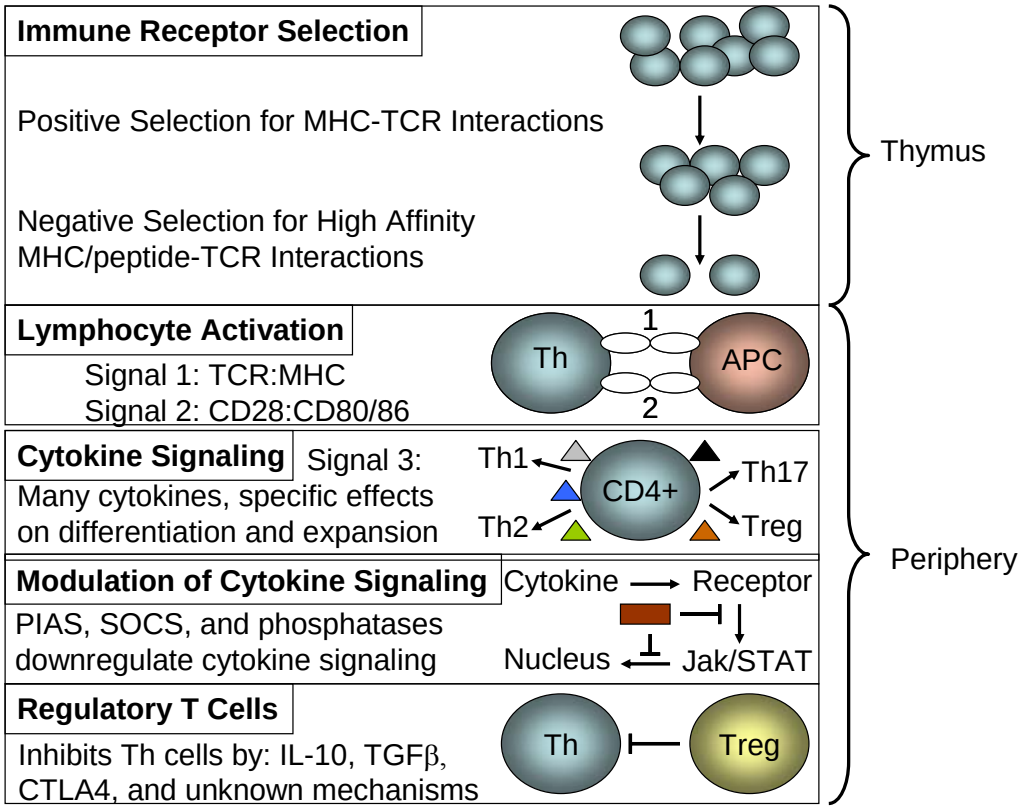

Figure 1. Different layers of T cell regulation

Th cell precursors traffic to the thymus where they undergo positive and negative selection. In the periphery, Th cell activation is initiated when TCR:MHC Class II interaction occurs (Signal 1). Th cells are only fully activated upon ligation of their TCR together with a second signal, termed "costimulation", usually through CD28 ligation with CD80 or CD86. Cytokines serve as a third signal to modulate Th activation and differentiation, and these signals are regulated by intracellular proteins, such as SOCS. Inappropriately activated Th cells are normally inhibited by Tregs via a number of different mechanisms. Abbreviations: MHC- major histocompatibility complex; TCR- T cell receptor; APC- antigen presenting cell; CD4+- CD4+ T cell, probably a Th cell, but not known for sure; STAT- signal transducer and activator of transcription; PIAS- protein inhibitors of activated STATs; SOCS- suppressor of cytokine signaling. Note: unlabelled blue spheres in top panel represent Th cell thymic precursors, colored triangles in Cytokine Signaling panel represent cytokines, and the brown rectangle in Modulation of Cytokine Signaling panel represents inhibitors of signaling such as PIAS, SOCS, and phosphatases.

1.2 TH CELLS

CD4+ T cells are critical modulators and regulators of the immune system, and can be divided into 2 main groups, Th cells and Tregs. Th cells and Tregs both express CD4 and αβ T cell receptors (TCRs), and require MHC Class II for their development in the thymus. However, their overall functions oppose each other. Th cells are vital for the initiation of adaptive immune responses since they secrete cytokines and activate ligands on many other cell types. In contrast, Tregs inhibit immune responses, and are critical to the prevention of inappropriate responses such as allergic and autoimmune diseases.

1.2.1 Th Cell Development

Th cells are generated in the thymus from precursors that traffic from the bone marrow (1, 2). These cells recombine highly polymorphic segments of their DNA to create TCRs that are unique for each cell (1, 2). The cells then undergo positive selection based on weak/moderate affinity of TCR binding to MHC Class II proteins. As a result, cells with TCRs that are incapable of interaction with MHC Class II do not receive survival signals and subsequently undergo apoptosis. This is followed by negative selection to delete T cells expressing TCRs that have high affinity for MHC Class II: self peptide complexes, since these cells have the potential to be self-reactive. Interestingly, some cells with high affinity TCRs may differentiate into Tregs (3).

The positive and negative selection processes eliminate most self-reactive cells, and are referred to as “central tolerance”. However, some proteins are not expressed by the thymic stroma, and many harmless antigens from food or commensal flora are likewise not present in

the thymus. Therefore, additional mechanisms collectively termed “peripheral tolerance” are required to control pathogenic Th cells that have not been deleted in the thymus.

1.2.2 Th Cell Activation

After release from the thymus, Th cells circulate through the blood and lymph. When Th cells percolate through lymph nodes, they interact with APCs such as B cells, macrophages, and most importantly, DCs. APCs present exogenous peptides complexed with MHC Class II to Th cells. In most cases, the peptides are derived from self proteins and the Th cells have TCRs with low affinity for the peptide-MHC Class II complex due to negative selection in the thymus. However, in the case of infection, a fraction of Th cells will have TCRs that recognize foreign antigen presented by APCs. TCR activation alone is insufficient to activate the Th cell; but APCs can provide costimulation through molecules such as CD80 and CD86, which ligate CD28, and inducible costimulator (ICOS), which binds to ICOS ligand (ICOS-L). In the case of CD28, costimulation enhances TCR signaling via several pathways, including Ras and Akt (2). CD28 induces additional signaling pathways independent of the TCR, such as activation of Itk, a tec kinase (2). However, costimulatory signals in the absence of TCR ligation do not cause Th cell activation. In order for APCs to provide costimulation, they must be activated by TLR ligand binding, cytokine stimulation, or through stimulatory ligands such as CD40, which interacts with CD40-L on activated Th cells (1, 2). Together, TCR cross-linking and costimulation induce Th cell activation, resulting in cytokine production, upregulation of activating ligands such as CD40-L, and initiation of adaptive immunity. In addition, activated Th cells undergo rapid clonal expansion followed by trafficking from lymph nodes by most of these cells to the inflammed

tissues while the remaining cells aquire a memory phenotype and are capable of being rapidly reactivated if the same antigen is encountered again. Frequently, Th cell activation is described by the "3 signal" system, where "signal 1" is TCR ligation, "signal 2" is costimulation, and "signal 3" is cytokine stimulation, which modulates Th cell differentiation and will be discussed in the next section.

1.2.3 Th Cell Differentiation

Upon activation, Th cells secrete cytokines to initiate an adaptive immune response that is appropriate for the particular pathogen infecting the host. In order to activate a protective response, the immune system must determine the correct combination of cells to activate. This complex decision involves interplay between many factors that influence both Th cells and APCs including the presence of TLR agonists, and the concentration, composition, and size of antigens. In response to a pathogen, Th cells differentiate and secrete particular clusters of cytokines that generally promote protective responses against classes of infections. There are four major differentiation pathways for CD4+ T cells, specifically, Th1, Th2, Th17, and Treg cells (Figure 2). Th1, Th2, and Th17 will be discussed below. Although some types of Tregs seem to differentiate from Th cells, this has not been confirmed, so Tregs will be discussed in a separate section.

When Th cells are activated in the presence of interleukin 12 (IL-12) or interferon gamma (IFNγ), they differentiate into Th1 cells and express T-box expressed in T cells (T-bet), the defining transcription factor for this Th cell subset (4-6). T-bet induces expression of IFNγ, and upregulates the IL-12 receptor β (IL-12Rβ). As a result, T-bet-/- mice have severely reduced Th1

responses (4). In general, Th1 cells secrete IFNγ, promote CD8 T cell responses, and induce protective immune responses against intracellular pathogens such as *Listeria monocytogenes, Mycobacterium tuberculosis*, and viruses. Importantly, Th1 responses are self-reinforcing, because IFNγ enhances Th1 differentiation and upregulates the inducible IL-12Rβ. This increases IL-12 signaling, and IL-12 promotes additional IFNγ production. Further, IL-12 antagonizes the other Th cell subsets by inhibiting expression of the critical Th2 transcription factor GATA binding protein 3 (GATA3), and by promoting IFNγ production, which inhibits Th17 differentiation (7-11). Predominance of Th1 immune responses can lead to immune pathology, which in the past has been associated with several autoimmune diseases and delayed-type hypersensitivity responses. However, more recently, experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA), two diseases initially believed to be Th1-mediated, are exacerbated in animals with genetic defects in IFNγ signaling, and are now thought to be caused by the newly discovered Th17 lineage (7, 12-15). Thus, the contribution of Th1 cells to disease is being reevaluated.

While high TCR signal strength and expression of several costimulatory molecules, such as CD40 can promote Th1 differentiation, IL-12 and IFNγ may be considered the most important signals for inducing Th1 cells (2). Mice deficient in the IL-12R and IL-12 signaling molecules, such as signal transducer and activator of transcription 4 (STAT4) demonstrate significant defects in Th1 responses, as do those deficient in IFNγ and IFNγ-associated signaling molecules such as STAT1 (5, 16-20).

If Th cells are activated under conditions with low IL-12, or in the presence of IL-4, such as during most helminth infections, Th2 responses will be favored (21). Th2 cells secrete IL-4, IL-5, IL-9, and IL-13, and express GATA-3, a critical transcription factor that promotes Th2

cytokine production and inhibits IL-12 signaling by downregulating IL-12Rβ expression (11, 22, 23). These cells promote B cell antibody production, particularly IgE, and are important for eosinophil responses and protection against helminths. Th2 cells secrete IL-4 initiating a positive feedback loop due to promotion of Th2 differentiation and inhibition of Th1 and Th17 biasing (7, 8). Unfortunately, positive feedback mechanisms can allow excessive Th2 responses in individuals with a genetic predisposition to increased Th2 biasing. Such biasing can be observed in patients with either gain of function mutations in the IL-4Rα that cause enhanced IL-4 signaling, or decreased inhibitory/regulatory function, leading to allergic disease and asthma (24-26).

Although IL-4 strongly promotes Th2 differentiation, mice that lack the IL-4R or its main signal transduction molecule, STAT6, can generate Th2 responses to certain pathogens (27). Since IL-13 also requires the IL-4R and STAT6 for signaling, it is also dispensable for Th2 responses to certain infections. Thus, non-cytokine influences, including costimulatory molecules such as ICOS, and weak TCR signal strength may be important to Th2 biasing *in vivo* (28). On the other hand, GATA-3 is required for Th2 development, and Th2 responses are inhibited in Th cells that express a dominant negative form of GATA-3 and in GATA-3 conditional knockouts (29, 30).

In the past 2 years, a new Th cell lineage, Th17, has been described (7, 9, 10, 31-33). These cells promote inflammation, and recent data strongly implicates Th17 in the pathogenesis of many autoimmune and inflammatory diseases, such as arthritis and multiple sclerosis, which were previously thought to be Th1-mediated (7, 12-15). Th17 cells secrete IL-17 and TNFα and are induced by activating Th cells in the presence of TGFβ and IL-6 along with TCR ligation and costimulation. Recently, retinoic acid-related orphan receptor γt (RORγt) was shown to be a key

Th17 transcription factor, and mice with RORγt-deficient T cells do not develop Th17 cells (34). Th17 is inhibited by Th1- and Th2- associated cytokines, but it is unknown whether Th17 cells antagonize Th1 and Th2 differentiation (7, 32, 35). Due to the recent discovery of the Th17 lineage, their precise role *in vivo* in protective and disease settings remains to be elucidated.

In contrast with the other three differentiation pathways of CD4+ T cells, Tregs inhibit Th cell expansion and adaptive immune responses, and usually show minimal proliferation. However, little is known about the converse, whether there is inhibition of Tregs by Th1, Th2, and Th17. Mice and humans with deficiencies in Tregs, show severe autoimmune and inflammatory disease (25, 26). Tregs can be generated as a separate lineage in the thymus, or induced from CD4+ T cells, and will be discussed in more detail in the next section.

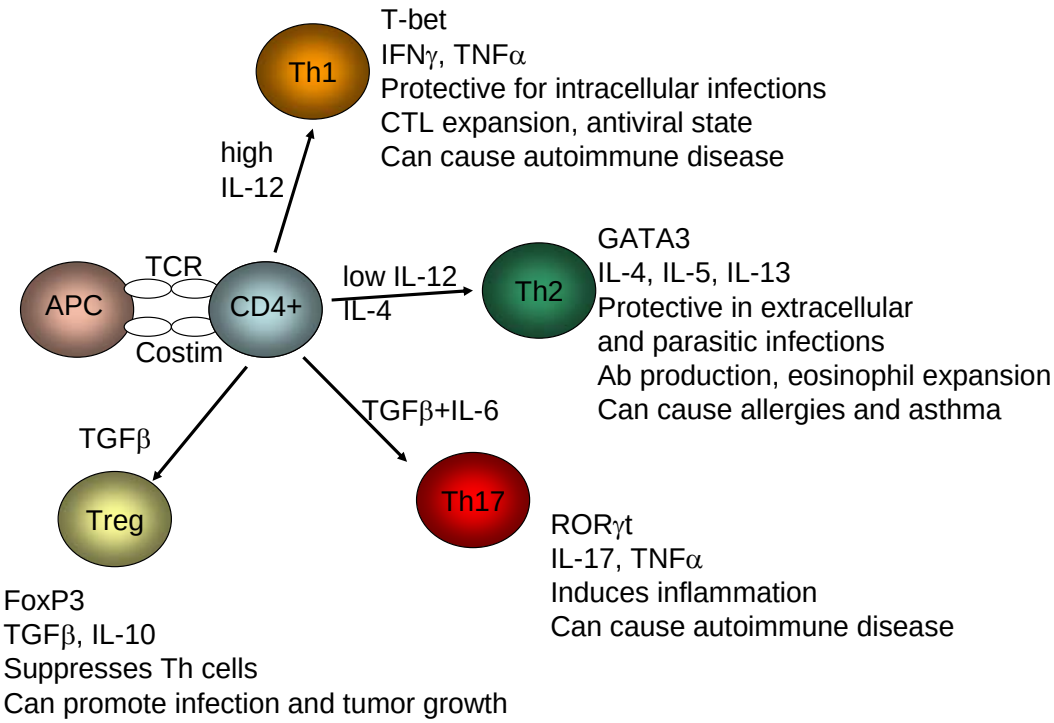

Figure 2. Four major pathways of CD4+ T cell activation/differentiation

Upon Th cell activation by TCR ligation in the presence of costimulation, CD4+ T cells proliferate and differentiate based in large part on the cytokines in the microenvironment. If CD4+ T cells are activated in the presence of a high concentration of IL-12, they differentiate into Th1 cells, which produce IFNγ and TNFα. When CD4+ T cells differentiate in a microenvironment with low IL-12 or high IL-4, Th2 biasing occurs, and the cells produce IL-4, IL-5, and IL-13. The recently discovered Th17 lineage develops upon CD4+ T cell activation in the presence of TGFβ and IL-6. Th17 cells secrete IL-17 and TNFα. Tregs can be induced by stimulating CD4+ T cells, with TGFβ during activation. Tregs produce IL-10 and TGFβ. Note: T-bet is a vital Th1 transcription factor; GATA3 is a critical Th2 transcription factor; RORγt is an important Th17 transcription factor; FoxP3 is a definitive Treg transcription factor; CTL are cytotoxic CD8 T lymphocytes; Costim is a costimulatory molecule; and Ab is antibody.

1.3 REGULATORY T CELLS

Regulatory T cells (Tregs) include a large and diverse group of T cells capable of inhibiting immune responses. Tregs can express CD4, CD8, or both, but most investigation to date has focused on CD4+ Tregs, and this document will focus on these as well (1, 2). A major function of Tregs is to suppress Th cell responses through either secreted mediators or direct cell-cell contact. Because Th cells are constantly stimulated with weak signals from self antigen, Tregs are crucial for the prevention of autoimmune disease, and mice that lack naturally occurring Tregs (NatTregs) due to thymectomy develop severe autoimmunity (36). In addition, Tregs influence immune responses to pathogens, such as prevention of immunopathology, or maintenance of chronic infections (37, 38). The different types of Tregs, their suppressive mechanisms, and regulation by forkhead box protein P3 (FoxP3), a critical transcription factor for the development of certain lineages of Tregs, will be discussed below.

1.3.1 Types of Tregs

Tregs can be thymically derived NatTregs, or adaptive Tregs generated from Th cells via various stimuli. The multiple types of Tregs exhibit diverse mechanisms of suppression. All of the Tregs discussed below express CD4, and most of them also express cytotoxic T lymphocyte-associated antigen 4 (CTLA4) as well as CD25, the high affinity IL-2 receptor alpha chain (Figure 3).

NatTregs are anergic, thymically derived cells that are found in the absence of any exogenous stimulation or treatment, and they express CD4, CD25, and FoxP3. Unfortunately, CD4 and CD25 are also expressed by activated Th cells, and FoxP3 is an intracellular protein, so

isolation of these cells in viable form is difficult. Other molecules have been associated with Tregs in certain situations, such as CCR4 and CCR5, and CD127 is expressed by CD25+ effector T cells, but not Tregs (3, 39-42). However, additional work is required to find extracellular markers of Tregs that are useful for their identification and isolation under a broad range of conditions. Although only present in small numbers, NatTregs are the most studied type of Treg. NatTregs suppress Th cell proliferation through a contact-dependent mechanism. However, there are conflicting data as to whether suppression is antigen-specific (43, 44), and details of the precise mechanism of suppression remain to be elucidated. Nonetheless, evidence points towards a role for cell surface TGFβ (csTGFβ) (45), and possibly CTLA4 (46). However, NatTregs are able to suppress Th cells *in vitro*, even in the absence of TGFβ or CTLA4 signaling, suggesting that there are several cooperative inhibitory mechanisms (47, 48). Interestingly, unstimulated NatTregs are only moderately suppressive, and require stimulation through their TCR in combination with IL-2 in order to exhibit maximum suppressive activity (49). Paradoxically, addition of exogenous IL-2 to co-cultures of NatTregs and Th cells abrogates suppression, suggesting a dominant effect of IL-2 on Th cells that makes them resistant to suppression (50).

A major logistical hurdle in the study of NatTregs is the relatively small number of cells present in an unmanipulated animal. In contrast, TGFβ induced Tregs (IndTregs) can be induced in significant numbers *in vitro*. IndTregs also express CD4, CD25, and FoxP3, and can be generated by activating CD4+ T cells in the presence of TGFβ (51). Similarly to NatTregs, IndTregs inhibit Th cells through a contact-dependent mechanism *in vitro*, which can be csTGFβ-dependent, but is antigen nonspecific (51). IndTregs suppress allergic airway inflammation in mice mediated by house dust mite extract, implying a mechanism of antigen nonspecific suppression (51). Although much of the work on IndTregs has been performed in

mice, increasing evidence indicates that these cells may be even more important in humans (52). Upon activation with plate-bound anti-CD3 and soluble anti-CD28 antibodies, a small but significant proportion of human CD4+ T cells upregulate FoxP3 expression, and these cells suppress proliferation of freshly isolated Th cells (53). Thus, if human CD4+ T cells are activated with APCs in the presence of a specific antigen, it is possible that targeted IndTregs could be generated for treatment. As mentioned above, antigen specificity may not be required, but it may still enhance IndTreg potency, a distinction which remains to be elucidated.

In contrast to *in vitro* differentiated IndTregs, antigen induced Tregs (AgTregs) are generated *in vivo* by repeated administration of antigen in the absence of adjuvant. This treatment parallels the exposure of humans to harmless foreign antigens, such as food, allergens, or commensal organisms. Phenotypically, AgTregs are indistinguishable from IndTregs and activated NatTregs. They express CD4, CD25, and FoxP3, and they suppress Th cells via expression of csTGFβ (54). Importantly, mice adoptively transferred with AgTregs are tolerized to the antigen upon challenge (54). It is currently unclear whether AgTregs are comprised of expanded NatTregs, IndTregs, an additional lineage, or some combination of cell types. Also, the degree of antigen specificity in AgTreg suppression remains to be elucidated.

The AgTregs discussed above probably use csTGFβ for suppression (54). In contrast, another TGFβ-dependent Treg, known as Th3, functions via secreted TGFβ instead of the cell surface form. Like the other TGFβ-dependent Tregs described above, Th3 cells express FoxP3 (55). Th3 cells are frequently found in the gut, but are able to migrate to the rest of the body where they can inhibit disease, such as in the central nervous system in experimental autoimmune encephalitis (56). Th3 cells are generated by oral administration of antigen, and are characterized by production of TGFβ, IL-10, and sometimes, IL-4 (57). Th3 differentiation is

enhanced by IL-4, IL-10, and TGFβ (57). It is possible that Th3 cells serve an important role as the source of IL-10 and TGFβ for the generation of other types of Tregs. However, Th3 suppression seems to be mainly TGFβ-dependent (57).

Th3 cells bear similarities to another type of Treg, the T regulatory type 1 (Tr1) cell. Both lineages produce IL-10 and TGFβ, but Th3 cells release IL-4, whereas Tr1 cells secrete IFNγ and IL-5, but not IL-4 (2, 57, 58). Also, Tr1 cells usually lack FoxP3, whereas Th3 cells can express FoxP3 (55, 59). A more subtle difference that remains somewhat controversial is the relative importance of the suppressive cytokines TGFβ and IL-10, with Th3 cell function being more dependent on TGFβ, and Tr1 suppression being IL-10 and TGFβ dependent (2, 58, 60). Interestingly, Tr1 suppressive function is also contact dependent (2, 60). Although all Tregs are anergic, Tr1 cell anergy is partially dependent on autocrine and paracrine production of IL-10 (58). Thus, when IL-10R blocking antibody is added to cultures of Tr1 cells, proliferation is enhanced (58). *In vivo*, Tr1 cells have been found in cases of allograft tolerance in patients with high levels of IL-10 (61). Generation of Tr1 cells *in vitro* is achieved by activation of CD4+ T cells in the presence of IL-10, and is further enhanced by treatment with IFNα (61).

An additional IL-10 dependent Treg type can be differentiated *in vitro* by stimulating naïve CD4+ T cells through their TCR in the presence of vitamin D (vitD) and dexamethasone (dex) (62). These Tregs proliferate significantly and do not produce IL-5 or IFNγ, which differentiates them from Tr1 cells (62). Importantly, exogenous IL-10 does not enhance their development, but blocking endogenous IL-10 inhibits their differentiation, implying that only low level IL-10 production is required (2, 62). As with Tr1 cells, IL-10 secretion seems to be required for suppressive function of vitD/dex Tregs (62).

A number of other methodologies have been used to generate/activate T cells with regulatory properties including: vitD and mycophenolate mofetil induced Tregs, NatTreg induced Tregs, and immature DC (iDC) induced Tregs (2). It is not known whether these represent distinct lineages from those described above or if there is some degree of overlap between them. These possibilities remain to be more fully understood.

In addition to the wide variety of CD4+ Tregs discussed above, CD8+ T cells with suppressive abilities have been described. These cells inhibit adaptive immune responses in an MHC Class I-dependent manner, and lack expression of CD28, which is found on some, but not all, CD8+ cytotoxic T cells (2).

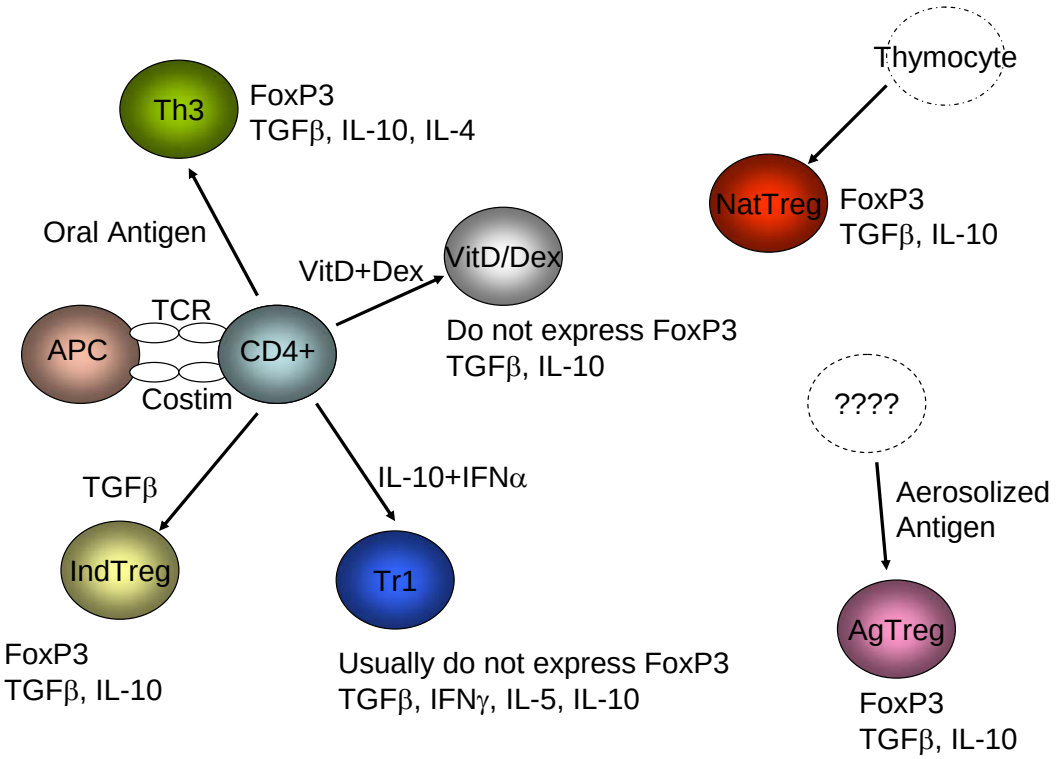

Figure 3. Types of CD4-expressing Treg

There are several types of Treg, mostly derived from CD4+ T cells, with an important exception. Administration of oral antigen to mice in the absence of adjuvant promotes Th3 differentiation. Activation of CD4+ T cells in the presence of Vitamin D and dexamethasone, a steroid, causes development of VitD/Dex Tregs. Tr1 cells are generated by stimulating CD4+ T cells with IL-10, and this process is significantly enhanced by administration of IFNα. IndTregs are induced by activating CD4+ T cells in the presence of TGFβ. NatTregs are thymically derived cells in a separate lineage from Th cells. AgTregs are generated by treatment of mice with aerosolized antigen, but it is unclear whether they are derived from NatTregs, Th cells, or a new lineage. Abbreviations: IndTreg- TGFβ induced Treg; NatTreg- naturally occurring Tregs; AgTregs- antigen induced Tregs; vit D- vitamin D; dex- dexamethasone.

1.3.2 Suppressive Mechanisms

Although Tregs are widely accepted as important inhibitors of immune responses, analyses of Treg function have proven difficult because there are several suppressive pathways that may be partially redundant. These include secreted TGFβ, csTGFβ, IL-10, and CTLA4 (Figure 4). Traditionally, suppression has been defined as inhibition of Th cell proliferation under the assumption that this correlates with Th cell cytokine production. However, Treg/Th cell co-cultures containing exogenous IL-2 show significant Th proliferation, but also significantly reduced IL-2 mRNA production, indicating that proliferation and cytokine production can be independently modulated (63). In this discussion, suppression refers specifically to proliferation, not cytokine production, since, at this point, most published data focuses on Th cell proliferation.

While most Tregs express both TGFβ and IL-10, they can be divided into two partially overlapping groups based upon the cytokine that is most important for their function, either TGFβ or IL-10. The first group is the most studied, and consists of cells that are more dependent on TGFβ for their function. Some of these cells suppress via secretion of TGFβ, but most utilize a contact dependent mechanism that is likely to involve csTGFβ (45, 51, 54). Stimulation of Tregs through their TCR promoted TGFβ secretion and upregulation of csTGFβ, and stimulation of CTLA4 on Tregs enhanced TGFβ production further (45). This study provides a possible explanation for the requirement of TCR stimulation in Tregs in order to gain maximal suppressive function, and the requirement of cell-cell contact for suppression (64). However, the importance of TGFβ remains controversial, due to the observation that *in vitro* Tregs are able to suppress Th cells deficient in TGFβ-signaling-associated Sma- and Mad- related protein 3 (Smad3) or dominant negative TGFβR transgenic animals, neither of which are responsive to

TGFβ (48). *In vivo*, there appears to be a more defined role for TGFβ, since TGFβ signaling was required for Treg protection in a murine model of diabetes (65). Furthermore, animals that lack TGFβ die within 3-4 weeks from severe wasting and inflammation, confirming a role for TGFβ in the prevention of autoimmune disease (66, 67). In parallel, mice that lack some types of Tregs due to mutations in the vital transcription factor, FoxP3, also die at 3-4 weeks from autoimmune disease (26). Nonetheless, animals expressing a CD4-specific dominant negative TGFβR survive approximately 4 times longer (68). Thus, intact TGFβR signaling is required in CD4 T cells in order to prevent autoimmune disease, but is clearly less important than the overall presence of Tregs, implying a role for other mechanisms of suppression. Alternatively, in contrast to a potential role in suppression, analyses of Tregs in TGFβ-/- mice showed reduced numbers of peripheral, but not thymic, Tregs, implicating TGFβ in Treg development, maintenance, induction, or thymic egress (69).

In contrast to TGFβ-dependent Tregs, cells that suppress primarily by producing IL-10 do not express FoxP3 (60). The function of IL-10 dependent Tregs remains controversial, with descriptions of contact dependent suppression that was not reduced by IL-10R antibody blockade and observations by others of contact independent suppression that was abrogated with IL-10R antibodies *in vitro* (58, 60). On the other hand, there is agreement that IL-10 is required *in vivo*, especially in the gut, since IL-10-/- mice develop inflammatory bowel disease (IBD) that can be prevented by administration of IL-10 (70, 71). Unlike TGFβ, IL-10 has not been observed on the cell surface, so suppression by IL-10 is abrogated by the addition of IL-10R blocking antibody (58).

Activated Th cells and most Tregs express CTLA4, which binds to CD80 and CD86, the ligands for CD28, but with higher affinity (72). In contrast to costimulation provided by CD28,

CTLA4 transduces an inhibitory signal to Th cells, and although both receptors are expressed on the cell surface, most CTLA4 is stored in endocytic vesicles, whereas CD28 is not (72). Tregs usually express CTLA4, but its functional role has remained controversial for several reasons. First, CTLA4 and CD28 signaling influence development and generation of both Tregs and Th cells, so it is difficult to interpret changes in suppression since there may be abnormal cell development. Several studies have demonstrated that CD28-/- mice have reduced numbers of Tregs (73, 74). Over-expression of CTLA4 also causes a reduction in the number of Tregs (47). Second, as mentioned above, Tregs probably use several partially redundant mechanisms of suppression. Some groups have found that signaling via CTLA4 is dispensable for Treg suppression *in vitro* (47), but others have not (46). *In vivo*, CTLA4 seems to be much more important for the prevention of inappropriate activation of Th cells, and CTLA4-/- mice have severe autoimmune disease. However, this is at least partially due to lack of CTLA4 on Th cells, and obfuscates the role of CTLA4 expression on Tregs *in vivo* (75). Third, studies using blocking antibodies showed conflicting results to those from CTLA4-deficient cells, possibly due to effects caused by antibody binding or inappropriate doses (46, 47).

Clearly, CTLA4 is involved in the inhibition of Th cells, even if it is not always required by Tregs for suppression, since knockout mice develop severe, fatal, autoimmune disease due to chronic Th cell activation (75). If Tregs use CTLA4 to suppress Th cells under certain conditions, it raises the possibility that Th cells express a ligand for CTLA4. Recent work has provided evidence that Th cells, and not just APCs, express CD80 and CD86, and that stimulation of these receptors on Th cells provides a strong inhibitory signal (76, 77). Others have demonstrated that Th cells from CD80/86-/- mice are resistant to WT Treg suppression, which can be reversed by CD80/86 over-expression in the Th cells (78). These studies imply that

CTLA4 expressed by Tregs binds to CD80 and CD86 on Th cells and suppresses either 1) through direct suppression, or 2) by maintaining close cell-cell contact between Tregs and Th cells to enhance suppression through other mechanisms, such as csTGFβ. Nonetheless, more investigation is required to confirm the involvement of CD80/86 expression on Th cells in Treg suppressive mechanisms.

Contact Dependent	Secreted
csTGFβ	TGFβ
CTLA4	IL-10

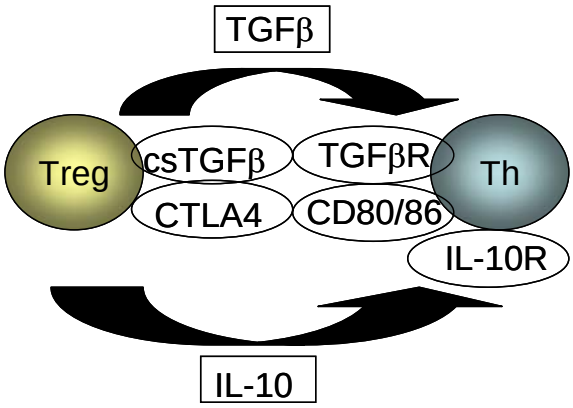

Figure 4. Treg suppressive mechanisms

Although other undiscovered mechanisms may be involved in Treg suppression, four pathways are critical to normal Treg function, csTGFβ, CTLA4, TGFβ, and IL-10. csTGFβ, which is expressed on the Treg surface, and secreted TGFβ both ligate the TGFβR on Th cells to cause inhibition. CTLA4 on the Treg surface binds to CD80 and CD86 on Th cells to transduce inhibitory signals. IL-10 is secreted by Tregs and stimulates the IL-10R on Th cells to cause inhibition.

1.3.3 FoxP3

Most TGFβ-dependent Tregs express FoxP3, which is the defining transcription factor for several types of Treg. The importance of FoxP3 is highlighted by the severe autoimmune, allergic, and inflammatory disease that characterizes humans with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) disease and scurfy mice, both of which are caused by lack of functional FoxP3 (25, 26). Its role in Treg function was confirmed when it was shown that FoxP3 over-expression in Th cells conferred suppressive function (79-81). Further, compared to vector-expressing control Th cells, FoxP3 transduced Th cells exhibited increased expression of CD25 and CTLA4, which are both normally expressed by Tregs.

Due to its importance in Treg function, FoxP3 is under intense scrutiny, but relatively little is known about how it promotes suppressive function. However, recent studies have shown that nuclear factor of activated T cells (NFAT) and NFκB, important molecules involved in TCR signal transduction, can bind to FoxP3, resulting in inhibition of cytokine gene expression (82, 83). These observations support earlier work that demonstrated potential FoxP3 recognition sequences near NFAT binding sites, which suggested why over-expression of FoxP3 significantly reduced proliferation and cytokine production in Th cells following activation (84).

While FoxP3 is reported to regulate cytokine production and proliferation, regulation of FoxP3 itself remains poorly understood. Among the few known modulators of FoxP3 are IL-2 and TGFβ, such that mice with impaired IL-2 or TGFβ signaling contain reduced numbers of FoxP3-expressing cells in the periphery. This leads to autoimmune disease even though thymic Treg numbers are normal (69, 85). FoxP3 expression is maintained by IL-2 through the STAT5

signaling pathway. The importance of this pathway *in vivo* has been demonstrated in a patient with a STAT5b mutation who had reduced FoxP3 expression in Tregs (86, 87). The critical role of IL-2 for maintaining FoxP3 expression likely explains why most Tregs express CD25, the high affinity IL-2Rα. The other cytokine known to regulate FoxP3, TGFβ was recently found to promote upregulation of FoxP3 in Th cells (51). As a result, it is unclear whether the decreased number of peripheral Tregs in mice that lack TGFβ signaling is due to reduced maintenance of FoxP3 expression or the absence of *de novo* induction in Th cells, especially since these mice have normal thymic FoxP3 expression.

1.3.4 Treg Therapy

Tregs are under intense scrutiny as potential therapeutic targets of immune response regulation. In the case of inadequate immune responses, such as certain infectious diseases and cancers, inhibition of Tregs may allow development of enhanced protective adaptive responses. On the other hand, patients with undesirable immune responses, including allograft rejection, autoimmune diseases, or allergies may benefit from increased Treg responses.

In situations where inhibition of Treg function might be desirable, the primary therapeutic approach has been to deplete cells with an anti-CD25 monoclonal antibody. Such an approach is sometimes, but not always, effective in enhancing responses to infectious diseases. For example, Tregs promote chronic infections by *L. major*, but also prevent lethal immunopathology in a model of *P. carinii* infection (37, 38). Thus, Treg depletion may be beneficial in *L. major* infections, but fatal in cases of *P. carinii* infection. Treg depletion is being studied as a treatment for several types of cancer because many tumors exhibit Treg infiltration,

which is correlated with poor clinical outcomes (39, 40, 88-91). Unfortunately, current approaches to reduce Treg function involve depletion of cells using anti-CD25, which is expressed by both Tregs and activated Th cells. Thus, anti-CD25 treatments designed to promote immune responses by depleting Tregs also target the antigen specific Th cells whose function the therapy is designed to enhance. Furthermore, non-specific elimination of a large proportion of Tregs may also lead to the initiation of autoimmune and allergic diseases. In addition, most molecules known to be expressed on the surface of Tregs are also expressed by activated Th cells. As a result, potential Treg-inhibitory therapeutic strategies will be beset with the caveats described above until Treg specific molecules can be identified.

In contrast to cancer and infectious diseases, where Tregs may inhibit desirable immune responses, increased Treg responses are potentially beneficial in organ transplantation, autoimmune diseases, and allergies. Most current conventional treatments for these conditions involve nonspecific inhibition of immune responses. Due to their ability to inhibit antigen specific Th cells without causing global immunosuppression, Tregs themselves are being studied as potential therapeutic agents. One possible caveat may be that Treg-mediated treatment might require antigen specificity, at least for some diseases (44). In one recent report, TCR transgenic Th cells specific for a particular islet antigen were transduced with FoxP3, and when adoptively transferred, potently inhibited progression of established diabetes *in vivo* (44). However, the approach was unsuccessful when TCR transgenic Th cell specific for a different islet antigen were adoptively transferred. This may prove to be a major obstacle to the use of NatTregs therapeutically due to the compounding problems of small NatTreg total cell numbers and the even smaller percentage of antigen specific NatTregs.

Alternatively, the two main difficulties of using NatTregs for treatments can be overcome by using induced Tregs (IndTregs) because a large number of cells can be generated, and, in some systems, antigen specificity seems to be unnecessary (51). However, for logistical reasons, the most promosing Treg directed therapies may involve administration of purified protein antigen or antibody, to promote Treg function *in vivo*. A novel therapy under development utilizing administration of anti-CD3 antibody to induce tolerance during the early stages of autoimmune diabetes, has shown clinical efficacy in phase I and II clinical trials (92-98). This same antibody was similarly effective in early clinical trials in the treatment of psoriatic arthritis (99). Despite the recent progress in cell transfer and antibody immunotherapy, the most common tolerizing treatments involve oral administration of antigen, which has been used successfully in humans to ameliorate many autoimmune and allergic diseases, possibly through induction of Th3 cells (100). Oral administration has been shown to reduce allergies to nickel (101, 102), house dust mites (103), and several types of pollen (104-107). Importantly, some antigens may require administration in protective capsules, as oral grass pollen was not protective in an early trial, possibly due to degradation by the digestive tract (108). Oral tolerance has also demonstrated success in patients with rheumatoid arthritis (109-112), and shows potential in multiple sclerosis (113). In trials of oral antigen administration in patients with uveitis, there was evidence of efficacy, although the data were not statistically significant (114). Unfortunately, results were mixed in patients with diabetes (115, 116). Thus, current data, especially for oral tolerance, supports the hope that immunomodulatory therapy will mature into a widely used clinical tool. However, the determination of appropriate antigens to deliver and methods of antigen preparation, such as encapsulation, require additional study.

The concept of Tregs as potential therapeutic targets is clear, but important questions remain to be addressed before widespread use of Tregs in the clinic. Little is known about regulation of Tregs, especially by cytokines, which are frequently present in significant concentrations in disease states. In addition, alternative methods of antagonizing Tregs that do not result in cell depletion or simultaneous inhibition of Th cells would be the most advantageous for treating patients with infectious diseases or malignancies. Due to the difficulties involved in Treg transfer, most Treg-targeted therapies that enter the clinic in the next few years will probably involve administration of antigen or antibodies instead of cells, to induce expansion of Tregs *in vivo*.

1.4 CYTOKINES

The previous sections discussed regulation of Th cells by intrinsic mechanisms such as TCR receptor selection, and by the function of regulatory T cells. This section describes cytokines, and their critical role as modulators of Th cells. Cytokines are small molecules that are usually newly synthesized, unlike many other secreted immunological communication molecules such as histamine, which are stored in granules (1, 2). Cytokines serve as an important method of communication among immune cells, and between the immune system and other cell types including epithelial cells, endothelial cells, and hepatocytes. As mentioned above, cytokines can induce CD4+ T cell differentiation into Th1, Th2, Th17, and Treg cells, and promote their function, yet also serve a vital role in modulation of adaptive immune responses. As a result, they continue to be actively investigated for potential therapeutic efficacy. Among notable

successful applications of cytokine-based immunotherapy is treatment with anti-TNFα antibody. Blockade of TNFα by either infliximab or etanercept has proven to be a highly efficacious treatment for IBD and RA (117-123). Many other diseases, such as atopy, are associated with functional mutations in genes for cytokines or their receptors, in this case, IL-4Rα, which increases IL-4 signaling and Th2 biasing (24). Therefore, cytokines are clearly important regulators of the adaptive immune system, and could be exploited for therapeutic benefit. Significant progress continues to be made in understanding cytokine signaling, describing the effects of cytokines, and elucidating cellular negative feedback pathways, all of which could eventually suggest novel approaches to therapy.

1.4.1 Signal Transduction

Most cytokines signal primarily through activation of STATs, although the vast majority of cytokines also signal through additional pathways, which are less studied, such as insulin receptor substrate (IRS) proteins, phosphatidylinositol 3 kinase (PI3K), and the Ras/mitogen activated protein (MAP) kinase cascade (1, 2). However, a few cytokines signal through unique transduction pathways. Among these, TGFβ, which signals through the Smad pathway, is one of the most important, and is involved in the present work (124).

STAT-dependent cytokine signaling is initiated upon cytokine binding to a specific receptor, which causes association of Janus protein kinases (Jaks) with the intracellular portion of cytokine-receptor chains (Figure 5) (125). Jaks can phosphorylate other Jaks as well as docking sites for many other proteins on the receptor cytoplasmic tail (125). STAT proteins bind

to the receptor, in the domains phosphorlyated by Jaks. Upon binding, STATs are themselves phosphorylated by the Jaks (125). The phosphorylated STATs (pSTATs) form homodimers or heterodimers, and translocate to the nucleus (125). Until recent years, only dimerized pSTATs were considered to influence transcription. However, increasing evidence suggests that some unphosphorylated, monomeric, STATs can also translocate to the nucleus and influence gene expression (126). Still, the importance of unactivated STAT monomers *in vivo* remains poorly understood.

Four Jak proteins, Jak1, Jak2, Jak3, and Tyk2, and seven STAT proteins, STAT1-STAT4, STAT5a, STAT5b, and STAT6, are expressed in mammals (2, 126). Since many cells express all four Jaks, and several cytokines recruit the same Jaks to their receptors, cytokine specificity is conferred primarily by differential recruitment of particular STATs to cytokine receptors. For instance, both Jak1 and Jak3 are associated with the IL-2 and IL-4 receptors, but IL-2 primarily activates STAT5a and STAT5b, whereas IL-4 mainly causes STAT6 phosphorylation (2). An additional level of signaling complexity is found for several cytokines, such as IL-6, which activate multiple STATs, in this case STAT1 and STAT3 (127, 128). This opens the possibility of both homodimer and hererodimer formation (127). The ratios and compositions of the STAT1 and STAT3 dimers are clearly important because cytokines with very different effects can activate the same STATs. For instance, IL-10, an immunosuppressive cytokine, activates STAT3, but IFNγ, which is a potent immune activator, signals primarily through STAT1 (129-131). Interestingly, IL-6 can be pro-inflammatory or anti-inflammatory depending on the situation, and these effects could be due to activation of STAT1 and STAT3, respectively (128, 132).

In addition to different ratios of STATs, cytokines exert specific effects through many other pathways, such as insulin receptor substrate (IRS) and phosphatidylinositol 3 kinase (PI3K) activation, and the Ras/ mitogen associated protein (MAP) kinase pathway (2). The IRS pathway will be described briefly due to its involvement in IL-4 signaling, which is an important component of the investigations described in this document. Upon stimulation by cytokines, such as IL-4, the receptor is activated and phosphorylated at key tyrosine residues that allow recruitment of IRS proteins. After binding to the cytokine receptor, IRS proteins are phosphorylated, and serve as adaptors by recruiting many other molecules, especially PI3K (2, 133-136), which reportedly activates Akt, promoting cell survival and inhibition of pro-apoptotic c-Jun N-terminal kinase 2 activation in a Th cell line (136). Others found that IRS2 was unnecessary for the anti-apoptotic effects of IL-4, but was involved in IL-4-induced proliferation (135). This was confirmed by studies in mice of a mutated IL-4R that lacked the tyrosine required for IRS2 recruitment, but was still able to activate STAT6 (137). Upon stimulation, Th cells with the mutant receptor proliferated significantly less in response to IL-4 than cells with a WT receptor. Therefore, IL-4 has a reduced ability to induce proliferation in the absence of IRS activation. Thus, IL-4 effects on proliferation may be less dependent on STAT6 than are other IL-4-mediated responses.

In contrast to the IRS2, PI3K, and Ras pathways described above, which signal concurrently with STATs, receptors that signal through Smads may not transduce signals through STATs. One of the few cytokines that utilizes the Smad pathway is TGFβ (124). Upon ligand binding, the TGFβR forms a multimer of four separate chains, which have intrinsic kinase activity (124). The complex undergoes autophosphorylation, and this enables binding of either Smad2 or Smad3 (124). After recruitment to the receptor, Smad2 and Smad3 are themselves

phosphorylated (124). They then form homodimers or heterodimers, which bind to Smad4 to form a trimer (124). The Smad2/3-Smad4 complex enters the nucleus to modulate transcription (124). In the case of BMP receptors, Smad1, Smad5, and Smad8 are activated, and form complexes with Smad4, which translocate to the nucleus and influence transcription (124). Unlike STATs, which act primarily as transcription factors, the Smad proteins include two members that function as competitive inhibitors, Smad6 and Smad7 (124). Although Smads transduce signals for relatively few cytokines, the importance of TGFβ has increased their profile as important modulators of immunity.

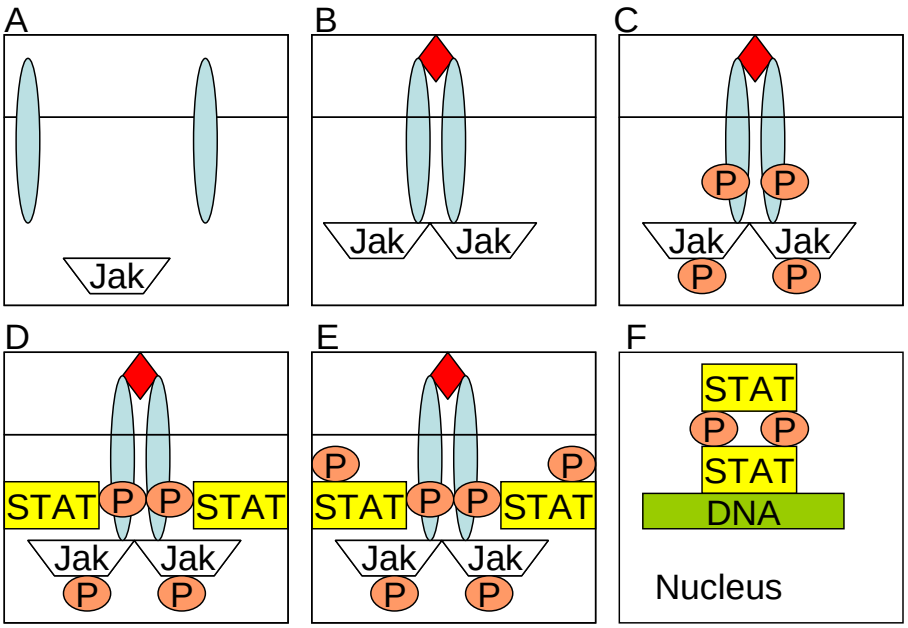

Figure 5. Cytokine signaling

A, undimerized receptor that has not bound cytokine. B, cytokine causes receptor dimerization and Jak recruitment. C, Jaks phosphorylate each other or autophosphorylate. Then, Jaks phosphorylate STAT binding sites on the receptor. D, STATs are recruited to phosphorylated receptor. E, Jaks phosphorylate receptor-bound STATs. F, phosphorylated STATs dimerize, enter the nucleus, and bind DNA. Legend: blue oval- cytokine receptor chain, red diamond- cytokine, horizontal line- cell membrane (above line is extracellular), orange circle- phosphate.

1.4.2 Important Cytokines

Cytokines are important modulators of immune responses, and intense investigation has led to the discovery of many new cytokines in the past few years. This text will focus on a few cytokines of great importance in the regulation of CD4+ T cell responses (Table 1).

IL-2 is one of the best understood cytokines, and is required for normal Treg development (138). The fact that most types of Tregs express CD25 (IL-2Rα chain) strongly implies that IL-2 is important to their function, and this is supported by data indicating that IL-2 promotes NatTreg FoxP3 expression, survival, proliferation, and suppressive function (49, 63, 85, 86). Thus, in mice that genetically lack IL-2 cytokine, or any component of the IL-2R, severe autoimmune disease develops due to a deficiency in NatTregs (85). The IL-2R is comprised of three chains, the inducible CD25 chain, the IL-2Rβ chain, and the common gamma chain (γc), which is also a component of the receptors for IL-4, IL-7, IL-9, IL-15, and IL-21. IL-2 binds weakly in the absence of CD25 to the low affinity receptor, which is constitutively expressed in CD4+ T cells, and is comprised of the other two receptor chains. Upon cytokine binding, Jak1 and Jak3 are activated, and induce signaling through STAT5a and STAT5b. There is a high degree of homology between STAT5a and STAT5b, and the two are partially redundant (139). However, the two STAT5 molecules are not completely interchangeable, and defects in STAT5b in humans are linked with immunodeficiency and autoimmune disease, due to decreased numbers of NatTregs (87). IL-2 is clearly important for NatTreg function, and culture of NatTregs with TCR stimulation and IL-2 *in vitro* increases their suppressive function (49). However, addition of exogenous IL-2 to NatTreg/ Th cell co-cultures abrogates suppression (50).

This suggests that either IL-2 has opposing effects on Th cells and NatTregs, or that IL-2 promotes FoxP3 while inhibiting NatTreg suppression through another mechanism that is rapidly reversible upon removal of the cytokine.

Similarly to IL-2, IL-4 signals through a receptor incorporating γc, so, it can replicate many effects of IL-2 stimulation (49). The IL-4R is formed by γc and the IL-4Rα chain, which is also part of the receptor for IL-13. Ligation of the IL-4R causes Jak1 and Jak3 activation, followed by activation of STAT6, which dimerizes and translocates to the nucleus. In Th cells, IL-4 promotes Th2 differentiation and proliferation. Mice that lack IL-4Rα or STAT6 demonstrate a severe deficiency in the initiation of Th2 immune responses, but no other deficits (140-143). However, in certain *in vivo* infection models, these mice are still capable of generating Th2 responses, highlighting the role of other signals in Th2 differentiation (27). An important characteristic of Th2 responses in general, and IL-4 in particular, is antagonism of Th1 and Th17 responses (35). Thus, dysregulation of IL-4 can lead to Th1-, Th2-, or Th17-associated pathology. Importantly, IL-4Rα alleles associated with more efficient signaling predispose humans to atopy, asthma, and other Th2-mediated diseases, whereas alleles associated with less efficient signaling result in lower serum IgE, and therefore less atopy (24, 144). While the role of IL-4 in the induction of Th2 responses has been studied in great detail, the influence of IL-4 on Tregs remains poorly understood. Overall, IL-4 seems to influence Tregs similarly to IL-2. Specifically, IL-4 promotes NatTreg survival and proliferation, but inhibits suppression when added exogenously to NatTreg/ Th cell co-cultures (63, 145, 146).

Th1 responses provide a counterbalance to Th2 biasing, and are required for protective immunity to many intracellular pathogens. Unlike Th2 differentiation, which can be initiated and expanded by a single cytokine (IL-4), efficient Th1 biasing *in vivo* is initiated by IL-12 and

amplified by IFNγ (5, 16-20, 147). After providing TCR ligation and costimulation to activate naïve Th cells, DCs can produce IL-12 to promote Th1 differentiation. Ligation of the IL-12R leads to activation of Jak2 and Tyk2, and signals are subsequently transduced to the nucleus through STAT4. This promotes IFNγ production, which leads to Jak1 and Jak2 phosphorylation, STAT1 activation, and eventual Th1 differentiation. In mice that lack IL-12, IFNγ, or their receptors, Th1 responses are markedly inhibited, but animals are still capable of mounting responses to some viruses and intracellular pathogens (147). The effects of IL-12 and IFNγ on protective adaptive responses has been characterized in depth, but their roles in Treg development, phenotype, and function remain to be elucidated.

Th1 and Th2 cytokines generally antagonize each other and inhibit Th17 biasing, but the influence of the recently described Th17 lineage and Th17-produced cytokines on Th1 and Th2 responses is unclear (7, 31, 32, 35). Th17 cells produce IL-17 and TNFα, and are associated with inflammation and some autoimmune diseases (7, 12-15). The Th17 cell type is being studied with great interest, but its precise role in these processes, remains unclear. Several landmark papers in the past few years have demonstrated that neither IL-17 nor IL-23, which is also associated with Th17 responses, drives differentiation of naïve Th cells into Th17 cells (31-33). Surprisingly, TGFβ, which is considered to be an immunosuppressive cytokine, and which can induce differentiation of CD4+ T cells into IndTregs, promotes Th17 differentiation in combination with IL-6 (31-33). Although IL-23 is unable to promote Th17 biasing, it does seem to be vital for Th17 survival and expansion (7). Thus, a combination of IL-6, primarily a pro-inflammatory cytokine, and TGFβ, mainly an anti-inflammatory cytokine, leads to differentiation of Th17 inflammatory cells.

The recent excitement surrounding the identification and description of Th17 cells has focused attention on IL-6, which is associated with cancer, inflammation, and autoimmune disease (148). Mice that lack IL-6 have defective acute-phase responses and enhanced sensitivity to many infections, such as vaccinia and *Listeria monocytogenes* (149). Interestingly, IL-6 has also been implicated in the inhibition of Treg suppressive function, although this remains controversial (49, 150, 151). IL-6 signaling can be mediated via a membrane bound receptor (mIL-6R) or a soluble receptor (sIL-6R). IL-6R family members are among the few known soluble receptors able to transduce signals, since soluble receptors usually act as non-signaling antagonists (148). IL-6 provides signals by binding to mIL-6R or sIL-6R, and forming a complex with gp130, which is a common component of the receptors for IL-6, IL-11, leukemia inhibitory factor, oncostatin M, and many others (2). Importantly, gp130 is vital for signaling, whereas the mIL-6R and sIL-6R seem not to be directly involved in signaling, but rather enable IL-6 to activate gp130 (148). Upon ligation with IL-6 bound to one of its receptors, gp130 activates Jak1, Jak2, and Tyk2, which results in activation of STAT3 and STAT1. As mentioned earlier, the precise ratio and composition of STAT dimers induced by IL-6 may allow significant modulation of the signal the cell receives (127, 132, 148, 152). Also, CD4+ T cells can express preferential inhibitors of STAT1 and STAT3, such as SOCS1 and SOCS3, respectively, to fine-tune their responses. The importance of the STAT1:STAT3 activation ratio in IL-6 signaling is demonstrated by the fact that, surprisingly, STAT3 is also an important signal transduction molecule for IL-10, an anti-inflammatory cytokine that is associated with Tregs and tolerance (55, 70, 100, 153).

As mentioned above, IL-10 is produced by Tregs, promotes Tr1 differentiation, and inhibits Th cells. Ligation of the IL-10R causes Jak1 and Tyk2 activation, which in turn leads to

STAT3 dimerization. IL-10 inhibits Th1 cells and promotes differentiation of Tr1 cells (58, 61). Several types of cells including Tregs and Th2 cells, produce IL-10, and mice that lack IL-10 spontaneously develop IBD, supporting a role for IL-10 as an important anti-inflammatory cytokine (70).

Similar to IL-10, TGFβ is a vital tolerogenic cytokine that strongly inhibits Th cell expansion and IL-2 production (154). In the absence of TGFβ or its receptors, mice develop severe autoimmune disease and have significantly reduced peripheral, but not thymic, Treg numbers (69). A unique feature of TGFβ is that it does not signal primarily through STATs. Upon ligand binding, the TGFβR causes phosphorylation of Smad2 and Smad3, which form homodimers and heterodimers that complex with Smad4, and translocate to the nucleus (124). Activation of CD4+ T cells in the presence of TGFβ induces IndTregs, but the presence of IL-6 inhibits IndTreg differentiation and instead promotes the generation of Th17 (31).

Table 1. Cytokine/STAT KO phenotypes

Adapted from 2003 Fundamental Immunology by Paul.

Knockout Gene	Phenotype
IL-2	Treg deficiency, severe autoimmune disease
IL-4	Almost complete abrogation of Th2 differentiation *in vitro*, able to mount Th2 responses to certain pathogens *in vivo*, reduced anti-tumor immunity
IL-6	Diminished acute-phase responses, decreased resistance to multiple types of infection
IL-10	Inflammatory bowel disease, enhanced immune responses
IL-12	Defects in Th1 biasing, protective responses remain for a limited number of intracellular pathogens
IFNγ	Similar to IL-12-/- mice
TGFβ	Severe autoimmune disease
STAT1	Significantly reduced responses to many viruses
STAT3	Embryonically lethal
STAT4	Similar to IL-12-/- mice
STAT5a/b	Reduced Th proliferation, dramatic decrease in the number of naturally occurring Tregs
STAT6	Similar to IL-4-/- mice

1.4.3 Cytokine Regulation

Due to the importance of cytokines as immune modulators, cells tightly regulate cytokine-mediated effects at the level of cytokine production, receptor expression, signal transduction, and signal modulation. There are many mechanisms that inhibit cytokine signaling pathways including: phosphatases, protein inhibitors of activated STATs (PIAS), and suppressors of cytokine signaling (SOCS). The SHP phosphatases and SHIP phosphatases play important roles in deactivation of many cytokine signaling pathways to prevent constant activation, but are not cytokine-specific (2). The PIAS group of proteins has four members, PIAS1, PIAS3, PIASx, and PIASy. PIAS proteins not only inhibit STAT binding to cytokine receptor chains, but are also capable of other mechanisms of inhibition, such as through E3 ubiquitin ligase activity. However, the best known inhibitors of cytokine signaling are the SOCS proteins, which will be discussed in more detail below.

1.4.3.1 SOCS Family

The SOCS family (also known as CIS/SSI/JAB) consists of eight members, SOCS1-7 and CIS, which are characterized by a central SH2 domain and C-terminal SOCS box (155-159). Other proteins also contain SOCS boxes, such as Von-Hippel-Lindau protein (VHL) and Elongin A, and these domains allow E3 ligase complex formation, which promotes ubiquitination and proteasomal degradation. Thus, SOCS proteins can act as adaptors to induce degradation of a variety of targets, including receptors, Jaks, and STATs, leading to inhibition of cytokine signaling. In addition, SOCS proteins can bind to signaling molecules directly leading to their

inhibition without degradation. The mechanism of suppression depends upon the particular SOCS protein-signaling molecule pairing.

The best studied SOCS proteins are CIS, SOCS1, SOCS2, SOCS3, and, to a lesser extent, SOCS5. These proteins will be generally reviewed, and a later section will be devoted solely to SOCS3 (Table 2 and Table 3). CIS is induced by a number of cytokines, many of which have receptors whose signaling is also inhibited by CIS, such as IL-2, IL-3, erythropoietin (EPO), GH, and prolactin (156, 160). All of these cytokines signal through STAT5, and CIS inhibits STAT5 activation by blocking STAT5 docking sites (160). The phenotype of STAT5-/- mice and animals that over-express CIS are similar, each showing defects in growth hormone signaling and mammary gland development (161). On the other hand, CIS-deficient animals are phenotypically normal, suggesting possible compensatory mechanisms to downregulate STAT5 signaling (162).

Although it can inhibit some of the same cytokines as CIS, SOCS1 is best known as a critical inhibitor of IFNγ signaling (163). Thus, SOCS1-deficient animals die by one month of age due to severe, systemic inflammation. The phenotype can be significantly improved, although not eliminated, by administration of neutralizing antibody to IFNγ, or by simultaneous deletion of the gene for IFNγ (163). SOCS1/RAG2 double knockout mice, which lack Th cells, have significantly reduced pathology and lengthened lifespans, further strengthening the conclusion that SOCS1 is critical for inhibition of IFNγ signaling in Th cells (164). SOCS1 acts by inhibition of Jaks and other molecules activated by cytokines, such as IRS1 (156, 165). SOCS1 also inhibits signaling by IL-4 and IL-12, and SOCS1-deficient Th cells show more extreme Th1 or Th2 biasing than cells from normal mice upon treatment with either cytokine (166-168).

Less is known about the role of SOCS2 in the immune system, and the most thoroughly described effect of SOCS2 is its inhibition of STAT5 activation following growth hormone (GH) stimulation (155, 156, 158, 159). Additionally, recent work has suggested that SOCS2 may be important for Treg development and/or function, since microarray analyses have shown increased SOCS2 expression in Tregs, compared to Th cells (169-171). This is supported by data demonstrating that over-expression of FoxP3 in Th cells is associated with upregulation of SOCS2 (170). Another potentially important function of SOCS2 is the enhanced degradation of SOCS3 in the presence of IL-2 (172).

Unlike the other SOCS proteins, which do not seem to skew Th cell differentiation towards a particular lineage, SOCS3 and SOCS5 are associated with Th2 and Th1 responses, respectively (173-177). Over-expression of SOCS5 seems to promote Th1 biasing in mice by binding to the IL-4R and inhibiting IL-4 signaling (177). However, SOCS5-deficient Th cells appear to differentiate into Th1 and Th2 cells normally, so the importance of SOCS5 *in vivo* remains unclear (178).

The multiple functions of SOCS3 have proven more laborious to elucidate because of the large number of cytokines that it regulates, the variety of inhibitory mechanisms used, and because of embryonic lethality of SOCS3-/- mice (162). Nonetheless, SOCS3 is one of the most studied SOCS proteins in immunology, probably as a result of its association with many diseases. SOCS3 will be discussed in-depth in the next section.

Table 2. Cytokines inhibited by SOCS and cytokines that induce SOCS expression.

Adapted from Naka *et al*. *Trends in Immunology*. 2003.

Protein	Induces Expression of SOCS	Inhibited by SOCS
CIS	IL-2, IL-3, IL-6, IL-9, IFNα, TNFα, EPO, TSLP, GH, prolactin	IL-2, IL-3, EPO, GH, prolactin
SOCS1	IL-2, IL-4, IL-6, IL-7, IL-9, IL-13, IFNα, IFNβ, IFNγ, LIF, TNFα, EPO, TPO, TSLP, G-CSF, GM-CSF, M-CSF, GH, prolactin, insulin, CNTF, cardiotropin, TSH	IL-2, IL-4, IL-6, IL-7, IL-12, IL-15, IFNα, IFNβ, IFNγ, LIF, TNFα, EPO, TPO, TSLP, GH, prolactin, insulin, leptin
SOCS2	IL-6, IFNα, IFNγ, LIF, GH, prolactin, insulin, CNTF, cardiotropin	IL-6, GH, IGF1
SOCS3	IL-1, IL-2, IL-6, IL-9, IL-10, IL-13, IFNα, IFNγ, LIF, EPO, GM-CSF, GH, prolactin, insulin, leptin, CNTF	IL-2, IL-4, IL-6, IL-9, IL-11, IFNα, IFNβ, IFNγ, LIF, EPO, GH, prolactin, insulin, leptin
SOCS5	IL-6	IL-4, IL-6
SOCS6	Unknown	Unknown

Table 3. Phenotypes of SOCS KO and over-expressing mice.

Adapted from: *Annual Reviews in Immunology*. 2004., *Nature Immunology*. 2003., and *Trends in Immunology*. 2004.

Protein	Deficiency	Over-expression
CIS	Increased hematopoetic precursors	Th2 bias, reduction of NK and NKT cells, decreased IL-2 signaling
SOCS1	Neonatal inflammation and death, mainly due to increased IFNγ signaling	Larger number of Th cells, increased Th activation
SOCS2	Gigantism caused by altered GH signaling	Paradoxically, gigantism caused by altered GH signaling
SOCS3	Lethal *in utero* due to placental defects, altered IL-6 signaling in hepatocytes and macrophages	Lethal anemia *in utero*, atopy, enhanced Th2 responses
SOCS5	No effect on Th cell differentiation	Increased Th1 biasing due to reduced IL-4 signaling
SOCS6	Slightly reduced growth	Unknown

1.4.3.2 SOCS3

SOCS3 is a 27kD protein that is associated with enhanced Th2 responses, possibly due to inhibition of STAT4 activation; and that is dysregulated in cancer, hepatitis C infection, allergic conjunctivitis, arthritis, asthma, and atopic dermatitis (174, 175, 179-182). SOCS3-/- mice die *in utero* due to placental defects, necessitating the use of several alternative systems such as tetraploid rescue and cell specific knockout approaches to elucidate its functions (130, 131, 183-185). Neurons, hepatocytes, macrophages, Th cells, and several other cell types express SOCS3, and its expression can be induced by many cytokines, including IL-2, IL-6, IL-10, IFNα, IFNγ, and EPO (155, 156, 158, 159). SOCS3 inhibits signaling by IL-2, IL-6, IFNα, IFNγ, EPO, and other cytokines by interacting with cytokine receptors and preventing docking of STATs (155, 156, 158, 159).

SOCS3 is expressed in two isoforms, with different stabilities that may allow modulation of SOCS3 protein levels through altenative splicing. The half-life of SOCS3 can be decreased by phosphorylation, and the shorter isoform that is transcribed during stress conditions has a longer half-life (186-190). Phosphorylation of SOCS3 in its SOCS box also enables binding to Nck and Crk-L, which are involved in TCR signal transduction to the cytoskeleton (187). Interestingly, others have shown that SOCS3 can use its SH2 domain to interact with phosphotyrosines on CD28 (191). Thus, SOCS3 may be involved in Th activation due to interaction with TCR and costimulatory signal transduction pathways.

Much of the initial immunological research on SOCS3 focused on its association with Th2 responses (173-176, 191). Several groups have shown that SOCS3 is expressed at higher levels in Th2 cells than in Th1 cells, and over-expression of SOCS3 in Th cells undergoing

differentiation seems to enhance Th2 development possibly by binding to the IL-12R, and inhibiting STAT4 activation (174).

In addition to its influence on Th1/Th2 biasing, two particularly important functions of SOCS3 are its regulation of IL-2 and IL-6 signaling. SOCS3 inhibits IL-2-mediated STAT5 activation by binding to the IL-2R, and antagonizing STAT5 binding and phosphorylation (173, 189, 190). Further, over-expression of SOCS3 inhibits IL-2-mediated proliferation of Th cells (173). Also, SOCS3 expression is inversely proportional to Th cell proliferation (173).

In contrast to the relatively straightforward regulation of IL-2 by SOCS3, regulation of IL-6 signaling by SOCS3 is quite complex. As mentioned earlier, IL-6 causes activation of STAT3 and STAT1. Several groups described alterations in IL-6 signaling in SOCS3-deficient cells, with prolonged activation of STAT3, and less, but still significant, enhancement of STAT1 activation (129-131). Interestingly, lack of SOCS3 does not increase the normal pro-inflammatory effects of IL-6. Instead, IL-6 stimulation promotes anti-inflammatory signals similar to those of IL-10, which activates STAT3, and causes transcription of a set of IFNγ-inducible genes that are also anti-inflammatory. These data imply that SOCS3 modulates the ratio of activated STAT3:STAT1 generated by IL-6 signaling, and may explain the highly variable effects described for IL-6 in different cell types or under different conditions (127, 128, 132, 148, 152).

Clearly, SOCS3 plays a critical role in the modulation of cytokine signals. As a result, many diseases are associated with abnormal expression of SOCS3, which will be discussed below. In cancer, as with other diseases associated with SOCS3 dysregulation, SOCS3 can be under- or over-expressed, depending on the particular form of malignancy. For instance, patients with hepatocellular carcinoma (HCC) frequently demonstrate decreased expression of SOCS3 in

their tumors, whereas other malignancies, such as breast carcinomas, often show elevated expression of SOCS3 (179, 192, 193). Over-expression of SOCS3 was also observed in hepatocytes infected with HCV. The HCV core protein directly induces SOCS3 expression in hepatocytes, and SOCS3 inhibits IFNα signaling (180, 181). Since IFNα increases resistance to viral infection and propagation, this is important evidence of direct SOCS3 involvement in modulating disease progression.

Elevated SOCS3 expression is also found in Th2-mediated pathological conditions such as asthma and atopic dermatitis (AD), as well as in a murine model of allergic conjunctivitis (AC) (174, 175). However, this does not definitively prove a role for SOCS3 in promoting the disease; rather, it merely establishes a correlation of its expression with disease. In order to prove that SOCS3 is involved, over-expression, loss-of-function, and heterozygous approaches were used. In a murine model of asthma, SOCS3 over-expression exacerbated disease, and the expression of dominant-negative SOCS3 in Th cells inhibited Th2 differentiation (174). Similarly, Th2 biasing was reduced in Th cells from SOCS3+/- heterozygous mice (174). In agreement with the manuscript above, induction of AC in mice induced an increase in SOCS3 expression (175). T-cell-specific SOCS3 over-expression enhanced disease, whereas disease severity was diminished in SOCS3 heterozygotes and animals that over-expressed dominant negative SOCS3 (175).

Rheumatoid arthritis (RA) is an inflammatory disease where Th17 and IL-6 play prominent roles. Over-expression of SOCS3 in the joints of mice strongly inhibited progression of already established disease in collagen-induced and antigen-induced models of RA (182). Interestingly, over-expression of WT SOCS3 showed more therapeutic efficacy than the introduction of a dominant negative STAT3. This implies that upregulation of SOCS3 may prove

useful in the treatment of RA, and that SOCS3 is probably inhibiting signaling by other STATs as well as STAT3.

In addition to its role as an inhibitor of chronic inflammation, SOCS3 was shown to reduce acute inflammation caused by sepsis. Cell-penetrating SOCS3 (cpSOCS3), which is a chimera of SOCS3 and a short peptide tag that is cell-permeable, was administered to mice intravenously (IV), and provided protection in a model of sepsis (194). In the future, cpSOCS3 could be used to treat diseases where SOCS3 expression is deficient. In contrast, strategies such as SOCS3 siRNA may prove to be therapeutically beneficial to reduce SOCS3 expression when high expression is deleterious such as in HCV infection, breast cancers, and allergies.

In contrast to Th cells, the effects of SOCS3 expression in Tregs remain poorly studied. However, several microarray-based studies of NatTregs versus Th cells, and IndTregs versus Th cells, suggest that SOCS2 expression in FoxP3+ cells is elevated compared to cells that lack FoxP3 (169-171). Since SOCS2 can enhance IL-2-mediated SOCS3 degradation (172), and Tregs express CD25, the high affinity IL-2R, we hypothesized that Tregs may lack SOCS3 expression. Interestingly, others demonstrated that SOCS3-deficient Th cells secreted elevated levels of the Treg-associated cytokines IL-10 and TGFβ after *in vitro* stimulation, cells the authors suggested might be Th3 regulatory cells (185). However, the number of NatTregs and the expression of FoxP3 were not altered in T cell-specific SOCS3-deficient mice (185). Therefore, in spite of the importance of SOCS3 in immune regulation, and investigations of SOCS3 as a therapeutic target, its expression and function in Tregs have not been thoroughly evaluated.

1.4.4 Cytokine Therapy

Cytokines play a central role in the pathogenesis of many diseases, and are vital modulators of immune function. As a result, cytokines and their signaling pathways have become important targets for the development of new therapeutic agents. Currently, most treatments involve direct administration of cytokines, such as IL-2, or neutralizing antibodies and receptor antagonists, as in the case of anti-TNFα (117, 119-123, 195-197). However, treatments that inhibit cytokine signal transduction are under development, such as the use of STAT3 decoys and cpSOCS3 (194, 198, 199).

Many of the early cytokine therapies involved direct administration of the particular cytokine itself. A combination of TNFα and IFNγ was injected into cancer patients after the observation that both cytokines induced tumor cell death *in vitro*; but, as with many direct cytokine therapies, the side-effects were very severe (200). In this case, toxicity was severe enough to discontinue use, even in cancer patients with advanced disease. On the other hand, single cytokine therapy with IL-2, IFNα, or IFNγ, are used to treat certain cancers and infections (180, 195-197, 201). Nonetheless, these cytokines frequently have severe side-effects. The toxicity associated with cytokine-based therapy, especially following systemic administration, has dampened enthusiasm for further investigation of direct treatment with cytokines.

Enhancing cytokine signaling through their direct administration has proven to be poorly tolerated by patients. However, antibody or receptor antagonist inhibition of cytokines frequently, but not always, shows more moderate toxicity. Infliximab, etanercept, and adalimumab, which all target TNFα, are among the best known anti-cytokine therapeutics, and are beneficial to patients with IBD and RA (117-123, 202-204). Anti-TNFα therapy is tolerated

well in most patients, although it can increase the risk of infection, and can cause reactivation in patients infected with *Mycobacterium tuberculosis* (205-209). Similar to TNFα, IL-6 is associated with inflammatory diseases; but in contrast to the anti-tumor effects of TNFα, IL-6 is a growth factor for many tumors (198, 199, 210). Due to the involvement of IL-6 in many diseases, tocilizumab, an IL-6R blocking antibody, has been developed as a therapeutic agent with potential as a treatment for inflammatory and malignant diseases (210-219).

In contrast to TNFα and IL-6, which are important in many inflammatory diseases, IL-4 is critical in the induction of allergic diseases and asthma. Clinical trials are underway to determine the efficacy of nuvance, a soluble IL-4R, as a treatment for asthma (220, 221). Thus, therapeutic inhibition of cytokines is an area of intense research due to potentially high efficacy and relatively low side-effects. Although these treatments usually demonstrate low toxicity, this is not always the case; and there are several drawbacks, including: 1) some patients do not tolerate the infusions; 2) since these agents are proteins, they must be administered intravenously; 3) patients can develop immune responses against the antibody or receptor antagonist, reducing or eliminating treatment efficacy; and 4) relatively high concentrations of agent are required to neutralize the cytokine or block its receptor.

The cytokine-targeted therapeutic approaches described above rely on modulation of extracellular components of the signal transduction pathway, i.e. the cytokine and its receptor. Several preliminary therapeutic approaches are designed to act on intracellular signaling molecules, which may prove more potent than extracellular targets because they are further downstream and will inhibit signaling by several cytokines simultaneously. Highlighting its importance in many diseases, the main cytokine targeted by three different approaches was IL-6. One group created an oligonucleotide sequence containing a STAT3 binding site that inhibited

IL-6-induced transcription, proliferation, and survival in IL-6-dependent tumor cells *in vitro* and *in vivo* (198, 199). Although injections would still be required for therapeutic use, intratumoral administration may allow for the use of lower doses; and due to the small size of the agent, patients are less likely to initiate immune responses against the treatment compared to antibody- and receptor antagonist-based approaches. Another manuscript described two reagents, a dominant-negative STAT3 (dnSTAT3) adenoviral construct, which has a mutation at either a tyrosine that is phosphorylated by gp130 or a mutation at the DNA binding domain, and a SOCS3 adenoviral construct. Both the dnSTAT3 and SOCS3 viruses inhibited disease in a murine model of rheumatoid arthritis (182). Interestingly, SOCS3 over-expression ameliorated disease more potently than dnSTAT3, implying that additional signaling pathways are probably being inhibited by SOCS3. The potential for simultaneous inhibition of multiple signaling pathways could be a significant advantage of SOCS3 therapy over specific cytokine or STAT-directed approaches. While adenoviral SOCS3 therapeutic strategies will require significant study before approval for clinical use, the generation of cpSOCS3 may enable relatively rapid progress to the bedside. Animals injected with cpSOCS3 were protected in a model of sepsis, demonstrating the potential efficacy of cpSOCS3 *in vivo* (194). Nonetheless, a significant drawback to the use of cpSOCS3 is the cost of production, especially during early studies, in comparison with antibody, oligonucleotide, or adenoviral approaches. However, the most likely initial use of SOCS3 in the clinic will not be as a therapy, but rather as a prognostic marker. For example, in patients infected with HCV, hepatocyte SOCS3 levels inversely correlate with clinical responsiveness to IFNα therapy (180, 181). In addition, many other diseases demonstrate

dysregulated SOCS3 expression, such as asthma and atopic dermatitis, which were mentioned above, suggesting possible use as a clinical marker of disease progression and a prognostic indicator.

1.5 IMMUNE PATHOLOGY

The adaptive immune system is critically important for survival due to the ubiquitous presence of pathogens and microbes that could lead to lethal infection in the absence of an immune response. Unfortunately, in industrialized countries, where infectious disease has become less prevalent, pathologic autoimmune responses are increasing in frequency (2, 222). Immunopathology can involve unnecessary or excessive responses, such as those to harmless or self antigens in the case of grafts, allergies, and autoimmune disease. On the other hand, immunopathology can be induced by insufficient or tolerogenic responses to tumors and infections. Cytokines are increasingly recognized as important causes of pathology, and therefore, as potential therapeutic targets (117-123, 195-197, 200, 201, 220, 223). The two sections below provide examples of insufficient and excessive cytokine responses that lead to cancer and asthma, respectively.

1.5.1 Cancer

In cancer patients, Th cells specific for tumor antigens are frequently found, but the cells are usually unresponsive to stimulation *in vitro*, and clearly do not induce an adequate response *in*

vivo (224). This can be explained by several factors that promote tolerogenic responses to tumors. First, the vast majority of proteins expressed by transformed cells are self antigens, with only a few, if any, identifiable tumor-specific antigens. Thus, most Th cells with TCRs capable of recognizing tumor-expressed self antigens are eliminated in the thymus. Second, many cancers produce anti-inflammatory cytokines such as IL-10 and TGFβ that directly inhibit Th cell responses even if Th cells specific for tumor antigens are present. Third, tumors frequently have large numbers of Tregs in the local microenvironment (39, 40, 88-91). This may be due to induction of Tregs by tumor-produced cytokines, or to increased trafficking of Tregs to the tumor as a result of necrosis, or other factors produced by the tumor itself. Importantly, Treg numbers inversely correlate with disease prognosis for many malignancies (39, 40, 88-91, 225-227).

Cytokines are important players in the inhibition of anti-tumor immunity, but they can also participate in the promotion of tumor growth, as is evident in IL-6-dependent expansion of renal cell carcinoma, cervical cancer, and multiple myeloma (210). The importance of cytokine signaling in tumors is highlighted by the high frequency of dysregulated SOCS expression. For example, many breast carcinomas demonstrate elevated SOCS3 expression, whereas HCC frequently shows reduced SOCS3 expression, compared to the surrounding tissue (179, 192, 193). The opposing effects of SOCS3 on different tumors may be due to the particular cytokines present in the tumor microenvironment.

1.5.2 Asthma

In contrast to tumors, where adaptive immunity is desirable, but tolerance predominates, autoimmune and allergic diseases are caused by a failure of tolerance. Autoimmune diseases such as rheumatoid arthritis, lupus, type I diabetes, IBD, multiple sclerosis, and Hashimoto's thyroiditis are all caused by the initiation of adaptive immune responses to self antigens, and are usually mediated by Th1 or Th17 cells, although Th2 responses can be involved (2, 7). On the other hand, allergies develop when an immune response is initiated against a harmless environmental antigen, and almost always demonstrate a Th2 bias (2). Whereas autoimmune disease is usually insidious and progressive, allergic diseases are frequently mild, but can lead to rapidly fatal hypersensitivity responses (2). Allergic responses can be fatal if they involve the generation of high titers of IgE, which leads to systemic mast cell and/or basophil degranulation, and anaphylaxis, or mucosal swelling and inflammation that causes asphyxiation (2, 222).

One of the most common allergic diseases is asthma, which affects more than 15 million people in the United States (222). It is characterized by airway hyperresponsiveness (AHR), swelling, inflammation, and mucus hypersecretion (Figure 6). Although it usually can be controlled and is often mild, asthma can be severe, and even fatal, in some patients. Elevated serum IgE, Th2 cytokines, and eosinophilic infiltrate are key immunological indicators of asthma, especially in murine models.

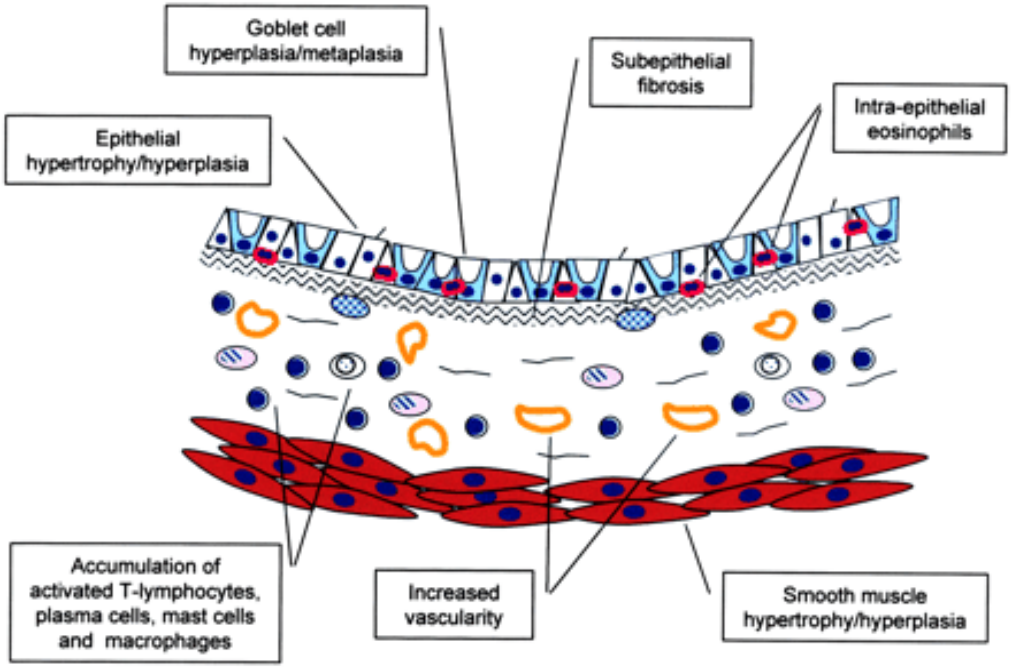

Figure 6. Asthma pathology

Adapted from Kumar, *et al.*, *American Journal of Respiratory Cell and Molecular Biology*, 2002. Asthma leads to increased airway resistance through several mechanisms. Epithelial cell, goblet cell, and smooth muscle hypertrophy and hyperplasia increase wall thickness and mucus volume while decreasing airway diameter. Increases in vascularity enhance leukocytic infiltration of the lungs, which leads to tissue damage that increases pathology further.

In general, murine models of asthma are acute inflammatory situations, and mice frequently have remission of disease in chronic situations (228). This is a major obstacle to research, because human asthma is a chronic condition. However, acute murine models are able to replicate many of the signs of asthma, such as AHR, elevated serum IgE, Th2 cytokines in the airways and parenchyma, and pulmonary inflammatory infiltration characterized by a major eosinophilic component.

The importance of Th2 responses in the development of asthma is supported by data that the incidence of IL-4Rα mutations which promote Th2 biasing is elevated in asthmatics (24). Investigations in genetically modified mice also highlight the role of Th2 cells in asthma. As would be expected, both IL-4Rα-/- and STAT6-/- mice are highly resistant to allergic airway inflammation due to a profound deficiency in Th2 differentiation (140-143). In contrast, surprising data has come from Y500F IL-4Rα knock-in mice. These mice have a mutation in the IL-4Rα chain that allows signaling by STAT6, but not IRS2 (137). As mentioned earlier, IRS2 signaling is thought to be important for IL-4-mediated proliferation, and a lack of IRS2 activation in the knock-in mice was expected to reduce induction of asthma. However, these mice demonstrated exacerbated disease, even though the Th cells proliferated less in response to IL-4 *in vitro*. There are two possible interpretations of these observations. First, IRS2 may be influencing Tregs or Th cell/Treg interactions to promote adaptive immunity. Second, the major influence of IRS2 signaling may be in another cell type than Th cells. Currently, more work needs to be conducted to determine which possibility is correct.

Clinically, AHR, not Th2 immunity *per se*, may be the more important aspect of asthma, since it is responsible for many of the symptoms, and especially for severe and fatal cases of

status asthmaticus (222). AHR can be measured in patients and in animals by challenge with a bronchoconstrictor such as methacholine, followed by spirometry. This provides measures of airway resistance, and in many cases, a breakdown of resistance in large vs. small airways. Although allergic inflammation was traditionally considered predictive of AHR, the two parameters correlate with each other frequently, but not always (229). Exactly how immune responses influence AHR, and whether certain immunological markers do predict AHR severity, remains to be elucidated.

In order to investigate asthma, particularly its immunological components, one of the most common murine models is the ovalbumin (OVA)/alum system. This involves intraperitoneal (i.p.) immunization with OVA in a suspension of alum, which is a common Th2 adjuvant (54). Animals are rested and then challenged with aerosolized OVA. Following aerosol, the mice are analyzed by taking AHR measurements, bronchoalveolar lavages (BALs), and lung histology. Unlike AHR measurement, which requires sophisticated instruments, BALs require minimal equipment, making their use more common in asthma research. Since BALs from normal lungs in humans and mice have low levels of Th2 cytokines, and almost no eosinophils, these serve readily as markers for disease together with serum IgE levels (222).

A major limitation of the OVA/alum system is the artificial nature of the sensitization. Because alum is extremely viscous, it can not be administered intratracheally (i.t.) or intranasally (i.n.). As a result, OVA/alum is delivered i.p., and does not sensitize through the lung. The natural course of asthma sensitization involves inhalation of allergens into the lung. Recently, our laboratory has published work using i.n. administration of OVA/cholera toxin (CT) in order to sensitize mice. CT is not viscous, and is known to be a Th2 adjuvant (230). The OVA/CT system has allowed induction of asthma through the airways, which is the natural route. The

main disadvantage of the OVA/CT model is that it does not provoke the same degree of airway inflammation as the OVA/alum system. Thus, the OVA/CT model is superior for the study of priming, because these events take place in the lung-draining lymph nodes, whereas OVA/Alum is more useful in the analysis of later responses, such as those following antigen challenge (Table 4).

Table 4. OVA/Alum vs. OVA/CT immunization systems

	OVA/Alum	OVA/CT
Immunization method	i.p., artificial route of exposure	i.n., similar to natural exposure
Source of APCs	Peritoneal DCs, peritoneal macrophages	Lung DCs, pulmonary macrophages
Site of priming	Peritoneal lymph nodes	Lung lymph nodes
Inflammatory response	Severe	Moderate
Used to study	Inflammation	Priming

Despite the ubiquitous nature of allergens, most people do not develop asthma. This lack of hypersensitivity is achieved through several mechanisms, including ignorance, anergy, and dominant tolerance. Immunologic ignorance refers to the processes by which an allergen is prevented from being presented to Th cells by, for example, elimination of antigen by coughing, mucus transport, sequestration, and APC degradation. Many of the innate immune cells in the lung are pulmonary macrophages (pMO), which are poor APCs with significantly reduced antigen presentation capacity compared to DCs (1, 2). Thus, pMOs may promote immunologic ignorance, but due to their low levels of costimulation, these cells could also promote anergy, which occurs when Th cells receive TCR stimulation in the absence of costimulation. Anergic cells will not produce cytokine, even if they receive costimulation later. In contrast to ignorance and anergy, which are known as recessive tolerance due to a lack of immune responses, dominant tolerance is an active process that prevents responses to an allergen following the

tolerizing event, even in the presence of adjuvant. Dominant tolerance can be mediated by many mechanisms including those involving Tregs and DCs. For instance, our laboratory uses repeated OVA aerosols in the absence of adjuvant to tolerize mice to OVA. These tolerized animals are resistant to airway inflammation induced by an OVA/alum protocol similar to the one described above (54). The tolerance protocol causes induction of AgTregs, which are described above. These cells suppress immune responses *in vitro* and *in vivo* via a mechanism utilizing csTGFβ. Furthermore, adoptive transfer of AgTregs protects recipients from the OVA/alum protocol (54).

1.6 SUMMARY

The importance of immune regulation is becoming increasingly clear. Much research and many new treatments have focused on immune modulation, and the ones that are directed at cytokines, have shown significant potential for the treatment of certain diseases, such as IBD. Simultaneously, the Treg field has expanded rapidly, and the potency of these cells has made them obvious therapeutic targets if technical difficulties can be overcome, particularly low cell number and antigen specificity.

Treg-based immunotherapy will involve the administration of these cells into patients with inappropriate inflammation, or modulation of these cells by soluble factors. However, regulation of Tregs by cytokines remains poorly understood. Understanding this regulation is critical for immunotherapy since cytokines could be secreted in the microenvironment where Tregs are administered, especially in patients with significant inflammation who may benefit most from treatment. The best studied cytokine in the Treg field, IL-2, mediates a paradoxical

response where NatTreg FoxP3 expression and proliferation are increased, but suppressive function is decreased when administered to NatTreg/ Th cell co-cultures. This same pattern appears to hold for certain other cytokines, especially IL-4 and IL-6, which have also been implicated in the modulation of NatTreg function. IL-4 shares the γc with the IL-2R. Hence, it is not surprising that there is some overlap in signaling, but there are also important differences. Unlike IL-2, IL-4 is critical for Th2 differentiation, and Th2 cells are central to the pathogenesis of asthma and allergic diseases. Therefore, understanding the influence of IL-4 on Tregs is necessary for the development of Treg-targeted therapy in atopic diseases. Whereas IL-4 is required for Th2 differentiation, IL-6 in combination with TGFβ significantly enhances Th17 biasing. Th17 cells and IL-6 are important in the pathology of several cancers, asthma, and many autoimmune and inflammatory diseases. Yet, the role of IL-6 in the modulation of Tregs is poorly understood.

While little is known about cytokine modulation of Tregs, even less is known about how these cells regulate cytokine signaling. Many groups have suggested that SOCS proteins may serve an important role in Tregs, but this remains to be adequately investigated. Increasingly, SOCS proteins are being implicated in the pathology of cancer, autoimmune disease, allergies, and other diseases. In particular, SOCS3 is emerging as a therapeutic target, since SOCS3 expression is altered in many diseases, and elevated expression of SOCS3 correlates with poor response to IFNα treatment in HCV infection. However, in order to develop SOCS3 therapies, understanding the regulation of Tregs by SOCS3 is vital because SOCS3 may be a useful target for the modulation of Tregs, and therapies such as cpSOCS3 may inadvertently act on Tregs, and as a result, cause unwanted side-effects. Interestingly, SOCS3 regulates two cytokines that modulate Treg function, IL-2 and IL-6, which implies that SOCS3-targeted therapy may have

significant effects on Treg function. If SOCS3 will be targeted by future therapies, it is important to characterize its expression and function in Tregs. Therefore, a critical challenge in the next several years will be elucidating the roles of cytokines and SOCS proteins in the modulation of Tregs.

Due to the importance of SOCS3 and the cytokines IL-4 and IL-6 in disease, and the lack of information on their influence on Treg function, this dissertation investigates the role of IL-4, IL-6, and SOCS3 in Treg modulation. The effect of these three regulators on FoxP3 expression, proliferation, and Treg suppressive function are characterized. These findings may prove highly useful in the design of Treg-targeted therapies.

1.7 SPECIFIC AIMS

Aim 1: To characterize the roles of IL-2, IL-6 and SOCS3 in the modulation of Treg function

Hypothesis 1: IL-2 and IL-6 enhance Treg proliferation, FoxP3 expression, and function, whereas SOCS3 inhibits these cytokines and as a result, opposes these effects

IL-2 and IL-6 are important cytokines that have been shown to affect Treg function, and are both regulated by SOCS3. Further, IL-6 and SOCS3 are currently under investigation as potential therapeutic targets. Thus, Chapter 2 describes: 1) IL-2 and IL-6 modulation of Treg proliferation, function, and FoxP3 and CTLA4 expression, 2) SOCS1, SOCS2, and SOCS3 expression in Tregs and Th cells, and 3) Treg function, proliferation, and FoxP3 and CTLA4 levels following SOCS3 over-expression.

Aim 2: Determine the influence of IL-4 on Tregs during experimentally induced allergic airway inflammation

Hypothesis 2: IL-4 enhances Treg proliferation and FoxP3 expression, and increases Th cell resistance to suppression in a STAT6-dependent fashion

Asthma is a common allergic disease caused by Th2 responses to allergens, and IL-4 is a critical cytokine in the induction of Th2 differentiation. IL-4 inhibits Treg suppression *in vitro*, but little is known about its role *in vivo* and the signaling pathways involved. Chapter 3 evaluates: 1) Treg numbers in the lungs of mice with airway inflammation compared to tolerized animals, 2) the ability of WT and IL-4Rα-/- AgTregs to inhibit airway inflammation, 3) IL-4 modulation of Th cell responsiveness to suppression, 4) IL-4-mediated effects on Treg suppression, proliferation, and FoxP3 expression, and 5) whether the influence of IL-4 on Tregs and Th cells is dependent on STAT6.

2 DEFICIENT SOCS3 EXPRESSION IN CD4+CD25+FOXP3+ REGULATORY T CELLS AND SOCS3-MEDIATED SUPPRESSION OF REGULATORY T CELL FUNCTION

2.1 ABSTRACT

Naturally occurring CD4+CD25+FoxP3+ regulatory T cells (Tregs) suppress T helper (Th) cell-mediated immune responses. The cytokines IL-2 and IL-6 are known to influence Th cell and Treg function. However, their relative effects on T helper (Th) cells versus Tregs are not well understood. Stimulation with IL-2, and to a lesser extent, IL-6, enhanced Treg proliferation, FoxP3 and CTLA4 maintenance, and Treg suppressive function. In contrast, when IL-2 or IL-6 was added to Treg/Th cell co-cultures, suppression was inhibited. Because the molecule SOCS3 negatively regulates Th cell responses to IL-2 and IL-6, we investigated SOCS3 expression in Tregs. Interestingly, unlike Th cells, Tregs were found to be deficient in SOCS3 protein expression. The significance of this finding lies in the need for Tregs to rapidly respond to these cytokines to prevent unwarranted immune responses to self-antigens. Over-expression of SOCS3 in Tregs decreased their proliferation, FoxP3 and CTLA-4 expression and suppressive function. Thus, upregulation of SOCS3 expression may be a useful therapeutic approach in diseases where inhibition of Tregs is desirable.

2.2 INTRODUCTION

Naturally occurring Tregs differentiate in the thymus, express CD4, CD25 (IL-2Rα), CTLA-4, and FoxP3 (the defining transcription factor), and serve as suppressors of Th cell proliferation *in vitro* and *in vivo* through a contact dependent mechanism (2, 46, 49, 52, 63, 72). The importance of these cells was reinforced by the observation that humans and mice that lack FoxP3 do not have Tregs, and as a result, have severe, fatal, autoimmune disease (25, 26, 52). The critical role of FoxP3 was confirmed by studies showing that over-expression of FoxP3 in Th cells imparts a suppressive phenotype (79). However, regulation of FoxP3 expression in Tregs is poorly understood. IL-2 seems to promote FoxP3 expression, since Tregs gain maximal suppressive ability after TCR stimulation and pretreatment with IL-2 (49, 63, 87). Surprisingly, the addition of exogenous IL-2 in Treg/Th cell co-cultures abrogates suppression of Th proliferation (63). This paradoxical response implies that IL-2 induces opposing responses in Tregs and Th cells. Because of the potent effect of IL-2 on Tregs, interest has increased in the influence of other cytokines on Treg function, as well. One such cytokine is IL-6, which is being actively investigated as a promoter of Th2 and Th17 responses (151, 231). Like IL-2, IL-6 has been shown to reduce Treg suppressive function during the induction of adaptive immunity (150, 151, 232) and also to promote Treg proliferation (233).

Due to the importance of IL-6 in many areas of biology, an increasing amount of research is being devoted to physiological inhibitors of IL-6 signaling. Thus, significant attention has been focused on the suppressor of cytokine signaling (SOCS) family of proteins, which are negative feedback inhibitors of cytokine signaling (155). In particular, SOCS3 inhibits signaling of many cytokines, including IL-2 and IL-6, and is being studied as a therapeutic target for hepatitis C, cancer, asthma, and rheumatoid arthritis (129-131, 173, 174, 180, 182, 190, 194).

Recently, a SOCS3 chimera, known as cell penetrating SOCS3 (cpSOCS3), was developed that penetrates cell membranes to treat low SOCS3 expression (194).

SOCS3 is expressed in naïve and activated Th cells (155, 174) and is associated with Th2 responses (174). However, its role in Treg biology remains poorly understood. Because it regulates IL-2 and IL-6 signaling in Th cells, we have proposed that SOCS3 is expressed minimally in Tregs, to allow rapid responses to these cytokines. Here, we show that naïve Th cells express high levels of SOCS3 protein, but unstimulated Tregs do not. To determine whether low SOCS3 expression in Tregs could be exploited to modulate their function, SOCS3 was over-expressed in Tregs using a retroviral expression system. We show that SOCS3 over-expression in Tregs reduces FoxP3 and CTLA4 expression concomitant with reduction in Treg cell proliferation and suppressive function.

2.3 MATERIALS AND METHODS

2.3.1 Mice

All primary cells were isolated from male Balb/cByJ mice (Jackson Laboratories) which were used at 7-9 wks of age. Mice were housed in the University of Pittsburgh Department of Laboratory Animal Resources, and were used in accordance with approved IACUC protocols and AALAC regulations.

2.3.2 Cell isolation and Th cell stimulation

Tregs and Th cells were isolated from the spleens of male Balb/cByJ mice using magnetic bead isolation kits (Miltenyi Biotec). Where necessary CD4+ T-cell depleted, gamma irradiated (2000 rad) APCs were used as a means of costimulation. Several experiments also included exogenous IL-2 (50 U/mL) or IL-6 (20 ng/mL). In other experiments, plates were coated for 4 h at 37^0C with anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) monoclonal antibodies (BD Biosciences). Plates were washed once with PBS, and then cells were cultured in the presence of IL-2 (50 U/mL, Roche). This stimulation constituted the "neutral' activation, whereas some cultures were skewed towards Th1 using anti-IL-4 (2 μg/mL, BD Biosciences) and rIL-12 (7.5 ng/mL, Peprotech), and others were skewed towards Th2 using anti-IFNγ (2 μg/mL, BD Biosciences) and rIL-4 (40 U/mL, Peprotech).

2.3.3 Pretreatment with cytokines

For pretreatment with cytokines, cells were stimulated with plate bound anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL). The plates were coated as described above. Cells were incubated in complete medium alone for 24 h, or with IL-2 (50 U/mL) or IL-6 (20 ng/mL), then cells were washed with PBS and used for further culture without any additional cytokine.

2.3.4 [^{3}H]-thymidine incorporation assay

Cells were cultured in complete medium with soluble anti-CD3 (2 μg/mL) in 96 well, round bottom plates with $3x10^4$ gamma irradiated APCs per well (Th cells were added at 1 Th cell: 1 APC in suppression assays). Tregs were added in ratios of 1 Treg: 1 APC for fresh Tregs, or 1 Treg: 4 APC for treated Tregs, due to limited Treg numbers. Cells were cultured for 36 h with [^{3}H]-thymidine (1 μCi/well, Perkin Elmer) included for the final 18 h of culture. [^{3}H]-thymidine incorporation was assessed by harvesting DNA onto glass fiber filters followed by liquid scintillation counting (Wallac). All conditions were done in triplicate and are expressed as mean counts per min (cpm) plus or minus the standard deviation (SD).

2.3.5 CFSE dilution assay

Th cells were labeled with carboxyfluoroscein succinimidyl ester (1 μM, CFSE, Invitrogen) for 15 min at 37^0 C and washed twice with PBS/FCS (PBS, pH 7.2 plus 2% FBS). Labeled Th cells ($1x10^5$/well) were cultured in complete medium in 96 well plates with equal numbers of irradiated APCs and soluble anti-CD3 (2 μg/mL) in the presence of varying ratios of Tregs and either no cytokine, IL-2, or IL-6. After 3 d, cells were stained with allophycocyanin-(APC) labeled anti-CD4 monoclonal antibody, phycoerythrin-(PE) labeled anti-CD25 monoclonal antibody, and propidium iodide (PI) to exclude dead cells (BD Biosciences). Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, Immunocytometry Systems). Dead cells and APCs were excluded by light scatter, CD4 expression, and PI staining. Th proliferation of live (PI negative) CD4 Th cells was

determined by CFSE dilution. The percent divided cells was determined by dividing the number of CD4 cells undergoing at least one cell division by the total number of live CFSE-labeled cells and multiplying the result by 100.

2.3.6 Western blotting

Total cell extracts were made using non-denaturing cell lysis buffer with 1% Triton-X (Cell Signaling), and equal amounts of protein were analyzed by Western blotting techniques as previously described (22). Membranes were probed with anti-murine SOCS3 monoclonal antibody (MBL), or anti-murine SOCS1 monoclonal antibody (MBL) at 1:1000 dilutions, or anti-murine SOCS2 polyclonal antibody (Novus) at a 1:500 dilution. Equal loading of protein was monitored by probing for comparable amounts of β-actin (Novus).

2.3.7 Quantitative PCR of SOCS3

Freshly isolated Tregs and Th cells were treated with trizol (Invitrogen) to lyse the cells and denature RNAses. RNA was isolated using an RNeasy kit (Qiagen), and treated with DNAse. After first strand cDNA synthesis (Applied Biosystems) of 200 ng and 500 ng of RNA, quantitative RT-PCR was performed by the University of Pittsburgh genomic core facility using primers for FoxP3, SOCS3, and β-glucuronidase (GUS) (Applied Biosystems). The CT value

was normalized to GUS expression using the ΔΔCT method as described by Applied Biosystems, and these values were compared between Tregs and Th cells. Th values were arbitrarily set to one.

2.3.8 Construction of retroviral vector and infection of regulatory T cells

SOCS3 cDNA was amplified using SOCS3 PCR primers (forward:5'-AACTGGATCCTGCGCCATGGTC-3'; reverse:5'-GGAATTCCTTAAAGTGGAGCAT-3'), and was inserted into a MIGR1 retroviral vector using BamH1 and EcoR1 restriction endonuclease sites (234). To generate retrovirus, MIGR1-SOCS3 or MIGR1 vector was purified and used to infect a packaging cell line (Phoenix cells). Infection of cells was performed as previously described (235). Tregs were infected with MIGR1-SOCS3 retrovirus or MIGR1 vector control virus, and GFP expression was assessed by flow cytometry to determine infection efficiency. After infection, cells were maintained in the presence of IL-2 either for 2d, and sorted based on GFP expression; or for 3 d, and analyzed for FoxP3 expression by flow cytometry. Sorted cells were used for proliferation assays.

2.3.9 Analyses of FoxP3 and CTLA4 expression

FoxP3 and CTLA4 expression in cells was assessed using intracellular staining and flow cytometry. Cells were washed, and resuspended in Cytofix/Cytoperm buffer (BD Biosciences) for 15 min at 4^0C. After washing with PBS, the cells were resuspended in permeabilization

buffer (BD Biosciences), and were incubated with APC-labeled anti-FoxP3 monoclonal antibody (eBioscience) or PE-labeled anti-CTLA4 monoclonal antibody (BD Biosciences) for 30 min. Finally, cells were washed with PBS, resuspended in pH 7.2 PBS with 2% FBS, and analyzed by flow cytometry. Dead cells were excluded by forward versus 90^0 light scatter characteristics. FoxP3 analyses in non-infected cells was similar except that buffers were from eBioscience instead of BD; and, cells were also stained with PerCP-labeled anti-CD4 and FITC-labeled anti-CTLA4. It should be noted that the eBioscience buffers inhibited GFP fluorescence, and FITC-labeled anti-CTLA4 could not be used for infected cells since CTLA4-FITC emission overlaps that of GFP.

2.3.10 Statistics

Results show mean values ± S.D. All p values were determined using 2 tailed Student's t-test, and differences were considered significant where $p \leq 0.05$. Calculations pertain to individual representative experiments with samples done in triplicate.

2.4 RESULTS AND DISCUSSION

2.4.1 Prior exposure to IL-2 and IL-6 promotes Treg suppressive potential, but not when these cytokines are present during Th/Treg co-culture

Tregs inhibit Th cell proliferation, but this suppressive activity can be reduced by the addition of exogenous IL-2 (63). This is in spite of the fact that Tregs express high levels of CD25, a main component of the high affinity IL-2 receptor (46). Tregs are also known to express the IL-6 receptor (151); and recently several groups have implicated IL-6 as an inhibitor of Treg function during the induction of adaptive immune responses (150, 151, 232). In agreement with these reports, exogenous IL-2 and IL-6 inhibited suppression of Th cell proliferation by Tregs as examined in a [^{3}H]-thymidine incorporation assay (Figure 7A) with IL-2 demonstrating much greater potency when each cytokine was added at an optimal dose. However, both cytokines promoted Treg proliferation; and again, IL-2 showed far greater effects than IL-6, when these cells were cultured without Th cells (Figure 7B). Treg proliferation was markedly less than that of Th cells. Nevertheless, in order to directly show that exogenous cytokine inhibited suppression, Th cells were labeled with CFSE, and co-cultured with Tregs (Figure 7C). Using flow cytometry, we were able to evaluate proliferation of Th cells, and since the Tregs were unlabeled, their proliferation could not impact the data. The CFSE suppression assay confirmed that IL-2 and IL-6 inhibited suppression of Th cells by Tregs. Taken together, these results suggested that either Treg function was inhibited by IL-2 or IL-6 despite their ability to promote Treg proliferation, or that effects of the cytokines on the Th cells were able to override the suppressive function of the Tregs. In order to determine which of these possibilities was correct, we investigated whether IL-2 and/or IL-6 affected FoxP3 and CTLA4 expression. This would be

expected to result in altered potency of Tregs, since lower FoxP3 expression correlates with decreased suppressive function (236). Similarly, CTLA4 seems to be an important mechanism of suppression in many systems (72, 237) if not all (47). Furthermore, CTLA4 expression often correlates with that of FoxP3 (238, 239). When Tregs are initially isolated, FoxP3 expression is fairly uniform (>90% of CD25-expressing cells; data not shown). However, when these cells are cultured, the percentage of FoxP3+ cells tends to decrease (236). When IL-2 was added to the Tregs in culture, FoxP3 and CTLA4 expression were better maintained (Figure 7D). However, unlike IL-2, IL-6 had small effects on the maintenance of FoxP3 and CTLA4 expression in the Tregs (Figure 7D). Since IL-2 and IL-6 both enhanced Treg proliferation, and neither IL-2 nor IL-6 decreased FoxP3 or CTLA4 expression, we investigated whether either cytokine improved Treg suppression. In these experiments, the Tregs were pretreated with either cytokine prior to inclusion in a suppression assay. We found that preincubation of Tregs for 24 h with IL-2 or IL-6 in the presence of plate bound anti-CD3 and anti-CD28 enhanced their suppressive function, compared to incubation without cytokine, and that the effects of IL-2 and IL-6 were comparable (Figure 7E). What these experiments allowed us to infer is that IL-2 and IL-6 modulate both Tregs and Th cells, but the effect on Th cells is dominant in the presence of strong Th cell activation signals, since the suppressive function of Tregs is abrogated when these cytokines are added to co-cultures of the two cell types. Interestingly, IL-6 appears to enhance Treg function, even though it is not particularly effective at enhancing FoxP3 or CTLA4 expression (Figure 7B and D). Thus, while most studies to-date have focused on the ability of IL-2 and IL-6 to inhibit Treg activity when added to Treg/Th co-cultures, our data show that these cytokines can significantly enhance Treg suppressive effects, which may be important in maintaining normal immune homeostasis in the absence of foreign antigens and danger signals. *In vivo*, we propose

that Treg suppression would be favored in the presence of weak activation stimuli, such as self-antigens, whereas during an infection, significantly stronger signals would favor Th cell expansion and differentiation.

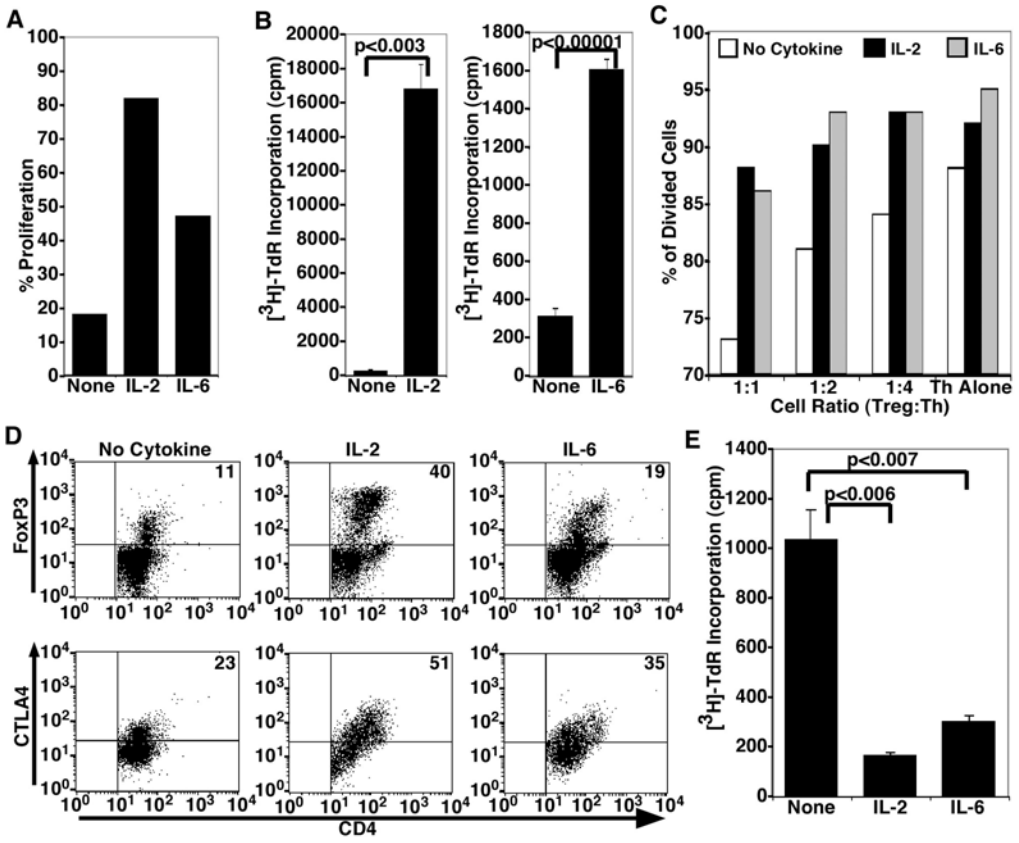

Figure 7. IL-2 and IL-6 have opposing effects on Tregs and Th cells.

CD4+CD25+ Tregs and CD4+CD25- Th cells were isolated from naïve Balb/cByJ male mice. Cells were cultured in the presence of soluble anti-CD3 and irradiated CD4-depleted splenocytes (APCs). (A)-(D), Wells received either IL-2, IL-6, or no exogenous cytokine. (A), Th cells were cultured alone or in the presence of Tregs, and proliferation was analyzed by measuring [^{3}H]-thymidine incorporation. Because exogenous cytokine was present, baseline Th cell proliferation was different. Therefore, the graph shows the percentage of cell proliferation in co-cultures compared to proliferation of Th alone under either condition to normalize for the influence of cytokine on Th cell proliferation. It should be noted that in all experiments the incorporated radioactivity in the Th cells, in the absence of Tregs, was proportional to the number of cells used as previously observed (232). (B) Tregs were cultured with IL-2, IL-6, or no exogenous cytokine, in the absence of Th cells, and proliferation was analyzed by thymidine incorporation. (C) Conditions were as described in panel A except that Th cells were labeled with CFSE, and proliferation was analyzed by flow cytometry instead of by estimation of thymidine incorporation. (D) Tregs were cultured for 3d as described in panel B, and FoxP3 and CTLA4 expression was analyzed by flow cytometry.

Numbers represent the percentage of gated cells in the upper right quadrant. (E) Tregs were cultured for 1 day with plate bound anti-CD3 and anti-CD28 in the presence of IL-2, IL-6, or no exogenous cytokine. The Tregs were next used in a suppression assay with freshly isolated Th cells. The incorporated radioactivity of Th cells alone was 24,400 cpm. (A-E) Results shown are representative of three independent experiments. Error bars were not calculated in A because data was normalized, and in C because data was aquired by flow cytometry.

2.4.2 Differential SOCS3 protein expression in Tregs and Th cells

Although the full suppressive potential of Tregs is compromised in the presence of IL-2 and IL-6, with seemingly dominant effects of the cytokines on Th cells, Tregs do respond to these cytokines as evidenced by increased potency when pre-exposed to them (Figure 7E).

A major mechanism of limiting responses to cytokines in Th cells is via negative feedback regulation by SOCS proteins. One such protein, SOCS3, is a negative feedback inhibitor that reduces signaling by many cytokines, including IL-2 and IL-6 (129-131, 155, 180, 190). The enhanced potency of Tregs upon preexposure to IL-2 and IL-6 prompted us to investigate the status of SOCS3 expression. Our data showed that SOCS3 is expressed in Th cells in a biphasic manner (Figure 8A). Unstimulated Th cells from naïve mice expressed appreciable amounts of SOCS3, but the expression was reduced by two days post-stimulation under either Th1- or Th2-promoting conditions (Figure 8A). At five days after stimulation, both Th1 and Th2 cells had regained SOCS3 expression, although Th1 cells tended to express less SOCS3, in agreement with previous reports (174). It may be that the observed biphasic pattern of SOCS3 expression allows Th cells to be less responsive to cytokines initially, which could serve to prevent inappropriate activation. However, after activation, downregulation of SOCS3 expression would presumably allow Th cell expansion and differentiation in response to

cytokines, since SOCS3 inhibits IL-2 signaling and inversely correlates with Th cell proliferation (173, 190). Finally, according to this scenario, upregulation of SOCS3 again after differentiation could serve to inhibit uncontrolled proliferation and signaling in the effector Th cells. In contrast to Th cells, Tregs were found to express little or no SOCS3 protein, either when freshly isolated, or in response to *in vitro* stimulation (Figure 8B). We have been unable to induce SOCS3 expression in Tregs using several different activation conditions (data not shown). Contrary to the dramatic differences observed in protein levels, Tregs and Th cells expressed similar amounts of SOCS3 mRNA (Figure 8C). As a control, FoxP3 mRNA expression was also compared between Tregs and Th cells, and as expected, a significantly higher level was detected in Tregs compared to that in Th cells (Figure 8C). Figure 8C seems to support reports which indicate that SOCS3 is regulated at the level of protein stability (188). We were initially surprised by the discrepancy between SOCS3 mRNA and protein expression, but this lack of corerspondence can be explained by several factors. The overall genetic program of CD4 T cells, either Tregs or Th cells, tends to be broadly similar. In Th cells, SOCS3 protein is inversely related to IL-2 signaling, so it would not be surprising to find that the same is true in Tregs (173). Since IL-2 is critical for maintenance of normal numbers of Tregs in the periphery, it is strongly implied that Tregs receive low levels of homeostatic IL-2 signaling (85). Therefore, similar to Th cells, Tregs produce SOCS3 mRNA, but maintain low levels of SOCS3 protein in situations where responsiveness to IL-2 is required. Further, under certain conditions such as infection, Tregs may rapidly upregulate SOCS3 protein to reduce their function due to the presence of pre-made transcripts. Given that Tregs and Th cells express relatively similar levels of SOCS3 mRNA, it will be interesting to elucidate the mechanisms that maintain low/undetectable levels of SOCS3 protein in Tregs.

We also analyzed expression of SOCS1 and SOCS2 proteins in unstimulated Tregs and Th cells (Figure 8D). SOCS2 was expressed by Tregs as described by others (170, 171, 240), but SOCS1 was expressed at barely detectable levels in Tregs. SOCS1 is best known as an inhibitor of IFNγ signaling, but it can also inhibit IL-2 and IL-6 signaling (241, 242). On the other hand, SOCS2 is associated with inhibition of growth hormone and growth factors, and possibly, it potentiates IL-2 signaling, as it is increased during IL-2 signaling at the same time as SOCS3 is being downregulated in Th cells (173, 243). It is interesting to note that among the three SOCS proteins studied, the greatest difference in expression level between Tregs and Th cells was observed in the case of SOCS3, which is the most critical regulator of IL-2 and IL-6 signaling. This is especially important in the case of IL-2, because, as mentioned above, it is required for Treg homeostatic maintenance, and inhibition of IL-2 signaling in all Tregs in the periphery could lead to a significant reduction in the number and functional capacity of these cells.

Thus, lack of SOCS3 in Tregs may allow a prompt response to cytokines such as IL-2 and IL-6 for cell expansion and potentiation of their immunosuppressive function. While IL-2 is a product of Th cells secreted during constant provocation by self-antigens, IL-6 is a product of many more cell types, and has been linked to autoimmunity (2, 182). Hence, the significance of these two cytokines in augmenting Treg function in the absence of strong Th cell activation signals induced by foreign antigens.

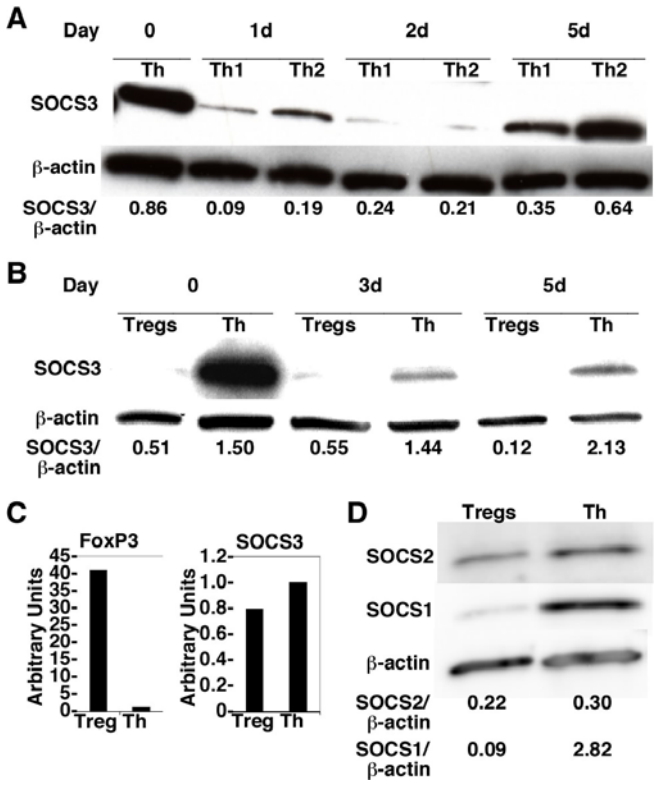

Figure 8. SOCS3 expression is much lower in Tregs in comparison to Th cells.

(A) Freshly isolated Th cells were stimulated under Th1 or Th2 biasing conditions and total cell extracts were made at t=0, 1 d, 2 d, and 5 d. Extracts were analyzed by Western blotting for SOCS3 expression, and then reprobed for β-actin to ensure equal protein loading in each lane. (B) Tregs and Th cells were stimulated under neutral conditions and total cell extracts were made at t=0, 3 d, and 5 d, and total cell extracts were made and analyzed as in A. (C) SOCS3 and FoxP3 mRNA expression was evaluated in unstimulated Tregs and Th cells by quantitative RT-PCR techniques, and normalized to the housekeeping gene β-glucuronidase (GUS), which was equally expressed in Tregs and Th cells. Graphs show the fold change of FoxP3 or SOCS3 in Tregs compared to that in Th cells, which was arbitrarily set to one. (D) Cell extracts prepared from unstimulated Tregs and Th cells were analyzed for expression of SOCS2 and SOCS1 proteins by Western blotting techniques, and reprobed for βactin as a loading control as in panel A. (A, B, and D) Band intensity was quantified and the ratio of the indicated band intensities were calculated. Results shown are representative of two independent experiments.

2.4.3 SOCS3 over-expression inhibits proliferation, FoxP3 and CTLA4 expression, and suppressive function of Tregs

As shown in Fig 1E, pretreatment of Tregs with IL-2 or IL-6 promoted their suppressive function. Because SOCS3 expression was barely detectable in Tregs, we were curious whether its forced expression would inhibit cytokine-mediated effects. Thus, we developed a recombinant retrovirus that would allow SOCS3 protein expression in Tregs (Figure 9A). We have previously used recombinant MIGR1 retrovirus to infect Th cells (235), and the bicistronic vector induced expression of SOCS3 and GFP proteins. Figure 9B shows expression of SOCS3 in Phoenix cells infected with SOCS3-encoding retrovirus, confirming the ability of the construct to induce expression of SOCS3 protein.

SOCS3-expressing Tregs, sorted based upon coexpression of GFP, exhibited reduced proliferation compared to cells infected with empty vector (expressing GFP alone) in response to stimulation with anti-CD3 and APCs (Figure 9C). A reciprocal relationship between SOCS3 expression and cell proliferation has been also noted with Th cells (173). The effect of SOCS3 expression was even more pronounced in the presence of IL-2 (Figure 9C). Although IL-2 promoted FoxP3 and CTLA4 maintenance in uninfected cells (Figure 7C), SOCS3 expression in Tregs inhibited maintenance of FoxP3 and CTLA4 expression in the presence of IL-2 (Figure 9D). Importantly, SOCS3-expressing Tregs exhibited reduced suppressive activity compared to sorted control, vector infected, Tregs (Figure 9E). Thymidine incorporation was used to measure proliferation because the Tregs expressed GFP, which has an overlapping emission spectrum with CFSE. Therefore, SOCS3 expression inhibited Treg proliferation, FoxP3 and CTLA4 expression, and suppressive function. These data highlight the importance of a high level of CD25 expression in Tregs to allow IL-2 signaling since its blockade by SOCS3 impairs their

suppressive function. SOCS3 antagonism of Tregs observed in this study is in line with a recent finding of increased Th cell TGF-β and IL-10 production in mice with SOCS3-deficient T cells, both cytokines being associated with Tregs and tolerance (185). In recent studies, we have shown membrane-bound TGF-β to play an important role in tolerance induction by inhaled antigen (54, 244).

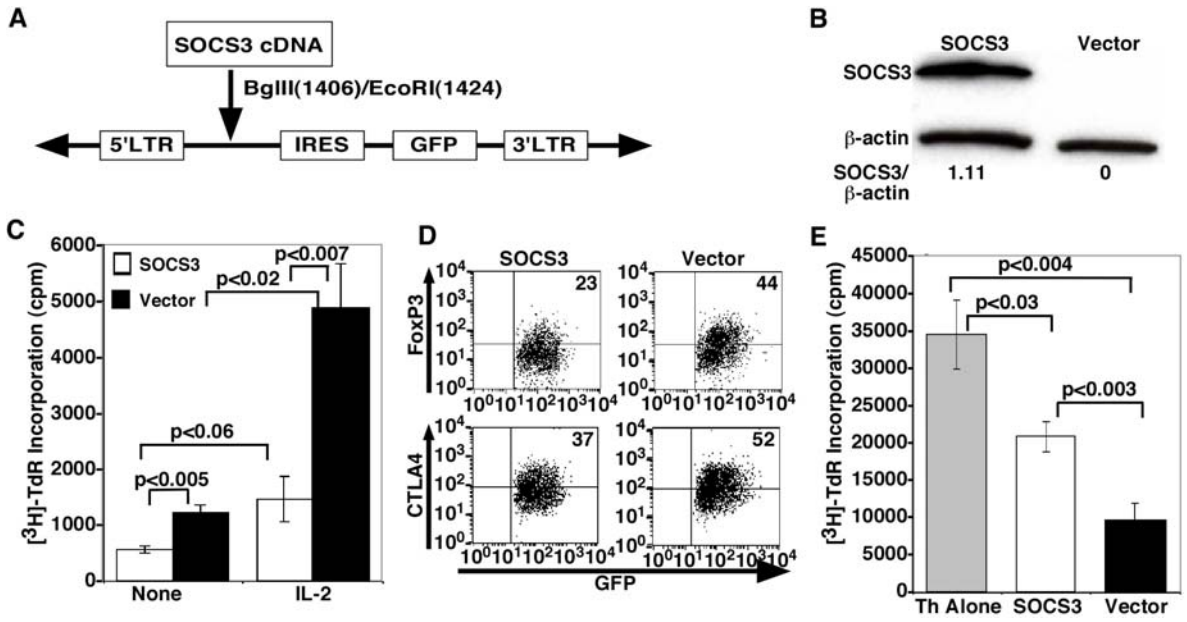

Figure 9. SOCS3 over-expression in Tregs reduces their proliferation, FoxP3 and CTLA4 expression, and suppressive function.

(A) Schematic of the SOCS3-MIGR1-GFP bicistronic construct. (B) Phoenix cells were infected with SOCS3-expressing or control retrovirus, and total extracts were made. Extracts were analyzed for SOCS3 by immunoblotting, and reprobed for β-actin as a loading control. Band intensities were quantified and ratios of SOCS3 and β-actin were calculated. (C)-(E) Tregs were infected with SOCS3-expressing or MIGR1 vector control retrovirus. Following infection, cells were maintained in IL-2 (40 U/mL) for 2 d before staining or sorting. (C) Infected Tregs were sorted, rested for 2 d in media containing 40 U/mL of IL-2, and GFP+ cells were stimulated with anti-CD3 (2 μg/mL) and APCs in the presence or absence of IL-2, and proliferation was analyzed by [^{3}H]-thymidine incorporation as in Fig 1B. (D) Unsorted, infected Tregs were stained for FoxP3 or CTLA-4 expression. Cells were gated on light scatter and GFP expression to exclude uninfected cells and debris. (E) Infected Tregs were sorted, rested for 2 d in media containing IL-2 (40 U/mL), and GFP+ cells were used in suppression assays with anti-CD3 (2 μg/mL) and freshly isolated Th cells and APCs, at a ratio of 1 Treg: 4 Th: 4 APC, as described in the Methods section. (C-E) Results shown are representative of two independent experiments. Abbreviations: LTR- long terminal repeat; IRES- internal ribosomal entry site; GFP- green fluorescent protein.

2.5 CONCLUDING REMARKS

Certain diseases, such as some hepatocellular carcinomas, are associated with pathologically low levels of SOCS3 (193). In many cases of hepatocellular carcinoma, hepatocytes express abnormally low SOCS3, and tumor cells express even less (193). This may enable the cancer to expand in a cytokine-dependent manner. Thus, a therapy that increases SOCS3 levels in the region of the tumor might antagonize the cancer, and inhibit intratumoral Tregs thereby promoting an adaptive immune response. Recently, cell penetrating SOCS3 (cpSOCS3) has been developed, which is a fusion protein containing SOCS3 and a short peptide tag that allows penetration of the cell membrane in many cell types through a poorly understood mechanism (194). Upon intravenous administration, cpSOCS3 enters cells and is biologically active (194). Thus, SOCS3 could be targeted specifically to Tregs, using cell specific markers that remain to be discovered, or markers that correlate with pathology in particular diseases, such as CCR5 or CCR4. Recently, CCR5 was shown to be associated with Tregs that homed to sites of *Leishmania major* infection, which allowed disease persistence (41). Similarly, when CCR4 expressing Tregs were depleted with antibody in an *in vitro* model of Hodgkin's lymphoma, Treg migration was reduced (39).

Taken together, our observation of SOCS3 deficiency in Tregs, combined with the ability of SOCS3 to suppress Treg function, raises the exciting possibility that Treg-targeted cpSOCS3 might serve as an adjunct therapy for cancer, certain infections, and other diseases where reduction of Treg function would be beneficial.

2.6 ACKNOWLEDGEMENTS

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2.7 SUPPLEMENTAL DATA

2.7.1 Methods

Analyses of phosphoSTAT1 (pSTAT1), pSTAT3, and pSTAT5 by flow cytometry

Analyses of cell signaling in response to cytokines were performed as previously described (245). Cytokine signal transduction was monitored by intracellular staining combined with flow cytometry using antibodies specific for phospho-STAT1, phospho-STAT3 or phospho-STAT5 (all Alexa 488-labeled), or total STAT1 (Alexa 647-labelled) (BD Biosciences). Antibody concentrations used were recommended by BD Biosciences. After staining, the cells were washed with PBS, and analyzed by flow cytometry.

2.7.2 Results

Tregs and Th cells show differential STAT activation

Although the full suppressive potential of Tregs is compromised in the presence of IL-2 and IL-6, with seemingly dominant effects of the cytokines on Th cells, Tregs do respond to these cytokines as evidenced by increased potency when pre-exposed to them (Figure 7). Since IL-2 and IL-6 signal via the JAK/STAT pathway (2), we wondered whether these cytokines elicit differential STAT activation in Th cells versus Tregs that might relate to the effects of these cytokines on the two cell types. STAT5 is one of the most important molecules involved in IL-2 signal transduction, and upon activation, it becomes phosphorylated and is transported to the nucleus where it promotes transcription of IL-2 responsive genes (2). Tregs contained much more tyrosine phosphorylated STAT5 (pSTAT5) than Th cells following a brief stimulation with anti-CD3 and APCs in the presence of IL-2, as determined by flow cytometry using a monoclonal antibody specific for pSTAT5 (Figure 10A). Because Tregs express CD25, which is part of the high affinity IL-2R, and unstimulated Th cells do not, it is not surprising that STAT5 activation is high in Tregs and barely detectable in unstimulated Th cells (46). Thus, Tregs probably respond to IL-2 more quickly than naïve Th cells.

Analogous to pSTAT5-mediated IL-2 signal transduction, IL-6 signals are delivered to the nucleus via phosphorylation of STAT1 and STAT3. Therefore, we examined pSTAT1 and pSTAT3 in Tregs and Th cells following IL-6 stimulation. Tregs and Th cells expressed similar levels of pSTAT3 in response to IL-6, but the Tregs expressed less pSTAT1 than Th cells (Figure 10B). STAT1 and STAT3 oppose each other in many biological processes including cell growth, survival and inflammation and their relative abundance is thought to guide many of these processes (128, 132, 246). Our data shows that Tregs have lower STAT1 activation in

response to IL-6 compared to that in Th cells resulting in a higher pSTAT3:pSTAT1 ratio, which implies a comparatively anti-inflammatory signal in the Tregs as compared to that in Th cells. The lower pSTAT1 level in Tregs was not due to a lower level of total STAT1 (Figure 10C), or greater expression of SOCS1, a negative regulator of STAT1 (Figure 8).

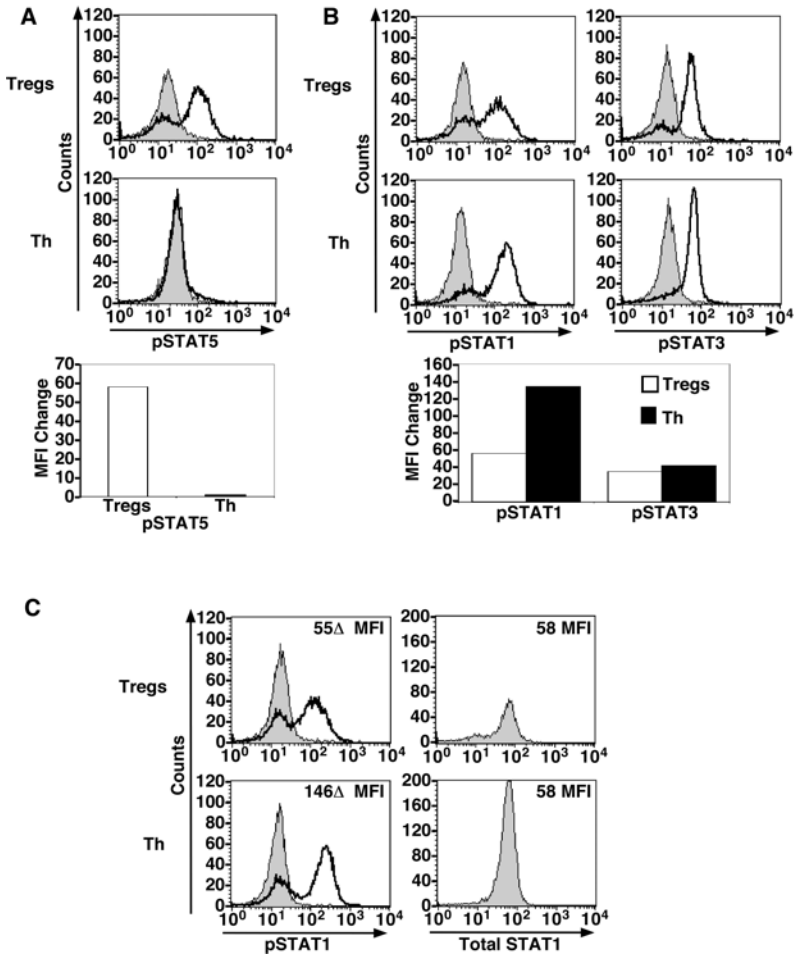

Figure 10. STATs are differentially activated in Tregs and Th cells in response to cytokines.

(A) is the column of 3 panels on the left, (B) is the column of 3 panels on the right. Tregs or Th cells were cultured with anti-CD3 (2 μg/mL) and APCs (1 T cell: 1 APC) for 30 min in the presence or absence of (A), IL-2 ($1.35x10^5$ T cells/well), (B), IL-6 ($1.5x10^5$ T cells/well), or (C), IL-6 ($1.35x10^5$ T cells/well). Cells were fixed in 1.5% paraformaldehyde, permeabilized with 100% methanol, washed, and stained for (A), pSTAT5, (B), pSTAT1 or pSTAT3, and (C), pSTAT1 or total STAT1. Graphs show the mean fluorescence intensity (MFI) change between cells stimulated in the presence (line) and absence (filled) of cytokine. (C), numbers on left panels indicate MFI change, and right panels show MFI arbitrary values. Data is representative of 2 independent experiments.

2.7.3 Discussion

We demonstrate that Tregs are able to rapidly respond to IL-2, whereas Th cells are not, as assessed by STAT5 activation, which can be explained by expression of the high affinity IL-2R (CD25) in Tregs, but not in unstimulated Th cells. Therefore, we propose that Tregs are able to expand and increase their suppressive function to inhibit Th responses, particularly in situations with low levels of cytokine and weak TCR and costimulatory signals, as would be typical during Th cell stimulation by self-antigens. Interestingly, STAT5b was recently shown to be important for the accumulation and function of Tregs in humans (87). We therefore contend that during stimulation by self-antigens, weak activation signals promote Treg dominance. In contrast, in the presence of foreign antigens such as pathogens, stronger activation signals downstream of TCR and costimulatory molecules lead to Th cell dominance by increasing Th cell proliferation and causing resistance to Treg-mediated suppression. Thus, in our experiments, stimulation with anti-CD3 and APCs in combination with exogenous IL-2 or IL-6 is able to overwhelm the Treg suppressive response, as would occur during induction of adaptive immunity by infectious agents. However, similar stimulations without exogenous cytokine allowed suppression, with low levels of endogenous cytokine probably favoring suppression by Tregs, similar to tolerogenic stimulations with antigen in the absence of adjuvant.

IL-6 signaling in Tregs suggests an additional level of regulation that has been investigated recently, which involves altering the ratio of pSTAT3:pSTAT1 (129-131). Stimulation of Tregs with IL-6 caused similar STAT3 activation, but less STAT1 activation, resulting in a higher pSTAT3:pSTAT1 ratio compared to that in Th cells, a difference that

was not due to differences in total STAT1 expression. While activation of both STAT1 and STAT3 were previously thought to promote inflammation, more recently, anti-inflammatory roles of STAT3 have also been recognized. For example, STAT3-deficient macrophages are resistant to the anti-inflammatory effects of *Toxoplasma gondii* infection (247). In addition, STAT3 has been shown to inhibit inflammation downstream of IL-27 (246). Importantly, STAT3 activation in macrophages seems to induce an anti-inflammatory response in the absence of SOCS3, but a pro-inflammatory response if SOCS3 is present (152). Therefore, IL-6 may provide an anti-inflammatory signal to Tregs, which lack SOCS3, but a pro-inflammatory signal to Th cells, which express SOCS3.

Low SOCS3 expression in Tregs emphasizes the critical role of IL-2 signaling in these cells, since IL-2 signaling is inhibited by SOCS3. We have shown that high SOCS3 expression was antagonistic to Tregs, probably due to inhibition of IL-2 signaling. Overexpression of SOCS3 in Tregs reduced their proliferation, especially in response to IL-2, as well as their FoxP3 expression and suppressive function. These data highlight the importance of a high level of CD25 expression in Tregs to allow cytokine signaling via the JAK/STAT pathway since its blockade by SOCS3 impairs their suppressive function.

3 MODULATION OF TREG FUNCTION AND FOXP3 EXPRESSION BY IL-4

3.1 ABSTRACT

Regulatory T cells (Tregs) are important inhibitors of adaptive immune responses, and initial attempts at modulation of allergic airway inflammation by Tregs in mice have yielded some success. We observed that Tregs were present in similar numbers in the lungs of both tolerized mice and those with pulmonary inflammation. The apparent lack of Treg function in lungs with inflammation may be explained by the presence of soluble factors, such as cytokines, that inhibit Treg suppressive function directly or by increased Th cell resistance to suppression. One possible mediator of this effect is the cytokine, interleukin 4 (IL-4), which is critical to the development of asthma and other Th2 diseases. Unfortunately, little is known about modulation of Tregs by IL-4. We show that exogenous IL-4 inhibits Treg-mediated suppression of Th cells *in vitro*. However, Tregs that lack the IL-4 receptor (IL-4R) demonstrate similar or reduced ability to ameliorate inflammation *in vivo*. Detailed examination *in vitro* using knockout mice and retroviral overexpression techniques revealed that IL-4 did not directly influence Treg suppression. Instead, IL-4 increased Th cell resistance to suppression. Furthermore, IL-4 increased Treg expansion, Treg FoxP3 maintenance, and Th cell proliferation; and decreased *de novo* expression of FoxP3 in CD4+ T cells upon activation in the presence of TGFβ. Finally, these effects of IL-4 were due to activation of STAT6.

3.2 INTRODUCTION

Asthma is a common disease that affects over 15 million people in the United States; and is characterized by airway inflammation, mucus hypersecretion, elevated serum IgE, and airway hyperresponsiveness (AHR) (222). An important underlying cause of asthma is Th2-mediated inflammation with eosinophilic infiltration of the airways. The success of immunomodulatory treatments such as steroids and oral administration of antigen, strongly suggests an immunological contribution to the allergic form of disease (248). The central role for Th2-type responses is supported by the altered rates of disease in humans with mutations in their IL-4R (24, 144). Specifically, gain of function mutations in the IL-4R that lead to increased signaling correlate with atopy (24). Conversely, alleles that cause diminished signaling via the IL-4R lead to reduced serum IgE levels (144).

The IL-4R is composed of the IL-4Rα chain and the common gamma chain (γc), which is shared with receptors for IL-2, IL-7, IL-9, IL-15, and IL-21 (1, 2). Engagement of the IL-4R primarily induces signaling through signal transducer and activator of transcription 6 (STAT6), which, upon activation, translocates to the nucleus and initiates transcription of specific genes. Importantly, mice that lack IL-4R or STAT6 are highly resistant to experimentally induced allergic airway inflammation, and show significant defects in Th2 differentiation (140-142, 249).

Current treatments for Th2-associated diseases usually involve the use of immunosuppressive agents such as steroids, administered on a regular basis, which can have undesirable side-effects. Tregs suppress immune responses *in vitro* and *in vivo*, and individuals with defective Treg function develop severe autoimmune disease (25). Thus, Tregs have potential for use in future therapeutic interventions aimed at controlling aberrant immune responses. There are several types of Tregs, including naturally occurring (NatTregs), TGFβ

induced (IndTregs), and antigen generated (AgTregs). All three cell types express CD4, CD25 (IL-2Rα, part of the high affinity IL-2R), and FoxP3, and are characterized by their ability to inhibit T helper (Th) cell proliferation through a TGFβ-dependent mechanism (45, 51, 54). These three types of Treg are distinguished by the processes by which they are generated. NatTregs are thymically derived cells found in mice and humans in the absence of any treatment. In contrast, IndTregs are induced by activating CD4+ T cells *in vitro* in the presence of TGFβ (51). AgTregs are generated by, for example, repeated exposure of mice to aerosolized ovalbumin (OVA) in the absence of adjuvant, and provide protection against experimentally induced airway inflammation in response to OVA (54). Since these three types of Tregs are phenotypically similar, and there is no accepted method for identifying them *in vivo* or *ex vivo*, it is possible that AgTregs may be derived from NatTregs, IndTregs, or a separate lineage.

Importantly, regulation of Tregs in asthma remains poorly understood, especially their modulation by cytokines such as IL-4, which are critical regulators of immune responses. We found that Tregs were present in similar numbers in the lungs of mice following the induction of inflammation or tolerance. Therefore, allergic airway inflammation is probably not due to deficiencies in Treg numbers or trafficking. Instead, soluble factors, such as cytokines, might modulate Treg function. NatTreg suppression of Th cells in co-culture is inhibited by IL-4, which is central to the development of Th2 responses and asthma (49, 140-142, 145, 146, 249, 250). Consequently, we investigated the role of IL-4 signaling in Tregs *in vivo* using a model of allergic airway inflammation, and observed that adoptive transfer of AgTregs could inhibit disease. Unexpectedly, lack of IL-4 signaling in AgTregs reduced their effectiveness in the inhibition of disease under certain conditions. *In vitro*, we characterized the influence of IL-4 on FoxP3 maintenance/induction, proliferation, and NatTreg-mediated suppression and also

investigated the role of STAT6 using knockout and over-expression approaches. Interestingly, IL-4 does not alter NatTreg suppressive function on a per-cell basis, but may promote overall suppression indirectly by increasing NatTreg proliferation through a STAT6-dependent mechanism.

3.3 MATERIALS AND METHODS

3.3.1 Mice

Animals were housed in the University of Pittsburgh Department of Laboratory Animal Resources. Mice were used in accordance with AALAC regulations under approved IACUC protocols.

3.3.2 Cell isolation and Th cell stimulation

Tregs (CD4+CD25+), Th cells (CD4+CD25-), and antigen presenting cells (APCs) were isolated from the spleens of 7-12 wk old male Balb/cByJ mice (Jackson Laboratories). Spleens were minced, filtered through 70 μM nylon screens, and mononuclear cells were enriched by density gradient centrifugation (LSM, MP Biomedicals). Tregs and Th cells, were isolated using a regulatory T cell magnetic bead isolation kit (Miltenyi Biotec). CD4- cells were obtained as a byproduct of the T cell isolation, and these cells were used as APCs. Unless indicated, all cells

were cultured in complete medium: Click's medium supplemented with 10% FBS (Gemini), gentamicin (50 mg/L, Gemini), L-glutamine (2 mM, Gibco), and 2-mercaptoethanol (55 μM, Gibco). T cells were activated with agonistic soluble anti-CD3ε monoclonal antibody (2 μg/mL; BD Biosciences) and gamma irradiated (2000 rad) APCs as a source of costimulation, in most experiments. Some experiments included addition of exogenous IL-2 (50 U/mL) or IL-4 (20 ng/mL) as indicated.

3.3.3 Pretreatment with cytokines

In cytokine pretreatment experiments, plates were coated for 4 h at 37^0C with anti-CD3ε (5 μg/mL) and anti-CD28 (2 μg/mL) monoclonal antibodies (BD Biosciences). Plates were washed once with PBS, and then cells were cultured in the presence of IL-4 (20 ng/mL) or with no exogenous cytokine (Peprotech). After 24 h, cells were harvested from the plates, washed with PBS to remove cytokines, and then were used for suppression assays with no additional cytokine added.

3.3.4 [^{3}H]-thymidine incorporation assay

Cells were cultured in round bottom, 96 well plates with soluble anti-CD3ε (2 μg/mL) and $3x10^4$ gamma-irradiated APCs per well (if used in an assay, Th cells were added at 1 Th cell: 1 APC). Freshly isolated Tregs were added at a ratio of 1 Treg: 1 APC, or in the ratios indicated in the figures. Cells were cultured for 48 h, and then pulsed with [^{3}H]-thymidine (1 μCi/well, Perkin

Elmer) and incubated for an additional 18 h. Incorporation of [^{3}H]-thymidine was measured by harvesting cell debris and DNA onto glass fiber filters, followed by liquid scintillation counting (Wallac). All conditions were performed in triplicate, and data is presented as mean counts per min (cpm) plus or minus the standard deviation (SD).

3.3.5 CFSE dilution assay

Carboxyfluoroscein succinimidyl ester (1 μM, CFSE, Invitrogen) was used to label Th cells for 15 min at 37^0 C followed by two washes with PBS/FCS (PBS, pH 7.2 plus 2% FBS). Cells were cultured in 96 well plates in complete medium ($3x10^4$ labeled Th cells/well), an equal number of irradiated APCs, and soluble anti-CD3 (2 μg/mL) in the presence varying ratios of Tregs with or without IL-4. After 3 d, cells were stained with propidium iodide (PI), allophycocyanin-(APC) labeled anti-CD4 monoclonal antibody, and phycoerythrin-(PE) labeled anti-CD25 monoclonal antibody (BD Biosciences). A FACSCalibur flow cytometer with CellQuest software was used to analyze cells (BD Biosciences, Immunocytometry Systems). Dead cells and APCs were excluded by light scatter and PI staining. Proliferation of live (PI negative) Th cells was determined by CFSE dilution. The percentage of divided cells was determined by dividing the number of Th cells undergoing at least one cell division by the total number of live CFSE-labeled cells and multiplying the result by 100.

3.3.6 Western blotting

Nondenaturing cell lysis buffer with 1% Triton (Cell Signaling) was used to make total cell extracts, and Western blotting techniques were used to analyze equal amounts of protein, as described previously (22). Membranes were probed with anti-STAT6 monoclonal antibody (Cell Signaling) at a 1:1000 dilution. Expression of β-actin (Novus) was used to confirm equal protein loading.

3.3.7 Construction of retroviral vector and infection of regulatory T cells and Th cells

A constitutively active STAT6 (caSTAT6) retroviral construct was generously provided by Dr. Jinfang Zhu (251). caSTAT6 or empty vector was purified, and a packaging cell line (Phoenix cells) was infected to generate retrovirus. Infection of Tregs and Th cells was performed as previously described (235). caSTAT6 retrovirus or empty vector control virus was used to infect cells, and evaluation of GFP expression by flow cytometry was used to determine infection efficiency. Following infection, cells were maintained in the presence of IL-2 either for 2 d, then sorted based on GFP expression using a FACSAria cell-sorter (BD Immunocytometry Systems), or for 3 d, then analyzed for FoxP3 expression by flow cytometry. Cells purified by cell sorting were used in proliferation assays similar to those described above.

3.3.8 Analyses of FoxP3 expression

Expression of FoxP3 in uninfected and retrovirally-infected Tregs was determined by intracellular staining and flow cytometry. Cells were washed, and resuspended in Cytofix/Cytoperm buffer (BD Biosciences) at 4^0C. After 15 min, cells were washed with PBS, resuspended in permeabilization buffer (BD Biosciences), and incubated with APC-labeled anti-FoxP3 monoclonal antibody (eBioscience). After 30 min, cells were washed with PBS, resuspended in pH 7.2 PBS with 2% FBS. Finally, cells were analyzed by flow cytometry.

3.3.9 Isolation of Treg from tolerized mice

Animals were tolerized by exposure to aerosolized 1% OVA in PBS daily (20 min/day) for 10 consecutive days, followed by 10 d of rest (54). Mononuclear cells were isolated from spleens of tolerized mice by density gradient as described above, and CD4+ cells were purified using magnetic bead negative selection (Miltenyi Biotec). From this population, CD4+CD25+ Tregs were obtained by cell sorting. Tregs were suspended in PBS, then adoptively transferred ($2x10^5$ cells per recipient) to naïve animals for experiments described in the following sections.

3.3.10 Prevention of experimentally induced airway inflammation

A model for the prevention of experimentally-induced airway inflammation was described in detail previously (54). Briefly, mice received i.p. injections of Tregs from OVA tolerized mice or sham injections, followed by an i.p. injection of OVA/Alum to prime the animals 15 minutes later. After 6 d, animals were administered an i.p. injection of OVA/Alum as a boost. Mice were rested for 1 wk, then subjected to challenge with 1% aerosolized OVA (20 min/day) for 7 consecutive days.

3.3.11 Treatment of experimentally induced airway inflammation

A model for the treatment of experimental airway inflammation was similar to the prevention model described above, except that animals received an i.p. injection of Tregs from tolerized mice or a sham injection on the first day of the 7 d challenge with 1% aerosolized OVA.

3.3.12 Bronchoalveolar lavage (BAL)

At 24 hours after the seventh aerosol challenge in the models described above, BAL was conducted on the animals, and cell differentials were completed, as previously described (54).

3.3.13 Lung cell isolation

Lungs were removed, homogenized, and incubated at 37^0C for 2 h in 10mL digestion buffer: 10mL RPMI/10%FBS, 6.56 mg collagenase A, 10 μl 10mg/mL DNase I. Digest was filtered through a 70 μm screen, and cells were separated by a series of Percoll gradients (20%, 35%, 45%, and 55%). Cells isolated by this procedure were stained for expression of CD4, CD25, and FoxP3 as described above, and analyzed by flow cytometry.

3.3.14 Statistics

Results shown are mean values ± S.D. All p values were determined using a 2 tailed Student's t-test, and differences were considered significant where $p \leq 0.05$. Calculations pertain to individual representative experiments with samples done in triplicate.

3.4 RESULTS

3.4.1 The number of Tregs is similar in the lungs of tolerized mice and those with inflammation

Although Tregs can act as potent inhibitors of the immune system, they are unable to maintain tolerance in patients with autoimmune and allergic diseases, for reasons that remain to be elucidated. Surprisingly, we found statistically similar numbers of Tregs in the lungs of mice during allergic airway inflammation and tolerance (Figure 11). As expected, the number of Th cells was higher in the lungs of animals undergoing inflammation compared to tolerized mice (Figure 11).

Our laboratory has shown that Th2-mediated airway inflammation can be prevented in a murine model of asthma by the adoptive transfer of AgTregs (54). Thus, at least under certain conditions, Tregs are able to inhibit airway inflammation. Taken together, this suggests that soluble factors present in the microenvironment may account for the inhibition of Treg function. Due to its importance in Th2 responses and asthma, and its ability to inhibit Treg-mediated suppression *in vitro*, we hypothesized that IL-4 could be involved in the modulation of Treg function in asthma.

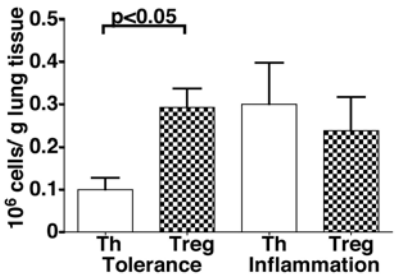

Figure 11. Treg numbers are similar in tolerized and inflammatory lungs.

Mice were tolerized by exposure to 10 consecutive days of aerosol OVA in the case of tolerance, or 2 immunizations with OVA/Alum followed by OVA aerosol challenge for 7 d for the inflammation group. Lung T cells were isolated, stained for CD4, CD25, and FoxP3, and analyzed by flow cytometry.

3.4.2 Tregs are able to prevent development of airway inflammation regardless of their ability to respond to IL-4

In order to determine if IL-4 has any effect on the *in vivo* development of AgTregs, we tolerized WT and IL-4Rα-/- mice, isolated AgTregs from spleens, and transferred these cells into WT recipients (Figure 12). Immediately following the AgTreg adoptive transfers, recipient animals were subjected to a model of experimentally induced asthma, and then analyzed for airway inflammation. AgTregs from both types of mice inhibited airway inflammation almost completely (Figure 12). Therefore, IL-4 signaling did not appear to be necessary for AgTreg development and/or function *in vivo*.

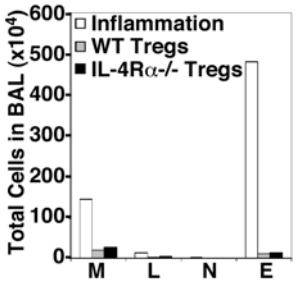

Figure 12. Adoptive transfer of Tregs from WT or IL-4Rα-/- mice prevents airway inflammation.

WT or IL-4Rα-/- mice were tolerized, and their Tregs were isolated. WT recipient animals received i.p. injections of $2x10^5$ Tregs from WT or IL-4Rα-/- mice or a sham injection of PBS. After 15 min, mice were subjected to the airway inflammation model, starting immediately with an OVA/Alum priming injection, followed by an OVA/Alum boost, and then an OVA aerosol challenge. Animals were subjected to BAL, and cell differentials were determined. Inflammation: sham injected WT recipients; WT Tregs: WT recipients of WT Tregs; IL-4Rα-/- Tregs: WT recipients of IL-4Rα-/- Tregs. Abbreviations: M- macrophages; L- lymphocytes; N- neutrophils; E- eosinophils. Data is representative of 2 independent experiments.

3.4.3 Tregs promote a moderate reduction in airway inflammation when administered following immunization, with a requirement for IL-4 signaling in Tregs

Since WT and IL-4Rα-/- AgTregs were able to prevent induction of allergic airway inflammation (Figure 12), we wanted to determine whether AgTregs could alter the course of disease following priming. To that end, recipient animals received i.p. priming and boosting injections of OVA/Alum, and then on the first day of OVA aerosol challenge, AgTregs were administered (Figure 13). Unlike the situation where prophylactic administration of AgTregs reduced inflammation by approximately 10-fold (Figure 12), transfer of WT AgTregs after priming reduced the number of eosinophils by one half, whereas IL-4Rα-/- AgTregs barely decreased eosinophil numbers in the BAL fluid (Figure 13). Thus, AgTregs potently prevent disease, but have a more modest effect on established airway inflammation. In contrast to the similar effects of WT and IL-4Rα-/- AgTregs in the "prevention" experiment, IL-4 signaling was required for AgTreg-mediated *inhibition* of disease (Figure 13). Thus, the ability to respond to IL-4 seemed to enhance AgTreg-mediated inhibition, especially in a relatively "high IL-4 environment" found in the "treatment" experiment.

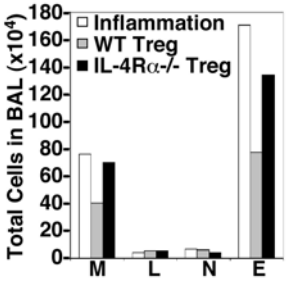

Figure 13. WT Tregs moderately inhibit airway inflammation, but IL-4Rα-/- Tregs do not.

WT recipient mice were subjected to the airway inflammation model as in previous figures. Briefly, animals were immunized i.p. two times with OVA/Alum 5 days apart. One week after immunizations, on the first day of OVA aerosol challenges, animals received Tregs that were isolated from tolerized WT and IL-4Rα-/- mice. Animals were subjected to an additional six days of OVA aerosol. On the day following the last aerosol, mice were subjected to BAL and cell differentials were determined. Inflammation: sham injected WT recipients; WT Tregs: WT recipients of WT Tregs; IL-4Rα-/- Tregs: WT recipients of IL-4Rα-/- Tregs. Abbreviations: M- macrophages; L- lymphocytes; N- neutrophils; E- eosinophils. Data is from a single experiment.

3.4.4 Exogenous IL-4 inhibits Treg-mediated suppression *in vitro*, and modulates proliferation and FoxP3 expression of Tregs and Th cells

In order to determine the mechanism for the unexpected enhancement of AgTreg function by IL-4, the effects of IL-4 on Tregs and Th cells were investigated *in vitro* (Figure 14). Th cells were labeled with CFSE, and co-cultured with NatTregs in the presence or absence of IL-4 (Figure 14A). In agreement with others (145, 146), exogenous IL-4 increased Th cell proliferation when cultured alone, and decreased suppression in colcultures with NatTregs, which implies a dominant effect of IL-4 on Th cells (Figure 14A). Preactivation of NatTregs for 1 d prior to use in co-culture experiments was not influenced by the addition of exogenous IL-4, indicating that short-term treatment with IL-4 does not have any effects on NatTreg potency (Figure 14C). In order to explain why WT AgTregs were more efficient inhibitors of disease than IL-4Rα-/- AgTregs, proliferation of NatTregs in 3 d cultures was characterized in the presence or absence of IL-4 (Figure 14B). Administration of IL-4 increased NatTreg proliferation by approximately 30-fold, implying that IL-4 enhances AgTreg inhibition of disease by increasing the number of Tregs (Figure 14B). Because FoxP3 is a critical transcription factor in Tregs, the influence of IL-4 on FoxP3 maintenance in NatTregs in culture was evaluated (Figure 14D). Approximately 90% of freshly isolated Tregs express FoxP3, but after 3 d in culture, the percentage of cells expressing FoxP3 decreased substantially, as reported previously (236). Addition of IL-4 allowed for considerable maintenance of FoxP3 expression (Figure 14D). On the other hand, IL-4 inhibited *de novo* production of FoxP3 during *in vitro* generation of IndTregs, which involved activation of CD4+ T cells in the presence of TGFβ (Figure 14E). This may be a result of

increased Th2 differentiation, or due to direct inhibition of FoxP3 induction by IL-4 in CD4+ T cells. Hence, IL-4 promoted the maintenance of FoxP3 expression in cells that already expressed it (NatTregs), but inhibited expression in cells initially lacking it (CD4+ T cells).

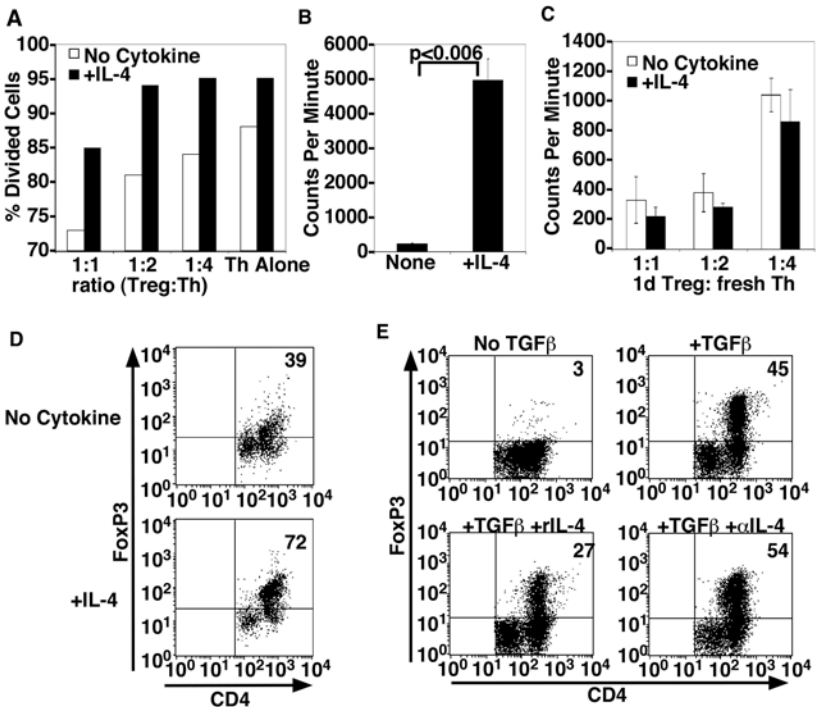

Figure 14. Exogenous IL-4 reduces Treg function *in vitro*, but not due to direct effects on Tregs.

(A-E) Tregs and Th cells were isolated from naïve WT mice. CD4-depleted splenocytes were irradiated (2000 rads), and used as APCs ($3x10^4$ per well) in conjunction with anti-CD3 (2 μg/mL) to stimulate Th cell/Treg co-cultures (A, C) or Tregs alone (B, D). (A-E) Cells were stimulated in the presence or absence of IL-4 (20 ng/mL), as indicated. (A) Th cells were labeled with CFSE and cultured with Tregs in the indicated ratios for 3 d. Cells were stained and analyzed by flow cytometry as described in the Methods section. The Y-axis shows the percentage of Th cells that divided 1 or more times. (B) Tregs were cultured in the presence or absence of IL-4 (20 ng/mL) for 2 d, pulsed with [^{3}H]-thymidine for 18 hr, and analyzed by liquid scintillation counting. (C) Tregs were isolated and stimulated for 1 d with IL-4 (20 ng/mL) and plate-bound anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL). Tregs were removed from the plate, washed, and co-cultured with Th cells at the indicated ratios in a [^{3}H]-thymidine incorporation assay similar to (B) NOTE: in (C), no exogenous IL-4 is present during co-culture. (D) Tregs were cultured for 3 d and FoxP3 expression was analyzed by flow cytometry. (E) $1x10^6$ CD4+ T cells were stimulated for 3d with plate-bound anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) in 24 well plates. In addition, TGFβ (2 ng/mL), IL-4 cytokine (20 ng/mL), and neutralizing anti-IL-4 antibody (2 μg/mL) were added as indicated. rIL-4:

recombinant IL-4 cytokine; αIL-4: neutralizing anti-IL-4 antibody. FACS analysis is described in the Methods section. Numbers in FACS plots are the percentage of cells in the quadrant. (A-E) Data is representative of at least 2 independent experiments.

3.4.5 IL-4 effects on FoxP3 expression are mediated via a STAT6-dependent mechanism

Signal transduction in response to IL-4 requires binding to the IL-4R, which is comprised of IL-4Rα (CD124) and the γc (CD132). Following receptor ligation, several pathways are activated with the STAT6 pathway being among the most important. Consequently, using STAT6-/- and IL-4Rα-/- animals, it was found that IL-4-mediated NatTreg maintenance of FoxP3 (Figure 15A) as well as inhibition of *de novo* FoxP3 synthesis (Figure 15B) both required IL-4Rα, and were STAT6-dependent.

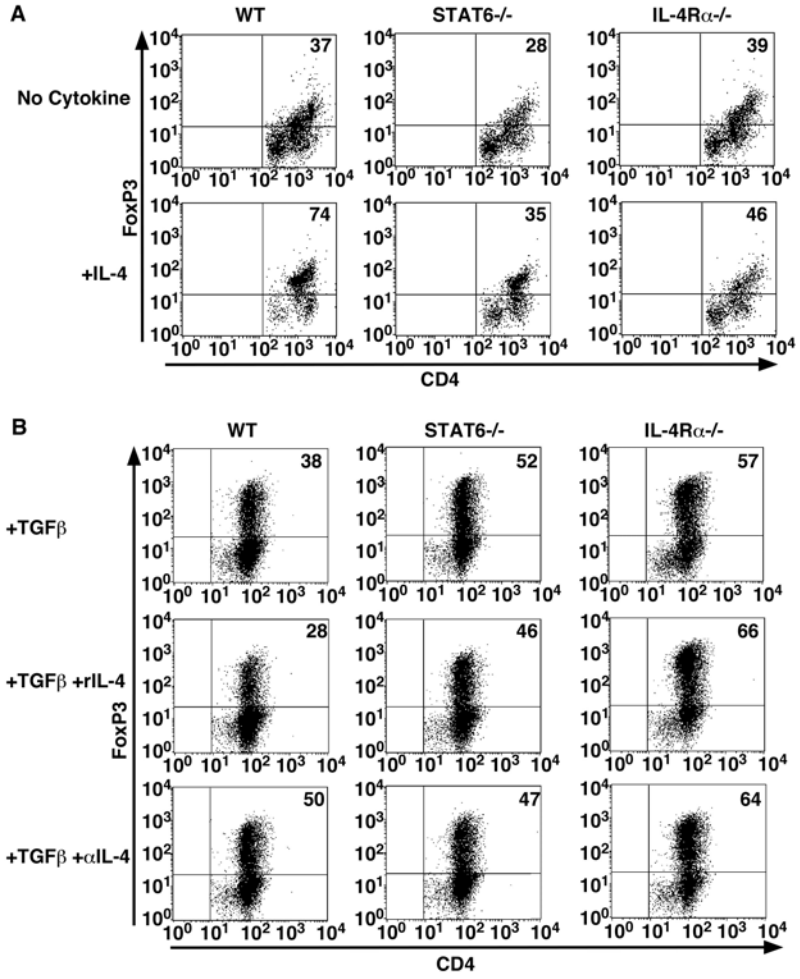

Figure 15. IL-4 enhances FoxP3 maintenance in naturally ocurring Tregs, but inhibits TGFβ induced Treg generation in a STAT6-dependent manner.

(A) Tregs ($3x10^4$ per well) or (B) CD4+CD25- T cells ($1x10^6$ per well), were isolated from WT, STAT6-/- and IL-4Rα-/- mice, and stimulated with (A) APCs ($3x10^4$ per well) and anti-CD3 (2 μg/mL) in a round-bottom 96 well plate or (B) plate bound anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) in a 24 well plate. (B) TGFβ (2 ng/mL), IL-4 cytokine (20 ng/mL), and neutralizing anti-IL-4 antibody (2 μg/mL) were added as indicated. (A and B) Cells were cultured for 3 d and FoxP3 expression was analyzed by flow cytometry. Abbreviations: rIL-4- recombinant IL-4 cytokine; αIL-4- neutralizing anti-IL-4 antibody. FACS analysis is described in the Methods section. Numbers in FACS plots are the percentage of cells in the quadrant. Data is representative of 3 independent experiments.

3.4.6 Treg expansion, Th cell proliferation, and Th cell resistance to suppression are enhanced by STAT6-dependent responses to IL-4

IL-4-mediated effects on FoxP3 expression strongly suggested functional alteration of the cells as well. To investigate this, the influence of IL-4 on cellular proliferation and Treg function were assessed. IL-4 enhanced proliferation of both Th cells (Figure 16A) and NatTregs (Figure 16B) provided that the cells expressed IL-4Rα and STAT6, indicating that IL-4-induced proliferation is STAT6-dependent. Further, the effect of IL-4 on proliferation is significantly greater for NatTregs than Th cells. However, Th cells do proliferate readily in the absence of IL-4, whereas NatTregs do not, and overall proliferation is also an order of magnitude greater with Th cells. CFSE suppression assays indicated that IL-4 inhibited suppression through direct effects on the Th cells (Figure 14). An additional experiment that included all possible combinations of NatTregs and Th cells from WT, STAT6-/- and IL-4Rα-/- mice was conducted to directly evaluate this conclusion (Figure 16C and -D). In Figure 16C and -D, each set of three different-colored bars represents the suppressive function of all three types of Treg on a single type of Th cell, which is indicated on the X-axis. Bars demonstrated similar heights within each triplicate, indicating that the type of Treg had little influence on suppression regardless of the presence of IL-4 (Figure 16C and -D). Thus, in the presence (Figure 16D) or absence (Figure 16C) of exogenous IL-4, NatTregs from all three types of animal showed equal suppressive function, with the exception of marginally enhanced suppression by WT NatTregs (Figure 16C and -D). On the other hand, the first triplicate was greater than the other two, and was increased by the presence of IL-4, demonstrating that IL-4 decreased suppression when administered to WT Th cells, but showed no effect on STAT6-/- and IL-4Rα-/- Th cells (Figure 16C and -D). Therefore,

WT Th cells increased their resistance to suppression in response to IL-4 in a STAT6-dependent manner (Figure 16C and -D). Together, our data support the conclusion that IL-4-induced STAT6 activation promotes proliferation of both NatTregs and Th cells, increases Th cell resistance to suppression, but has no direct effects on NatTreg suppressive function.

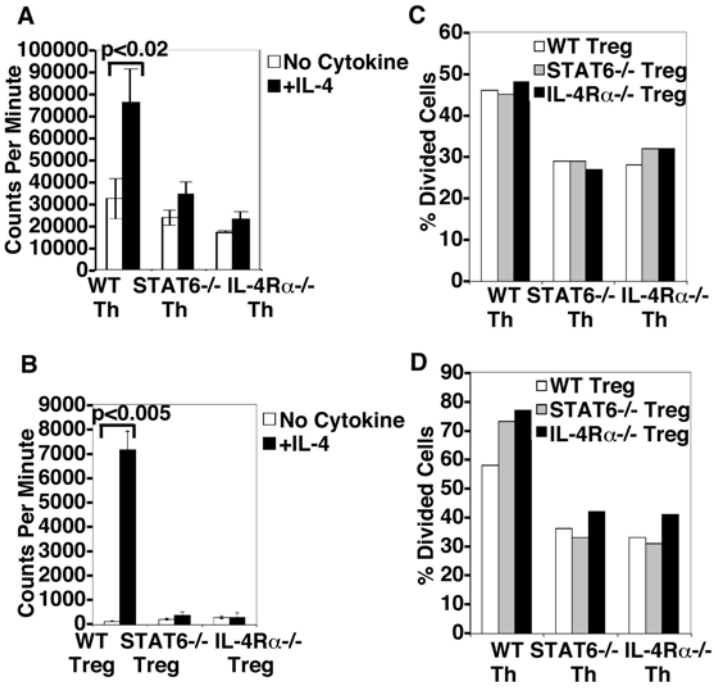

Figure 16. IL-4 promotes proliferation of Th cells and Tregs, and inhibits Treg function by increasing Th cell resistance to suppression through a pathway that requires STAT6.

(A) Th cells or (B) Tregs were isolated from WT, STAT6-/-, or IL-4Rα-/- mice. (A) Th cells ($3x10^4$ per well) and (B) Tregs ($3x10^4$ per well) were stimulated with anti-CD3 (2 μg/mL) and APCs ($3x10^4$ cells per well) in a [^{3}H]-thymidine incorporation assay, as described in the Methods section. (C and D) Th cells and Tregs were isolated from WT, STAT6-/-, or IL-4Rα-/- mice. Th cells were labeled with CFSE and the indicated combinations of cells were used for a CFSE dilution assay. Assays were conducted in the (C) absence or (D) presence of exogenous IL-4 (20 ng/mL). After 3 d, cells were analyzed by flow cytometry as described in the Methods section. (C and D) bar shading represents the type of Treg used for the assay, and X-axis labeling indicates the type of Th cell. (A-D) Data is representative of 2 independent experiments.

3.4.7 STAT6 signaling is sufficient for promotion of Treg and Th cell proliferation and enhancement of Th cell resistance to Treg-mediated suppression

While STAT6 appeared to be necessary for IL-4-mediated effects on Tregs and Th cells, it was not known if STAT6 was sufficient for these effects. Therefore, cells were infected with a bicistronic retroviral construct that induced expression of GFP and a constitutively active form of STAT6 (caSTAT6) (Figure 17). Expression of caSTAT6 increased proliferation of Th cells (Figure 17A) and NatTregs (Figure 17B), with the greater relative effect being on NatTregs, in agreement with observations using WT cells following IL-4 stimulation (Figure 16A and -B). FoxP3 expression was also higher in GFP+ NatTregs infected with caSTAT6 compared to GFP+ cells infected with control vector (Figure 17C). Importantly, caSTAT6 infected NatTregs showed only minimal, if any, enhancement of suppressive function, further supporting the findings that IL-4 did not directly influence NatTreg suppression (Figure 17D). Furthermore, Th cells infected with caSTAT6 retrovirus exhibited greater resistance to Treg-mediated suppression than those infected with control vector retrovirus (Figure 17E).

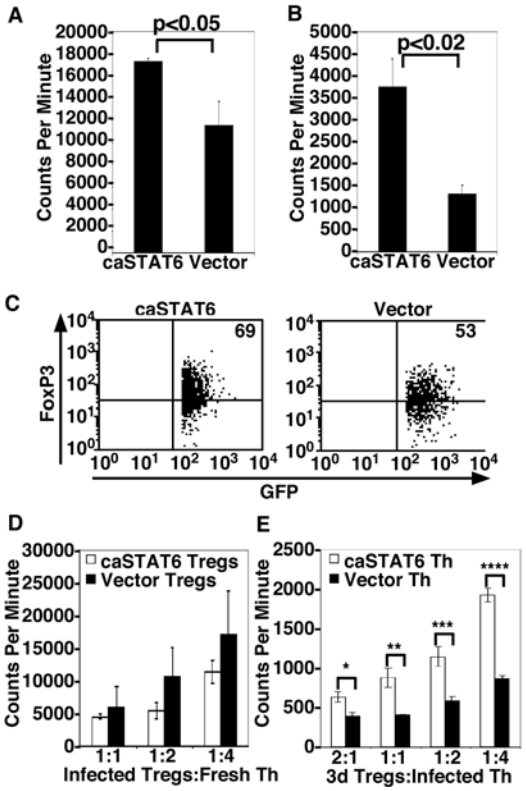

Figure 17. STAT6 signaling is sufficient to promote Treg and Th cell proliferation, FoxP3 maintenance, and Th cell resistance to suppression.

(A) Th cells or (B) Tregs were isolated from STAT6-/- mice and infected with caSTAT6 or vector control retrovirus. GFP+ cells were sorted, rested for 2 d, and stimulated with APCs and anti-CD3 in a thymidine incorporation assay. (C) Tregs were isolated, infected, and sorted as in (B), and FoxP3 expression was evaluated using flow cytometry. Cells were gated using GFP expression and light scatter to exclude debris and uninfected cells. (D) Tregs or (E) Th cells were infected and sorted as in (A and B). (D) infected Tregs were co-cultured in varying ratios with freshly isolated Th cells in a [^{3}H]-thymidine incorporation assay. (E) infected Th cells were combined with Tregs in the indicated ratios in a thymidine incorporation assay. Before use in the assay, Tregs were isolated and cultured for 3 d in the presence of IL-2 (50 U/mL) and plate-bound anti-CD3 and anti-CD28 to activate them and increase their potency. Th cell proliferation is relatively low in (E) because the cells were stimulated for several days during the infection procedure, which decreases their ability to proliferate upon exposure to additional activating stimuli. Abbreviations: *=p<0.008; **=p<0.03; ***=p<0.007; ****=p<0.0004. (A-E) Data is representative of 2 independent experiments.

3.5 DISCUSSION

In the developed world, asthma is an increasingly common disease that can lead to significant morbidity and mortality (222). Steroids have proven moderately successful in the treatment of many patients, but this often requires daily administration for the lifetime of an individual, raising the possibility of unwanted side effects. In addition, some cases do prove to be resistant to steroid-based therapy. Therefore, the development of novel treatment modalities is warranted. One possibility is the use of Tregs in therapeutic approaches. The Treg field has had a resurgence in recent years due to the potential of Tregs to provide lasting tolerance in patients with transplants or autoimmune disease (2). Thus, Tregs also show great promise in the treatment of allergic diseases and asthma.

We have shown previously that adoptive transfer of Tregs can prevent development of experimentally induced airway inflammation (54). In this manuscript, we demonstrate that Tregs are present in similar numbers in the lungs of animals with tolerance or allergic airway inflammation (Figure 11). This raises the possibility that secreted factors, such as cytokines, are released into the pulmonary microenvironment to modulate Treg function or Th cell responsiveness to suppression, since significant numbers of Tregs are present, but do not inhibit the response. In addition, once an inflammatory response has been established, Th cells increase in number relative to Tregs, which may further reduce Treg-mediated inhibition. In murine models of asthma, IL-4 signaling is required for initiation of disease, and genetic defects in IL-4 signal transduction provide resistance to induction of Th2 pulmonary inflammation (140-142, 249). Due to its importance in the initiation of Th2 responses, we investigated whether IL-4 was involved in the regulation of Treg function. We show that a lack of IL-4 signaling in Tregs has no significant influence on their ability to prevent disease (Figure 12). Interestingly, in a more

therapeutically relevant system, adoptive transfer of Tregs into animals that had already been primed and boosted, decreased BAL fluid eosinophilia, a major component of airway inflammation, suggesting the potential for Treg treatment of asthma (Figure 13). Further, although WT Tregs reduced eosinophilia, IL-4Rα-/- Tregs did not (Figure 13). The differential function of WT compared to IL-4Rα-/- Tregs in the treatment model contrasted with fairly similar degrees of inhibition observed between the two in the prevention model. This discrepancy might be due to higher IL-4 concentrations in these mice compared to animals that received Tregs before priming and boosting. Also, both types of Tregs efficiently prevented inflammation, so it is possible that a difference would have been detected at a lower dose of adoptively transferred cells. These results clearly show that IL-4 signaling does not inhibit Treg function, but rather probably enhances it, *in vivo*.

In contrast with the *in vivo* data, addition of IL-4 to Treg/ Th cell co-cultures inhibited suppression *in vitro* (Figure 14A). Analyses of STAT6-/- and IL-4Rα-/- cells (Figure 16) and cells over-expressing caSTAT6 (Figure 17) prove that IL-4 does not directly alter Treg suppressive function. Rather, STAT6-dependent signaling induces proliferation of both Tregs and Th cells, promotes maintenance of FoxP3 expression, antagonizes *de novo* FoxP3 expression, and increases Th cell resistance to suppression. Thus, IL-4 signaling *in vivo* probably leads to Treg expansion, which could explain the enhanced suppression of disease observed with WT Tregs compared to IL-4Rα-/- Tregs following adoptive transfer (Figure 13). We propose that IL-4-mediated enhancement of Treg proliferation did not significantly influence the outcome of the *in vitro* assays because by the time Treg numbers increased enough to enhance

suppression, there was not enough time left in the assay to observe these effects (Figure 14 and Figure 16). On the other hand, exogenous IL-4 increased Th resistance to suppression *in vitro* (Figure 14 and Figure 16).

While Th cells proliferated more vigorously than Tregs overall, IL-4 did increase Treg proliferation by approximately 20-fold, compared to only a 2-fold expansion of Th cells (Figure 16A and B). This suggests that IL-4 may serve as an important signal for Treg expansion following engagement of TCR and costimulatory molecules. Conversely, Th cells undergo dramatic expansion following TCR and costimulatory molecule engagement, and IL-4 has a less potent effect on expansion of activated cells. This may be because many Tregs are specific for self antigens, which would be encountered constantly, such that, alternative signals may be necessary to fully activate them. Since Tregs produce minimal levels of IL-4, paracrine sources of cytokine would be necessary for their proliferation. This has been implied by others in the case of IL-2, which increases Treg proliferation and enhances suppressive function if the cells are pre-treated, but abrogates suppression when included in co-culture with Th cells (49, 50, 252). Thus, activated Th cells can provide a feedback mechanism to Tregs promoting suppression, but these same signals also increase Th cell resistance to suppression. Presumably, upon pathogen clearance, Th cells would no longer receive signals through the TCR and costimulatory molecules, and resistance to suppression would wane, thus allowing the expanded Tregs to further downregulate the response. The regulation of FoxP3 expression by IL-4 provides further support for this idea. Cells that already express FoxP3 presumably are able to provide protective suppressive function, so IL-4 promotes FoxP3 maintenance in these cells. On the other

hand, during an adaptive immune response to an invading pathogen that results in IL-4 production, *de novo* synthesis of FoxP3 is inhibited in naïve CD4+ T cells undergoing differentiation.

In this model, Tregs and CD4+ T cells receiving the same signal will initiate opposing responses. As a result, the balance between initiation of Treg-mediated tolerance vs. adaptive immunity is likely determined by a number of factors including the strength of the signal, through the TCR, co-stimulation, and the type and quantity of cytokines produced.

3.6 ACKNOWLEDGEMENTS

We would like to thank Amgen, for providing the material transfer agreement for the caSTAT6 reagent, and Dr. William Paul and Dr. Jinfang Zhu for generously providing the caSTAT6 and control vector retroviral reagents.

3.7 SUPPLEMENTAL DATA

In order to characterize the influence of cytokines on TGFβ-mediated IndTreg generation from naïve CD4+ T cells, we included IL-2, IL-4, IL-6, IFNγ, or a combination of anti-IL-4 and anti-IFNγ neutralizing antibodies with TGFβ in *in vitro* cultures (Figure 18). FoxP3 expression was increased significantly by anti-IL-4 plus anti-IFNγ, enhanced slightly by IL-2, decreased by IL-4

and IFNγ, and dramatically reduced by IL-6. Thus, the cytokines that promote differentiation of Th1, Th2, and Th17 all appear to inhibit generation of IndTregs in response to TGFβ. IL-6, which is involved in the generation of Th17 cells, caused the greatest reduction in the percentage of FoxP3+ cells. Interestingly, Th17 cells are increasingly associated with immune pathology and autoimmune and inflammatory diseases, which may be explained by increased IL-6 production that enhances Th17 biasing while antagonizing IndTreg development. As a result, it has been proposed that IL-6 serves as a factor that balances Treg versus Th17 differentiation (31).

Since NatTregs are difficult to obtain in large numbers, therapeutic administration of Tregs is likely to involve IndTregs, which can be generated in significant numbers. However, Figure 18 shows that a number of cytokines inhibit IndTreg generation, and therapeutic use of IndTregs would involve their administration to patients with presumably high levels of systemic cytokines due to inflammation associated with their disease. Thus, we analyzed the effect of cytokines on *in vitro* generation IndTregs if added initially, or after 2 d of culture. On day 3 after isolation, cells were analyzed for FoxP3 expression. We found that exposure to IL-2 for the final 1 d of culture enhanced FoxP3 expression somewhat, and IL-4, IL-6, and IFNγ all had no effect (Figure 19). This contrasts with the inhibition of FoxP3 expression by these cytokines if present for all 3 d of IndTreg generation. Therefore, the cytokines tested appear to antagonize FoxP3 expression during early stages of IndTreg generation, but do not alter its expression at later stages. Consequently, we conclude that IndTregs likely will maintain FoxP3 expression and suppressive function upon transfer into patients with elevated cytokine concentrations.

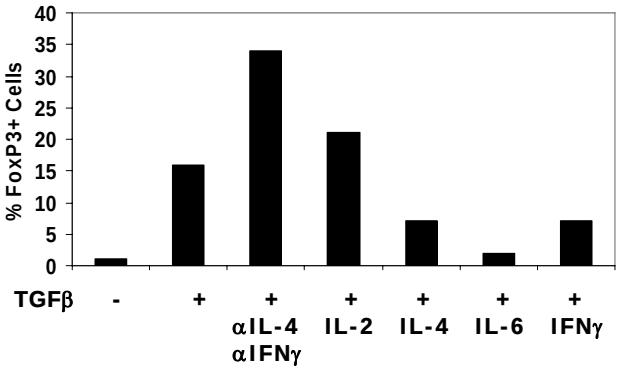

Figure 18. Cytokines modulate IndTreg induction

IndTregs were generated by activating CD4+ T cells with plate-bound anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL), in the presence or absence of TGFβ (20 ng/mL), IL-2 (50 U/mL), IL-4 (40 U/mL), IL-6 (20 ng/mL), IFNγ (200 U/mL), neutralizing anti-IL-4 antibody (2 μg/mL), and neutralizing anti-IFNγ (2 μg/mL) antibody, as indicated. All reagents were added at the start of the induction, and after 3 d of culture, cells were analyzed for FoxP3 expression by flow cytometry. Data is representative of 2 independent experiments.

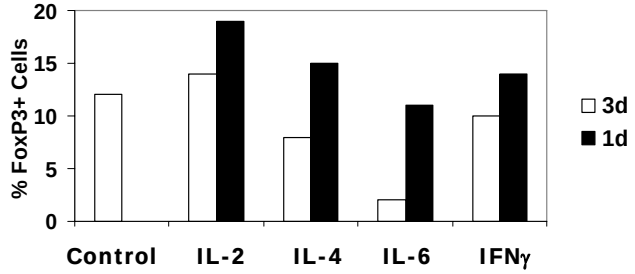

Figure 19. Inhibition of FoxP3 expression requires cytokine during early stages of induction

IndTregs were generated by activating CD4+ T cells with plate-bound anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL), and TGFβ (20 ng/mL). The indicated cytokines were present for all 3 d, or the last 1 d of generation. IL-2 (50 U/mL), IL-4 (40 U/mL), IL-6 (20 ng/mL), and IFNγ (200 U/mL). On day 3 after isolation, cells were analyzed for FoxP3 expression by flow cytometry. White bars: cytokine present during induction for all 3 d. Black bars: cytokine administered after 2 d, and present for only 1 d. Data is representative of 2 independent experiments.

4 CONCLUSIONS

The immune system is characterized by inducible rapid clonal proliferation, which is amplified by positive feedback loops involving cytokines. Positive feedback is uncommon in biology due to the tendency towards uncontrollable expansion. The vast majority of self-reactive T cells are eliminated before exiting the thymus, but some potentially pathogenic T cells are released into the periphery. For example, some harmless antigens, such as those in food, are not present in the thymus, so these cells must be regulated extrathymically. As a result, regulation of Th cells is complex and occurs on many levels, in order to reduce the likelihood of aberrant pathogenic Th cell responses. This dissertation focuses on a group of cells that are critically involved in immune regulation, Tregs; and on the modulation of their development and function by cytokines.

Initial evidence of Tregs was found in 1969, when mice thymectomized at 3 d of age, but not 1 d or 7 d, developed severe autoimmune disease (36). Others found that adult thymectomized mice would develop thyroiditis if exposed to sublethal irradiation that depleted T cells (253). However, following initial progress (254, 255), the field advanced slowly for years. After years of minimal investigation, Tregs returned to a prominent position following the discovery in 1995 of a population of CD4+CD25+ T cells that exhibited suppressive function (256). Since then, the Treg field has expanded rapidly, many new lineages of Treg have been described, and several mechanisms of suppression have been uncovered. Nevertheless, many of the finer points of how Tregs themselves are regulated remain poorly understood.

Cytokines are vital regulators of the immune system that can expand, contract, or bias a response. One of the few known regulators of Tregs is the cytokine IL-2 (49, 63). In particular, IL-2 enhances proliferation of Tregs and Th cells, and its role in the regulation of Treg function has been studied in some degree of detail (49, 50, 63, 85, 86, 257). Exogenous IL-2 promotes FoxP3 expression and enhances Treg suppressive function, but its addition to co-cultures of Tregs and Th cells abrogates suppression. Thus, IL-2 has a dominant effect on Th cells. Because IL-2 is important in the modulation of Tregs, other cytokines that are associated with disease, such as IL-6, have received increasing scrutiny as potential regulators of Tregs in recent years (150, 151, 233). IL-6 is associated with inhibition of Treg function *in vitro* and *in vivo* in the lung, but its precise effects on Treg phenotype and function had remained to be fully characterized. We showed that IL-6 promotes Treg proliferation, increases suppressive function, and marginally increases FoxP3 maintenance. However, similarly to IL-2, addition of IL-6 to Treg/Th cell co-cultures reduces suppression, although the effects of IL-6 are less robust than those of IL-2. Therefore, IL-6 also has a dominant effect on Th cells that overrides its promotion of Treg function.

Interestingly, both IL-2 and IL-6 modulate Treg function, and both are regulated by SOCS3 (155-159). SOCS3 dysregulation is associated with several human diseases; and as a result, SOCS3 is being investigated as a potential therapeutic target (174, 175, 179-182, 194). Taken together, this led us to examine expression of this important regulator in Tregs. We found that unstimulated Tregs expressed barely detectable amounts of SOCS3, whereas freshly isolated Th cells expressed it abundantly. Therefore, we hypothesize that SOCS3 protein levels remain low in Tregs to allow rapid responses to cytokines. On the other hand, Th cells express high concentrations of SOCS3 to delay their ability to respond to cytokine. In our model, in the

absence of pathogens, Tregs rapidly respond to cytokine to induce enhanced suppressive function in order to inhibit inappropriate immune responses following weak Th cell activation by harmless antigens. Swift responses to cytokines by Tregs are particularly important to control aberrant immune responses because Th cells vastly outnumber Tregs and have greater proliferative potential. Conversely, in the presence of foreign pathogens, Th cells receive significantly stronger TCR activation and increased costimulation. When these are combined with greater cytokine signals, Th cells become resistant to Treg-mediated suppression. In this case, cytokines still promote Treg proliferation, FoxP3 expression, and suppressive activity which may aid in the inhibition of an adaptive immune response once a pathogen is cleared. This proposal is further supported by our observation that SOCS3 over-expression in Tregs inhibits FoxP3 expression, suppression, and proliferation, especially in response to IL-2. The information above is summarized in Table 5.

Table 5. Comparison of Treg and Th cell cytokine responsiveness

	Tregs	Th Cells
SOCS3 Expression	Low	High
Cytokine Response Kinetics	Rapid	Delayed
Cytokine Response Functional	Significant Proliferation, Increased Suppression	Elevated Proliferation, Enhanced Resistance to Suppression
Endogenous Cytokine Production	Low	High
Proliferation Requirements	TCR +/- Costimulation + Cytokine	TCR + Costimulation +/- Cytokine

Our findings are important because SOCS3 is being pursued as a therapeutic target. An initial approach may involve systemic administration of cpSOCS3 to treat a variety of diseases, which could enter Tregs in addition to the target cells, producing unwanted effects (194). Thus, understanding the influence of SOCS3 on Tregs will enable more informed development of

cpSOCS3 therapy. Further, SOCS3 over-expression in Tregs may actually prove to be clinically useful in the treatment of certain malignancies and infectious diseases due to its inhibition of Treg function. Whereas current Treg-antagonist therapies involve anti-CD25-mediated depletion of cells (258, 259), SOCS3 expression in Tregs reduces function, but does not eliminate the Tregs. Therefore, Treg-targeted SOCS3 therapy could inhibit Treg suppression without dramatic reductions in cell number that may cause autoimmune disease. In addition, if cpSOCS3 is targeted to Tregs, the protein half-life is short enough to enable rapid reversal of its effects if toxicity is observed (194).

A significant obstacle to Treg-targeted SOCS3 therapy is the absence of established extracellular markers for Tregs. These could be used to isolate Tregs from patients, or to direct cell-specific therapeutics to the Tregs through the use of antibody-coated microcapsules that specifically recognize Tregs. Although FoxP3 is an accepted marker of Tregs, it is unsuitable for use as a marker for the isolation of live cells due to its nuclear localization. At present, CD25 is the most widely used Treg marker, despite its presence on activated Th cells. A promising new marker is CD127, which is found on Th cells that express CD25 (3, 42). This could allow for isolation of Tregs from patients by significantly reducing Th cell contamination. Unfortunately, since CD127 is expressed on Th cells but not Tregs, it obviously cannot be used in the targeting of therapeutic agents to Tregs. Suitable markers for such Treg-targeted therapy remain to be described.

After analyzing the modulation of Tregs by two molecules associated with inflammation, IL-6 and SOCS3, we wanted to examine the role of an important Th2 cytokine in Treg regulation. IL-4 is a critical initiator of Th2 differentiation that is required for development of asthma and allergies, and mutations in the IL-4Rα are associated with an increased incidence of

atopy in humans (24). This finding has led to the development of nuvance, a soluble IL-4R, to treat asthma, which is currently being studied in phase II clinical trials (220, 221). Our laboratory has demonstrated that experimental asthma can be prevented by adoptive transfer of Tregs (54, 244, 260), however, the influence of IL-4 on Tregs in this setting remained to be elucidated. We showed that Tregs were present in comparable numbers in the lungs of tolerized mice and those with experimentally induced airway inflammation. Thus, inflammation is not caused by a lack of Tregs in the lung, which suggests that secreted cytokines present in the lung may inhibit Treg function. However, adoptive transfer of Tregs from either WT or IL-4Rα-/- mice both prevented induction of airway inflammation to a similar degree. Additionally, when cells were transferred following immunization to more closely approach a "treatment" scenario, WT Treg recipients experienced less pulmonary inflammation than IL-4Rα-/- Treg recipients. Therefore, IL-4 signaling increased Treg function *in vivo*. Detailed analyses *in vitro* revealed that IL-4 enhanced maintenance of FoxP3 expression and dramatically amplified proliferation of Tregs, but did not influence suppressive activity directly. On the other hand, IL-4 inhibited suppression in Treg/Th cell co-cultures, which indicated that IL-4 has dominant effects on Th cells that make them resistant to suppression. Paradoxically, IL-4 inhibited *de novo* synthesis of FoxP3 during IndTreg induction. The use of STAT6-/- and IL-4Rα-/- cells showed that the observations described above were STAT6 dependent, and retrovirally-mediated overexpression of caSTAT6 confirmed this conclusion.

These data are therapeutically relevant for two reasons. First, IL-4 seems to enhance Treg function *in vivo*. Therefore, clinical use of IL-4 neutralizing agents such as Nuvance™ to treat asthma may have unintended effects on Tregs. Neutralization of IL-4 could decrease NatTreg numbers by limiting their proliferation, but it would also probably increase IndTreg generation.

Thus, the net effect on Tregs remains unclear. Second, adoptive transfer of Tregs after immunization reduced airway inflammation. While Treg "treatment" was not nearly as effective as transfer prior to immunization, the results were still significant. In the future, combination with other therapies, such as oral administration of antigen, may allow partial restoration of tolerance and reversal of disease in patients with asthma.

Initially, the effect of IL-4 on FoxP3 expression in NatTregs vs. IndTregs seemed contradictory. IL-4 enhanced maintenance of FoxP3 expression in NatTregs, but decreased *de novo* FoxP3 production during IndTreg generation. We hypothesize that this system of regulation evolved to enhance contraction of immune responses following pathogen clearance while preventing differentiation of responder CD4+ T cells into IndTregs (Figure 20). According to this model, any cells that already express FoxP3 prior to cytokine stimulation are assumed to be Tregs that will increase in number and potency to control an aberrant immune response. In the presence of adequate stimulation, such as during infection, Th cells become resistant to Treg-mediated suppression. Otherwise, Tregs inhibit Th cells to prevent inappropriate activation. Conversely, in cells that lack FoxP3 and recognize antigens from an infectious agent, cytokine signaling inhibits IndTreg differentiation resulting in development of potentially protective Th1, Th2, or Th17 cells. In the absence of such cytokine signaling, IndTregs may develop.

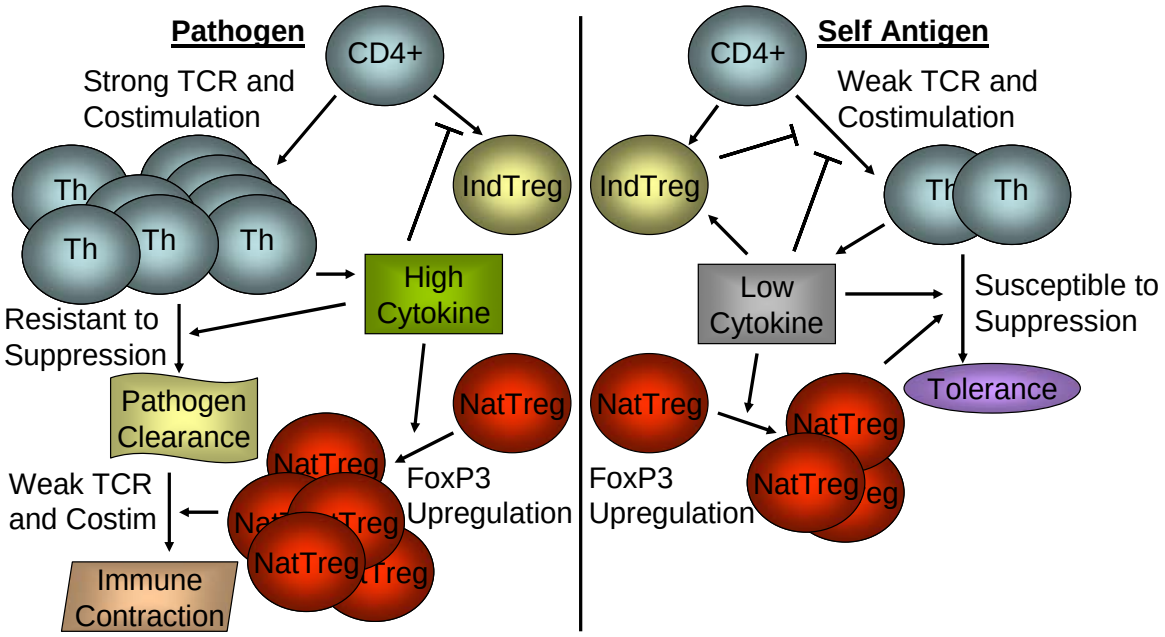

Figure 20. Model of Th cell stimulation by pathogen vs. self antigen

Following stimulation by an APC presenting antigens from a pathogen, CD4+ T cells proliferate and secrete significant amounts of cytokines. This cytokine production acts to inhibit IndTreg differentiation, and strong activation signals combined with autocrine and paracrine cytokine signaling induce CD4+ T cell resistance to Treg suppression. At the same time, high doses of cytokine induce NatTreg proliferation and FoxP3 maintenance to enable NatTregs to downregulate the response once the pathogen is cleared. On the other hand, CD4+ T cells that are activated by self antigen undergo minimal proliferation and produce low levels of cytokine. As a result, IndTreg differentiation is not inhibited, which results in IndTreg antagonism of further CD4+ T cell activation. Also, due to their sensitivity to cytokines, partly as a result of low SOCS3 levels, NatTregs expand and enhance FoxP3 expression in the presence of relatively low concentrations of cytokine. Weakly activated CD4+ T cells are highly responsive to suppression by IndTregs and NatTregs. Therefore, tolerance is initiated. Note: low cytokine levels do not induce differentiation of IndTregs and inhibit effector Th cell differentiation. Rather, low levels of several cytokines are a prerequisite of IndTreg generation, and high cytokine concentrations anhance effector Th cell differentiation and proliferation.

Taken together, our data demonstrates that cytokines and their regulators can have potent effects on Treg phenotype and function. Cytokines may be elevated in the microenvironments where Tregs could be used therapeutically, and SOCS3 is currently being studied as a therapeutic target. Therefore, further understanding the influence of cytokines and cytokine signaling inhibitors on Tregs is critical to the development of future therapies.

Table 6. Summary of findings

	IL-2	IL-4	IL-6	SOCS3	caSTAT6
NatTreg FoxP3 expression	+++	+++	+/-	-	+
NatTreg proliferation	+++	++	+/-	-	+
NatTreg suppressive function	+	+/-	+	-	+/-
IndTreg FoxP3 expression: during induction	+/-	-	---	unknown	Unknown
IndTreg FoxP3 expression: after induction	+	+/-	-	unknown	unknown
Th cell proliferation	++	++	-	-	+
Th cell resistance to suppression	+++	++	++	unknown	+

PUBLICATIONS

Pillemer, B. B., H. Xu, T. B. Oriss, Z. Qi, and A. Ray. 2007. Deficient SOCS3 expression in CD4(+)CD25(+)FoxP3(+) regulatory T cells and SOCS3-mediated suppression of Treg function. *Eur J Immunol 37:2082*.

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Chapter 2 was adapted from our paper in the *Eur J of Immunol* (252).

Pillemer, B. B., H. Xu, T. B. Oriss, Z. Qi, and A. Ray. 2007. Deficient SOCS3 expression in CD4(+)CD25(+)FoxP3(+) regulatory T cells and SOCS3-mediated suppression of Treg function. *Eur J Immunol*. 2007. 37:2082. Advance Online Publication. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

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