STAT PROTEIN REGULATION OF FOXP3 EXPRESSION AND INFLAMMATORY CYTOKINE PRODUCTION IN T HELPER CELL SUBSETS

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DEDICATION

To my wife, Kate, and my family

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ABSTRACT

John Thomas O'Malley

STAT PROTEIN REGULATION OF FOXP3 EXPRESSION AND

INFLAMMATORY CYTOKINE PRODUCTION IN T HELPER CELL SUBSETS

The differentiation of naïve CD4+ T cells into subsets of T helper cells (Th) is an essential process that impacts host defense and the pathogenesis of immune-mediated diseases. Signal transducers and activators of transcription (STAT) proteins, activated downstream of instructive cytokines, dictate and perpetuate the lineage decision of Th cells through both positive and negative effects. This is accomplished by regulating transcription factors, surface receptors and promoting epigenetic changes in gene expression through chromatin remodeling. Transforming growth factor-β1 (TGF-β1) can induce Foxp3 in developing Th cells and these Foxp3-expressing adaptive T regulatory cells (aTregs) are able to suppress inflammation in vitro and in vivo. To define the mechanism by which STAT proteins regulate Th cell pro- and anti-inflammatory phenotypes, we examined T cells deficient in *Stat3*, *Stat4*, and *Stat6* as well as T cells expressing two STAT4 isoforms after being cultured in the presence or absence of TGF-β1 and cytokines known to be instructive in Th cell development. The negative effects of STAT proteins are demonstrated by our results indicating STAT3, STAT4 and STAT6 proteins activated downstream of the instructive cytokines IL-6, IL-12 and IL-4, respectively, negatively regulate the development of TGF-β induced Foxp3 and aTreg development. STAT3, STAT4, and STAT6 utilize a

common mechanism to inhibit aTreg generation by inhibiting STAT5, a positive regulator of Foxp3 expression, from binding to the *Foxp3* gene. STAT proteins positively effecting inflammatory immunity are demonstrated by our analysis of STAT4 isoforms and their ability to regulate the production of proinflammatory cytokines downstream of IL-12. STAT4β, a STAT4 splice isoform that lacks a C-terminal domain, and STAT4α, a full-length isoform are both capable of mediating inflammatory cell development. However, STAT4β promotes greater inflammation in vivo than STAT4α independent of its ability to repress Foxp3. Instead, the inflammation correlates with STAT4 isoform-dependent expression of inflammatory cytokines. Thus, cytokine-stimulated STAT proteins orchestrate T helper cell pro- and anti-inflammatory cell phenotypes.

Mark H. Kaplan, Ph.D., Chair

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LIST OF ABBREVIATIONS

Ag	Antigen
AHR	Aryl Hydrocarbon Receptor
AP-1	Activator Protein-1
APC	Antigen Presenting Cell
aTregs	adaptive T regulatory cells
CD	Cluster of differentiation
ChIP	Chromatin Immunoprecipitation
COX-2	Cyclooxygenase-2
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DAPA	DNA Affinity Purification Assay
EAE	Experimental Autoimmune Encephalomyelitis
EAU	Experimental Autoimmune Uveitis
EBI3	Epstein-Barr virus (EBV)-induced gene 3
ER	Estrogen Receptor
ERM	Ets Related Molecule
FICZ	6-formylindolol[3,2-b]carbazole
Foxp3	Forkhead Box Protein 3
GATA-3	GATA binding protein 3
G-CSF	Granulocyte-Colony Stimulating Factor
GI	Gastrointestinal
GITR	Glucocorticoid-induced Tumor Necrosis Factor Receptor
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
gp130	Glycoprotein 130
hCD4	human CD4
Hlx	H2.0-like Homeobox-1
IBD	Inflammatory Bowel Disease
ICER	Inducible cAMP Early Repressor
ICS	Intracellular cytokine staining
ICOS	Inducible T-cell Co-stimulator

ICOSL	Inducible T-cell Co-stimulator Ligand
IFN-γ	Interferon-gamma
IFN-γR	Interferon-gamma receptor
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 receptor
IL-4R	Interleukin-4 receptor
IL-6R	Interleukin-6 receptor
IL-12R	Interleukin-12 receptor
IL-21R	Interleukin-21 receptor
IL-23R	Interleukin-23 receptor
IL-27R	Interleukin-27 receptor
iTregs	inducible T regulatory cells (another name for aTregs)
JAK	Janus Kinase
JH	Janus Kinase homology domain
LAT	Linker for Activation of T cells
MAPK	Mitogen Activated Protein Kinase
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor kappa B
NIK	Nuclear Factor kappa B Inducing Kinase
NK cell	Natural Killer cells
nTregs	Natural T regulatory cells
PAMP	Pathogen Associated Molecular Patterns
PD-1	Programmed Death 1
PD-1L	Programmed Death 1 Ligand
PLCγ	Phospholipase C gamma
PRR	Pattern Recognition Receptor
pSTAT	Phospho-STAT
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction

RA	Rheumatoid arthritis
RARα	Retinoic Acid Receptor alpha
RBC	Red blood cell
ROR	Retinoic-acid-related Orphan Receptor
RORc	Gene name for RORγt
SH2	Src homology domain 2
SMAD	Mothers against Decapentaplegic Homolog
SNPs	Single Nucleotide Polymorphisms
SOCS-3	Suppressor of cytokine signaling-3
STAT	Signal Transducer and activator of transcription
T-bet	T-box expressed in T cells
Tbx21	Gene name for T-bet
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCR	T cell receptor
TGF	Transforming growth factor
TGF-βR	Transforming growth factor beta receptor
Th	T-helper
Th1	T-helper type 1
Th17	T-helper type 17
Th2	T-helper type 2
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
Tr1	T regulatory cell type 1
Traf6	TNF-receptor associated factor
Tregs	T regulatory cells
Tyk2	Tyrosine kinase 2
WT	wild-type
α-CD28	anti-CD28
α-CD3	anti-CD3
α-IFN-γ	anti-interferon-gamma
α-IL-4	anti-IL-4

INTRODUCTION

Innate and adaptive immunity

A functioning immune system from the simplest to most complex multicellular organisms relies on being tolerant to self and quick to respond and neutralize pathogens. When a pathogen breaches protective barriers, the immune system is activated and attempts to contain the offender. To accomplish this, the immune system uses an expansive array of mechanisms and cell types to balance the equally important jobs of sentinel, orchestrator, and executioner. The immune system can be classified into two arms called innate and adaptive immunity. Innate immunity consists of immediate responders to an offending pathogen. Although considered to be the more ancient of the two arms, it is a first line of defense to acute injury and invasion.

Most infections are avoided due to epithelial barriers in the skin, gut, and lungs which prevent pathogens from colonizing tissues. If a pathogen breaches those barriers, the next line of defense are the inflammatory cells of the innate immune system which include granulocytes (neutrophils, mast cells, eosinophils, and basophils), γδ T cells, Natural Killer (NK) cells, and phagocytes (neutrophils, dendritic cells, and macrophages) which perform the orchestrator and executioner jobs of the immune system. Granulocytes like neutrophils and mast cells release a plethora of toxic materials and chemoattractant molecules upon stimulation that can recruit other immune cells to the microenvironment (1, 2). γδ T cells are a unique subset of T cells that express a T cell receptor (TCR) that is made up of one γ-chain and one δ-chain. They are found in highest abundance in the gut mucosa (3). They are considered part of the innate immune system since they do not require the processing of an antigen and its presentation in the context of a MHC Class I or II molecule and they respond within hours to a foreign invader by releasing cytotoxic granules and cytokines to neutralize the invader (4). However, their placement as a cell of the innate immune system is becoming less clear as they also seem to play a role in adaptive immunity (5).

NK cells are a type of cytotoxic lymphocyte and they are characterized by the absence of a TCR and surface immunoglobulins (Ig) but the presence of the surface markers CD16 (FcγRIII) and CD56 in humans, and NK1.1/NK1.2 in certain strains of mice. These cells are activated by cytokines, Fc receptors, other activating cell surface receptors or the absence of cell surface inhibitory receptors (6). Once activated, these cells detect cells that are missing "self" MHC molecules and lyse them. Therefore, these cells are extremely important in the anti-tumor and anti-viral response (7). In summary, the orchestration and activation of these cell types attempt to neutralize the offender before the adaptive arm of the immune system is induced.

Phagocytes perform the role of sentinel by engulfing microbes and cellular debris and presenting their findings to other immune cells. It is only fitting that these cells are called professional antigen presenting cells (APCs). Specifically, neutrophils, dendritic cells and macrophages are able to phagocytose self and non-self proteins. Once inside these cells, lysosomes and phagosomes fuse allowing the phagocytosed material to be digested into peptides. These peptides can then be loaded onto major histocompatibility complex class I and II molecules which present the peptide to CD8+ T cytotoxic and CD4+ T helper lymphocytes, respectively (8, 9).

The immune system contains a number of checks and balances to protect the organism from attacking itself while still being able to defend and destroy pathogens. While the presentation of peptides is constantly ongoing even in the absence of infection, the presentation of peptides in the context of MHC Class I and II molecules is exponentially enhanced when the APCs are activated. The mechanism of activating APCs and other cells of the innate and adaptive immune system is based on pattern recognition receptors (PRR) such as C-type lectins and toll-like receptors (TLR) that recognize repeating carbohydrates and proteins appropriately called pattern associated molecular patterns (PAMPs) that are shared by pathogens but not present normally in the host. When PAMPs bind

the PRRs of APCs, the APCs secrete cytokines and upregulate the cell surface expression of antigenic peptides within the groove of MHC molecules (10, 11).

This presentation of antigenic peptide to T cells in the context of a MHC Class I or II molecule to T cells bridges the innate and adaptive immune system. Ultimately, there is constant oversight by the adaptive immune system sampling antigens and responding appropriately if the peptide presented activates their respective TCR (12). Importantly, the innate immune system has limited memory or specificity such that a repeat infection by the same pathogen will not result in either a quicker or more robust immune response by the cells of the innate immune system.

The second arm of the immune system is called adaptive immunity and is orchestrated by a type of leukocyte called a lymphocyte. This type of immunity is complex in design and allows the immune system to respond to specific antigens. This arm has the ability to establish memory so that a repeat pathogen infecting the organism will stimulate an exponentially more robust and quicker response than the original pathogen infection (13, 14). The adaptive immune system consists of B and T cells which possess receptors that recognize either unprocessed antigen in the case of B cells or processed antigen in the context of MHC Class I and II molecules in the case of T cells. This system is highly adaptable because of somatic hypermutation and V(D)J recombination of antigen gene receptor segments (15). This allows for an extremely diversified set of antigen receptors that are uniquely expressed on each individual lymphocyte. Importantly, the antigen receptors of B and T cells are heritable allowing for memory B and T cells, the key to long-lived specific immunity.

T cell development

B and T cells are derived from the same pluripotent hematopoietic stem cells. B cells produce antibodies and are responsible for the humoral immune response while T cells produce cytokines and cytotoxic granules that aid in both humoral

and cell-mediated immunity. While B and T cells are both produced from stem cells in the bone marrow, T cells are unique in that they travel to and develop in the thymus where they are culled if they are too reactive to self-antigens and/or cannot recognize MHC molecules (16). The T lymphocytes that survive this process emerge from the thymus and enter secondary lymphoid tissues looking for their specific antigen.

The peripheral lymphoid organs contain a mixture of T cells that can be categorized in at least three stages of differentiation. The first stage is naïve cells that are within the lymphatic system but have not yet encountered their cognate antigen. The second stage is effector cells that have been activated by their cognate antigen, and are in the process of eliminating the pathogen. The last stage of differentiation is memory cells which are long-lived pathogen specific cells.

T cells are divided into two major subsets based on surface expression of either CD4 or CD8, which they obtain by specific signals during development in the thymus(17). CD8+ T cells are referred to as cytotoxic lymphocytes and can induce the death of cells that are infected with viruses or are damaged or dysfunctional. They recognize antigenic peptide in the context of a MHC Class I molecule. The CD4+ T cells are termed T helper (Th) cells and they play a role in establishing and maximizing the capabilities of the adaptive immune response. Th cells recognize their cognate antigenic peptide in the context of a MHC Class II molecule. They are the orchestrators of the immune response such that they direct and mangage other cells to perform their jobs of phagocytosis and cytotoxicity. Both CD4+ and CD8+ T cells require costimulation in addition to their recognition of cognate antigen to become activated. If there is no costimulation, then the T cells become anergic and either undergo apoptosis or become unresponsive to subsequent stimulation (18). This is another example of the intricate balance needed to allow for immune system activation to “non-self” but tolerance to “self”.

CD4+ Th cells manage immune responses by communicating with other cells either through cell-cell contact or their production of cytokines. Cytokines are proteins and glycoproteins that, like hormones and neurotransmitters, act as intercellular mediators that differ from classical hormones because they are produced by cells or tissues rather than specialized glands. They can have local effects that are autocrine (activates the secretory cell itself), paracrine (activates nearby cells), or they can act distally in an endocrine manner. Cytokines are critical to not only the function of T helper cells but all of the cells involved in innate and adaptive immunity (19). They dictate the type of immune response by binding to other cells, initiating a signaling cascade, and producing an end effect such as cytokine secretion and upregulation and downregulation of surface molecules of the stimulated cell. A cytokine is, in effect, a unique molecular signature that can affect different cell types in different ways. For example, interferon-γ (IFN-γ) produced by a T helper cell can mediate MHC Class II upregulation and IL-12 secretion in macrophages while in B cells it can directly promote antibody isotype switching to IgG2a (20).

Certain cytokines or cytokine combinations promote pro-inflammatory effects while others induce anti-inflammatory responses. With a normally functioning immune system, expression of cytokines and cytokine receptors are tightly regulated in order to promote optimal immune responses. Dysregulation of the balance between pro- and anti-inflammatory cytokines can result in inflammatory diseases or chronic infections, respectively.

Coordination of an immune response by Th cells

Th cells lie at the nexus of a coordinated immune response. They enhance and sculpt immune responses to most efficiently eradicate pathogens and are instrumental in downregulating the immune response to minimize damage to host tissues and to reestablish normal immune homeostasis. In order to orchestrate an immune response, the Th cell must be activated. In the process of activation, there are a series of checkpoints to ensure the naïve Th cell is being

appropriately activated by a foreign antigen. When a Th cell recognizes the antigen presented by an APC, the TCR-CD3 complex binds strongly to the peptide-MHC complex present on the surface of professional APCs. The surface molecule CD4 also binds to the β2 chain of MHC Class II, strengthening the binding and prolonging the chance for intercellular communication between the T cell and the APC (21). The second signal a naïve T cell must receive can conceptually be understood as a verification signal. This is a protective measure to ensure the Th cell is responding to a foreign antigen. In the absence of this second signal, the T cell becomes anergic and unresponsive to restimulation. The second signal involves an interaction between CD28 on the Th cell and the proteins CD80 or CD86 on the professional APC. Importantly, CD80 and CD86 are only present on the surface of a professional APC if that APC has received an activating signal through its receptors specific for PAMPs (11).

Once the naïve Th cell has both pathways activated, the Th cell is induced to proliferate and further stimulation of the proliferating T cells only require TCR-CD3 activation and not the second signal. The T cell proliferates, in part, due to its secretion of IL-2 and upregulation of the high affinity subunit of the IL-2R, CD25. Lastly, the maturation of the T cell occurs resulting in one of three terminal differentiation states; an effector Th cell, a memory Th cell, or a regulatory Th cell. In brief, an effector Th cell secrete cytokines important in the proinflammatory immune response, a memory Th cell retains antigen affinity of the originally activated T cell and can become an effector cell during a second immune response to the same antigen, and regulatory T cells (Tregs) are important in maintaining tolerance in the absence of a foreign invader and downregulating immune responses post-eradication of a pathogen. Effector Th and Treg cells control immune responses by entering into infected tissues and releasing chemoattractant cytokines (chemokines) to recruit other cells of the innate and adaptive immune response. The types of cells that are recruited to the tissues are partly dependent on the specificity of the cytokine secretion profile of the terminally differentiated Th cell. This cytokine profile has enabled Th cells

to be profiled into subsets based on their phenotype and the cytokines that they secrete.

Th cell subsets and the importance of STAT proteins in their development

The differentiation of naïve CD4+ T cells into subsets of Th cells is an essential process that impacts host defense and the pathogenesis of immune-mediated diseases. The innate immune system and the cytokines present in the microenvironment of a recently activated T helper cell control the differentiation to an effector T helper cell. Other factors that influence the differentiation of T helper cells, including antigen dose, route of infection, type of APC/costimulation, and genetic background contribute to effector Th cell cytokine production and responsiveness (22-24). Ultimately, the differentiation of the T helper cell determines the cytokine output of the clonally expanded cells which, in turn, directs the cell-mediated or humoral immune response in an effort to eliminate the infectious agent.

Th cells can differentiate into either proinflammatory or anti-inflammatory subsets dictated by the cytokine milieu. These cells are categorized into Th subsets according to their cytokine production, cell surface receptors, and transcription factor expression. There are three recognized subsets of proinflammatory T cells termed Th1, Th2, and Th17 cells. Anti-inflammatory Th cells called T regulatory cells (Tregs) are further subdivided into three subsets called T regulatory type 1 cells (Tr1), adaptive T regulatory cells (aTregs or iTregs) and natural T regulatory cells (nTregs). Signal transducer and activator of transcription (STAT) protein activation plays critical roles in the induction and effector function of these subsets (Table 1).

STAT activated	Effect on T helper cell development						
	Th1	Th2	Th17	nTreg		aTreg Foxp3 expression	Tr1
				Foxp3 expression	Suppressive activity		
STAT1	IFN-γ (+) IL-27 (+)	IFN-γ (-) IL-27 (-)	IFN-γ (-) IL-27 (-)	Not determined	Not determined	IFN-γ (-)	IL-27 (+) IFN-γ (-)
STAT3	IL-6 (-) IL-10 (-)	IL-6 (+) IL-10 (+)	IL-6 (+) IL-21 (+) IL-23 (+)	neutral	IL-6 (-) IL-10 (+)	IL-6 (-) IL-21 (-) IL-23 (-) IL-27 (-)	IL-10 (+)
STAT4	IL-12 (+)	IL-12 (-)	IL-12 (-) IL-23 (+)	neutral	neutral	IL-12 (-)	IL-12 (-)
STAT5a, STAT5b	IL-2 (-)	IL-2 (+)	IL-2 (-)	IL-2 (+)	IL-2 (-)	IL-2 (+)	IL-2 (+)
STAT6	IL-4 (-)	IL-4 (+)	IL-4 (-)	IL-4 (+)	IL-4 (-) murine IL-4 (+) human	IL-4 (-)	IL-4 (-)

Table 1. Known roles of STAT proteins, downstream of cytokines, in mediating T helper cell subset differentiation. Cytokines represented on this table indicate the STAT-activating cytokine that either positively (+) or negatively (-) regulates the indicated Th cell subset.

Cytokines are broadly grouped as Type I or Type II. Type I cytokines comprise a large group of cytokines, including most of the interleukins, hematopoietic molecules like granulocyte colony stimulating factor, the IL-6 family of proteins whose receptors all contain gp130, and other important proteins including leptin, growth hormone, and prolactin. The Type II cytokines include interferons (IFN-α/β/λ/γ) and IL-10 family members (25). Within this large family, a few of these cytokines have been experimentally determined to affect Th cell subset differentiation. This select group of cytokines is referred to as being instructive because they dictate and perpetuate the lineage decision of T helper cells. This is done by upregulating transcription factors, surface receptors and promoting epigenetic changes in gene expression through chromatin remodeling so that the clonally expanding T cells have the same armament of cytokines in an effort to optimally defend the host. The JAK-STAT pathway, activated downstream of the instructive cytokines, is essential for the differentiation of T helper cell subsets (25).

Overview of the JAK-STAT pathway

Activation of the JAK-STAT pathway promotes various biological responses including cell differentiation, growth, and survival. There are four mammalian Janus kinases (JAKs) named JAK1, JAK2, JAK3, and TYK2 (26-30). JAKs are a family of receptor associated protein tyrosine kinases that share 7 regions of extensive homology to each other, termed JAK homology (JH) domains. Among the substrates for JAKs are STATs. Seven mammalian STAT proteins have been characterized: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (31-40). In the absence of cytokine stimulation, STAT proteins exist as latent cytoplasmic transcription factors. Following cytokine-receptor stimulation, the JAKs are phosphorylated and, in turn, phosphorylate tyrosine residues on the associated cytoplasmic tail of the cytokine receptor chain. Subsequently, they are recruited to the phosphorylated receptor chains by virtue of their src-homology-2 (SH2) domain. Upon docking to the phosphorylated receptor, the STAT protein is phosphorylated by the JAK proteins. Through reciprocal

interactions of the phospho-tyrosine and SH2 domain, the STAT proteins homo- or heterodimerize and translocate to the nucleus where they bind DNA and promote gene transcription (25). All seven members of the STAT protein family are present within the cytoplasm of naïve Th cells. They exhibit specific activities upon cytokine receptor stimulation and, depending on the cytokine milieu, are synergistic and/or antagonistic to other STAT proteins in the induction and maintenance of T helper cell subsets.

A brief overview of the Th cell subsets and the in vivo consequences of STAT deletion and dysfunction

Th1 cells

IL-12 and IFN-γ induce naïve Th cells to become Th1 cells that secrete IFN-γ and lymphotoxin-α (22). Th1 cells stimulate macrophages to kill phagocytosed pathogens including mycobacteria and toxoplasma and recruit other leukocytes to the site. Th1 cells are essential for the cell-mediated immune response and for resistance to most viruses, bacteria, intracellular protozoa and fungal pathogens (41-45). Th1 cells can also mediate organ-specific autoimmunity and, when dysregulated, are pathogenic in autoimmune diseases such as rheumatoid arthritis (RA), type I diabetes, Inflammatory Bowel Diseases (IBD) and multiple sclerosis (MS) (46-51). STAT4 and STAT1 become activated downstream of IL-12 and IFN-γ, respectively, and are essential factors in the differentiation of Th1 cells.

Stat1-deficient mice exhibit the importance of STAT1 and Th1 cells in cell-mediated immunity and immunosurveillance. These mice are highly susceptible to both microbial and viral infections and have an increased susceptibility to tumors (52-54). In addition, mutations in the *STAT1* allele can make patients susceptible to viral and mycobacterial infections due to impaired IFN responses (41-43).

The importance of STAT4 in Th1 development and immune responses was defined by the generation of *Stat4-/-* mice (55-57). Control of Th1 differentiation is maintained both by the tissue-restricted expression of STAT4 and by the limited activation of STAT4 by only certain cytokines (58). STAT4 is expressed in a variety of cells including monocytes, dendritic cells, natural killer cells, macrophages, connective tissue type mast cells (CTMC), B cells, T cells, and spermatogonia, with tissue restricted expression to testes, heart, brain, thymus, and spleen (36, 58, 59). In mouse models of autoinflammatory diseases, *Stat4-/-* mice exhibit milder disease and decreased inflammation compared to WT mice in experimental autoimmune encephalomyelitis (EAE), arthritis, colitis, myocarditis, and diabetes (60-64). In humans, the *STAT4* locus has been associated with rheumatoid arthritis, asthma, sarcoidosis, and systemic lupus erythematosus (65-70). Interestingly, although IFN-γ is an important STAT4-induced immune mediator, STAT4 must regulate other genes that are critical for the development of inflammatory disease since IFN-γ-deficient and *Stat1*-deficient mice are not protected from EAE, myocarditis, or colitis (62, 63, 71-73). Thus, STAT4 plays a multifunctional role in Th1 cell development and disease pathogenesis.

Th2 cells

IL-4 is a critically important cytokine for Th2 differentiation. The major cytokines produced by Th2 cells include IL-4, IL-13, and IL-5 (22). In addition to its role in promoting Th2 development, IL-4 acts through STAT6 as an effector cytokine to stimulate immunoglobulin E (IgE) class switching in B cells. IgE production plays a major pathogenic role in allergy and atopy (74-76). IL-13 also promotes synthesis of IgE in humans and can recruit and activate basophils (77, 78). IL-5 activates and attracts eosinophils in both humans and mice (79, 80). Th2 cells, basophils and eosinophils are essential for controlling extracellular infections such as helminthic parasites and, when dysregulated, can initiate and perpetuate allergy and atopy (81-83). IL-4 and STAT6 are critical factors in establishing the humoral immune response.

The phenotype of *Stat6*-deficient mice underscores the involvement of STAT6 in allergic inflammation and immunity to parasites. *Stat6*-deficient mice have defects in IL-4 and IL-13 mediated functions such as the differentiation of Th2 cells and IgE-dependent gastrointestinal parasite expulsion (74, 81, 82, 84, 85). In the context of allergy, *Stat6*-deficient mice fail to develop bronchial eosinophilic inflammation and airway hyperreactivity in animal models of allergic asthma due to effects in both T cells and resident airway cells (86-88). Moreover, mice that have a constitutively activate STAT6 expressed in T cells are predisposed to spontaneous allergic airway inflammation and atopic dermatitis (89, 90).

In humans, STAT6 was found to be upregulated in patients with asthma (91). In addition, GT allele repeat polymorphisms in the *STAT6* gene have been associated with a significantly increased risk to atopic asthma (92). Indeed, the variation in the length of the GT repeat sequence influences the regulation of the *STAT6* promoter activity (93, 94). Increased STAT6 activation due to aberrant IL-4 signaling is also present in patients with rheumatoid arthritis (95). Thus, STAT6 plays a protective role in mediating the humoral immune response but, when dysregulated, contributes to the development of disease and atopy.

Th17 cells

Th17 cells are the most recent subset of proinflammatory T helper cells to be identified and are defined by their ability to secrete IL-17, IL-21, and IL-22. Murine T cells are induced to differentiate in the presence of TGF-β1 and IL-6 and the cytokines IL-1β and IL-23 are essential for the maintenance of the IL-17 secreting phenotype (96-101). In human naive CD4+ T cell cultures, IL-1β induces Th17 polarization which can be enhanced by IL-6 and IL-23. Interestingly, human Th17 differentiation was suppressed by TGF-β, which is in contrast to the requirement of TGF-β in murine Th17 development (102). In vivo, Th17 cells are found at the interface between external and internal environments such as the skin and the lining of the GI tract. Mediators of acute inflammation, Th17 cells secrete defensins and recruit neutrophils. IL-17 production by T cells

is protective to the host in response to bacterial and fungal infections (103). However, when Th17 cells are dysregulated, they are important pathogenic effector cells in a variety of autoimmune diseases including multiple sclerosis, rheumatoid arthritis and psoriasis (104). The development and function of Th17 cells require the activation of STAT3 downstream of IL-6, IL-21 and IL-23 signaling (98, 105-108).

While *Stat3*-deficient mice die during early embryogenesis (109), the conditional deletion of STAT3 from specific cell types has allowed investigators to decipher the role STAT3 plays in immune function. Mice with STAT3 deleted from bone marrow cells died 4-6 weeks after birth due to an overwhelming inflammation resembling Crohn's disease (110). This phenotype suggests that STAT3 is important in both the myeloid and lymphoid lineages to maintain immune homeostasis. When STAT3 is specifically deleted from lymphocytes, the cells exhibit impaired proliferation to IL-2 and IL-6 signaling (111, 112). Furthermore, STAT3 is essential for Th17 development since *Stat3*-deficient lymphocytes are unable to upregulate the Th17 transcription factors Retinoic acid receptor-related orphan receptors γ (RORγt) and α (RORα) and these mice are protected from Th17-mediated autoinflammatory diseases like EAE and experimental autoimmune uveitis (EAU) (98, 113-116).

In humans, mutations in STAT3 have been associated with hyper-IgE syndrome and recurrent infections with *Staphylococcus sp.* and candidiasis. The T cells of these patients are also unable to differentiate into Th17 cells (117-119). Dysregulated activation of STAT3 has been described in human psoriatic lesions and in patients with Crohn's disease (49, 120).

T Regulatory Cells

The function of proinflammatory Th cells discussed above is regulated by another category of Th cell subsets that are essential in maintaining tolerance and downmodulating immune responses. These anti-inflammatory cells are

subdivided into three groups called T regulatory type 1 (Tr1), natural T regulatory cells (nTreg) and adaptive or inducible T regulatory cells (aTreg or iTreg). Tr1 cells are characterized by surface expression of CD25 and IL-15Rα and are induced to differentiate in the presence of IL-10, IFN-α and immunosuppressive drugs like dexamethasone and secrete IL-10 upon activation. IL-15 and IL-2 are also positive regulators of Tr1 cells and help to maintain their IL-10 secreting phenotype (121, 122). These cells populate the intestines and are thought to play roles in oral tolerance, as mice deficient in IL-10 develop spontaneous inflammation of the large intestine (123).

nTregs comprise 5-10% of αβ+CD4+ T cells in the periphery. They are characterized by the expression of the high affinity IL-2Rα (CD25) and the expression of the transcription factor forkhead box protein 3 (Foxp3) upon exit from the thymus. The presence of IL-2 and the activation of STAT5 are required for these T cells to maintain Foxp3 expression in peripheral lymphoid organs (124, 125). Their TCRs are often specific for self-proteins and these cells are theorized to have escaped negative selection in the thymus, although this dogma has recently been brought into question (126). If activated, they secrete the immunosuppressive cytokines IL-10 and TGF-β. They inhibit the proinflammatory Th cell subsets and the action of CD8+ cytotoxic T lymphocytes. The autoinflammatory sequalae of mice and humans that have mutations in the *Foxp3* gene highly suggest that nTregs are essential to prevent autoimmunity (127).

Murine naïve T cells are induced to become Foxp3+ aTreg cells after activation in the presence of TGF-β and IL-2. These cells share the phenotypic and functional characteristics of nTregs in that they have high surface expression of CD25 and are functionally suppressive. aTreg cells are present in the intestine, secrete TGF-β when activated and are sufficient to prevent inflammation associated with inflammatory diseases (128-130).

In contrast to murine Th cells, the requirement for TGF-β in human CD4+ T cells has been controversial since TCR activation of human CD4+25- cells is sufficient to induce transient induction of Foxp3. However, this transient induction does not result in Th cells that are functionally suppressive (128, 131-134). Upon repeated TCR stimulations in the presence of TGF-β, human aTreg cells develop and are characterized by maintained expression of Foxp3 (134).

The deletion of STAT5 in lymphocytes underscores its importance in Foxp3 induction and maintenance of Treg function. STAT5 activation downstream of IL-2 is required for induction and maintenance of Foxp3 expression and Treg function. STAT5a and STAT5b are expressed by tandem genes, and have both distinct and overlapping functions (135). Deficiency of STAT5a or STAT5b individually does not have severe consequences on T or B cell development (136-139). However, analyzing mice in which the entire *Stat5a/b* locus is deleted reveals that STAT5 deficiency results in a Severe Combined Immunodeficiency phenotype with defective B and T cell development and almost complete penetrance of perinatal lethality (140). When STAT5 was deleted in T cells only, the mice exhibited reduced numbers of peripheral B and T cells and significantly reduced numbers of nTregs (141). In human patients, mutations in *STAT5B* have been described and these patients are characterized by short stature (due to growth hormone insensitivity) and recurrent pulmonary infections with *Pneumocystis carinii* (142). Therefore, STAT5 is critical for immune homeostasis in vivo.

The STAT protein network in effector Th cell development

The transcription factors required downstream of instructive cytokines engineer the induction and maintenance of the T helper cell subset phenotype. The stability and commitment of T helper cells is controlled by three factors; the cytokines present in the microenvironment, the acquisition and/or downregulation of cytokine receptors on the surface of the T cell, and heritable chromatin remodeling (Fig. 1). STAT proteins, their contribution to the stability and

commitment of T helper cell subsets, and their role in the counter-regulation of other STAT-activated pathways are discussed below.

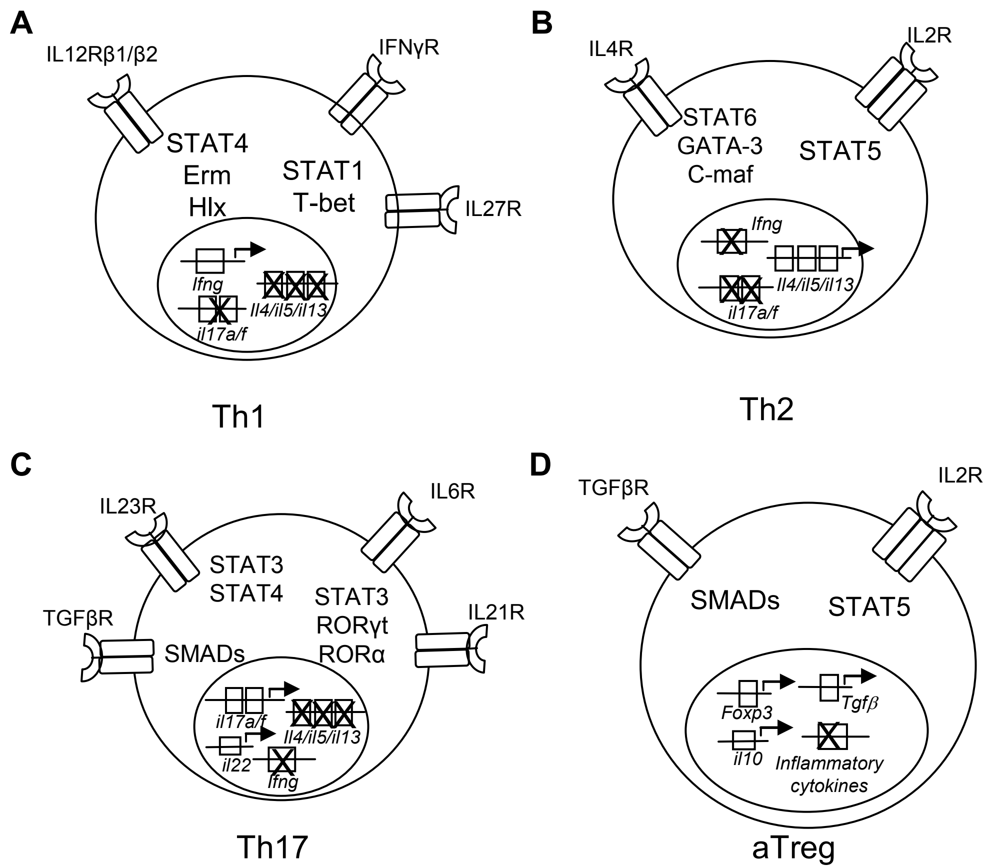

Figure 1. **Schematic summary of surface receptors, transcription factor, and chromatin remodeling in the (A) Th1, (B) Th2, (C) Th17 and (D) aTreg T helper cell subsets.** Surface receptors shown are the known receptors important for each subset. Transcription factors near the surface receptors represent transcription factors known to be activated and/or upregulated by ligation of the receptor by the cytokine. The X in the nucleus of cells represent repressive chromatin modifications at those loci while the arrows represent permissive transcription of those genes in the respective Th subset.

STAT1

STAT1 is a positive regulator of Th1 development when activated by the instructive cytokines IFN-γ and IL-27. IFN-γ binds a heterodimeric receptor composed of two chains, IFN-γR1 and IFN-γR2, and promotes the formation of STAT1 homodimers. IL-27 is a heterodimer composed of p28 and EBI3 that binds to the IL-27R chains WSX1 and gp130 to induce the activation of STAT1 (143). STAT1 activation by these cytokines induces expression of the Th1 transcription factor T-box expressed in T cells (T-bet). T-bet is important for Th1 differentiation because it can directly bind and transactivate the *Ifng* gene (144, 145) while also negatively regulating GATA-3 and inhibiting Th2 differentiation (146, 147). T-bet cross-regulates Th2 lineage commitment by physically interacting with GATA-3 and preventing its binding to target DNA (148). Moreover, T-bet expression is sufficient to induce IL-12Rβ2 expression and to positively regulate the expression of other Th1 specific genes like Hlx (149, 150). Hlx and T-bet act in a functional complex to synergistically increase IFN-γ secretion (150, 151). Interestingly, STAT1 downstream of IL-27 and STAT4 downstream of IL-12 can act synergistically in the expansion of naïve CD4+ T cells and the production of IFN-γ, suggesting the cooperative nature of STAT1 and STAT4 activation in the Th1 developmental program (143). IFN-γ and IL-12 further reinforce the development of Th1 cells by remodeling the Th2 genetic loci into heterochromatin (152, 153).

In addition to its roles in Th1 and Th2 development, STAT1 also inhibits the differentiation of other Th subsets. STAT1-induced T-bet inhibits the upregulation of Foxp3 in naïve T cells cultured in the presence of TGF-β (154). STAT1, downstream of IL-27, can also inhibit Th17 differentiation, possibly due to the upregulation of suppressor of cytokine signaling-3 (SOCS-3), which inhibits STAT3 activation, or the upregulation of T-bet, which has been shown to inhibit RORγt and the Th17 lineage (155-159). In vivo, STAT1, activated by IFN-γ and IL-27, is sufficient to inhibit the Th17-mediated disease experimental autoimmune

uveoretinitis (EAU) (160). Thus, STAT1 reinforces the Th1 phenotype by inhibiting the development of other Th subsets.

STAT3

STAT3 is activated downstream of IL-6, IL-21, IL-23, and IL-27. IL-6 and IL-21 are important instructive cytokines for the differentiation of the Th17 subset while the role of IL-23 is independent of differentiation but is essential for Th17 cells in vivo (161). IL-6 signals through the hexameric IL-6R which is composed of the common receptor chain gp130 and a unique subunit IL-6Rα. Binding of IL-6 to its receptor activates STAT1 and STAT3 (162). IL-21 binds to the unique subunit IL-21R and the common γ chain (IL-2Rγ) and engagement of the receptor activates STAT1 and STAT3 (163). However, while STAT1 is also activated by IL-6, IL-21 and IL-27, only STAT3 plays an essential, nonredundant role in the commitment of Th17 cells. STAT3 activation positively regulates the master transcription factors of the Th17 lineage RORγt and RORα (98, 108, 115, 116). In addition, STAT3 binds and induces transcription of the *Il17* and *Il21* genes (164, 165). STAT3 can stabilize the Th17 phenotype by upregulating the IL-23R thereby making the Th17 cells responsive to IL-23 (105, 108). IL-23 activates STAT3 and contributes to the maintenance of Th17 cells (98).

STAT3 regulates lineage decisions of other Th subsets. STAT3 is necessary and sufficient to repress Foxp3 transcription and aTreg generation in vitro (108). In addition, the roles of IL-6 and STAT3 activation in inhibiting Treg generation and function have been reiterated in vivo (113, 166). IL-23 has also been proposed to inhibit Treg generation in vivo but the STAT protein responsible is unknown (167). While STAT3 does not play a major role in Th1 development (168), STAT3 does positively regulate Th2 differentiation. STAT3, downstream of IL-6, can bind the c-Maf gene and transactivate the IL-4 promoter (169).

STAT3 also has functions upstream and downstream of IL-10 in several Th subsets. Not only is STAT3 the major STAT activated by IL-10, but STAT3 can

also mediate T cell production of IL-10 downstream of IL-6 and IL-27 in Th1, Th2 and Th17 cells (170). The role of STAT3 is presumed to be important in Tr1 cells since they are induced to differentiate in the presence of IL-10 and secrete IL-10 upon activation. In summary, STAT3 can mediate both pro- and anti-inflammatory immunity depending on the cytokine milieu.

STAT4

STAT4 is activated by the cytokines IL-12 and IL-23 in both humans and mice. IL-12 and STAT4 are required for the differentiation of Th1 cells while IL-23 and STAT4 play more important roles in the induction of IL-17 secretion from Th17 cells (55, 56, 98, 171). IL-12 is a heterodimeric cytokine composed of two subunits, p35 and p40, that bind the IL-12Rβ1 and β2 chains and activate Jak2 and Tyk2. STAT4 is subsequently activated and translocates to the nucleus where it induces permissive transcription of STAT4-dependent genes by recruiting chromatin remodeling complexes (172). Chromatin remodeling complexes modify histones with various modifications such as acetylation and methylation the can be either inhibitory or permissive to transcription. Activated STAT4 can initiate transcription by binding to STAT consensus sites in the genome, such as the *Ifng* promoter (173) and other STAT4 dependent Th1 genes including *Il18r1* (174), *Il12rb2* (175), *Irf1* (176), *Etv5* (177), *Nos2* (59) and *Ccr5* (178-180). STAT4 can induce as well as maintain heritable chromatin changes despite being transiently activated. As one example, STAT4 activation induces transient hyperacetylation of histones within the *Il18r1* locus thereby allowing the expression of IL-18Rα (181). In terminally differentiated Th1 cells, TCR ligation or IL-12+IL-18 stimulation produces IFN-γ (182). It is clear that IL-12 and IL-18-induced IFN-γ production depends strongly on STAT4, whereas TCR signaling can induce IFN-γ production, although at a reduced level, in *Stat4-/-* Th1 cells (149). By inducing IFN-γ, STAT4 also indirectly upregulates T-bet. Other STAT4 dependent transcription factors expressed in Th1 cells include ERM and Hlx. Hlx physically interacts with T-bet to promote heritable Th1 induction (150) while the exact role ERM plays in Th1 differentiation is still

unclear (177). Therefore, STAT4 initiates and maintains the development of Th1 cells downstream of the instructive cytokine IL-12.

IL-23 is a heterodimer, composed of the IL-12p40 subunit and a unique IL-23p19 subunit. IL-23 binds to the IL-23R composed of the IL-12Rβ1 chain and a unique IL-23R. Receptor expression is induced by IL-23 itself, as well as IL-6 and IL-21 (105, 106, 108, 183). STAT4, downstream of IL-23, promotes IL-17 production and is required for cytokine-induced IL-17 production (98).

STAT4 can also negatively regulate Th2 and aTreg differentiation. For example, *Stat4*-deficient T cells are more prone to develop into IL-4 secreting Th2 cells suggesting that STAT4 is a negative regulator of Th2 development (55). Indeed, T-bet and STAT4 stabilize the Th1 lineage by coordinately blocking GATA-3 function (146). In addition, IL-12 and STAT4 can inhibit the development of aTreg cells (154). Therefore, STAT4 is critically important for the development of fully functional Th1 cells and is also important for acute induction of IFN-γ and IL-17 in immune cells stimulated with IL-18 and IL-12 or IL-23, respectively.

STAT4 has been studied in detail with regard to the functions of various domains within the protein. Indeed, the functions of the N- and C-terminal domain have only recently been elucidated. Crystallographic studies have provided insights into the functional activities of certain STAT protein domains (184-187). For example, the crystal structure of the N-terminal domain of STAT4, comprising the first 124 amino acids, shows that it is composed of eight α-helices that assemble into a hydrophobic hook-like structure (185, 187). This hook-like structure interacts with the N-terminal domain of another STAT4 monomer to form higher order structures.

The canonical STAT recognition site on DNA is the palindromic sequence $TTCN_{3\text{-}4}GAA$ (188-190). Variations of the STAT consensus sequence result in different affinities of STAT4 binding. STAT4 can bind tandem low affinity STAT

consensus sequences by forming a higher molecular weight complex consistent with a tetramer formed by homotypic interactions of the N-terminal domain. However, the N-terminal domain is not essential for binding to a single high affinity STAT DNA-binding site (173). Thus, the N-terminal domain stabilizes the interaction of two STAT4 dimers binding tandem low affinity nonconsensus STAT binding sequences. This tetramerization augments transcription of the IFN-γ gene and perhaps other Th1 genes as well. It was subsequently discovered that the N-terminal domain of STAT4 is also required for STAT4 tyrosine phosphorylation and the formation of nonphosphorylated dimers before cytokine activation (191-193).

The function of the C-terminal transactivation domain of STAT4 has been studied by utilizing mice that express a STAT4 splice isoform that lacks the transactivation domain and a mutant STAT4 protein construct where a serine residue has been mutated to an alanine in the transactivation domain. These studies have given insight into the functional role of the transactivation domain. Alternatively spliced Stat4 transcripts, a full-length STAT4α and a STAT4β that lacks the C-terminal 44 amino acids of the transactivation domain were recently described (194). Primary T cells expressing either STAT4α or STAT4β were able to promote Th1 development in vitro. However, there were some differences in isoform function. IL-12 stimulation of STAT4α-expressing Th1 cells induced more IFN-γ production than Th1 cells expressing STAT4β, while STAT4β-expressing T cells proliferated more vigorously in response to IL-12 stimulation (194). Microarray analysis of Th1 cells expressing either isoform after IL-12 stimulation identified hundreds of target genes, most of which were mediated similarly by both isoforms. This suggests that the STAT4 transactivation domain is not always required for transcriptional activation. However, the microarray also showed that each isoform targeted a unique set of genes. This suggests that the transactivation domain confers some specificity to which genes are targeted upon IL-12 stimulation.

Present within the C-terminal domain of STAT4 is a serine residue at position 721 that, when phosphorylated by p38 MAPK, confers full transcriptional activity of STAT4 target genes (195-197). STAT4 serine phosphorylation, although necessary for IL-12 induced IFN-γ production, is dispensable for IL-12 mediated cell proliferation (198). This serine residue is not present in the STAT4β isoform and whether the STAT4β isoform behaves similarly to the S721A STAT4 protein is currently unknown. In addition, it is unclear whether the lack of a transactivation domain confers a differential ability of the STAT4β isoform to mediate in vivo inflammation.

STAT5

STAT5 is most widely appreciated as a transcription factor required for Foxp3 expression and maintenance in the aTreg and nTreg subsets. Indeed, STAT5, which is activated downstream of IL-2, and Smad proteins, activated downstream of TGF-β, are essential for the induction of Foxp3 in aTreg cells and the maintenance of Foxp3 in aTreg and nTreg cells. IL-2 binds to the IL-2R which is comprised of IL-2Rγ and β but higher affinity binding of IL-2 occurs when the IL-2Rα (CD25) is induced by T cell activation. Foxp3+ Treg cells help to maintain peripheral tolerance by a variety of mechanisms including inhibiting proinflammatory cytokine production. Foxp3 can inhibit IL-2 transcription by binding to the *Il2* gene (199). Foxp3 can also inhibit IFN-γ and IL-4 production by physically associating with and inhibiting the cytokine gene transactivators NFκB and NFAT (199, 200). In addition, Foxp3 upregulates a variety of cell surface molecules such as PD-1 (201), GITR (202), and CTLA-4 (202). These molecules transduce inhibitory signals following ligand engagement during cellular interaction with neighboring T helper cells. There is also increasing evidence that the constitutive expression of CD25 in nTreg cells serves as an “IL-2 sink” by soaking up excess IL-2 in the microenvironment. The lack of available IL-2 prevents other effector T cells from proliferating in response to this growth factor (203-206). Therefore, STAT5 plays important roles in Foxp3 induction (in the aTreg subset), Foxp3 maintenance (in the aTreg and nTreg subsets), and Treg

suppressive function (by STAT5-dependent upregulation of CD25 to enhance scavenging IL-2 from the microenvironment).

There are conflicting reports about the role STAT5 plays in the development of Th1 cells. One report states that Stat5a negatively regulates Th1 development by upregulating SOCS-3 and inhibiting IL-12 induced Stat4 phosphorylation and Th1 development (207). However, another report showed STAT5 can bind a distal region of the *Ifng* locus and this binding mediates IL-2 induced transcriptional enhancement of IFN-γ (208). Thus, whether STAT5 promotes or inhibits Th1 differentiation or function depends on the SOCS-3 expression levels within the T cells and the accessibility of the *Ifng* gene to STAT5 binding.

STAT5 has opposing effects on the development of Th2 and Th17 cells. Introduction of a constitutively active STAT5 is sufficient to promote Th2 differentiation even in the absence of IL-4 (209). Furthermore, STAT5a is required for Th2 cell differentiation and allergic airway inflammation in vivo (210, 211). In contrast, STAT5 is a negative regulator of Th17 development because STAT5-deficient mice exhibit increased Th17 cells (212). The mechanism relies, at least in part, by STAT5-induced Foxp3 protein physically interacting with RORγt and impeding its function (213). Therefore, STAT5 promotes anti-inflammatory T helper cell subsets by upregulating Foxp3 but, depending on the cytokine microenvironment, can impact the differentiation of other Th cell subsets as well.

STAT6

STAT6 is activated by the cytokines IL-4 and IL-13. Since T cells do not express the IL-13Rα, IL-4 is the major instructive cytokine for the development of Th2 cells. IL-4 interacts with the IL-4R, leading to dimerization with the common γ chain (IL-2Rγ). Binding of IL-4 to the dimerized receptor activates Jak1, Jak3 and STAT6. The activation of STAT6 programs developing T helper cells to become Th2 cells by remodeling the *IL-4/IL-13* locus into heritable euchromatin

(214), upregulating the Th2 master transcription factor GATA-3 (215, 216) and promoting IL-4 transcription (217). While GATA-3 induction is largely STAT6 dependent in Th2 cells, modest GATA-3 expression can be observed in the absence of STAT6 (218, 219). GATA-3 expression is maintained in Th2 cells (220), is necessary and sufficient to induce Th2 cytokine expression even in Th1 cells (221) and is sufficient to ensure heritable chromatin changes (222, 223).

STAT6 can also regulate other factors important for Th2 differentiation. STAT6 upregulates the Th2 specific transcription factor c-Maf that with JunB synergize to drive IL-4 expression (224-226). Interestingly, another mechanism by which STAT6 can direct Th2 differentiation is preventing the colocalization of the TCR and IFNγR at the Th-APC interface while influencing the colocalization of the TCR with the IL-4R (227).

STAT6 reinforces Th2 development by inhibiting Th1 transcription factors and cytokine receptors. STAT6-induced GATA-3 expression inhibits the expression of IL-12Rβ2 and can actively repress IFN-γ transcription by remodeling the *Ifng* locus into heterochromatin (228). C-Maf can also reciprocally regulate Th1 induction by inhibiting IL-12p35 induction by macrophages (229).

IL-4 and STAT6 have opposing roles in Foxp3 protein regulation in the nTreg and aTreg subsets. IL-4 and IL-13, through STAT6-dependent mechanisms, promote the generation of Foxp3 expressing T cells and expand Ag-specific nTregs (230). In addition, incubation of nTreg cells with IL-4 can enhance the amount of Foxp3 expressed per cell (231, 232). In contrast, addition of IL-4 can inhibit TGF-β induced Foxp3 expression and aTreg development in a STAT6-dependent manner in vitro (154, 233). The mechanisms involved in this inhibition include GATA-3-mediated transcriptional repression of Foxp3 (233). In addition, STAT6 can directly inhibit Foxp3 expression by binding a silencer element in the *Foxp3* gene (234). These results suggest that STAT6 activation can help to downmodulate inflammation when IL-4 is present in the microenvironment with

nTregs. However, STAT6 activation in proliferating T cells inhibits TGF-β induced Foxp3 and may aid in the development of inflammation by constraining aTreg development.

In Th17 development, STAT6 activation, at least partially through c-Maf induction, can inhibit IL-17 production from Th17 cells (235). Thus, while STAT6 is a positive regulator of Th2 differentiation and a negative regulator of Th1 and Th17 development, it plays a less clear role in the development and function of immunoregulatory T cells.

A comparative analysis of the transcriptional regulation and functional significance of Foxp3 in the aTreg and nTreg subsets

Although both aTreg and nTreg subsets express Foxp3, nTregs are induced to become Foxp3+ in the thymus while aTreg cells become Foxp3+ in the peripheral lymphoid organs in the presence of TGF-β and IL-2. Foxp3 is important in conjunction with other factors to confer suppressive function both in vivo and in vitro (236). However, it is still incompletely worked out whether the inducing factors of Foxp3 expression are similar between aTreg and nTreg subsets. With the development of GFP Foxp3 knock-in mice and mice with a nonfunctional Foxp3 protein (scurfy mice), some factors necessary to induce Foxp3 have been elucidated. Since the development of the thymic medulla during ontogeny correlates with an increase in Foxp3+ thymocytes (237), the medulla of the thymus is thought to be necessary for nTreg Foxp3 expression. Moreover, mutations in proteins that disrupt thymic medullary architecture, like NFκB inducing kinase (NIK) and TNF receptor associated factor 6 (Traf6), impair regulatory T cell generation (238, 239).

T cell receptor signaling molecules and Foxp3 expression

The strength of the TCR signal is an important determinant of Foxp3 expression in both nTregs and aTregs. High-affinity TCR-peptide-MHC interactions and subsequent downstream TCR signals in the thymus are essential for Foxp3

expression and natural regulatory T cell development (240). Developing thymocytes with dysfunctional or deleted proteins important in dampening down the strength of the TCR signal have increased propensities to become nTregs. For example, mice lacking Src homology containing phosphatase 1 (SHP-1), a tyrosine phosphatase that attenuates TCR proximal signaling, produce higher percentages of Foxp3+ nTregs in comparison to SHP-1 sufficient mice (241). In addition, TCR signaling through the membrane-associated adapter protein linker of activation in T cells (LAT) and the phospholipase C-γ (PLCγ) pathway is essential for the generation of Foxp3+ T cells (242). This implies that nTreg Foxp3 expression is induced by high-affinity TCR engagement.

In contrast to a strong TCR signal being needed for Foxp3 induction in nTregs, Foxp3 is induced most efficiently in the periphery with suboptimal TCR stimulation in aTreg cells. While TCR engagement is absolutely essential for induced Foxp3 expression (128, 132, 243), stimulating T cells with low doses of antigen promoted peripheral aTreg generation (244). Indeed, cbl-b, a ring-type E3 ubiquitin ligase, is essential for attenuating TCR and CD28 signals (245, 246) and its absence prevents TGF-β signaling from inducing Foxp3 expression (247). Therefore, Foxp3 induction is controlled differently in aTreg and nTreg differentiation.

The most well-characterized transcription factor important for Foxp3 expression and Treg function include Nuclear Factor of Activated T cells (NFAT). NFAT is necessary for Foxp3 expression in both humans and mice. Moreover, NFAT and Foxp3 act in concert to mediate nTreg-mediated suppression in vitro and in vivo (248-250). With regard to aTreg development, NFAT can also interact with Smad3 to bind an enhancer in the *Foxp3* gene to induce Foxp3 expression post TGF-β stimulation (251).

Co-stimulation and Foxp3 expression

Co-stimulatory signaling pathways have also been implicated as mediators of Foxp3 expression. CD28 expression is a positive regulator of Foxp3 induction in nTregs and aTregs (252-254). While the inhibitory surface molecule CTLA-4 is not required for Foxp3 expression in nTregs, it does seem to be required for Foxp3 expression in TGF-β induced aTreg cells (255, 256). The inhibitory costimulatory signaling pathway PD1-PD1L interaction also seems to be critical for Foxp3 expression independent of CD28 activation (257). The OX40 pathway inhibits aTreg generation and also inhibits Treg function (258, 259). The ICOS-ICOSL pathway is also important for the induction of Foxp3+ Treg cells (260, 261).

Other pathways of Foxp3 expression

Recently, diverse mechanisms of Foxp3 regulation in aTregs and nTregs have been described. For example, Vitamin A metabolites like all-*trans* Retinoic Acid signaling through the Retinoic acid receptor α (RARα) can enhance TGF-β induced Foxp3 expression in aTreg cells (262-264). In addition, binding of the dioxin receptor aryl hydrocarbon receptor (AHR) by its ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced Foxp3 expression through a TGF-β1 mechanism while AHR binding to another ligand 6-formylindolo[3,2-b]carbazole (FICZ) inhibited Foxp3 expression and induced Th17 cells (265). Physiological concentrations of estrogen have also been demonstrated to increase Foxp3 expression and stimulated the conversion of CD4+25- T cells into Foxp3+CD4+CD25+ T cells without the addition of TGF-β (266). Lastly, cyclooxygenase-2 (COX-2) and its product prostaglandin E2 can induce Foxp3 expression in nTreg and aTreg cells (267, 268).

Cytokines and Foxp3 expression

Cytokines present in the microenvironment also have influential roles in the positive and negative regulation of Foxp3. As discussed previously, TGF-β and IL-2 are important for Foxp3 expression and maintenance in aTregs while IL-4 is

also important for nTregs. TGF-β binds to the TGF-βRI and II and phosphorylates Smad2/3 which then shuttle into the nucleus and form complexes with Smad4. Smad2/4 or Smad3/4 complexes bind DNA and recruit coactivators that contain histone-acetyl transferase activity to activate Foxp3 expression (269). In addition, IL-35 is a cytokine composed of 2 subunits, EBI3 and p40. This cytokine is produced by aTreg cells, maintains Foxp3 expression, and is important for Treg mediated suppression (270, 271).

Negative regulators of Foxp3 expression

Interestingly, there is limited information on negative regulators of Foxp3 expression. Currently, the only molecules that have been shown to repress Foxp3 expression are STAT6, GATA-3, T-bet, STAT3, IL-6, inducible cAMP early repressor (ICER), and the *tax* gene of the human T lymphotrophic virus type I (HTLV-Tax) (108, 154, 272-275). It is interesting to note that many of the molecules that are influential in repressing Foxp3 are either part of the JAK-STAT pathway or transcription factors upregulated by the JAK-STAT pathway.

Summary

Each STAT, downstream of instructive cytokines, has positive and negative regulatory roles in the differentiation of particular Th subsets (Fig. 1). STAT1 and STAT4 are essential for the development of Th1 cells and cell-mediated immunity. STAT3 is vital for Th17 differentiation and also positively regulates Th2 differentiation. STAT5 is important for Foxp3 expression and the maintenance of Treg function. STAT6 is critical for the development of Th2 cells and the humoral immune response. Importantly, there are extensive crossregulatory networks of STAT proteins and the downstream proteins they induce that inhibit the other Th subsets while promoting a specific Th subset.

In addition to STAT proteins promoting specific Th cells subsets, the transcription factor Foxp3 is also essential in keeping immune homeostasis and tolerance. While the importance of Foxp3 in directing nTreg functions is well characterized,

the regulators of Foxp3 expression are only beginning to be elucidated. The factors that induce Foxp3 are diverse ranging from TCR stimulation to vitamin A metabolites. The importance of cytokines in Foxp3 induction has begun to be understood with the characterization of TGF-β induced Foxp3+ aTreg cells. Since STAT proteins play major roles in Th cell subset differentiation, it is not surprising that they also play positive and negative roles in Foxp3 expression. It is essential to understand these processes if we are to make immune modulation a tailored and specific therapy for the management of human disease.

Research Goals

Based on the synthesis of the information given above, the overarching goal of this research was to gain a better understanding of how STAT proteins regulate Th1, Th17 and aTreg cell development downstream of instructive cytokines. We wanted to reach this goal by asking two different questions. The first question involved taking a global analysis of the STAT protein family and asking how multiple STAT proteins can regulate TGF-β1-induced Foxp3 expression. The second question was how the N- and C-terminal domains of STAT4 mediate STAT4 phosphorylation, gene regulation and inflammatory cytokine production downstream of IL-12 and IL-23 signaling.

With regard to the first question, T cell responses to a cytokine milieu instruct the development of multiple effector phenotypes. IL-6, in the presence of TGF-β1, diverts the development of Foxp3+ aTreg cells toward pro-inflammatory Th17 cells. While TGF-β1 inhibits the development of Th1 and Th2 cells, the ability of Th1- and Th2- promoting cytokines to alter TGF-β1-primed aTreg development has not been fully defined. In addition, much is known about chromatin regulation and transcription factor downregulation of opposing Th cell subsets. However, not much is known about STAT protein regulation of the *Foxp3* gene. Therefore, we wanted to examine if stimulation with multiple Th instructive cytokines can inhibit TGF-β1-primed aTreg development through a common mechanism.

To address the second queston, we specifically focused on STAT4. The N-terminal domain of STAT4 is required for STAT4 activation and the tetramerization of two activated STAT4 dimers when two consensus STAT binding sequences are spaced approximately 10 DNA base pairs apart (173, 191-193). What is not known, however, is if the same portion of the N-terminal domain mediates tetramerization, tyrosine phosphorylation, and nonphosphorylated dimerization. Acquisition of such knowledge is important because if different portions facilitate STAT4 functions, then inhibiting specific regions of the N-terminal domain could inhibit STAT4 function to varying degrees. Thus, the goal is to determine if different portions of the N-terminal domain mediate tyrosine phosphorylation and tetramerization.

Furthermore, STAT4 can be expressed as two alternative splice forms, a full-length STAT4α, and a STAT4β isoform lacking a C-terminal transactivation domain. Each isoform is sufficient to program Th1 development in vitro through both common and distinct subsets of target genes. However, the ability of these isoforms to mediate inflammation in vivo has not been examined. Therefore, to determine if the presence or absence of the C-terminal tranactivation domain of STAT4 differentially mediates inflammation in vivo, we utilized a model of colitis that develops following transfer of naïve T cells expressing either the STAT4α or STAT4β isoform into SCID mice.

MATERIALS AND METHODS

Mice. The generation of *Stat1*-deficient (*Stat1-/-*), *Stat4*-deficient (*Stat4-/-*), *Stat6*-deficient (*Stat6-/-*), *Bcl6*-deficient (*Bcl6-/-*) and *T-bet*-deficient (*Tbx21-/-*) mice were previously described (52, 55, 84, 276, 277). *Stat4-/-* mice were maintained on both a C57BL/6 and Balb/c background and bred in our animal housing facility. C57BL/6 *Tbx21-/-* and Balb/c *Stat6-/-* mice were obtained from Jackson Laboratories (Bar Harbor, ME). Strain matched C57BL/6 or Balb/c wild type (WT) control mice were purchased from Harlan (Indianapolis, IN). For the colitis study, the B6.CB17-Prkdc^{scid}/SzJ (B6 SCID) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The STAT4α and STAT4β transgenic mice were maintained on a *Stat4-/-* C57BL/6 background. All experiments were approved by the IACUC. The mice were maintained in an SPF barrier facility. Eight to 14 week old female mice were used in the experiments.

STAT3 conditional mutants were generated as previously described and obtained from Dr. David Levy (278). Briefly, *Stat3* genomic DNA was isolated from a 129/SvJ genomic library utilizing a *Stat3* cDNA probe. A targeting construct was generated by inserting a loxP site in intron 15 as well as a thymidine kinase (TK)-neomycin resistant (neo^{r}) cassette flanked by two loxP sites in intron 21. STAT3-mutant embryonic stem (ES) cells were generated by inserting the targeting construct into mouse ES cells by electroporation. Neomycin resistant clones were isolated and assayed for homologous recombination by PCR using primers overlapping the DNA sequence in the TK-neo^{r} cassette as well as primers overlapping the loxP site inserted in intron 15. Cre-recombinase, an enzyme that recognizes loxP sites and excises intervening DNA elements between two loxP sites, was transiently transfected into ES clones and used to generated and isolate 'floxed' alleles where two loxP sites flank exons 16-21. Floxed mice (*Stat3*$^{fl/fl}$) were generated and mated to Cre-transgenic mice, where the expression of Cre is controlled by the CD4 promoter. *Stat3*$^{fl/fl}$ mice that express Cre (*Stat3*$^{CD4-/-}$) are deficient in normal STAT3

expression in T cell lineages due to Cre-mediated excision of exons 16-21 in the *Stat3* gene. STAT-mutant mice were maintained on a mix of B6/129 background. Wild type controls were *Stat3*$^{fl/fl}$ Cre-negative littermates.

CD4+ T cell and APC isolation. Spleens and lymph nodes were harvested from 8-14 week old *Stat1-/-, Stat4-/-, Stat6-/-, Stat3*$^{CD4-/-}$, STAT4α, STAT4β, and *Tbx21-/-* mice and strain matched wild-type controls. Spleens and lymph nodes were dissociated in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), penicillin-streptomycin, sodium pyruvate, non-essential amino acids, L-Glutamine, HEPES (all from BioWhitaker, Walkersville, MD) and 50 µM β-mercaptoethanol (Sigma, St. Louis, MO).

Cell suspensions were put into a 15 ml conical tube and subsequently transferred to another tube while avoiding the transfer of settled cellular debris. The cells were centrifuged at 1,500 rpm for 5 minutes at 4°C, cell pellets were re-suspended in 2-3 mL of red blood cell (RBC) lysis buffer (8.3 g/L NH_4Cl in 0.01M Tris-HCl; pH: 7.5). After 1-2 minutes of incubation at room temperature, 10 mL of supplemented RPMI were added to each tube and samples were centrifuged at 1,500 rpm for 5 minutes at 4°C. Cell pellets were washed once in MACS Buffer (2mM EDTA and 0.5% bovine serum albumin (BSA) in PBS) and re-suspended in 900 µL/spleen of MACS buffer. Total CD4+ T cells were isolated by positive selection according to manufacturers protocol (MACS isolation system, Miltenyi Biotec, Auburn, CA). In other experiments, Naïve CD4+ T cells were isolated by negative selection according to manufacturer's protocol (MACS isolation system, Miltenyi Biotec, Auburn, CA). In order to obtain APCs, splenocytes were incubated with CD4 (LT34 Miltenyi Biotech) and CD8 microbeads. After the microbead incubation, the flow through of cells from LS columns were collected and irradiated at 3000 rads to be used as APCs in T cell cultures.

Plate-bound anti-CD3 T cell culture. α-CD3 (145-2C11 clone isolated from hybridoma; 4 μg/mL) in 1 mL of PBS was added into each well of a 12-well tissue culture plate. Plates were incubated overnight at 4°C or placed in a 5% CO_2 incubator at 37°C for 2-6 hours generating plate-bound α-CD3 coated plates. The 4 μg/mL α-CD3 solution was aspirated and the wells were washed with 1 mL of PBS before $1x10^6$ CD4+ T cells/mL were added to the wells and activated with plate-bound α-CD3 and soluble α-CD28 (BD Biosciences, San Diego, CA; 1 μg/mL) and cultured in supplemented RPMI 1640. Adaptive Treg cells were differentiated with TGF-β1 (R+D Systems, Minneapolis, MN; 2ng/mL) and α-IL-4 (11B11 clone isolated from hybridoma; 10 μg/mL). TGF-β1+IL-4 cultures were generated with 2 ng/mL TGF-β1, IL-4 (Peprotech, 10 ng/mL), and α-IFN-γ (XMG or R46A2 10 μg/mL). Th17 cells were differentiated with 2 ng/mL TGF-β1, IL-6 (Peprotech, 100 ng/mL), and α-IL-4 (10 μg/mL). TGF-β1+IL-12 cultures were generated from 2 ng/mL TGF-β1, IL-12 (Peprotech, 5 ng/mL), and α-IL-4 (10 μg/mL). TGF-β1+IL-21 cultures were generated with 2 ng/mL TGF-β1, IL-21 (Peprotech, 10 ng/ml) and α-IL-4 (10 μg/mL). TGF-β1+OSM cultures were generated with 2 ng/mL TGF-β1, 75 ng/mL OSM, and α-IL-4 (10 μg/mL). TGF-β1+IL-10 cultures were generated with 2 ng/mL TGF-β, IL-10 (Peprotech; 10 ng/mL) and α-IL-4 (10 μg/mL). TGF-β1+IL-23 cultures were generated with 2 ng/mL TGF-β1, IL-23 (R+D Systems, 10 ng/mL) and α-IL-4 (10 μg/mL). Th1 cells were differentiated with 5 ng/mL IL-12 and α-IL-4 (10 μg/mL). Th2 cells were differentiated with 10ng/mL IL-4 and α-IFN-γ (XMG or R46A2 10 μg/mL). All cell cultures were maintained in a 5% CO_2 at 37°C. After 3 days of differentiation, each well of differentiating cells was expanded into one well of a 6-well plate without α-CD3, supplemented with 4 mL of fresh media containing half the original concentration of cytokines and neutralizing antibodies. After 2 more days of culture, cells were collected, washed, and counted. Cells were then analyzed by RT-PCR, flow cytometry, and ELISA (described below).

T cell culture with irradiated APCs. T cells ($1x10^6$ CD4+ cells/mL) and $5x10^6$ irradiated APCs/mL were cocultured in 1 well of a 12-well plate with soluble α-

CD3 (either 2 μg/mL in the Stat4α/β experiments or 4 μg/mL in the Foxp3 studies), 0.5 μg/mL of soluble α-CD28, cytokines and neutralizing antibodies at the same concentrations as described above. After 3 days of differentiation, each well containing differentiating cells was expanded into one well of a 6-well plate, supplemented with 4 mL of fresh media containing half the original concentration of cytokines and neutralizing antibodies except that no additional α-CD3 or α-CD28 were added. After 2 more days of culture, cells were collected, washed, and counted. Cells were then analyzed by RT-PCR, flow cytometry, and ELISA (described below).

Detection of cytokines using ELISA. T cells differentiated for 5 days ($1x10^6$ CD4+ T cells/mL) were washed and stimulated with either medium alone, plate-bound α-CD3 (2 μg/mL for Stat4α/β experiments and 4 μg/mL for Foxp3 studies), IL-12 (5ng/mL), IL-12+IL-18 (Peprotech 25 ng/mL), IL-23 (4 ng/mL)+IL-18 (25ng/mL) for 24 hours to generate supernatants for analysis using ELISA. Cell-free supernatants were used to measure cytokine levels. To test for cytokine secretion, 2 μg/mL of α-IL-2, α-IL-4, α-IL-6, α-IL-10, α-IL-17, α-IFN-γ, α-TNF-α, α-OSM, or α-GM-CSF capture antibodies (BD Biosciences) were dissolved in 0.1 M $NaHCO_3$ (pH 9) and used to coat a 96 well Immunosorbent plate. Plates were incubated at 4°C overnight and washed three times in ELISA Wash buffer (0.1% Tween-20 in PBS) and blocked for at least two hours at room temperature in FACS/ELISA buffer (100 μL/well; 2% BSA and 0.1% NaN_3 in PBS). FACS/ELISA buffer was removed and supernatants and cytokine standards (R+D Systems) were added and incubated overnight at 4°C. Plates were washed three times with ELISA wash buffer and incubated at room temperature with 1 μg/mL of biotinylated detection antibodies dissolved in FACS/ELISA buffer for at least two hours. Plates were washed three times with ELISA wash buffer and incubated with streptavidin alkaline phosphatase (1:2000 dilution; Sigma) in FACS/ELISA buffer for at least one hour. Cytokine levels were determined by measuring the absorbance at 415 nm (BIO-RAD microplate reader model 550) following the addition of Sigma 104 phosphatase substrate (5 mg/ml; Sigma)

dissolved in ELISA substrate buffer (10% diethanolamine, 0.05 mM $MgCl_2$, 0.02% NaN_3, pH 9.8).

Cell surface staining and analysis using flow cytometry. T cells (0.1-1x10^6) suspended in supplemented RPMI 1640 were placed in 12x75 mm flow cytometry tubes. Following centrifugation at 1,500 rpm for 5 minutes at 4°C, media was removed and cells were washed once in FACS/ELISA buffer. Buffer was removed and purified α-mouse FcγIII/II receptor (1 μg; BD Biosciences) was added to each tube, mixed by vortex and incubated at 4°C for 15 minutes. For cytokine receptor staining, flourochrome conjugated antibodies of TGF-βRII (0.5 μg) and CD4 (0.5 μg) were added to tubes. Cells were incubated with antibodies at 4°C for 30 minutes. Cells were washed once in 1 mL of FACS/ELISA buffer and re-suspended in fixative buffer (FACS/ELISA buffer with formaldehyde) for 10 minutes at room temperature. Cells were washed one time in 1 mL of FACS/ELISA buffer and resuspended in FACS/ELISA buffer until analysis by flow cytometry. Samples were analyzed by flow cytometry on FACScan or FACS-Calibur machines (BD Biosciences) and data was analyzed using CellQuest or WinMDI software.

Intracellular staining and analysis using flow cytometry. CD4+ T cells were stimulated with plate-bound α-CD3 (2 μg/mL) or phorbol-myristate acetate (PMA) (50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) for 4 hours in the presence of GolgiPlug (BD Biosciences) or Monensin at a concentration of 3 μM. Cells were fixed and permeabilized using the eBioscience Fixation/Permeabilization Kit as recommended by manufacturer's protocol (eBioscience; San Diego, CA). Cells were stained for IL-2, IL-4, IL-17, IFN-γ, TNF-α and Foxp3 using fluorescently conjugated antibodies (BD Biosciences) for 30 minutes at 4°C. Cells were washed twice in Permeabilization buffer and resuspended in 200 μl of FACS/ELISA buffer. Samples were analyzed by flow cytometry using FACScan or FACS-Calibur machines and data were analyzed using WinMDI software.

Intracellular phospho-STAT staining and analysis using flow cytometry. CD4+ T cells ($0.2\text{-}1 \times 10^6$) were collected at the indicated time points and fixed for 10 minutes at room temperature with formaldehyde at a final concentration of 1.5%. Cells were pelleted by centrifugation and resupended in 100% methanol for 10 minutes at 4°C. Cells were then washed two times in FACS/ELISA buffer and stained with the indicated pStat Antibody (20 µL/1×10^6 cells) (All pStat Ab from BD Pharmingen). Cells were incubated in the dark for 30 minutes at room temperature. The cells were washed in FACS/ELISA buffer and the samples were analyzed by flow cytometry using FACScan or FACS-Calibur machines and data were analyzed using WinMDI software.

Generating whole cell protein lysates. At least 2×10^6 CD4+ T cells were lysed in 30-100 µL of protein lysis buffer (10% glycerol, 0.5% IGEPAL, 50 mM Tris pH 8.0, 0.1 mM EDTA, 150 mM NaCl, NaF, Sodium Orthovanadate, β-glycerol, DTT and protease inhibitors (aprotinin, leupeptin, pepstatin A, iodoacetamide, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), benzamidine). Cells were incubated on ice for 10-15 minutes and centrifuged at 14,000 rpm at 4°C in a microcentrifuge. Supernatant was transferred to a new pre-chilled tube and frozen at -80°C until use.

Generating nuclear and cytoplasmic protein lysates. Nuclear and cytoplasmic protein lysates were obtained following the manufacturer's instructions (Roche) and the lysates were stored at -80°C until use.

Measuring protein concentrations by Bradford Assay. A 1:4 protein dye reagent:ddH_2O ratio was used make the Bradford Reagent (Bio-Rad). In a 96-well flat-bottom plate, 100 µL of ddH_2O was added to each well. Subsequently, 1 µL of each lysate was added in duplicate to wells and serially diluted two-fold. Protein concentrations were compared to known concentrations of serially diluted BCG. Protein concentrations were determined by measuring absorbance at 595 nm using a microplate reader (Bio-Rad Model 680) and using linear regression

against the BCG standard curve to quantify the amount of protein in each sample.

SDS-PAGE and Western blot. Protein (50-100 μg) was added to SDS-PAGE loading buffer (200 mM Tris HCl pH 6.8, 40% glycerol, 8% SDS, 4% β-mercaptoethanol, 0.04% bromophenol blue). Samples were boiled for 5 minutes to denature proteins. Samples and Precision Blue plus marker (Bio-Rad) were electrophoresed on a 10-well pre-cast 4-12% gradient Bis-Tris polyacrylamide gels (Invitrogen) (power pack from Bio-Rad). The gel was run at 150 V for 1-2 hours and was then removed and proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) at 500 mAmps for 1.5 hours or 250 mAmps overnight at 4°C. Following transfer, the membrane was blocked in 1x TBST (Tris-Base, NaCl, Tween-20) supplemented with 3-5% (depending on the antibody used) nonfat powdered milk on a shaker for 1 hour.

Membranes were immunoblotted using primary antibodies to detect phospho-Stat3 (p-Stat3) (Upstate Cell Signaling, Charlottesville, VA), p-Stat4 (Zymed), p-Stat5 (BD Pharmingen), and p-Stat6 (BD Pharmingen), p-Smad2 (Cell Signaling, Boston, MA), p-Smad3 (Invitrogen), Stat3 (BD Pharmingen), Stat4 (Santa Cruz and BD Pharmingen), Stat5A/B (BD Pharmingen), Stat6 (BD Pharmingen), Foxp3 (eBioscience), β-actin (Calbiochem, San Diego, CA), and GAPDH (Meridian Life Sciences, Saco, ME). For detection of each protein, primary antibody was diluted in 1x TBST with 3-5% nonfat milk according to the manufacturer's recommendations, added to membranes and placed on a shaker for at least two hours at room temperature or at 4°C overnight. This was followed by washing the membrane three times in TBST for 5-minutes each. A 45 minute incubation with secondary horse-radish peroxidase (HRP) conjugated IgG (Bio-Rad) or IgM (Calbiochem) antibodies diluted in TBST with 5% nonfat milk was followed by 4 washes of TBST for 5 minutes each. Western lighting chemiluminescence reagent (PerkinElmer Life Sciences, Wellesley, MA) was used for detection of enzymatic activity and CL-Xposure film (Pierce, Rockford,

IL) was used to visualize the signal. To strip immunoblots, membranes were incubated with stripping buffer (42 mL ddH_2O, 5 mL 20% SDS, 3 mL 1 M Tris-HCl pH 6.8, 350 µl β-mercaptoethanol heated in a microwave for 20 seconds) for 15 minutes on a shaker at room temperature. Membranes were washed 3-4 times in TBST, blocked with 5% milk for 30 minutes to 1 hour and immunoblotted for another protein of interest.

Retroviral transductions. Wild-type C57BL/6 or $Stat3^{CD4-/-}$ CD4+ T cells were cultured under the indicated culture conditions for 24 hours. Cells were infected as described (159, 279) and cultured for 4 more days. Cells were either sorted for eGFP-positive cells, $H\text{-}2K^k$-positive cells or, in the case of IRF4, human CD4-positive cells (hCD4) and intracellularly stained for Foxp3. Cells were subsequently analyzed for Foxp3 expression either by Western blot or flow cytometry.

RNA isolation and cDNA conversion. For all RNA work, autoclaved centrifuge tubes and nuclease-free filtered pipette tips were used. Cells ($2\text{-}10x10^6$) were resuspended and lysed in 1 mL of TRIzol reagent (Invitrogen) and frozen at -80°C until further use. Once tubes were thawed, 200 µl of chloroform was added to each tube, vigorously shaken for 1 minute and incubated at room temperature for 3 minutes. Tubes were centrifuged at 14,000 rpm for 15 minutes at 4°C. The aqueous layer was added to a new tube and the organic layer was discarded. 500 µL of isopropanol was added to each tube, mixed, and stored at -20°C overnight to precipitate RNA. Samples were centrifuged at 14,000 rpm at 4°C for 15 minutes. The supernatant was discarded and the pellet was washed once with 80% cold ethanol. Ethanol was removed and the pellet was air dried for 10-15 minutes. RNA was dissolved in RNase-free diethylpolycarbonate (DEPC) treated ddH_2O. Samples were incubated in a 55°C water bath for 5-10 minutes to allow the RNA pellet to dissolve completely. Concentrations of RNA were quantified by analyzing 260 and 280 nm OD readings on a spectrophotometer (Eppendorf Uvette) according to manufacturer's instructions. 4 µg of RNA was

converted to cDNA using the First Strand Cloned AMV synthesis kit (Invitrogen) according to the manufacturer's protocol. cDNA samples were diluted to 100 µL final volume in DEPC treated ddH_2O.

Gene expression analysis using real time PCR. cDNA (20-40 ng/µL) (5 µL volume) was added to 12.5 µL to TaqMan Universal PCR Mastermix and 1.25 µL of TaqMan FAM-labeled TaqMan primers for the analysis of gene expression indicated in Results. All reagents for real time PCR were obtained from Applied Biosystems (Foster City, CA) and the manufacturer does not publish primer sequences for TaqMan PCR. Filter ddH_2O was added to a final volume of 25 µL. Samples were plated in duplicate in 96-well optical reaction plates and capped with optical reaction caps. Plates were vortexed and centrifuged at 3,000 rpm for 3 minutes. Plates were analyzed on an ABI 7500 Real Time PCR system (Applied Biosystems). Cycle number was normalized to expression of β_2-microglobulin. Cycle-threshold (Ct) values were obtained and the data were analyzed automatically using provided software and transferred to Microsoft Excel spreadsheets as instructed by the manufacturer.

Suppression assay. Spleen cells (1×10^6 cells/ml) were cultured as described previously for 5 days. Cells were harvested and extensively washed. For the suppressor assay, CD4+CD25- cells ($5x10^4$) were isolated from WT mice using the CD4+CD25+ Regulatory T cell Isolation Kit (Miltenyi Biotec) and these cells were incubated with 2 µg/ml soluble anti-CD3 in the presence of irradiated T cell depleted WT splenocytes ($5x10^4$) and increasing numbers of CD4+CD25+ cells from the indicated primary cultures isolated from control or gene-deficient mice. For the nTreg suppressor assays, CD4+CD25+ T cells were isolated using the CD4+CD25+ Regulatory T cell Isolation Kit (Miltenyi Biotec) and these cells were incubated with 2 µg/ml soluble anti-CD3 in the presence of irradiated T cell depleted WT splenocytes ($5x10^4$) and increasing numbers of CD4+CD25+ cells from the indicated primary cultures isolated from control or gene-deficient mice. Cultures were pulsed with 0.8 µCi [^{3}H]thymidine for the last 16-24 hours of a 72

hour incubation. Radioactivity incorporated was counted using a flatbed β counter (Wallac).

DNA Affinity Purification Assay. Whole cell extracts were prepared from cells cultured in the indicated conditions for 48 hours ± restimulation with the indicated cytokines for one hour. These extracts were precleared with streptavidin-agarose and then incubated with biotinylated oligonucleotides from the Foxp3 promoter containing STAT binding sites coupled to streptavidin-conjugated agarose beads for 16 hours at 4°C. The sequence of the biotinylated oligonucleotides was from the Foxp3 promoter containing STAT binding sites and the sequence was as follows: 5'/Bio/GCAGCTTCTGGGAGCCAGCCATTCTGAGACTCTCTGATTCTGATTCTG TGAATTT-3' Anti-sense: 5'- AAATTCACAGAATCAGAGAGTCTCAGAATGGCT GGCTCCCAGAAGCTGC-3' (Integrated DNA Technologies, Coralville, IA). Isolated proteins were subjected to SDS-PAGE and blotted onto the membranes. Bound proteins were identified with Western blotting using the indicated STAT antibodies.

Chromatin Immunoprecipitation. Crosslinking of protein-chromatin complexes was achieved by adding formaldehyde into cell cultures to a final concentration of 1% and shaking at room temperature for 10 min. Glycine was added to a final 0.125 M to quench the crosslinking. Cells were washed once with ice-cold PBS, resuspended in cell lysis buffer (5 mM Pipes pH 8.0, 85 mM KCl and 0.5% NP-40) and incubated for 10 min on ice. Protease and phosphatase inhibitors were added to all buffers. Nuclei were harvested after centrifugation at 5000 r.p.m for 5 min, resuspended in nuclear lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% SDS) and incubated 10 min on ice. An ultrasonic processor (Vibra-Cell) was used to shear genomic DNA (150-300-bp fragments), with eight sets of 10-s 50 W bursts. Cell extracts were diluted five-fold in ChIP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0 and 167 mM NaCl), precleared with salmon sperm DNA, BSA and protein A agarose bead slurry

(50%) at 4°C for 1 h. The supernatant was incubated in the presence or absence of 5 µg antibody (anti-Stat3; Santa Cruz, anti-Stat4; Santa Cruz, anti-Stat6; Santa Cruz, and anti-Stat5; Santa Cruz, anti-H_3K_9me3;Upstate) at 4°C overnight. The immunocomplex was precipitated with protein A agarose beads at 4°C for 1 h followed by centrifugation.

The supernatant from the control precipitation was used as input material. The beads were washed consecutively with low-salt wash buffer (0.1% SDS, 1% Triton-X 100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 150 mM NaCl), high-salt wash buffer (as above with 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris-HCl pH 8.0), and twice in 1 mM EDTA, 10 mM Tris-HCl pH 8.0. Bound DNA was eluted from the beads with elution buffer (2% SDS, 2 mM EDTA, 20 mM Tris-HCl), vortexing and incubating at 37°C before centrifugation. The supernatant was collected, supplemented with 2 mM EDTA, 20 mM Tris-HCl and 10 mg/ml proteinase K and incubated at 37°C. DNA crosslinks were reversed by incubating precipitates at 65°C for 16 h. DNA was purified by phenol/chloroform extraction, ethanol precipitated and resuspended in H_2O. DNA was then analyzed using qPCR. qPCR was performed with site-specific primer sets using ABI PRISM7500. To quantify chromatin immunoprecipitates, a standard curve was generated from serial dilutions of a known amount of DNA generated from sonicated T cells. To calculate ChIP results as a percentage of aTreg STAT5 binding, the amount of immunoprecipitated DNA from the isotype control antibody was subtracted from the amount of immunoprecipitated DNA from the specific antibody ChIP followed by normalizing against the amount of input DNA. Then, the amount of DNA precipitated from the aTreg cells using the STAT5 antibody was set at 100% and relative amounts of precipitated DNA from T cells cultured in the different conditions were calculated as a % of aTreg STAT5 binding. The sequence of the primers was as follows: TaqMan promoter probe, FAM-CCAGCCATTCTGAGACTCTCTGATTCTGTG-BHQ1; 5' TaqMan intron 1 probe, FAM-ACCTCCATACAGCTTCTAAGAAACAGTCAAACA-BHQ1.

Isolation of $CD45RB^{hi}$ and $CD45RB^{low}$CD4+ T cells and induction of colitis by cell transfer. Spleen, mesenteric lymph node, inguinal lymph node, and axillary lymph node cells were used as a source of CD4+ cells for reconstitution of SCID recipient mice. Spleens and lymph nodes were dissociated in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), penicillin-streptomycin, sodium pyruvate, non-essential amino acids, L-Glutamine, HEPES (all from BioWhitaker, Walkersville, MD) and 50 µM β-mercaptoethanol (Sigma, St. Louis, MO). Cell suspensions were put into a 15 ml conical tube and subsequently transferred to another tube while avoiding the transfer of settled cellular debris. The cells were centrifuged at 1,500 rpm for 5 minutes at 4°C, cell pellets were re-suspended in 2-3 mL of red blood cell (RBC) lysis buffer (8.3 g/L NH_4Cl in 0.01M Tris-HCl; pH: 7.5). After 1-2 minutes of incubation at room temperature, 10 mL of supplemented RPMI were added to each tube and samples were centrifuged at 1,500 rpm for 5 minutes at 4°C. Cell pellets were washed once in MACS Buffer (2mM EDTA and 0.5% bovine serum albumin (BSA) in PBS) and re-suspended in 900 µL/spleen of MACS buffer. CD4+ T cells were obtained from the spleens of donor animals and were enriched by magnetic sorting.

For cell purification, the following biotinylated anti-mouse antibodies were used to label non-CD4+ T cells isolated from donor mice: CD8a (Ly-2), CD45R (B220), CD49b (DX5), CD11b (Mac-1), and Ter-119, as well as Anti-Biotin MicroBeads. Isolation of $CD4^+$ T cells was achieved by depletion of the magnetically labeled cells using a MACS magnetic cell sorter (Miltenyi Biotec). The enriched CD4+ T cells were then labeled for cell sorting with Fitc-conjugated CD4 and PE-conjugated CD45RB (BD Pharmingen). Subsequently, cells were sorted under sterile conditions by flow cytometry for CD4+$CD45RB^{hi}$ on a FacsVantage (Becton Dickinson). The $CD45RB^{high}$ and $CD45RB^{low}$ populations were defined as the brightest staining 10-15% and the dullest staining 15-20% $CD4^+$ T cells, respectively. Intermediate staining populations were discarded. All populations were >99% pure on reanalysis. The purified $CD45RB^{hi}$CD4+ ($4x10^5$) cells diluted

in 100 μL of PBS were injected intraperitoneally into B6 SCID recipient mice. A separate group of B6 SCID mice received $CD45RB^{low}CD4+$ ($4x10^5$) cells. The recipient mice were weighed initially, then weekly thereafter. They were observed for clinical signs of illness, including hunched over appearance, piloerection of the coat, and diarrhea. Diseased animals were sacrificed 15 weeks after transfer.

Macroscopic and microscopic assessment of colon appearance. Once the animals were sacrificed, the entire large intestine length from cecum to rectum was measured. Tissue samples were taken from each segment of the colon (cecum, ascending, transverse, and descending colon and rectum) and fixed in 10% neutral buffered formalin. The samples were routinely processed, sectioned at 5 μm thickness, and stained with hematoxylin and eosin (H&E) for light microscopic examination.

The TJL histological scoring system. Four general criteria were evaluated in all sections: (1) severity, (2) degree of hyperplasia, (3) degree of ulceration, if present, and (4) percentage of area involved. Severity of lesion was graded as follows: 0 = normal, 1 = mild, 2 = moderate, 3 = severe. Mild lesions were small, focal, or widely separated multifocal areas of inflammation limited to the lamina propria. Moderate lesions were either multifocal or represented by locally extensive areas of inflammation extending into the submucosa. Severe lesions were ulcers covering large areas (>20 crypts) of the mucosa. Hyperplasia was graded as follows: 0 = normal, 1 = mild, 2 = moderate, 3 = severe. Mild hyperplasia consisted of morphologically normal lining epithelium that was two or more times thicker (length of crypts) than adjacent or control mucosa. Moderate hyperplasia was characterized by the lining epithelium being two or three times normal thickness, cells were hyperchromatic, numbers of goblet cells were decreased, and scattered individual crypts developed an arborizing pattern. Severe hyperplastic regions exhibited markedly thickened epithelium (four or more times normal), marked hyperchromasia of cells, few to no goblet cells, a

high mitotic index of cells within the crypts, and numerous crypts with arborizing pattern. Ulceration was graded as follows: 0 = no ulcer, 1 = 1-2 ulcers (involving up to a total of 20 crypts), 2 = 1-4 ulcers (involving a total of 20-40 crypts); and 3 = any group of ulcers that exceeds the previous criteria. The area involved by the disease process was estimated at intervals of 10% on a scale of 0%-100% of surface area. For analysis, area involved was coded as follows: 0 = 0%, 1 = 10%-30%, 2=40%-70%, 3 = >70%. The score was then determined from each slide by the following mathematical formula: ((inflammation score+ ulceration score+ hyperplasia score)x(Area involved score)) for a score range of 0-27. Scores from each section of the colon were averaged to determine the overall histological score per experimental group. Histological grades were assigned in a blinded fashion by the same pathologist

Cell preparation and cytokine analysis. Splenocytes and mesenteric lymph node cells were harvested from the reconstituted B6 Scid mice and single cell suspensions were obtained as described before. Viable cells were counted and determined by trypan blue exclusion. Surface and cytoplasmic staining and FACS analysis were performed as previously described. Cells ($1x10^6$ cells/mL) were plated on anti-CD3 coated plates (2 µg/mL) or restimulated with IL-12+IL-18 (1 ng/mL and 25 ng/mL, respectively), or IL-23+IL-18 (4 ng/mL and 25 ng/mL, respectively) for 72 hours. Supernatants were collected and assessed for cytokine production by ELISA. ELISA was performed as previously described.

In vitro T cell culture. For anti-CD3 differentiation with irradiated APCs, naïve CD4+ T cells were isolated by negative selection according to manufacturer’s protocol (MACS isolation system, Miltenyi Biotec, Auburn, CA). In order to obtain APCs, splenocytes were depleted of CD4+ and CD8+ cells by incubating with CD4 and CD8 microbeads (Miltenyi Biotech) and the flow through of cells from LS columns was collected. Cells were irradiated (30 Gy) and used as APCs in T cell cultures. Th1 cells were differentiated with 5 ng/mL IL-12 and α-IL-4 (11B11 10 µg/mL) and Th17 cells were differentiated with 2 ng/mL TGF-β1, 100 ng/mL

IL-6, 10 ng/mL IL-23, α-IFN-γ (XMG 10 μg/mL), and α-IL-4 (11B11 10 μg/mL) for five days. Cells were expanded on day 3 with 20 U/mL IL-2 and half the concentration of cytokines and neutralizing antibodies for the final two days of culture.

Plasmid construction and protein preparation of STAT4 N-terminal domain mutations. The plasmid pcDNA3.1/V5-His (Invitrogen) was modified for expression of wild-type and mutant Stat4 N-terminal deletion mutants by cloning the PCR products using the TOPO TA system. Stat4 cDNA was used as a template for the amplification by Pfx polymerase of wild-type and the ΔN Stat4 mutants by PCR and the following primers: wild-type Stat4 5'- GCC ACC ATG TCT CAG TGG AAT CAA -3', Δ10 5'- GCC ACC ATG GGA GTC CAA GTC CAA CAG -3', Δ21 5'- GCC ACC ATG TAT GAT GAC AAC TTT CCC -3', Δ30 5'- GCC ACC ATG CGG CAT CTG TTG GCC CAA TGG ATT GAA -3', Δ44 5'- GCC ACC ATG GCA GCT TCT AAC AAT GAA ACC -3', Δ51 5'- GCC ACC ATG GTG GAT CAA TTC TAT GAT -3', and hStat4 rev 5'- TCA TTC AGC AGA ATA AGG AGA CTT C -3'. PCR conditions were as follows: 30 cycles using the following three-step cycling conditions: 94°C for 15 sec., 55°C for 30 sec., and 68°C for 2 min. 30 sec. At the end of the cycle, 0.5 μL of Taq polymerase was added to the reaction and an extension cycle was initiated at 72°C for 10 min. The PCR product was then separated on an agarose gel and the band was excised and purified using GeneClean (Qiagen) following the manufacturer's instructions. The PCR products were then ligated into pcDNA3.1/V5-His.

For the Estrogen Receptor:STAT4 hybrids, the Estrogen receptor plasmid with an N-terminal myc tag was kindly provided by Dr. James Lyons. The plasmid pCEFL-N-mycERT2 (Invitrogen) was modified for expression of wild-type and mutant STAT4 N-terminal deletion mutants by the following methods. STAT4 cDNA was used as a template for amplification by Pfx polymerase of wild-type and the ΔN STAT4 mutants and the following primers: ERhStat4 5'-GGC GAA TTC ATG TCT CAG TGG AAT CAA -3', ERhStat4Δ21 5'-GGC GAA TTC TAT

GAT GAC AAC TTT CCC -3', ERhStat4Δ124 5'-ACG AAT TCG GGC CTC TAG AGA AAT CC -3', and ERhStat4rev 5'-GAA TTC TCA TTC AGC AGA ATA AGG AGA CTT C -3'. PCR conditions were as follows: 30 cycles using the following three-step cycling conditions: 94°C for 15 sec., 55°C for 30 sec., and 68°C for 2 min. 30 sec. At the end of the cycle, 0.5 μL of Taq polymerase was added to the reaction and an extension cycle was initiated at 72°C for 10 min. The PCR product was then run out on an agarose gel and the band was excised and purified using GeneClean (Qiagen) and following the manufacturer's instructions. The PCR products were then ligated into pcDNA3.1. TOP10 bacteria were transformed with the ligated product and a miniprep (Qiagen) was performed to isolate plasmid DNA. A restriction digest was then performed with EcoRI to cut Stat4 out of the plasmid. After treating the EcoRI cut pCEFL-N-mycERT2 with calf alkaline phosphatase, the hStat4 products were ligated into pCEFL-N-mycERT2.

Expression of hSTAT4, ΔNSTAT4, ERSTAT4, and ERΔNSTAT4 proteins in Cos7 cells. Cos7 cells were transfected with the Stat4 plasmids following the manfacturer's instructions for FuGene (Invitrogen). Briefly, 1-2x10^5 cells were plated in one well of a six-well plate 18 hours before transfection. The day of transfection, 3 μL of FuGene was dissolved in 97 μL of Serum-Free Dulbecco's Modified Eagle's Medium. 1 μg of plasmid was added to the mixture and incubated at room temperature for 15 minutes. Solution was then added drop-wise to the Cos7 cells and incubated for 48 hours before trypsinizing cells and making whole-cell protein lysates using previously described methods.

Inducible dimerization of ERSTAT4 plasmids with 4-OH tamoxifen. After transfection with FuGene (Invitrogen), 4-OH Tamoxifen was added to the wells. The 4-OH Tamoxifen powder was reconstituted in 100% ethanol and wells not receiving 4-OH Tamoxifen received 4 μL of ethanol. Cells were then incubated for an additional 24 hours before trypsinizing and making whole-cell protein lysates using previously described methods.

RESULTS

Stat protein regulation of Foxp3 expression in CD4+ T cells

TGF-β1-induced Foxp3 expression is important for the development of aTreg cells, which function as potent suppressors of inflammation in vitro and in vivo (128). The potent proinflammatory Th17 cells are induced to differentiate in the presence of TGF-β1 and IL-6. In addition, it has been shown that IL-6 through STAT3 dependent mechanisms can actively repress TGF-β1-induced Foxp3 (108, 274). However, it is unclear whether multiple STAT proteins are required for Foxp3 repression by IL-6. In addition, the temporal kinetics by which IL-6 represses Foxp3 has not been elucidated. Therefore, we wanted to examine what role STAT3 and STAT1 plays in the requirement of IL-6 inducing a TGF-β1 stimulated CD4+ T cell to go from an anti-inflammatory state (suppressive cell) to a proinflammatory state (IL-17 secretor).

Role of STAT1 in aTreg development

Upon activation, CD4+ T cells are known to secrete both IL-4 and IFN-γ early in differentiation (280). We wondered whether culturing cells in TGF-β1 affected the T cell secretion of IFN-γ and if IFN-γ was having a significant affect on Foxp3 expression and aTreg generation. First, we determined that the levels of IFN-γ in aTreg cultures were detectable by ELISA in the supernatants of 2-day activated CD4+ T cells (Fig. 2A). Next, we assessed if neutralizing IFN-γ during the culture period affected TGF-β1-induced Foxp3 (Fig. 2B). IFN-γ modestly inhibited Foxp3 expression at lower concentrations of TGF-β1 (1 ng/mL) while Foxp3 expression was not inhibited if TGF-β1 was at a higher concentration (2 ng/mL). This result suggested that IFN-γ can repress TGF-β1-induced Foxp3 but that the repression is sensitive to the concentration of TGF-β1. However, this experiment cannot rule out the possibility that 2 ng/mL of TGF-β1 inhibits IFN-γ secretion and that accounted for the lack of Foxp3 inhibition when IFN-γ was neutralized.

To address these possibilities, we added exogenous IFN-γ to aTreg cultured CD4+ T cells and observed that Foxp3 was not significantly repressed by the exogenous addition of IFN-γ (Fig. 2C). This suggested that TGF-β1-induced Foxp3 expression can overcome the repressive nature of IFN-γ if TGF-β1 is present at a high enough concentration.

Since adding IFN-γ slightly mitigated TGF-β1 induced Foxp3 when TGF-β1 was at a lower concentration, we wanted to determine if Foxp3 inhibition by IFN-γ was through a STAT1 dependent mechanism. Therefore, we cultured *Stat1-/-* CD4+ T cells in aTreg conditions and assessed the level of Foxp3 expression at the end of five days. Importantly, we saw a similar increase in Foxp3 expression in the *Stat1-/-* cells that we observed when IFN-γ was neutralized in STAT1 expressing CD4+ aTreg cultures (Fig. 2D). These experiments suggested that IFN-γ, through STAT1, can modestly repress TGF-β1 induced Foxp3 expression.

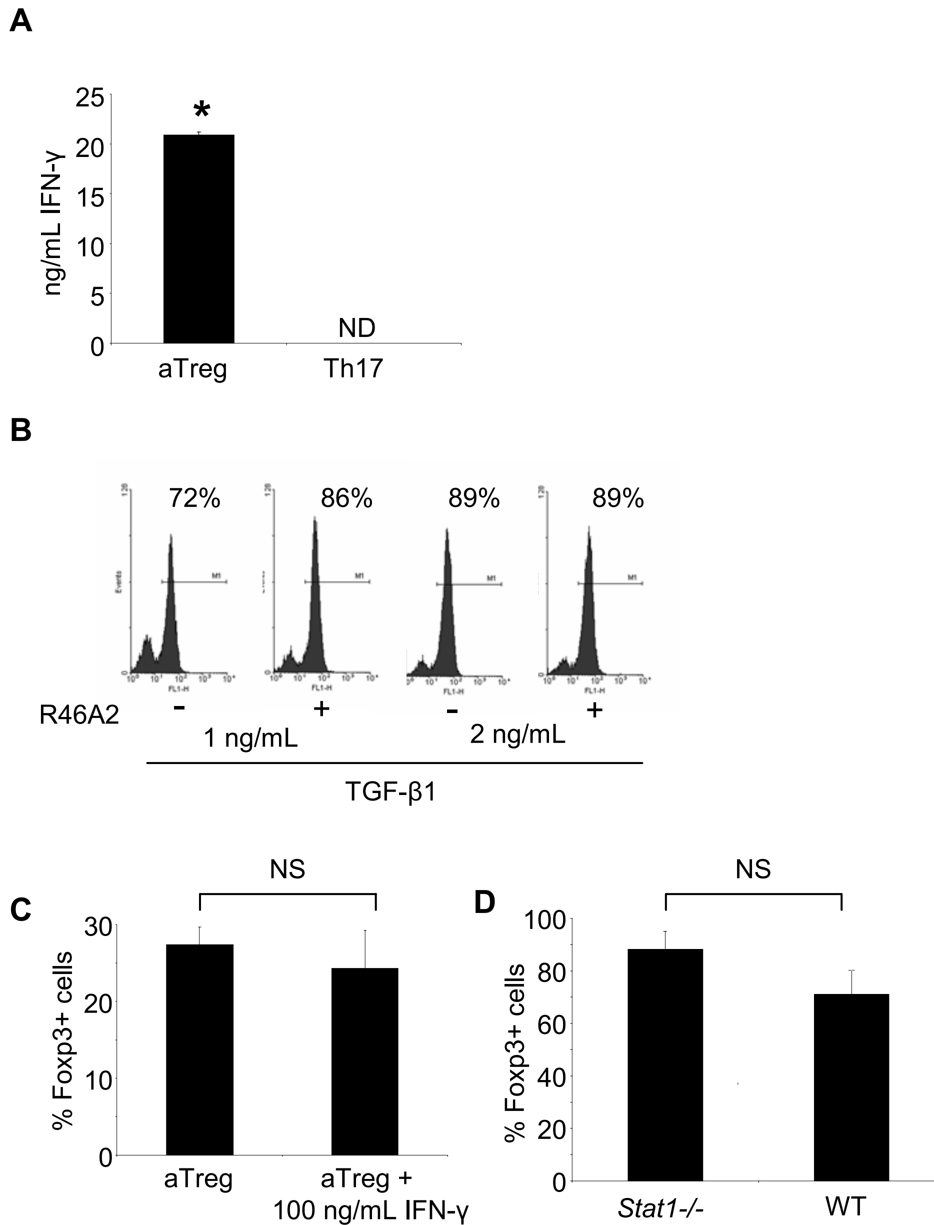

Figure 2. IFN-γ, through STAT1, can modestly repress TGF-β1-induced Foxp3. (A) Cell free supernatants were collected from naïve CD4+ T cells cultured under aTreg (TGF-β1 and anti-IL-4) or Th17 (TGF-β1+IL-6+anti-IL-4) conditions for 48 hours and analyzed for IFN-γ production by ELISA. Data are presented as mean ± SD of duplicates. ND=not detected. (B) Foxp3 expression was determined from cells cultured with 1 or 2 ng/mL TGF-β1 for 5 days with or without a neutralizing antibody (R46A2) specific for IFN-γ. Numbers represent % of live gated CD4+ T cells that are

Foxp3+. (C) Foxp3 expression was determined from five-day cultured aTreg cells in the presence or absence of 100 ng/mL IFN-γ. Data are presented as the mean % of Foxp3+ cells ± SD determined from replicates. (D) % of aTreg cultured cells expressing Foxp3 was determined from *Stat1-/-* or wild-type littermate controls. Data are presented as the mean % of Foxp3+ cells ± SD determined from replicates. Data are representative of 2 independent experiments. NS=not statistically significant ($p>0.05$) using unpaired Student's T-test.

Role of STAT3 in aTreg generation

Since there is impaired proliferation in *Stat3*-deficient T cells, we wanted to examine if there were any defects associated with TGF-β1-induced Foxp3 expression and aTreg development in the absence of STAT3 by culturing the cells in aTreg conditions for five days and intracellularly staining for Foxp3 (Fig. 3A). The *Stat3*-deficient aTregs had a modest increase in the percentage of Foxp3+ CD4+ T cells but the mean fluorescence intensity (MFI) of the Foxp3+ T cells was not significantly different. Since there was a modest increase in the number of cells expressing Foxp3 upon TGF-β1 stimulation but the amount of Foxp3 expressed per cell was no different, we hypothesized that the suppressive function of the $Stat3^{CD4-/-}$ aTreg cells would be similar to wild-type aTreg cells. To determine this, we utilized an in vitro suppressor assay comparing the ability of *Stat3*-deficient and wild-type 5-day cultured aTreg cells to suppress the proliferation of CD4+CD25- T cells (Fig. 3B). Having observed that there was no significant difference between the suppressive function of *Stat3*-deficient and wild-type aTreg cells, we concluded that STAT3 does not play a role in the ability of aTreg cells to function but can negatively regulate the number of cells that can become Foxp3+.

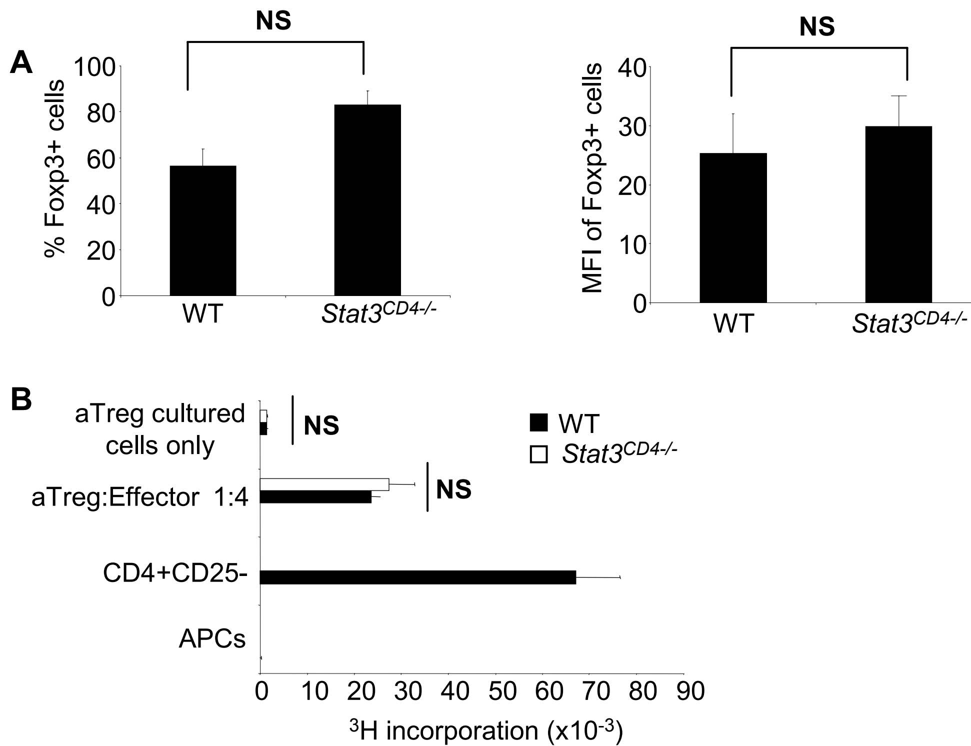

Figure 3. STAT3 is not required for aTreg suppressive functions. (A) Foxp3 expression was determined from five-day wild-type or *Stat3*$^{CD4-/-}$ CD4+ T cells cultured under aTreg conditions. Data are presented as both % of cells expressing Foxp3 (left panel) and the mean fluorescence intensity of the Foxp3+ cells (right panel). Results are represented as mean ± SD of replicates. NS=not statistically significant ($p>0.05$) using unpaired Student's T-test. (B) CD4+25- responder cells from wild-type littermate controls were stimulated in the presence of anti-CD3 and irradiated T cell depleted splenocytes in the presence or absence (CD4+25- cells alone) of WT or *Stat3*$^{CD4-/-}$ five day cultured aTreg cells (1:4 ratio of aTreg cultured cells:CD4+25- cells). Data are presented as the mean of the thymidine counts per minute (cpm) ± SD of triplicates. Data are representative of at least 3 independent experiments. NS=not statistically significant ($p>0.05$) using unpaired Student's T-test.

Opposing role of TGF-β1 and IL-6 in the development of Foxp3+ aTreg cells

The addition of IL-6 can mitigate the induction of Foxp3 by TGF-β1 (274). We wanted to examine whether there was an absolute or concentration dependent antagonism between IL-6 and TGF-β1 as it related to Foxp3 expression. To confirm that there was a dose-dependent upregulation of Foxp3 when T cells were cultured with TGF-β1 alone, CD4+ T cells were activated with anti-CD3 and anti-CD28 and cultured with increasing concentrations of TGF-β1 ranging from 1 ng/mL to 5 ng/mL for five days. At the end of the five days, the T cells were stained for intracellular Foxp3 expression (Fig. 4A). Since the peak of Foxp3 expression occurred when cells were stimulated with 2 ng/mL TGF-β1, this concentration was chosen as optimal for aTreg generation.

To determine if TGF-β1-induced Foxp3 expression inversely correlated with IL-6 concentration, we activated total CD4+ T cells in the presence of two doses of IL-6 while keeping the concentration of TGF-β1 constant (Fig. 4B). Since the 100 ng/mL IL-6 concentration showed the biggest effect on Foxp3 suppression, we used this concentration for the rest of the experiments.

Since IL-6 and TGF-β1 had antagonistic effects on Foxp3 expression, we wanted to test if there was a concentration of TGF-β1 that was refractory to IL-6 mediated Foxp3 suppression. To do this, we cultured the T cells in the presence of 100 ng/mL IL-6 and differing doses of TGF-β1 and assessed the level of Foxp3 expression at 72 hours. There was an inverse correlation between the concentration of TGF-β1 and IL-6 mediated Foxp3 repression such that the lower the concentration of TGF-β1, Foxp3 suppression by IL-6 was more efficient (Fig. 4C). In addition, IL-6 suppressed Foxp3 even when TGF-β1 was at the upper limit of concentrations tested (5 ng/mL). Therefore, the balance between TGF-β1-induced Foxp3 and IL-6 mediated Foxp3 suppression was dependent on the actual concentration of the cytokines stimulating the T cells.

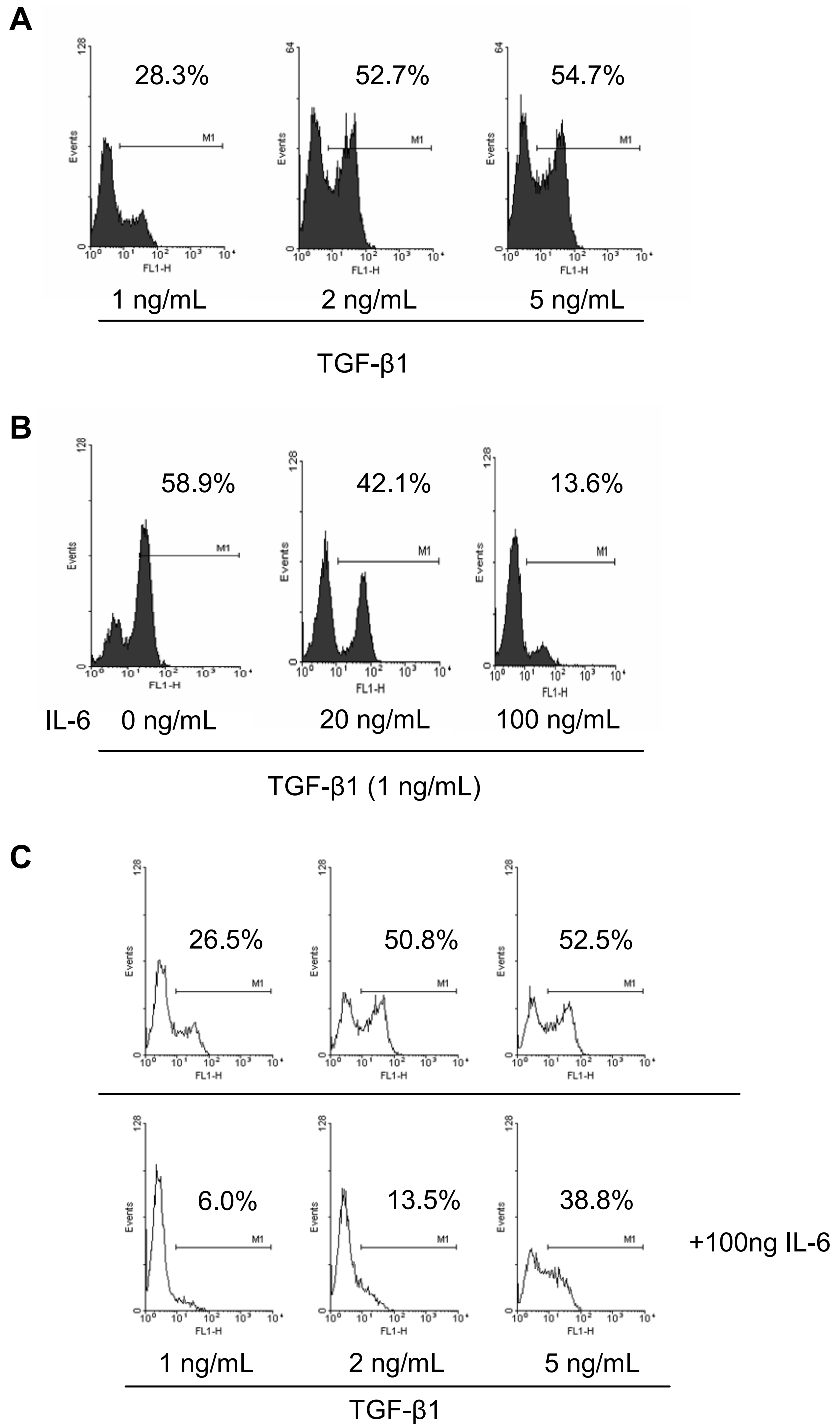

Figure 4. Foxp3 induction or repression are sensitive to the concentration of TGF-β1 and IL-6, respectively. (A) Foxp3+ T cells were determined after three days in culture with the indicated concentrations of TGF-β1 and anti-IL-4 (10 μg/mL). Numbers in histograms represent % of Foxp3+ T cells. (B) Foxp3+ T cells were determined after a five-day culture with the

indicated cytokines. Numbers in histograms represent % Foxp3+ T cells. (C) Foxp3 expression was determined from cells cultured with TGF-β1 ± IL-6 at the indicated concentrations. Numbers in histograms represent % Foxp3+ T cells. Data are representative of at least 2 independent experiments.

Previous studies showed that IL-6 and IL-21 can inhibit TGF-β1-induced Foxp3 expression and that this inhibition was STAT3-dependent (212, 274, 281, 282). However, it was not clear from those studies whether Foxp3 was inhibited from the outset or whether there was a dynamic downregulation of Foxp3 during the differentiation period. To test this, we performed time course studies examining Foxp3 induction using CD4+ T cells stimulated with anti-CD3 and anti-CD28 in the presence of either TGF-β1 alone (aTreg conditions) or with the combination of TGF-β1 and IL-6 (Th17 conditions). In the first 48 hours of culture, Foxp3 was similarly induced whether or not IL-6 was present (Fig. 5). After 48 hours of anti-CD3 stimulation, the presence of IL-6 in the culture decreased Foxp3 mRNA and protein levels while aTreg cultures maintained or increased Foxp3 expression throughout the 5-day culture period. The lack of induction of Foxp3 protein in cells cultured with IL-12 and anti-IL-4 (Th1 conditions) or in the absence of skewing cytokines (data not shown) demonstrates the requirement for TGF-β1 in the induction of Foxp3, consistent with previous studies (128).

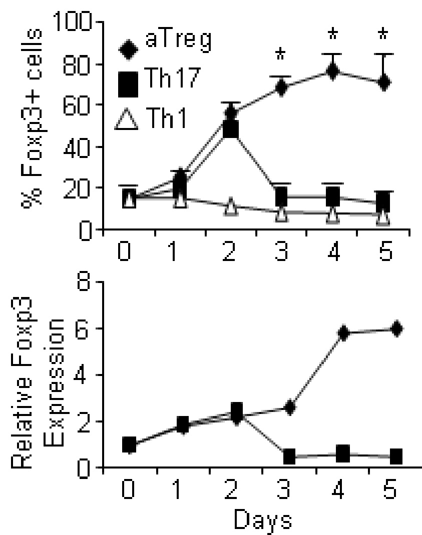

Figure 5. Temporal regulation of Foxp3. CD4+ T cells were activated with anti-CD3, anti-CD28, anti-IL-4 and 2 ng/ml TGF-β1 in the presence (Th17 conditions) or absence (aTreg conditions) of 100 ng/ml IL-6, or under Th1 conditions (IL-12 and anti-IL-4) for 5 days. Cells were collected daily and stained for Foxp3 or used for RNA isolation. Analysis of Foxp3 expression by qPCR was normalized to β_2-microglobulin expression and data are represented as fold induction relative to freshly isolated WT CD4+ T cells. Data are presented as mean % Foxp3+ ± SD from two independent replicates while qPCR data are represented as mean ± SD of duplicate samples and are representative of at least three independent experiments. *, significantly different (p<.05) from cells cultured under Th17 or Th1 conditions using unpaired Student's T-test. Foxp3 protein data are representative of 4 independent experiments.

IL-6 can repress Foxp3 only when present at the beginning of T cell differentiation

Since there was a similar initial induction of Foxp3 in both aTreg and Th17 culture conditions, we wanted to assess whether there was a temporal requirement for IL-6 mediated Foxp3 repression. In other words, we wondered

whether the presence of IL-6 was required at the time of TCR stimulation or whether adding IL-6 later in the culture was sufficient to downregulate Foxp3. As shown in Fig. 6A, Foxp3 was repressed as long as IL-6 was present within the first 24 hours of TCR stimulation. Interestingly, aTreg cells became refractory to IL-6 stimulation if added after 48 hours of TCR stimulation. We thought it was possible that aTreg cells downregulated the IL-6R (CD130) by 48 hours so that IL-6 could not initiate STAT3 activation. To address this possiblility, we analyzed surface level expression of IL-6Rα in aTreg cells at 24 hour intervals and found that the IL-6Rα expression recovered back to baseline levels by 48 hours and remained at those levels for the rest of the culture period (Fig. 6B and data not shown). Therefore, the lack of IL-6R did not account for this refractoriness. This suggests that IL-6 has a limited time to downregulate TGF-β1-induced Foxp3 that is independent of IL-6Rα expression levels.

Another possibility we examined for the aTregs being refractory to IL-6 mediated Foxp3 suppression was whether TGF-β1 inhibited IL-6 mediated upregulation of the Th17 transcription factor RORγt. To determine this, we examined RORγt mRNA levels at the end of the five-day culture when IL-6 was added at different time points. Interestingly, RORγt was not upregulated as efficiently if IL-6 was added 48 hours or later into culture (Fig. 6C). This result suggested that TGF-β1 was sufficient to inhibit RORγt upregulation if IL-6 was not present within the first 24 hours of T cell activation. Moreover, this observation also implied that RORγt may play a role in IL-6 mediated Foxp3 repression since there was an inverse correlation between Foxp3 and RORγt expression.

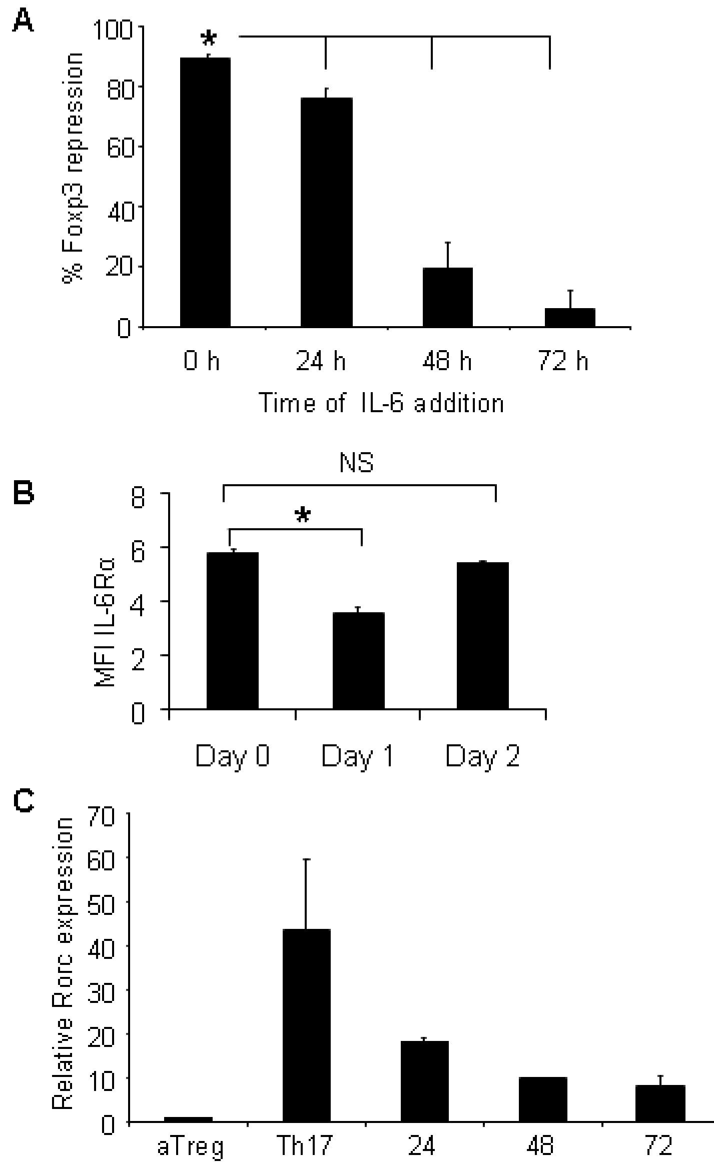

Figure 6. IL-6 is required in the first 24 hours post-TCR stimulation to repress Foxp3. (A) CD4+ T cells were activated with anti-CD3 and anti-CD28 and cultured under aTreg conditions. IL-6 (100 ng/mL) was then added to cells at 24 hour intervals from 0-72 h before intracellularly staining for Foxp3 expression after 5 days of culture. Data are presented as the mean % Foxp3 repression ± SD of experimental replicates. % Foxp3 repression=1-(% of Foxp3+ cells after IL-6 addition/ % Foxp3+ cells in aTreg conditions). *, Foxp3 repression at the 0 time point is significantly different ($p<.05$) than Foxp3 repression when IL-6 was added at any of the

other time points examined using unpaired Student's T-test. Data are representative of 2 independent experiments. (B) Cells cultured in aTreg conditions were collected at 24 and 48 hours and stained for IL-6Rα. Data are plotted as MFI of IL-6Rα ± SD of live gated CD4+ T cells. *, IL-6Rα expression levels at time 0 are significantly different ($p<.05$) than IL-6Rα levels at 24 hours using unpaired Student's T-test. NS=not significantly different ($p>.05$) using unpaired Student's T-test. (C) Analysis of Rorc expression by RT-PCR was normalized to β_2-microglobulin expression and data are presented as fold induction relative to five-day cultured aTreg cells. Data are presented as mean ± SD of individual experimental replicates.

Role of STAT3 in mediating the Th17/Treg lineage decision

STAT3, downstream of IL-6 and IL-21 signaling, is required to generate Th17 cells and repress Foxp3 expressing cells although it is unclear when STAT3 is required to repress Foxp3 and initiate Th17 differentiation (108, 141). To examine if the absence of STAT3 altered the temporal dynamics of Foxp3 repression, we performed a time course analysis comparing Foxp3 expression in Stat3$^{CD4-/-}$ and wild-type aTreg and Th17 cells. The absence of STAT3 completely prevented IL-6-mediated Foxp3 repression at all of the time points examined (Fig. 7A). Since *Stat3$^{CD4-/-}$* cells cultured under Th17 conditions still expressed Foxp3, we wanted to determine if IL-6-mediated STAT3 activation was also required to inhibit the suppressive function of aTregs. Therefore, we performed a suppressor assay to examine the ability of aTreg or Th17 cells to suppress the proliferation of activated CD4+CD25- T cells. *Stat3$^{CD4-/-}$* CD4+ T cells cultured in aTreg or Th17 conditions had a suppressive phenotype similar to wild-type aTreg cells. Thus, STAT3 is required downstream of IL-6 to inhibit Foxp3 expression and aTreg suppressor function (Fig. 7B).

These results suggested that expression of a constitutively active STAT3, even in the absence of endogenous STAT3, should be able to repress Foxp3. To

determine this, we activated *Stat3*$^{CD4-/-}$ CD4+ T cells in Th17 conditions and retrovirally transduced constitutively active STAT3 (STAT3C) or control virus at 24 hours post-TCR stimulation. After 5 days, we assessed Foxp3 mRNA and protein levels (Fig. 7C and D). Expression of STAT3C was able to repress Foxp3 transcription, with concomitantly higher levels of RORγt and IL-17 expression. Immunoblot analysis of protein extracts from these transduced cells demonstrated that STAT3C efficiently decreased Foxp3 protein levels. Interestingly, STAT3C transduced into cells cultured under aTreg conditions did not repress Foxp3 expression levels as efficiently as *Stat3*$^{CD4-/-}$ cells cultured under Th17 conditions suggesting that IL-6 stimulated pathways independent of STAT3 activation may play a role in Foxp3 repression (data not shown).

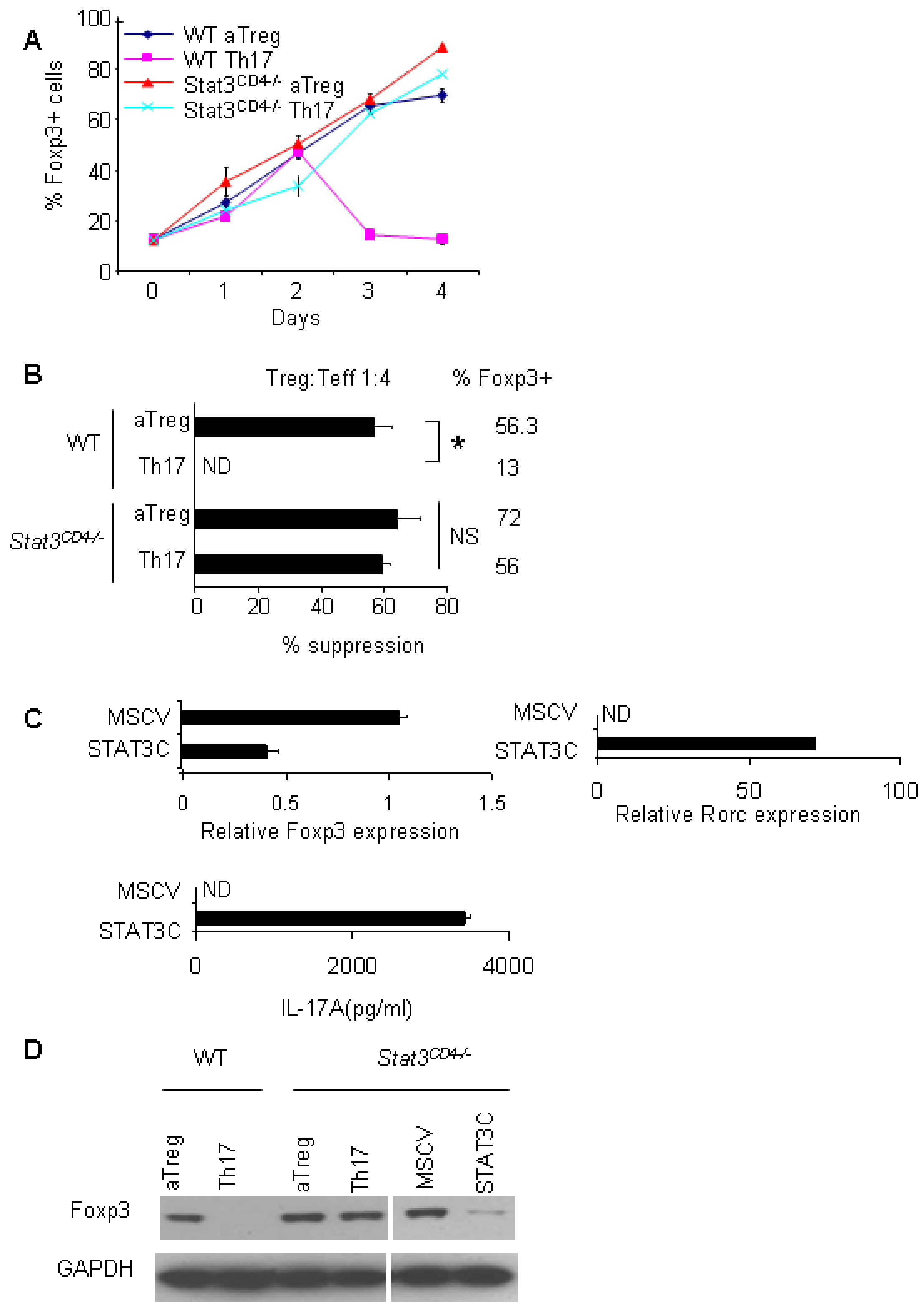

Figure 7. STAT3 is required downstream of IL-6 to inhibit Foxp3 expression and aTreg suppressor function. (A) Wild-type or *Stat3*$^{CD4-/-}$

CD4+ T cells were cultured under aTreg or Th17 conditions for 4 days. Cells were collected daily and stained for Foxp3. Data are presented as mean % Foxp3+ cells ± SD from two replicates. Data are representative of 3 experiments. (B) $5x10^4$ CD4+25- responder cells from Cre-negative littermate controls were stimulated in the presence of anti-CD3 and irradiated T cell depleted splenocytes in the presence or absence (CD4+25- cells alone) of cultured aTreg or Th17 cells isolated from either WT or $Stat3^{CD4-/-}$ CD4+ T cells (1:4 ratio of aTreg or Th17 cultured cells:CD4+25- cells). Percent suppression was calculated by comparing the mean of the proliferation of CD4+25- T cells with the proliferation of the co-cultured cells; % suppression=1- (mean proliferation of Th17 or aTreg cells cocultured with CD4+25- cells/mean proliferation of CD4+25- cells) ± SD of replicates. *, % suppression of wild-type aTregs is significantly different ($p<.05$) from wild-type Th17 cells using unpaired Student's T-test. ND=not detected. (C) CD4+ T cells from $Stat3^{CD4-/-}$ mice were cultured under Th17 conditions. After one day in culture, cells were transduced with control (MSCV-H-$2K^k$) or Stat3C-expressing retroviruses. On day 5 of differentiation, H-$2K^k$-positive cells were purified by magnetic selection and restimulated with 4μg/ml anti-CD3. Supernatants following 24 h of stimulation were tested for IL-17A levels using ELISA while the cells recovered from the 24 h stimulation were lysed in Trizol for RT-PCR analysis. ELISA data are presented as the mean ± SD of duplicate samples and are representative of two independent experiments. RNA was extracted from cells activated as described above and analyzed for Rorc and Foxp3 expression by real-time PCR. Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction to the WT aTreg condition. ND=not detected. (D) Immunoblot analysis of Foxp3 expression in WT and $Stat3^{CD4-/-}$ aTreg and Th17 cultures, and in $Stat3^{CD4-/-}$ Th17 cultures transduced with control or Stat3C-expressing retrovirus was performed using anti-Foxp3 and

GAPDH as a control. Data are representative of 2 independent experiments.

One thing that was unclear from the literature was whether all cytokines that activate STAT3 repressed TGF-β-induced Foxp3. To address the specificity of STAT3-activating cytokines in repressing Foxp3, we compared the ability of IL-6, IL-21, IL-10, IL-23 and Oncostatin M (OSM) in decreasing the generation of TGF-β1-induced aTreg cells. While IL-6 was the most potent cytokine in inhibiting Foxp3 expression, IL-21 was also capable of decreasing Foxp3 expression (Fig. 8), consistent with previous literature (107). In contrast, IL-23, IL-10 and OSM were not able to decrease the generation of Foxp3+ cells, despite their ability to modify the cytokine-secreting pattern of the cells in culture (Fig. 8 and data not shown). This suggests that there are may be additional requirements beyond STAT3 activation in the inhibition of Foxp3 expression.

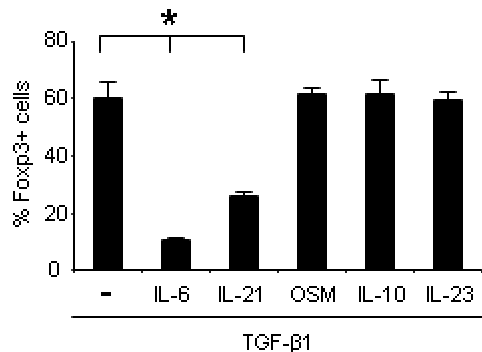

Figure 8. Cytokines that activate STAT3 are not sufficient to repress TGF-β1-induced Foxp3. (A) Foxp3 expression was determined in wild-type CD4+ T cells cultured for five days with 2 ng/mL TGF-β1 in the presence or absence of the indicated cytokines. The concentration of the cytokines used was as follows: IL-6 (100 ng/mL), IL-21 (50 ng/mL), Oncostatin M (OSM) (75 ng/mL), IL-10 (10 ng/mL) and IL-23 (10 ng/mL). Data are presented as mean % of Foxp3+ cells ± SD of replicates. *, Foxp3 expression in wild-type aTreg cells (designated as -) is significantly different ($p<0.05$) than cells cultured with IL-6 or IL-21 using unpaired Student's T-test.

Since the only STAT3-activating cytokines we examined that could repress Foxp3 were IL-6 and IL-21, we were interested in determining whether the STAT3-mediated Foxp3 repression downstream of IL-6 was direct or indirect. Therefore, we wanted to compare the temporal kinetics of the upregulation of Th17 genes and the downregulation of Foxp3 in the presence or absence of STAT3 to detect if there were upregulated STAT3-dependent Th17 genes that temporally coincided with Foxp3 repression. We performed qRT-PCR and compared the mRNA levels of the aTreg gene Foxp3 and the Th17 genes *Rorc, Il17a* and *Il17f* throughout the five-day culture between WT and *Stat3*$^{CD4-/-}$ cells cultured under Th17 conditions (Fig. 9A). Interestingly, wild-type aTreg and Th17 cells upregulated both Foxp3 and Th17 genes while *Stat3*$^{CD4-/-}$ Th17 cells did not show an induction of the Th17 genes in the first 48 hours of culture. In addition, Rorc expression was upregulated at the same time point that Foxp3 was transcriptionally repressed in the wild-type Th17 cells. In contrast, Rorc upregulation did not occur in the wild-type aTreg and *Stat3*$^{CD4-/-}$ Th17 cells. This suggested that the STAT3-dependent Foxp3 repression may result from the STAT3-dependent upregulation of Rorc.

To directly determine if RORγt could repress Foxp3 in the absence of endogenous STAT3, we transduced a RORγt-expressing or control retrovirus into *Stat3*$^{CD4-/-}$ CD4+ T cells cultured in Th17 conditions and assessed Foxp3 mRNA and protein levels (Fig. 9B and C). As observed with transduction of STAT3C, RORγt expression decreased Foxp3 mRNA levels and increased IL-17 mRNA. Thus, RORγt can repress Foxp3 transcription in the absence of endogenous STAT3. Interestingly, RORγt was not as efficient as STAT3C in repressing Foxp3 (Fig. 7 and Fig. 9C). One possible reason for more efficient Foxp3 repression by STAT3C was that we detected higher RORγt expression levels in the *Stat3*$^{CD4-/-}$ Th17 cells than retrovirally transducing RORγt (data not shown). Another possibility is that there is a STAT3-dependent, RORγt-independent mechanism of Foxp3 repression by IL-6.

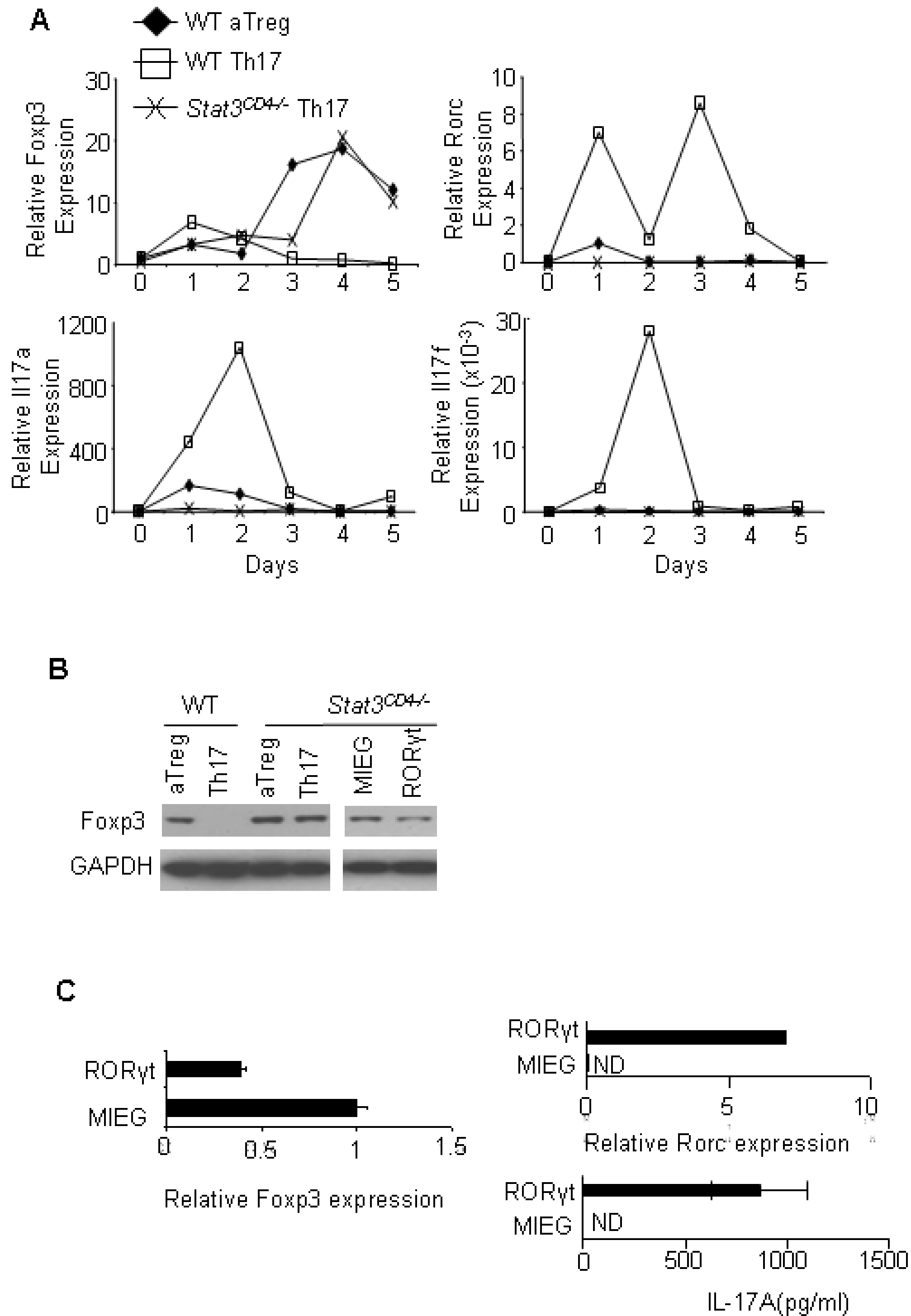

Figure 9. STAT3-dependent upregulation of RORγt is sufficient to repress TGF-β1 induced Foxp3. (A) Wild-type CD4+ T cells cultured under aTreg or Th17 conditions or *Stat3*$^{CD4-/-}$ CD4+ T cells cultured under Th17 conditions for 5 days were collected daily and used for RNA isolation.

Analysis of Foxp3 expression by RT-PCR was normalized to β_2-microglobulin expression and results are represented as fold induction relative to freshly isolated WT CD4+ T cells. Results are representative of 2 independent experiments. (B) and (C) CD4+ T cells from Stat3$^{CD4-/-}$ mice were cultured under Th17 conditions. After one day in culture, cells were transduced with control (MIEG-GFP) or RORγt-expressing retroviruses. On day 5 of differentiation, GFP-positive cells were purified by sorting and either lysed for RT-PCR and immunoblot analysis or restimulated with anti-CD3. (B) Immunoblot analysis of Foxp3 expression in *Stat3*$^{CD4-/-}$ Th17 cultures transduced with control or RORγt-expressing retrovirus was performed using anti-Foxp3 and GAPDH as a control. (C) RNA was extracted from cells activated as described above and analyzed for Rorc and Foxp3 expression by real-time PCR. Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction to the WT aTreg condition. Data are presented as mean ± SD of duplicate samples. Supernatants following 24 h of stimulation were tested for IL-17A levels using ELISA. ELISA data are presented as the mean ± SD of duplicate samples and are representative of two independent experiments. ND=not detectable.

Role of STAT1 in Foxp3 repression and the induction of the Th17 genetic program

STAT1 is a negative regulator of Th17 development (97, 156, 157). However, it was unclear whether STAT1, downstream of IL-6, also played a role in repressing Foxp3. To address these points, we cultured either *Stat1-/-* or wild-type CD4+ T cells in aTreg or Th17 conditions for 5 days and stained for Foxp3 at the end of the culture period. There was no significant difference between *Stat1-/-* and WT Foxp3+ cells in the aTreg or Th17 conditions (Fig. 10A). Consistent with previous literature, the absence of STAT1 resulted in an increase of IL-17 positive T cells as compared to the wild-type T cells in both aTreg and Th17 conditions (Fig. 10B). This suggested that STAT1 is required for negatively

regulating IL-17 production in both aTreg and Th17 subsets but IL-6 mediated Foxp3 repression was STAT1 independent.

To examine if the absence of STAT1 altered the temporal dynamics of Foxp3 repression, we performed a time course analysis comparing Foxp3 expression in *Stat1-/-* and wild-type CD4+ T cells cultured under aTreg and Th17 conditions (Fig. 10C). IL-6 repressed Foxp3 with similar kinetics in the presence or absence of STAT1 suggesting that STAT1 does not alter the kinetics of Foxp3 repression in Th17 development.

Since STAT1 had no effect on the repression of Foxp3 but did negatively regulate Th17 cell development, we wanted to determine if the gene expression patterns corresponded to the protein data. Therefore, we performed qRT-PCR for Foxp3 and Th17 genes in wild-type and *Stat1-/-* T cells cultured in aTreg and Th17 conditions (Fig. 10D). Interestingly, *Stat1-/-* Th17 cells had increased Foxp3 levels compared to wild-type aTreg cells. This increased message level did not correspond with the protein level, which was not significantly different than wild-type Th17 cells (Fig. 10C). These results suggested that STAT1 negatively regulated the transcription of aTreg and Th17 genes but this did not functionally result in decreased aTreg development as assessed by Foxp3 protein.

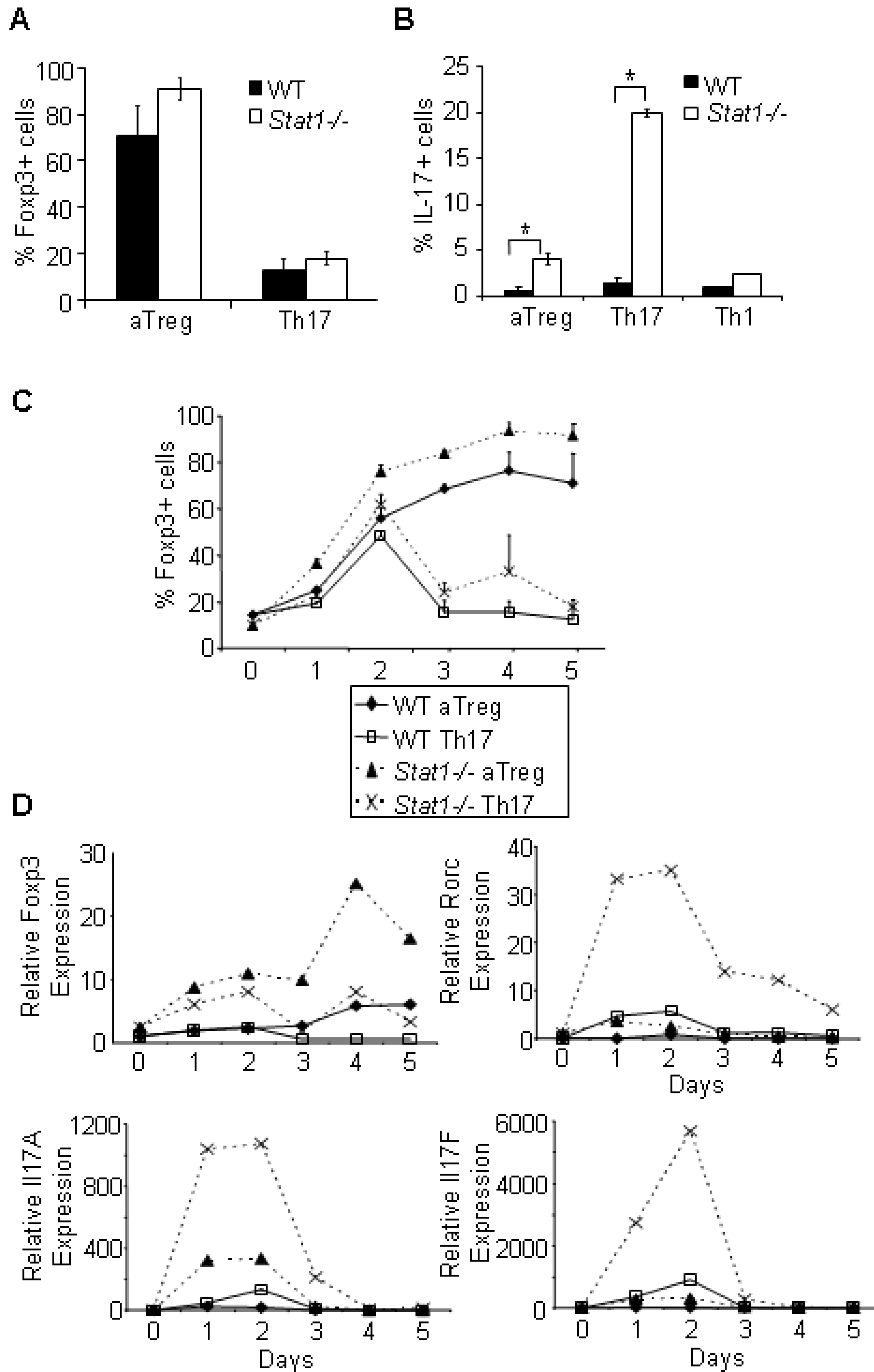

Figure 10. STAT1 is required to suppress the development of Th17 cells but does not play a role in Foxp3 repression upon IL-6 signaling. Percent of live gated CD4+ T cells that were Foxp3+ (A) or IL-17+ (B) was determined in *Stat1-/-* or wild-type T cells cultured under aTreg, Th17 or

Th1 conditions for five days. Data are presented as the mean % of Foxp3+ cells ± SD determined from replicates. *, IL-17 expression in wild-type aTreg or Th17 cells are significantly different (p<.05) than the *Stat1-/-* aTreg or Th17 cells, respectively, using unpaired Student's T-test. (C) Wild-type or *Stat1-/-* CD4+ T cells were cultured under aTreg or Th17 conditions for 5 days. Cells were collected daily and stained for Foxp3. Data are presented as the mean % of Foxp3+ cells ± SD determined from replicates. (D) RNA was extracted from cells activated as described in (C) and analyzed for the indicated genes by real-time PCR. Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction to freshly isolated WT T cells. Data are presented as the mean gene expression level ± SD of duplicate samples. Key is identical for (C) and (D). Data are representative of 2 independent experiments.

Since STAT1 and STAT3 are both activated by IL-6, we wanted to examine if the absence of one of the STAT proteins led to an increased activation of the remaining STAT protein. To accomplish this, we first confirmed that we could detect phospho-STAT3 (pSTAT3) by flow cytometry. To accomplish this, we stimulated CD4+ T cells with IL-6 for 1 hour and collected cells every 20 minutes. We did not detect any pSTAT3 at the 0 time point but did detect strong pSTAT3 from 20 minutes onward suggesting that the antibodies were specific for pSTAT3 (Fig. 11A). Subsequently, to determine if the absence of STAT1 increased IL-6 mediated STAT3 activation, we examined phosphorylated STAT3 levels at 24 hour intervals in *Stat1-/-* CD4+ T cells cultured in Th17 conditions. Interestingly, pSTAT3+ cells were increased in the *Stat1-/-* cells compared to wild-type cells cultured in Th17 conditions (Fig. 11B). These increased levels of pSTAT3 may partially explain the increased IL-17 levels seen in the *Stat1-/-* T cells. Alternatively, the increased pSTAT3 may result from increased STAT3-activating cytokines, such as IL-21, present in the culture of *Stat1-/-* Th17 cells compared to wild-type Th17 cells. The absence of STAT3 did not enhance STAT1 phosphorylation in the presence of IL-6 suggesting that the effect of increased

STAT3 activation due to *Stat1*-deficiency is specific (data not shown). Thus, mitigating STAT3 activation during IL-6 stimulation may be part of the mechanism by which STAT1 negatively regulates Th17 differentiation.

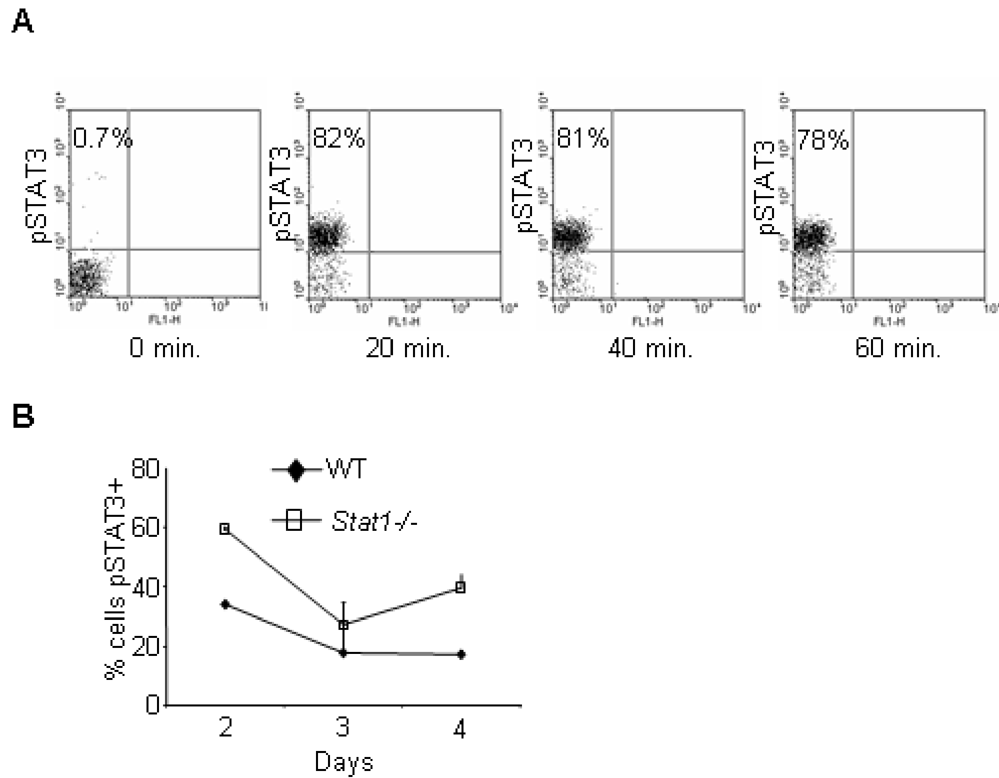

Figure 11. Absence of STAT1 increases IL-6-mediated STAT3 phosphorylation. (A) Total CD4+ T cells were stimulated with 100 ng/mL IL-6 for 1 hour and cells were collected every 20 minutes and intracellularly stained for pSTAT3. (B) Wild-type and *Stat1-/-* CD4+ T cells were cultured under Th17 conditions for 4 days. Cells were collected every 24 hours and stained for pSTAT3. Data are presented as the mean of % pSTAT3+ T cells ± SD. Data are representative of 2 experiments.

Role of T-bet in mediating the Th17/Treg lineage decision

T-bet, induced downstream of STAT1 activation, is an essential transcription factor for the generation of Th1 cells and the production of IFN-γ. T-bet is upregulated early in T helper cell differentiation and is recognized as a negative regulator of aTreg and Th17 development (154, 159). We wanted to examine the

role of T-bet in IL-6 mediated Foxp3 repression. To better discern whether the STAT3-dependent suppression of Foxp3 required T-bet, we examined the temporal kinetics of T-bet expression in the *Stat3*$^{CD4-/-}$ Th17 cells and compared it to wild-type Th17 cells (Fig. 12). We hypothesized that if T-bet was a factor in IL-6 mediated Foxp3 suppression, then T-bet would be suppressed in the *Stat3*$^{CD4-/-}$ Th17 cells compared to wild-type Th17 cells. However, the *Stat3*$^{CD4-/-}$ had similar expression levels of T-bet compared to the wild-type Th17 cells suggesting that the suppression of Foxp3 by IL-6 is independent of T-bet.

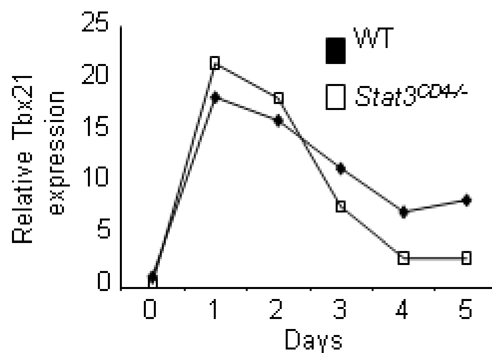

Figure 12. T-bet upregulation by IL-6 is independent of STAT3. RNA was extracted from wild-type and *Stat3*$^{CD4-/-}$ cells cultured under Th17 conditions at the indicated time points and analyzed for Tbx21 by qPCR. Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction to freshly isolated WT T cells. Data are represented as the mean ± SD of duplicate samples. Results are representative of 2 independent experiments.

To extend these findings, we examined the temporal kinetics of Foxp3 expression in *Tbx21-/-* and wild-type CD4+ T cells cultured under aTreg and Th17 conditions (Fig. 13A). Consistent with the literature, we observed an increased amount of Foxp3+ cells in the aTreg *Tbx21-/-* CD4+ T cells compared to the wild-type aTreg cells (154). However, we did not observe any difference in the ability of IL-6 to repress Foxp3 in either the kinetics or in the amount of cells expressing Foxp3. In addition, we verified the increased expression of IL-17 in *Tbx21*-deficient Th17 cells (Fig. 13B). Since *Tbx21*-deficient Th17 cells

repressed Foxp3 with the same kinetics as wild-type Th17 cells, it seemed unlikely that T-bet upregulation was required for IL-6 mediated Foxp3 repression.

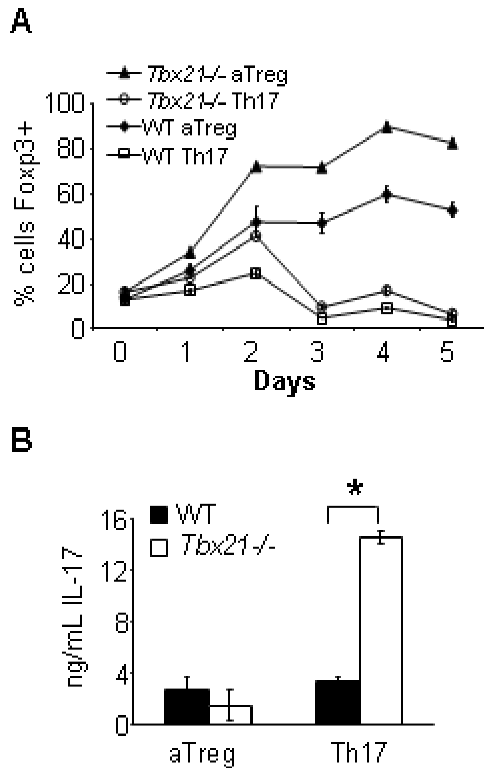

Figure 13. T-bet is not required for IL-6 mediated repression of Foxp3. (A) Wild-type or *Tbx21-/-* CD4+ T cells were cultured under aTreg or Th17 conditions for 5 days. Cells were collected daily and stained for Foxp3. Data are presented as the mean % of Foxp3+ cells ± SD determined from replicates. (B) 5-day aTreg or Th17 cultured cells from wild-type or *Tbx21-/-* were restimulated with anti-CD3 for 24 hours. Cell-free supernatants were collected and analyzed for IL-17 expression by ELISA. Data are presented as mean ± SD of duplicates. *, IL-17 expression levels in the wild-type Th17 cells are significantly different than the *Tbx21-/-* Th17 cells using unpaired Student's T-test. Results are representative of two independent experiments.

IL-4 and IL-12 can repress TGF-β1 induced Foxp3 through STAT-dependent mechanisms

The ability of IL-6 to divert the differentiation of aTreg cells into Th17 cells suggests that in an inflammatory cytokine environment, the development of aTreg cells is inhibited. It seemed logical that during the development of Th1- or Th2-mediated inflammation, aTreg development might also be inhibited. To test this hypothesis, we examined cells cultured in Th1 (IL-12 + anti-IL-4) or Th2 (IL-4 + anti-IFN-γ) conditions in the presence of TGF-β1 for Foxp3 expression and suppressor activity compared with cells cultured in aTreg (TGF-β1 + anti-IL-4) or Th17 (TGF-β1 + IL-6 + anti-IL-4) conditions. IL-4 repressed TGF-β1-induced Foxp3 expression and suppressor activity as efficiently as Th17 culture conditions (Fig. 14A). While a standard concentration of IL-12 (5 ng/mL) used to differentiate Th1 cells was not as efficient at repressing Foxp3 as Th17 culture conditions, IL-12 was able to decrease Foxp3 expression and suppressor activity. Similar results were observed when naïve Th cells were used to initiate the cultures (data not shown).

We next tested the requirement for STAT4 and STAT6 in IL-12- or IL-4-mediated repression of TGF-β1-induced Foxp3 (Fig. 14B). Inhibition of Foxp3 was STAT6 dependent in the IL-4+TGF-β1 cultured cells. The inhibition of TGF-β1-induced Foxp3 by IL-12 was partially dependent upon STAT4 (Fig. 14B). The effects of *Stat*-deficiency were specific as IL-4 functioned normally in *Stat3*- or *Stat4*-deficient cells and IL-12 functioned normally in *Stat3*- and *Stat6*-deficient cells. Similarly, STAT3 was required for IL-6-stimulated Foxp3 repression, but not for the effects of IL-4 or IL-12. These results suggested that STAT3, STAT4, and STAT6 can independently repress Foxp3 in the requisite activating cytokine environments.

Similar to STAT3C, we hypothesized that a constitutively activated STAT6 should be able to repress TGF-β1 induced Foxp3. To examine this, we tested the development of aTreg cells in cultures that express a constitutively active *Stat6*

transgene (termed STAT6VT) (90). Wild type or STAT6VT transgenic T cells were cultured under aTreg, TGF-β1+IL-4, Th17 and TGF-β1+IL-12 conditions. As shown in Fig. 14C, Stat6VT expressing cells had greatly attenuated expression of Foxp3 in aTreg conditions and the low levels observed were diminished further by the addition of other differentiative cytokines. Thus, active STAT6, even in the absence of other cytokine signals, is sufficient to inhibit Foxp3 expression.

A recent report has suggested that the ability of IL-12 to decrease the generation of Foxp3 cells is entirely through the production of IFN-γ and the induction of T-bet (154, 229). To test this in our system, we compared the Foxp3 expression between wild-type and *Tbx21-/-* CD4+ Foxp3+ cells cultured in aTreg and TGF-β1+IL-12 conditions. In contrast to Wei et al., Foxp3 repression by IL-12 was partially dependent on T-bet (153). Indeed, the ability of IL-12 to inhibit the development of Foxp3+ cells in the absence of T-bet was decreased by 50% but, nevertheless, showed significant Foxp3 repression compared to aTreg cells (Fig. 14D). Thus, both STAT4 and T-bet contribute to the ability of IL-12 to inhibit Foxp3.

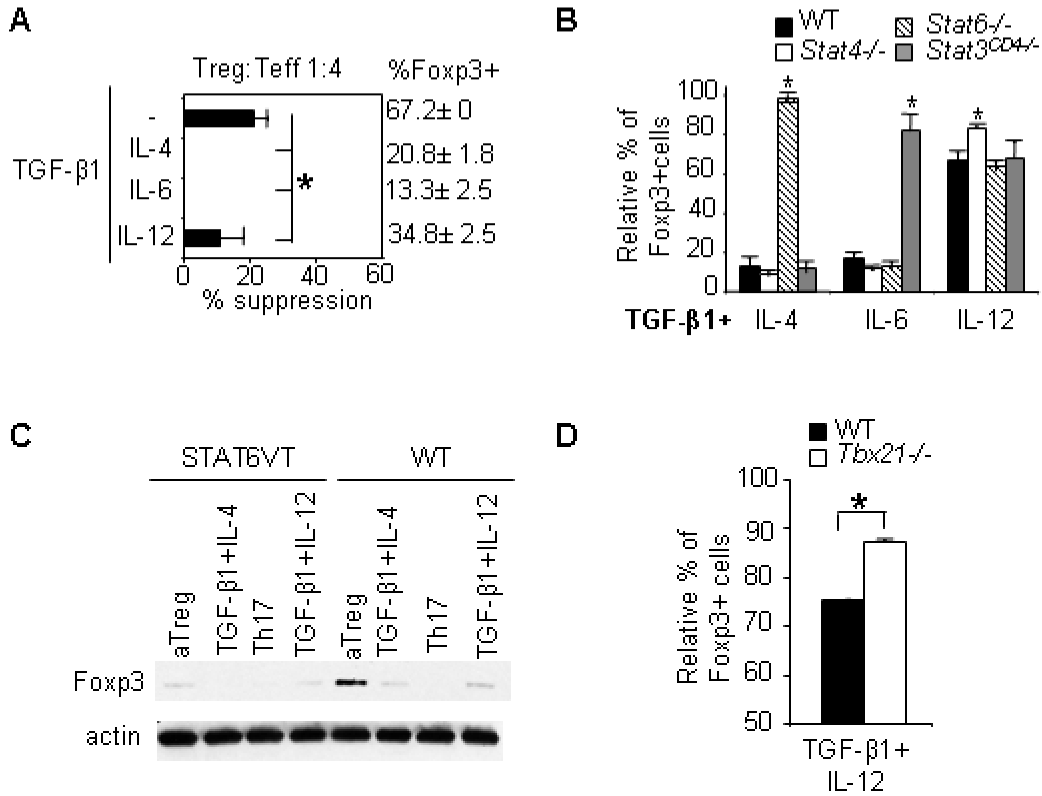

Figure 14. IL-4 and IL-12 repress Foxp3 and suppressive activity through STAT-dependent mechanisms. (A) CD4+25- responder cells from WT C57BL/6 mice were stimulated in the presence of anti-CD3 and irradiated T cell depleted splenocytes in the presence or absence (CD4+25- cells alone) of WT cells cultured in the indicated conditions (1:4 ratio of cultured cells:CD4+25- cells. Percent suppression was calculated by comparing the mean of the proliferation of CD4+25- T cells with the proliferation of the co-cultured cells; % suppression=1- (mean proliferation of Th17 or aTreg cells cocultured with CD4+25- cells/mean proliferation of CD4+25- cells) ± SD of triplicates. *, % suppression of wild-type aTreg cells (designated as -) is significantly different ($p<.05$) than the cells cultured with IL-4, IL-6 and IL-12 using unpaired Student's T-test. The data are representative of 2 independent experiments. (B) Five-day cultured cells from the indicated T helper cell culture conditions were assessed for the percentage of Foxp3+ cells ± SD from *Stat4*$^{-/-}$, *Stat6*$^{-/-}$ or *Stat3*$^{CD4-/-}$ mice

and WT strain matched controls. The relative % of Foxp3+ cells was calculated as % of Foxp3+ cells in cultures incubated with Th differentiative cytokine/ % of Foxp3+ cells in cultures with TGF-β1 alone x 100. Statistics were performed using an unpaired Student's T-test. Results are representative of 2-5 independent experiments and concentrations of IL-4 and IL-12 used are standard concentrations used to differentiate Th2 and Th1 cells, respectively. *, significantly different ($p<.05$) from WT cells cultured under the same conditions. (C) Purified CD4 T cells from wild type or Stat6VT transgenic were cultured as indicated in (A) and protein expression was determined using immunoblot for Foxp3 and actin as a control. (D) Foxp3 expression was determined in five-day TGF-β1 + IL-12 cultured CD4 T cells from wild type and *Tbx21-/-* mice and analyzed as in (B). *, % of Foxp3+ cells are significantly different ($p<0.05$) between wild-type and *Tbx21-/-* cells using unpaired Student's T-test. Results are representative of 2 independent experiments.

Altered cytokine production by cells cultured with TGF-β1 and instructive cytokines

TGF-β1 is a potent anti-inflammatory cytokine that can inhibit the development of Th1 and Th2 cells (283-286). Therefore, we wanted to explore the relationship between TGF-β1, Foxp3 expression, and Th subset specific proinflammatory cytokine secretion. More specifically, we wondered if the ability of TGF-β1 to suppress cytokine secretion was independent or dependent on its ability to upregulate Foxp3. To determine this, cells were cultured in Th2, TGF-β1+IL-4, Th1 or TGF-β1+IL-12 conditions and examined for their propensity to secrete Th1 or Th2 cytokines. Compared to aTreg cells, IL-4 and IL-12 increased the secretion of Th1 and Th2 cytokines even in the presence of TGF-β1, though levels were lower than those produced by cultures in the absence of TGF-β1 (Fig. 15). Despite Foxp3 repression by IL-4, we also observed IL-4-induced TGF-β1 production (Fig. 15), similar to previous reports of IL-4 enhancing TGF-

β1 from aTreg cultures (287). Thus, the combination of TGF-β1 and IL-4 or IL-12 generated T cell populations with low expression levels of Foxp3 and increased expression of effector cytokines compared to T cells cultured with TGF-β1 alone. However, the presence of TGF-β1 was sufficient to decrease the production of Th1 and Th2 cytokines even though those T cells expressed lower levels of Foxp3 compared to aTreg cells. These results suggested that the ability of TGF-β1 to inhibit IFN-γ and IL-4 production is not entirely dependent on Foxp3-mediated inhibition of Th1 and Th2 cytokine expression.

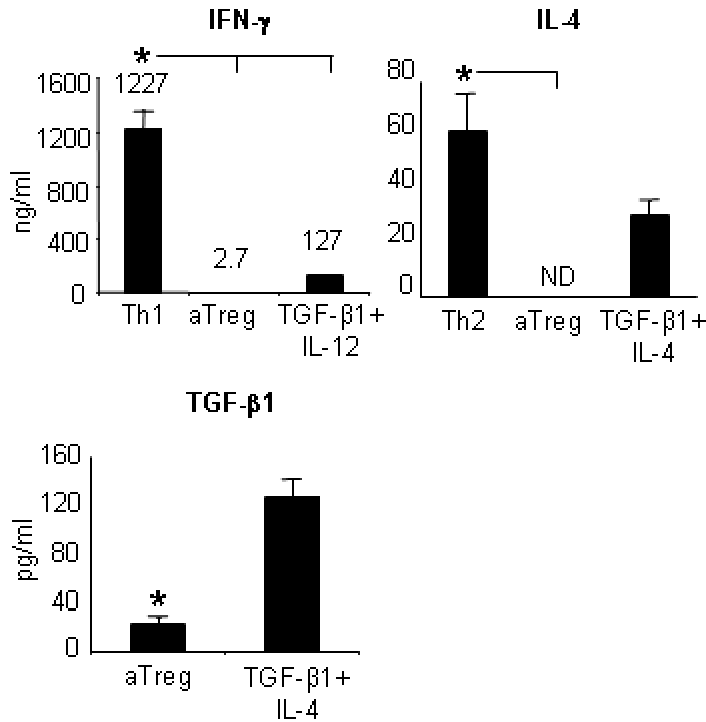

Figure 15. Cytokine production from Th cells incubated with TGF-β1 and instructive cytokines. CD4+ T cells cultured for five days under the indicated conditions were restimulated for 24 hours (IL-4 and IFN-γ) or 96 hours (TGF-β1) before cell free supernatants were collected for analysis of cytokine production using ELISA. Numbers above bars indicate the amount of measured cytokine. *, significantly different ($p<0.05$) from other conditions indicated by the line using unpaired Student's T-test. ND=cytokine level not detectable. Results are representative of 2 independent experiments.

Delayed addition of IL-4 and IL-12 can repress TGF-β1 induced Foxp3 with different kinetics

To begin to address if STAT4 and STAT6 inhibit the development of aTreg cells with similar kinetics as STAT3, we first wanted to determine when, during the differentiation period, Foxp3 was regulated. We examined Foxp3 induction using CD4+ T cells stimulated in aTreg, TGF-β1+IL-4 or TGF-β1+IL-12 conditions over a 5-day differentiation assay. Foxp3 expression was induced at day 2 and then significantly decreased in cultures where IL-4 or IL-12 had been added (Fig. 16A). Importantly, the kinetics of Foxp3 repression was similar to IL-6 (Fig. 5). This suggested the possibility that IL-4, IL-6 and IL-12 repressed Foxp3 by a common mechanism. Thus, we focused our analysis on the 48-72 hour time frame where Foxp3 was being actively regulated.

To determine if Foxp3 repression by IL-4 and IL-12 was subject to the same restricted time span to repress Foxp3 as observed for IL-6 (Fig. 4B), we performed a similar experiment where IL-4 or IL-12 were added at staggered 24 hour intervals from 0 to 72 hours after culture initiation (Fig. 16B). While the repression of Foxp3 by IL-12 was diminished when cytokine was added after 24 hours, IL-4 was effective at repressing Foxp3 when added at any point over the first 72 hours. The continued ability of IL-4 to repress Foxp3 was in contrast to IL-6 and IL-12 suggesting that IL-4 suppressed Foxp3 by a different mechanism than IL-6 or IL-12. However, it was still possible that the three cytokines upregulated a common factor that repressed Foxp3 but IL-4 stimulation was sufficient to upregulate this factor later in the culture while IL-6 and IL-12 stimulation could not. Thus, we decided to examine a variety of common factors upregulated downstream of STAT activation in an attempt to define a common mechanism by which STAT3, STAT4, and STAT6 repressed TGF-β1-induced Foxp3.

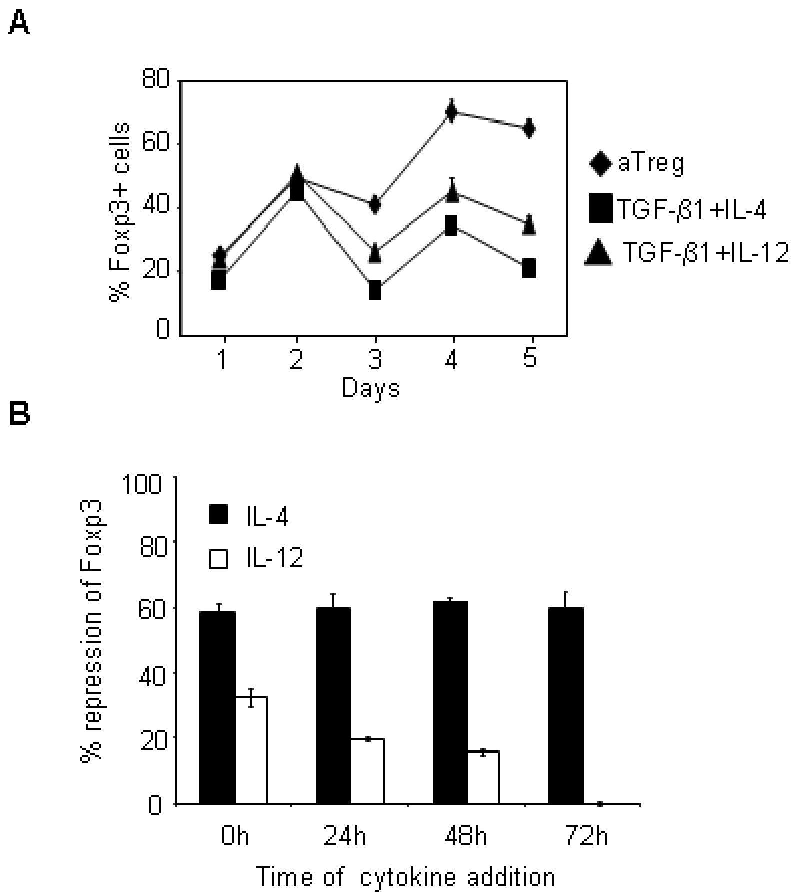

Figure 16. Delayed addition of IL-4 and IL-12 can repress Foxp3 with different kinetics. (A) Wild type CD4+ T cells were cultured in the indicated conditions, collected every 24 hours and stained for Foxp3 expression. Data are presented as mean % of Foxp3+ T cells ± SD from replicates. (B) CD4+ T cells were cultured with 2 ng/ml TGF-β1 with either 11B11 (for cells receiving IL-12) or R46A2 (for cells receiving IL-4). IL-4 or IL-12 was then added to cells at 24 hour intervals from 0-72 h before intracellularly staining for Foxp3 expression after 5 days of culture. Data are presented as the mean of % Foxp3 repression ± SD of replicates. Percent of Foxp3 repression was calculated as follows: % repression= 1-(% Foxp3+ cells after IL-4 or IL-12 addition/ % Foxp3+ cells in aTreg conditions). Results are representative of at least independent 3 experiments.

Induction of RORγt and IRF4 was not the common mechanism in STAT-dependent Foxp3 repression

Earlier, we observed that RORγt transduction could repress Foxp3 in *Stat3*$^{CD4-/-}$ T cells cultured in Th17 conditions. We wanted to examine whether the induction of RORγt was a common mechanism of Foxp3 repression in response to multiple cytokines. While RORγt is preferentially expressed in Th17 cells, expression has not been carefully examined during the differentiation of T cells with other cytokine combinations. We examined Rorc expression levels using qRT-PCR of RNA from cells cultured in aTreg, TGF-β1+IL-4, Th17 and TGF-β1+IL-12 conditions. TGF-β1+IL-4 and TGF-β1+IL-12 cultures had only minimal levels of RORγt compared to Th17 cultures (Fig. 17). Since IL-4 and IL-6 could repress Foxp3 to similar degrees and this did not correlate with RORγt expression, this suggested that IL-4 and IL-12 mediated Foxp3 repression is independent of RORγt induction.

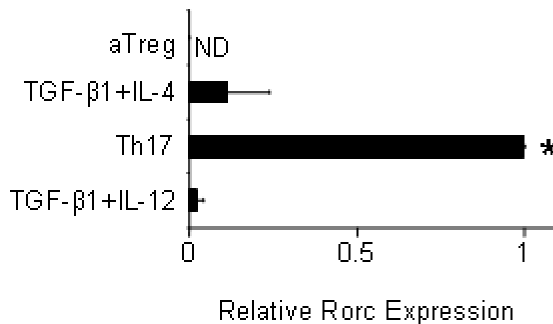

Figure 17. Induction of Rorc is not a common mechanism in STAT-dependent Foxp3 repression. Wild-type CD4+ cells were cultured in the indicated conditions for 3 days. Cells were collected, washed, and lysed in Trizol. RNA was extracted from cells and analyzed for Rorc expression by qPCR. Cycle number is normalized to β_2-microglobulin and results are represented as fold induction relative to the Th17 condition. Data are presented as the mean of replicates ± SD and is representative of independent 2 experiments. *, Rorc expression in Th17 cells is significantly different ($p<.05$) than all of the other conditions examined using unpaired Student's T-test.

We next wanted to examine the ability of the transcription factor Interferon Regulatory Factor-4 (IRF4) to mediate Foxp3 repression. It seemed a plausible candidate to examine since it is required for Th17 differentiation and *Irf4-/-* T cells have increased numbers of Foxp3 expressing cells compared to wild-type mice (288). To first determine if IRF4 can be induced by the instructive cytokines in a STAT-dependent manner, we examined IRF4 mRNA expression levels by qRT-PCR from RNA obtained from wild-type, *Stat6-/-*, and $\mathit{Stat3}^{CD4-/-}$ CD4+ T cells cultured in aTreg, TGF-β1+IL-4, Th17, and TGF-β1+IL-12 conditions (Fig. 18A, B and C). We observed an upregulation of IRF4 compared to aTreg cells in a STAT3 and STAT6 dependent manner. To examine if IRF4 directly repressed Foxp3, we transduced an IRF4-expressing retrovirus into wild-type and *Stat6-/-* TGF-β1+IL-4 cultured cells and examined intracellular Foxp3 at day 5. Since the bicistronic virus had a selectable hCD4 marker, we could easily differentiate transduced cells from non-transduced cells. We observed that IRF4 overexpression was unable to repress Foxp3 in the absence of STAT6 (Fig. 18D). Therefore, we concluded that IRF4 was not the common STAT-induced Foxp3 repressor.

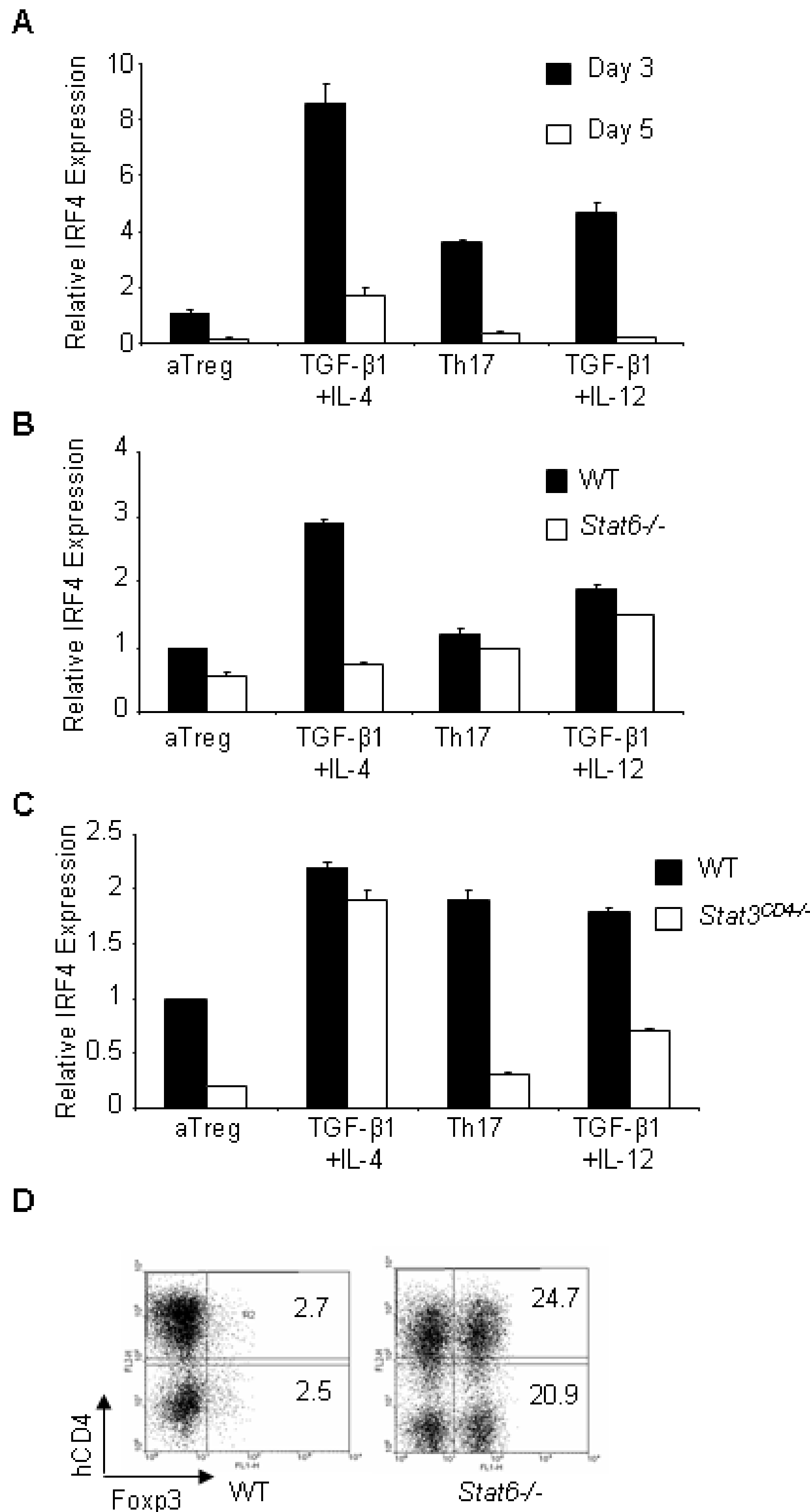

Figure 18. Foxp3 repression by STAT proteins is independent of IRF4 upregulation. (A) WT cells were cultured in the indicated conditions for 3

or 5 days. Cells were collected, washed, and lysed in Trizol. RNA was extracted from cells and analyzed for IRF4 expression by qPCR. Cycle number is normalized to β_2-microglobulin and results are represented as fold induction to the day 3 aTreg condition. Data are graphed as the mean of replicates ± SD and is representative of 2 independent experiments. WT and *Stat6-/-* (B) or WT and *Stat3*$^{CD4-/-}$ T cells (C) were cultured in the indicated conditions for 3 days. Cells were collected, washed, and lysed in Trizol. RNA was extracted from cells and analyzed for IRF4 expression by qPCR. Cycle number is normalized to β_2-microglobulin and results are represented as fold induction relative to the wild-type aTreg condition. Data are presented as the mean of replicates ± SD and is representative of 2 independent experiments. (D) CD4+ T cells from WT or *Stat6-/-* mice were cultured under TGF-β1+IL-4 conditions. After one day in culture, cells were transduced with control (MIEG-hCD4) or IRF4-expressing retroviruses. On day 5 of differentiation, hCD4 and Foxp3 were stained and analyzed by flow cytometry. hCD4+ T cells correspond to either control or IRF4 transduced cells. Numbers within quadrants represent % of total cells present in that quadrant.

Instructive cytokines do not alter Smad activation downstream of TGF-β1 signaling

We next wanted to examine if STAT activation altered the TGF-β signaling pathway as it related to Smad activation and TGF-βR2 expression. We hypothesized that STAT-dependent repression of Foxp3 could be due to a downregulation of the TGF-βR and a subsequent inhibition of Smad2 or Smad3 activation. To determine if the receptor expression was similar in the presence of instructive cytokines, we cultured cells in aTreg, TGF-β1+IL-4, Th17, or TGF-β1+IL-12 for 48 hours and examined surface receptor expression of TGF-βR2 in wild-type and *Stat3*$^{CD4-/-}$ CD4+ T cells (Fig. 19A). We observed that the addition of IL-4 and IL-6 modestly downregulated TGF-βR2 while IL-12 did not. Interestingly, the *Stat3*$^{CD4-/-}$ Th17 cultured cells had similar receptor expression

levels as the aTregs suggesting that the downregulation of the TGF-βR was STAT3-dependent. To analyze whether this downregulation functionally resulted in preventing TGF-β1 from activating Smad2 and Smad3, we took aliquots of cultured cells at 24 and 48 hours and determined whether the amounts of phosphorylated Smad2 and Smad3 differed between wild-type aTregs and cells cultured in Th17 or TGF-β1+IL-12 conditions by Western blot (Fig. 19B). Smad2 and Smad3 were phosphorylated to a similar degree as the aTreg cells when proinflammatory cytokines were present suggesting that the observed downregulation of TGF-βR2 did not translate to an inhibition of TGF-β signaling. This suggested that the STAT-dependent Foxp3 repression was independent of Smad activation.

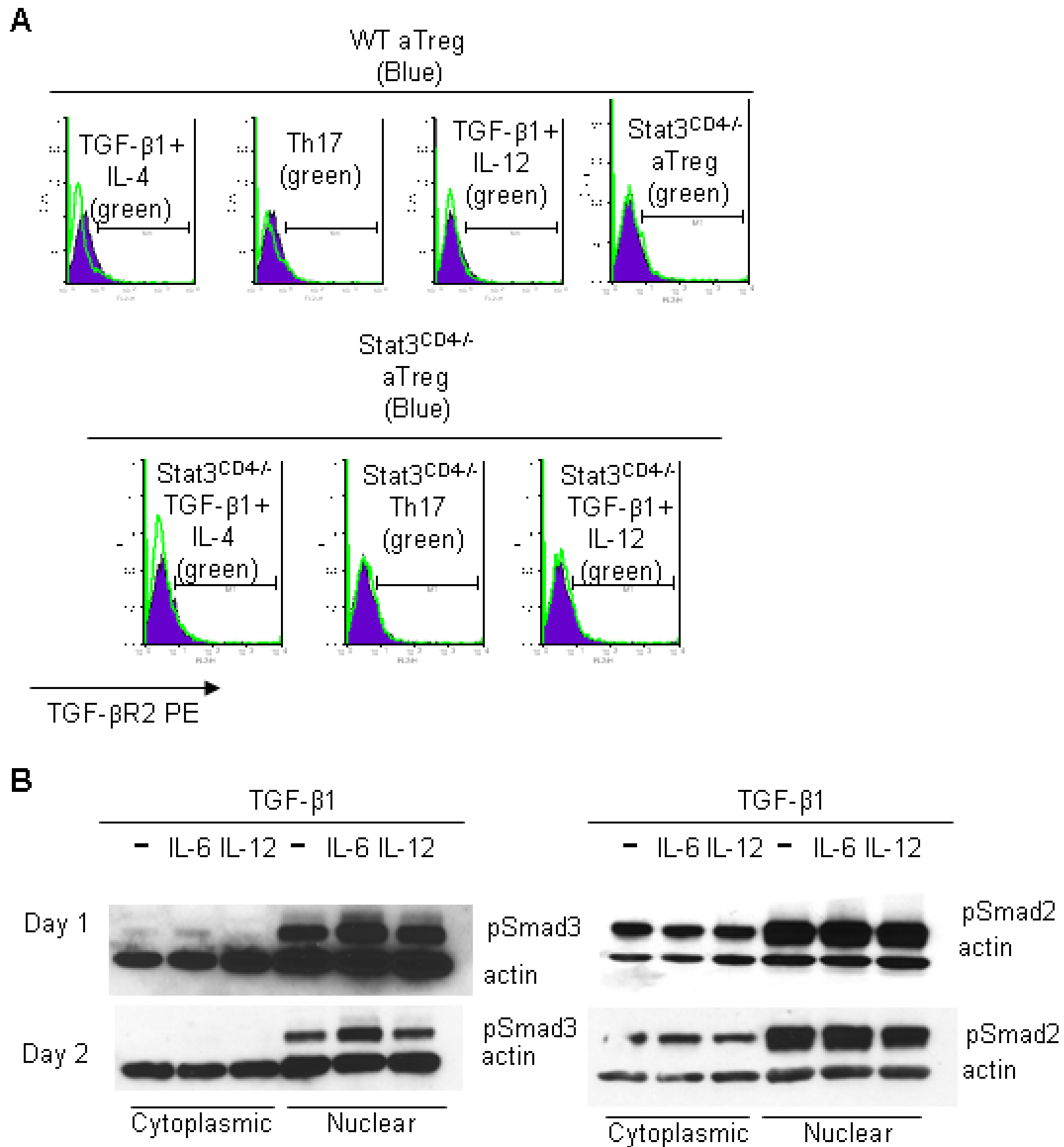

Figure 19. Smad activation downstream of TGF-β1 signaling is not altered in the presence of instructive cytokines. (A) Wild-type or *Stat3*$^{CD4-/-}$ CD4+ T cells cultured under the indicated conditions were surface stained for TGF-βR2 and analyzed by flow cytometry. Blue: wild-type aTregs (upper panel) or *Stat3*$^{CD4-/-}$ aTregs (lower panel). Green: the indicated conditions present within the histogram. (B) Wild-type cells cultured under the indicated conditions for 24 or 48 hours were collected and lysed into cytoplasmic and nuclear extracts. Immunoblot analysis of nuclear and

cytoplasmic extracts was performed using ant-pSmad3, anti-pSmad2, and actin as a loading control. -=no cytokine other than TGF-β1 added.

Cytokines that promote Th differentiation inhibit STAT5 binding to the **Foxp3** ***gene***

IL-2 in combination with TGF-β1 enhanced Foxp3 expression and repressed Th17 development (124, 131, 141, 212, 256, 289, 290). Moreover, neutralization of IL-2 inhibited the development of Foxp3+ cells (131). IL-2 functions through STAT5, which binds the promoter and first intron of the *Foxp3* gene to increase transcription (125, 141). In contrast, other STAT5-activating ©c cytokines like IL-7 and IL-15 cannot substitute for IL-2 in Foxp3 induction (131).

We hypothesized that a common mechanism for the STAT-dependent inhibition of Foxp3 was altering the IL-2-STAT5-dependent effects on Foxp3 expression. We first determined whether T helper-promoting cytokine treatment altered the production of endogenous IL-2 in vitro. In contrast to the effects of IL-4 and IL-6 on inhibiting Foxp3 expression, these cytokines increased the endogenous production of IL-2 (Fig. 20A). We then tested if additional exogenous IL-2 could prevent the downregulation of Foxp3 by IL-4, IL-6 or IL-12. The addition of IL-2 did not alter the repression of Foxp3 in the presence of any of the cytokines tested (Fig. 20B). Therefore, the STAT-dependent inhibition of Foxp3 was not due to a lack of IL-2 in the culture system.

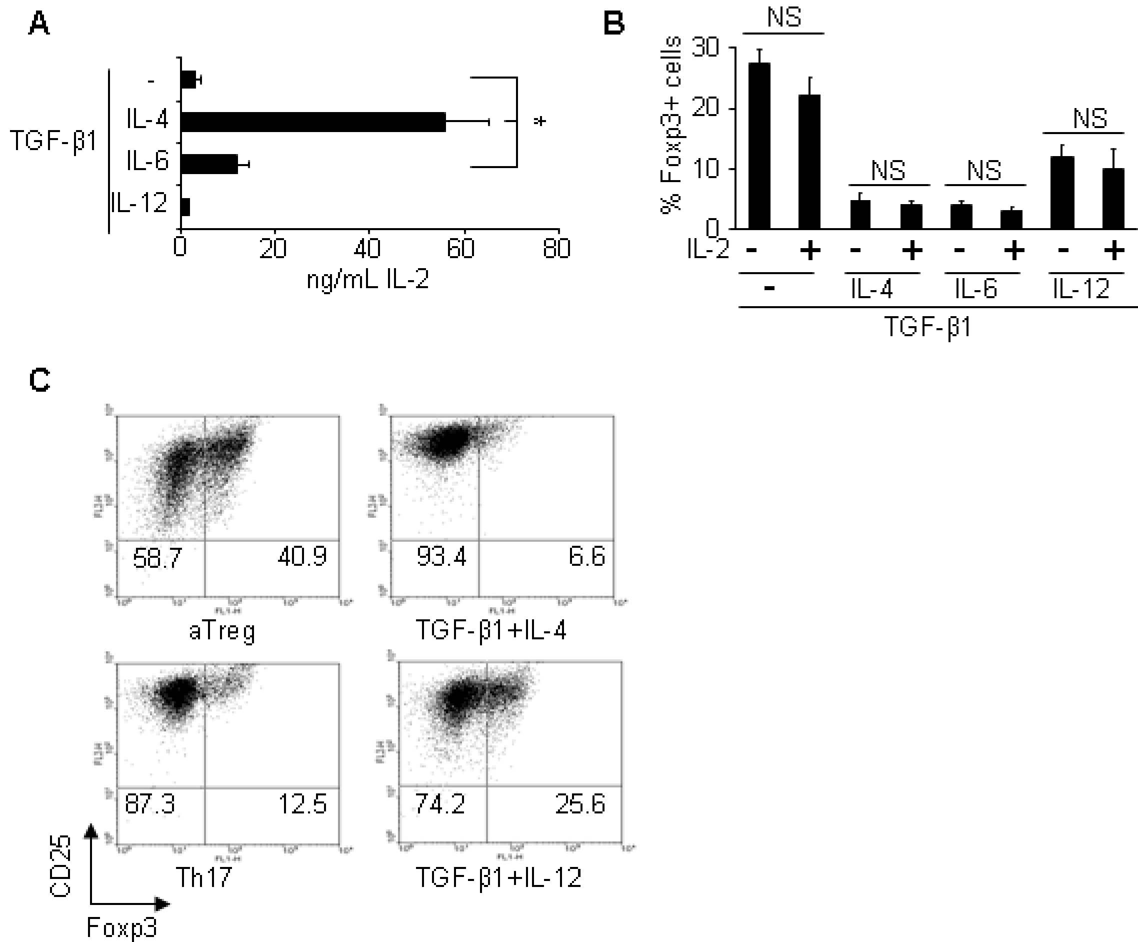

Figure 20. Normal IL-2 signaling and CD25 expression in cultures stimulated with Th instructive cytokines. (A) Cell-free supernatants were collected 48 hours after CD4+ T cells were plated in the indicated culture conditions. IL-2 production was tested using ELISA. Data are presented as mean ± SD of replicates. *, IL-2 production in cells cultured with IL-4 is significantly different (p<.05) from the other culture conditions underneath the line using unpaired Student's T-test. Results are representative of at least 2 independent experiments. (B) CD4+ T cells were cultured in the indicated conditions ± 100 U/ml hIL-2 for 3 days. After 3 days, cells were collected, washed, and stained for Foxp3 expression. NS=not statistically significant (p>0.05) between groups indicated by the line using unpaired Student's T-test. Data are presented as mean ± SD of replicates. Results are representative of 2 independent

experiments. (C) CD4+ T cells were cultured in the indicated conditions for 72 hours and stained with anti-CD25 and anti-Foxp3 for analysis by flow cytometry. Numbers represent % of cells present in the upper left and upper right quadrant above. Results are representative of 2 independent experiments.

We next wanted to examine if IL-2 signaling was affected by the presence of IL-4, IL-6 or IL-12 in these cultures. Expression of the inducible component of the IL-2R, CD25, was not compromised and was actually increased by the addition of IL-4 and IL-6 to cultures in both Foxp3+ and Foxp3- cells, concomitant with the decreased expression of Foxp3 (Fig. 20C). We then tested STAT5 phosphorylation levels during the first two days of culture by intracellular staining. Cells cultured with IL-4 and IL-6, which are the most effective in repressing Foxp3, had the highest levels of pSTAT5 (Fig. 21), correlating with CD25 expression (Fig. 20C) suggesting that activation of STAT5 was not compromised by the addition of Th instructive cytokines. Thus, IL-2 signaling was intact in aTreg cultures, even in the presence of other Th differentiative cytokines.

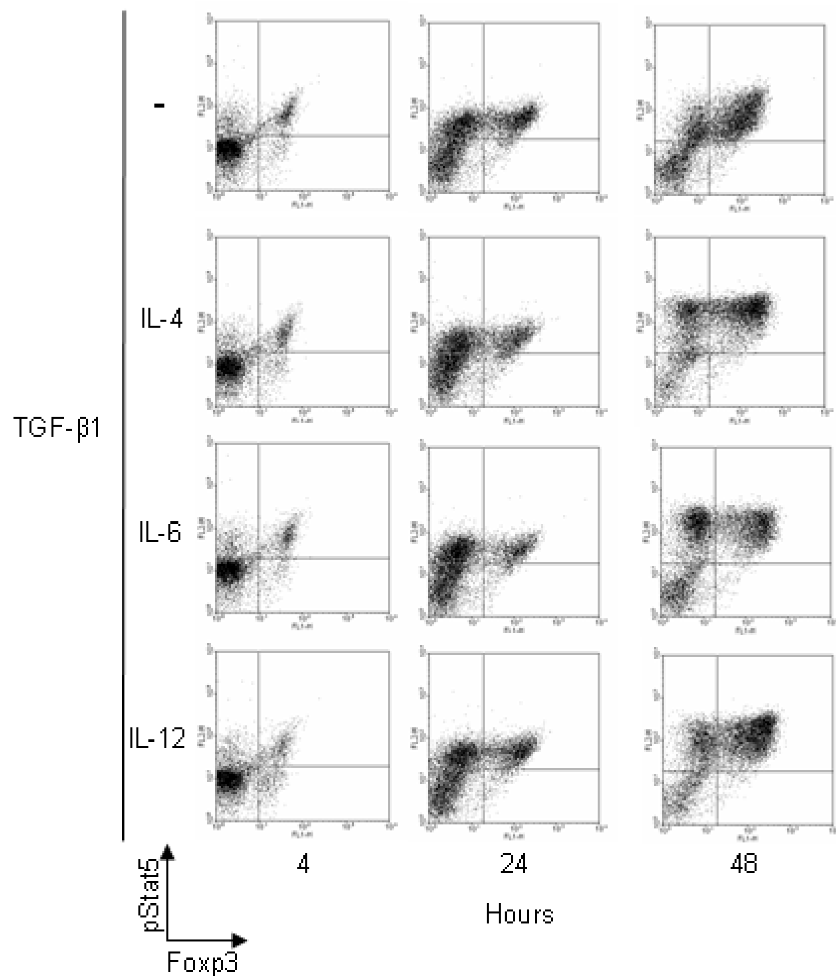

Figure 21. Normal pSTAT5 expression in cultures stimulated with Th instructive cytokines. CD4+ T cells cultured in the indicated conditions for 4, 24 or 48 hours were collected for staining intracellular pSTAT5 and Foxp3. Numbers of pSTAT5+ cells were not significantly different between the different culture conditions. Results are representative of 2-3 independent experiments.

Since altered IL-2 signaling did not account for the effects on Foxp3 expression, we hypothesized that the inability to maintain Foxp3 expression could be the result of activated STAT5 not being able to bind the *Foxp3* gene. To determine if STAT5 binding to *Foxp3* was decreased when aTreg cultures were exposed to

Th-promoting cytokines, we used chromatin immunoprecipitation (ChIP) to assess the level of STAT5 bound to the promoter and first intron in aTreg, TGF-β1+IL-4, Th17 or TGF-β1+IL-12 cultured cells at the 72 hour time point where there is active repression of Foxp3 (Fig. 3 and 16A). Compared to CD4+ T cells cultured under aTreg conditions, STAT5 binding to the *Foxp3* gene was inhibited when IL-4, IL-6 or IL-12 were present in the culture (Fig. 22A). To determine if chromatin at the Foxp3 locus was being altered by cytokine exposure, we performed ChIP for the H3K9me3 modification associated with gene repression (291-293). The addition of IL-4, IL-6 or IL-12 to TGF-β1-induced aTreg cultures resulted in a four-fold increase in this modification (Fig. 22B), suggesting that Th instructive cytokines participated in an active process to repress the *Foxp3* locus and that the decrease in STAT5 association with *Foxp3* resulted from decreased access to binding sites.

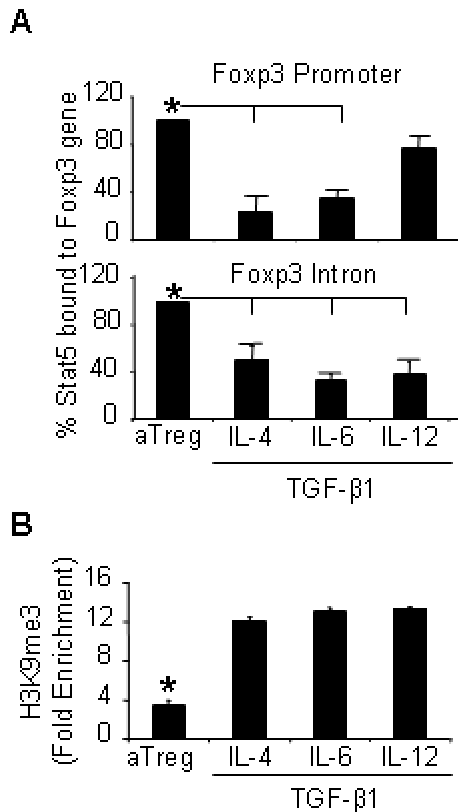

Figure 22. Decreased STAT5 binding to *Foxp3* following incubation with cytokines instructive in Th development. (A) Wild-type CD4+ T cells were cultured in the indicated conditions for 72 hours, collected, fixed, and

sonicated chromatin was precipitated with anti-Stat5. The amount of Stat5 bound to the indicated region in the aTreg culture conditions was set at 100% and the amount of Stat5 bound to the indicated regions in the other culture conditions was expressed in relation to the aTreg conditions. Data are presented as mean ± SD and are averages of 3 independent experiments. *, STAT5 bound to the Foxp3 promoter or intron is significantly different ($p<.05$) than STAT5 bound in the other conditions indicated by the line using unpaired Student's T-test. (B) Chromatin from cultures generated as in (A) was immunoprecipitated with antibodies to tri-methyl-H3K9. Results are expressed as fold-enrichment over ChIP with control IgG. *, the tri-methyl-H3K9 modification in the aTreg condition is significantly different ($p<.05$) than the modification in all of the other conditions examined using unpaired Student's T-test.

To explore possible mechanisms for decreased binding of STAT5 to the *Foxp3* gene, we wanted to determine whether STAT5 entered the nucleus in the presence of Th differentiative cytokines with similar efficiency with which activated STAT5 in aTregs can enter the nucleus. We obtained nuclear and cytoplasmic extracts of cells cultured for 48 hours in aTreg, Th17, and TGF-β1+IL-12 condtions and blotted for STAT5. Similar amounts of STAT5 were present in the nucleus suggesting that entry into the nucleus was not inhibited by IL-6 or IL-12 (Fig. 23A). To determine if STAT5 binding to the *Foxp3* promoter is altered when aTreg cultures are exposed to Th-promoting cytokines, we performed a DNA affinity precipitation assay (DAPA) using an oligonucleotide containing the sequence of the STAT5 binding site from the *Foxp3* promoter. The biotinylated oligonucleotide was used to precipitate bound protein using extracts from cells incubated in the various cytokine combinations for 48 hours. In aTreg extracts, STAT5 bound the oligonucleotide (Fig. 23B). In cultures stimulated with IL-4, IL-6 or IL-12, there was a decrease in the amount of precipitated STAT5 with a respective increase in the amount of STAT6, STAT3 and STAT4, suggesting that these factors interfered with the ability of STAT5 to

bind DNA. We demonstrated there is no overall defect in STAT5 activation (Fig. 20 and 21) and we have also observed that STAT5 binding to the CD25 promoter is not inhibited by these cytokines (data not shown), suggesting that the observed effects are gene-specific. While there could be a competition among the STAT proteins for binding to those sites, previous studies failed to identify significant STAT3 binding to the Foxp3 gene, suggesting that the effects of STAT3, STAT4 and STAT6 may be indirect. Importantly, stimulation with multiple Th instructive cytokines can inhibit TGF-β1-primed aTreg development and promote the development of inflammation through the common mechanism of prohibiting STAT5 access to bind the *Foxp3* gene.

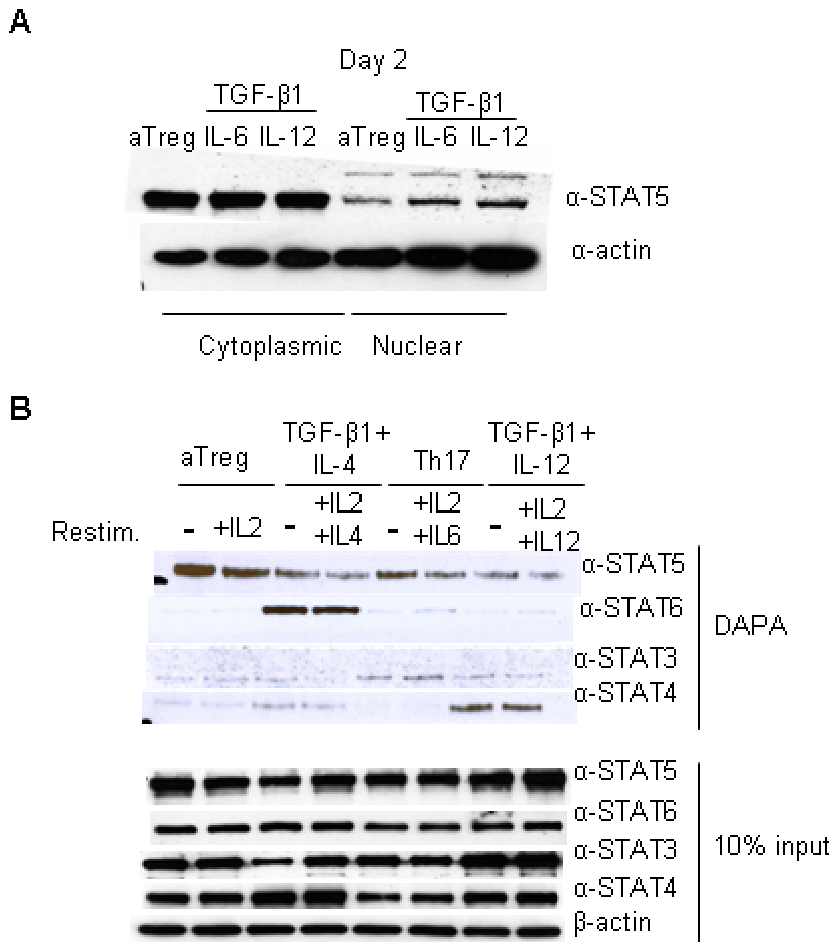

Figure 23. STAT3, STAT4, and STAT6 can directly compete with STAT5 to bind a *Foxp3* promoter oligonucleotide. (A) CD4+ T cells were cultured

in the indicated conditions for 48 hours. Cells were collected and lysed into cytoplasmic and nuclear extracts. Immunoblot was performed using anti-STAT5 and anti-actin as a loading control. (B) Whole cell extracts were prepared from cells cultured in the indicated conditions for 48 hours and cultured in the presence or absence of the indicated cytokines for one hour. These extracts were precleared with streptavidin-agarose and then incubated with biotinylated oligonucleotides coupled to streptavidin-conjugated agarose beads. Isolated proteins were subjected to SDS-PAGE and blotted onto membranes. Bound proteins were identified by Western blotting using the indicated STAT antibodies. Data are representative of 2 independent experiments.

Role of STAT proteins in nTreg Foxp3 expression and cytokine production

STAT proteins downstream of IL-4, IL-6 and IL-12 signaling suppressed Foxp3 expression and aTreg development. However, it was still unclear what role STAT proteins have on Foxp3 expression in the nTreg subset. Therefore, we undertook an analysis of nTreg function in the absence of STAT proteins. First, we wanted to characterize *Stat3*$^{CD4-/-}$ nTreg numbers in different lymphoid tissues, including thymus, spleen and mesenteric lymph nodes and compare them to wild-type littermate controls. Percentages of CD4+ T cells expressing Foxp3 in those three organs were similar suggesting that nTreg development and Foxp3 maintenance were not impaired in the absence of STAT3 (Fig. 24A). To compare the suppressive function between wild-type and *Stat3*$^{CD4-/-}$ nTregs we performed a suppressor assay after isolating nTregs from either genotype. *Stat3*$^{CD4-/-}$ nTregs were as suppressive as WT nTregs suggesting that STAT3 is not required for nTreg suppressor activity (Fig. 24B).

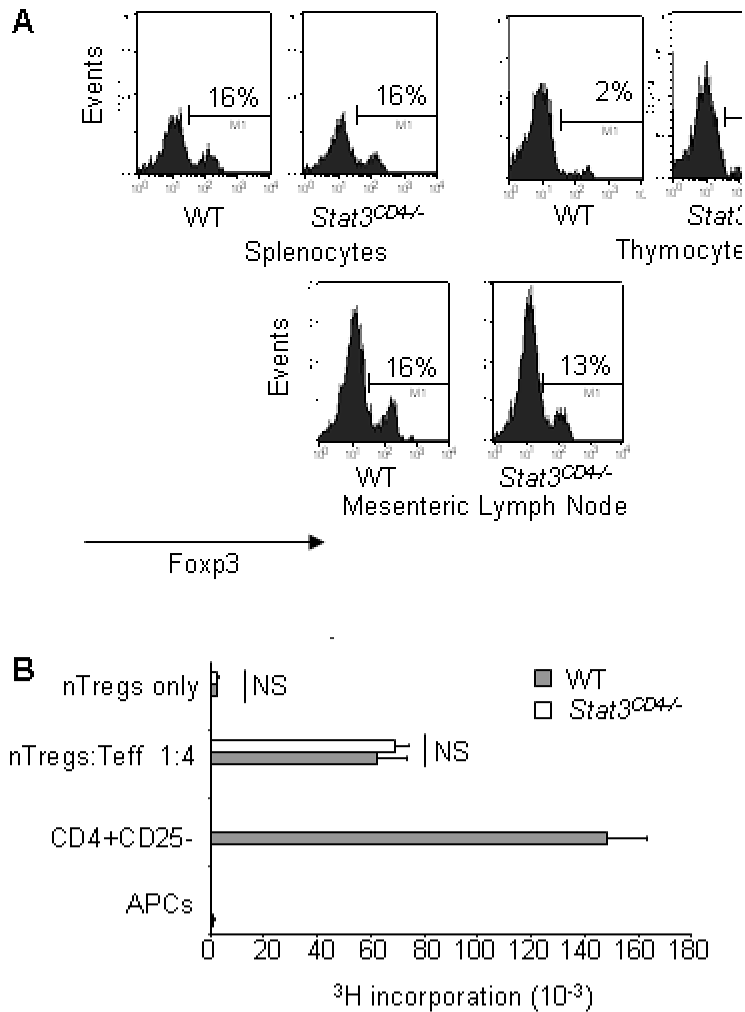

Figure 24. STAT3 is not required for nTreg development or suppressor activity. (A) WT or *Stat3*$^{CD4-/-}$ CD4+ T cells from the spleen, thymus and mesenteric lymph nodes were assessed for Foxp3 expression. Numbers within each histogram represent % of gated CD4+ live cells in each quadrant. Results are representative of 2 experiments. (B) CD4+25- responder cells from WT mice were stimulated in the presence of anti-CD3 and irradiated T cell depleted splenocytes in the presence or absence (CD4+25- cells alone) of WT or *Stat3*$^{CD4-/-}$ nTreg cells (1:4 ratio of nTreg:CD4+25- cell (Teff)). Data are presented as the mean cpm ± SD of triplicates. NS=not statistically significant (p>.05) using unpaired Student's T-test. The data are representative of 2 independent experiments.

We also characterized C57BL/6 and Balb/c nTreg function in the absence of STAT4 (C57BL/6 and Balb/c) and STAT6 (Balb/c) by performing suppressor assays. nTregs, in the presence or absence of STAT4 or STAT6, were equally suppressive suggesting that neither STAT4 nor STAT6 is required for nTreg suppressive function (Fig. 24).

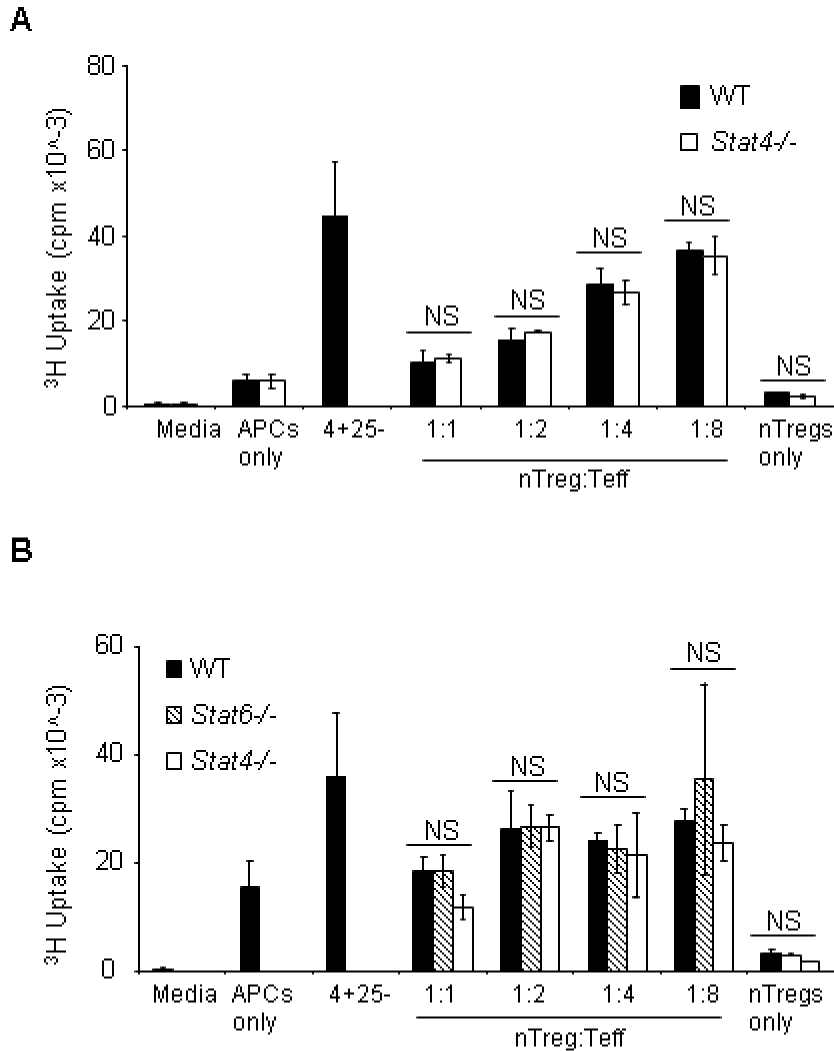

Figure 25. nTreg suppressor function is independent of STAT4 and STAT6. (A) CD4+CD25- cells ($5x10^4$) isolated from WT C57BL/6 mice were activated in the presence of mitomycin C treated T cell depleted WT splenocytes ($5x10^4$) and increasing numbers of CD4+CD25+ cells isolated from either WT or *Stat4-/-* mice (1:1, 1:2, 1:4 or 1:8 ratio of nTregs:CD4+CD25- cells (Teff)). The data are representative of 3

experiments and are expressed as mean cpm ± SD from triplicates. (B) CD4+CD25- cells ($5x10^4$) isolated from WT Balb/c mice activated and analyzed as in (A) with mitomycin C treated T cell depleted WT splenocytes ($5x10^4$) and increasing numbers of CD4+CD25+ cells isolated from WT, *Stat4-/-* and *Stat6-/-* Balb/c mice. NS=not statistically significant (p>.05) using unpaired Student's T-test. The data are representative of 3 independent experiments and are expressed as mean cpm ± SD from triplicates.

Since IL-4, IL-6 and IL-12 were able to suppress TGF-β1 induced Foxp3, we examined how those same cytokines affect Foxp3 expression and cytokine production in the nTreg subset. We isolated nTregs and cultured them in aTreg, TGF-β1+IL-4, Th17, and TGF-β1+IL-12 conditions for five days and examined Foxp3 expression at day 3 and day 5 of culture. Interestingly, Foxp3 expression in the presence of the instructive cytokines was maintained in the nTregs while it was repressed as expected in naïve CD4+ T cells (Fig. 26A). These results suggested that the maintenance of Foxp3 expression in the presence of the cytokines examined is regulated differently in the nTreg and aTreg subsets.

Since Foxp3 is known to negatively regulate proinflammatory cytokine production (236), we wanted to examine the cytokine profile of these five day cultured nTreg cells after an acute restimulation. Interestingly, nTregs cultured under any of the conditions except TGF-β1+IL-4 secreted significantly more IL-17 than naïve CD4+ T cells cultured under the same conditions (Fig. 26B). This result suggested that nTregs activated in the presence of TGF-β1, TGF-β1+IL-6 and TGF-β1+IL-12 secreted proinflammatory cytokines while maintaining Foxp3 expression. This suggested that high Foxp3 expression was not sufficient to repress proinflammatory cytokine secretion in nTreg cells. In contrast, nTregs cultured with TGF-β1+IL-4 not only maintained Foxp3 expression but also seemed to maintain their anti-inflammatory phenotype (Fig. 5A and B). This

suggested that, in the presence of TGF-β1, IL-4, unlike IL-6 and IL-12, may perpetuate anti-inflammatory properties of nTregs.

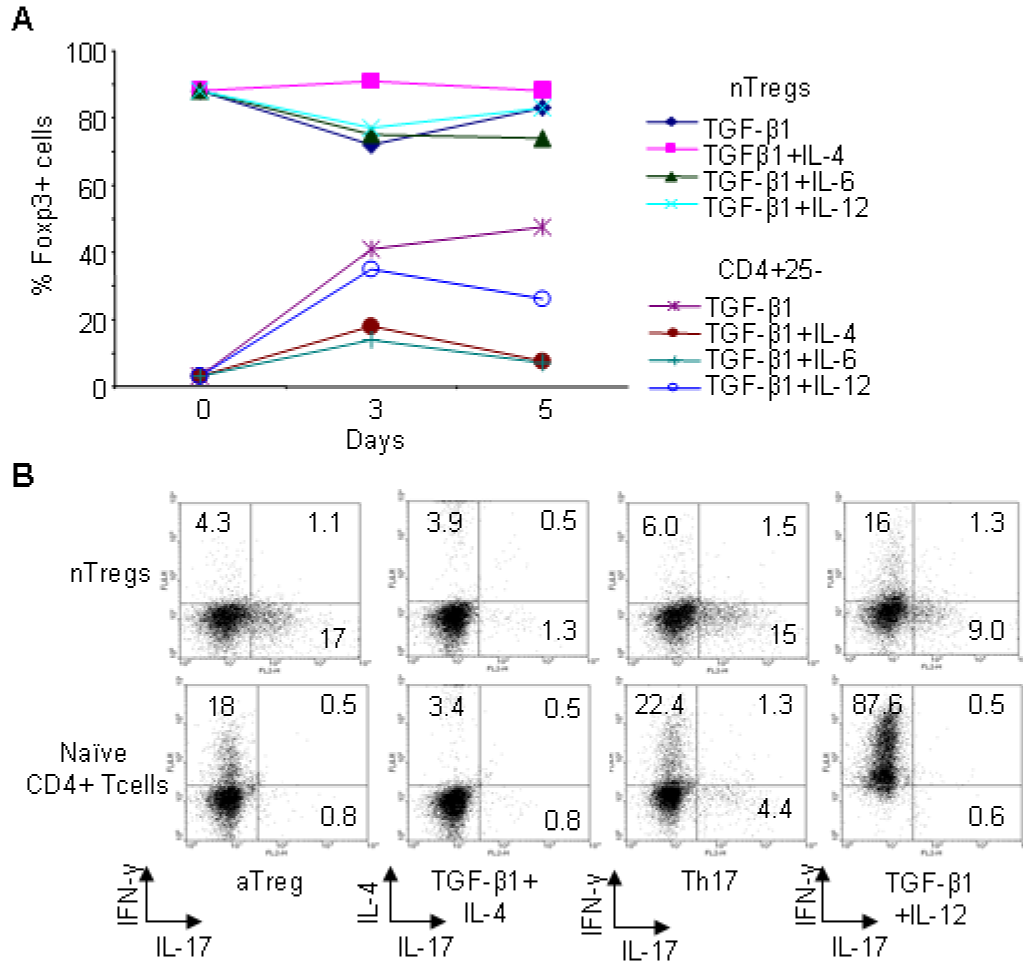

Figure 26. Instructive Th cyokines are unable to repress nTreg Foxp3 expression. (A) Wild-type nTreg (CD4+25+) or naïve (CD4+25-) T cells were cultured in the indicated conditions for five days and Foxp3 expression was determined by intracellular staining at day 3 and day 5 of culture. (B) Five-day cultured cells as in (A) were collected, washed, and restimulated with PMA+ionomycin for 4 hours and intracellularly stained for the indicated cytokines. Numbers within quadrants represent % of the total live gated CD4+ T cells in that quadrant.

We wanted to further characterize the role of IL-4 and STAT6 in the nTreg subset. Therefore, we incubated wild-type and *Stat6-/-* splenocytes in the

presence or absence of IL-4 for 24 hours and examined Foxp3 expression in CD4+ T cells (Fig. 27). Consistent with what was seen previously, IL-4 enhanced the Foxp3 expression levels per cell compared to splenocytes in the absence of IL-4 stimulation. Interestingly, the IL-4 enhancement of Foxp3 expression was STAT6 independent.

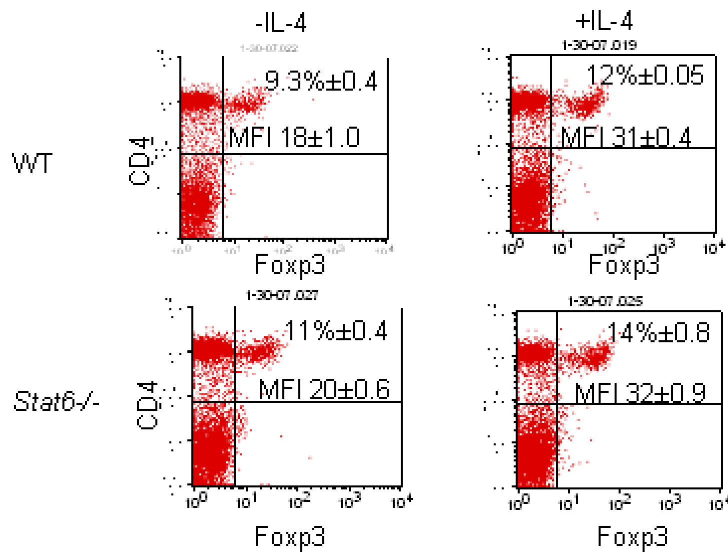

Figure 27. IL-4, independent of STAT6, increases Foxp3 expression in nTreg cells. Splenocytes from WT or *Stat6-/-* mice were stimulated in the presence or absence 10ng/mL IL-4 for 24 hours. Cells were then collected and stained for Foxp3. Data are presented as % of CD4+ T cells that are Foxp3+ ± SD and MFI of CD4+Foxp3+ T cells ± SD from triplicates.

To examine if STAT6 is functionally active in Treg cells, we confirmed that IL-4R is expressed (data not shown) and that pSTAT6 is induced by IL-4 treatment of the nTreg cells (Fig. 28). These results suggested that STAT6 does not play a role in IL-4 induced Foxp3 expression despite being functionally active.

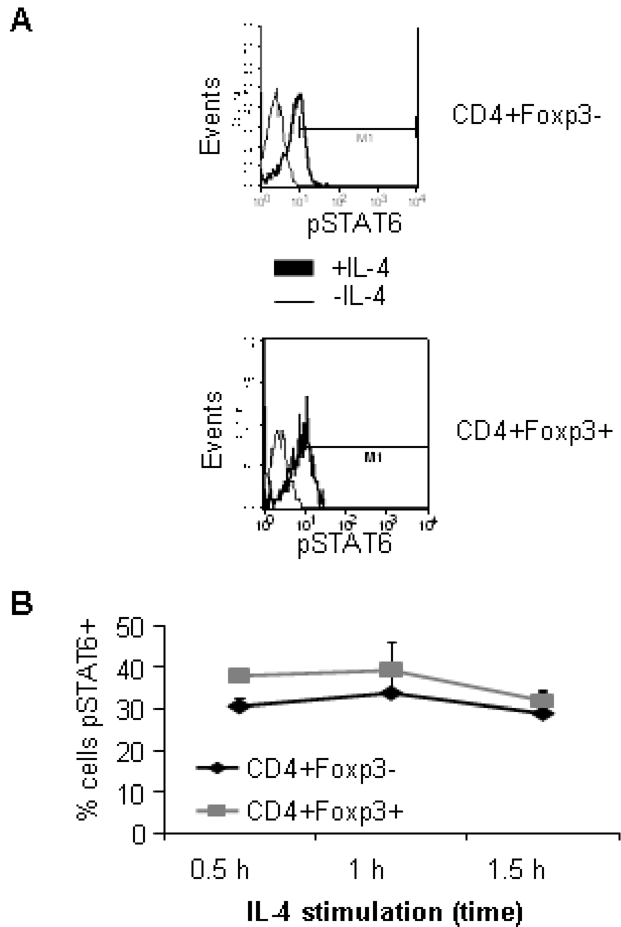

Figure 28. STAT6 is functionally active in nTreg cells. (A) and (B) Spleen cells were stimulated in the presence or absence of 10 ng/mL IL-4 for 30 min, 1 h, or 1.5 h. At each time point, cells were collected and stained for pStat6, CD4, and Foxp3. Histograms represent the 1 h time point and are gated on CD4+Foxp3- or CD4+ Foxp3+ as denoted. Error bars represent SD from duplicates. Results are representative from 2 independent experiments.

Natural Tregs, in the presence of IL-4, lose their suppressive function despite maintaining Foxp3 expression (294). Accordingly, we examined if the protein B cell lymphoma 6 (BCL6), a transcriptional repressor and inhibitor of GATA-3 expression and IL-4 production (295), had any effect on nTreg function. We performed a suppressor assay with *Bcl6-/-* and wild-type nTregs and observed that nTregs in the absence of BCL6 are significantly less repressive than wild-type nTregs (Fig. 29A). Since BCL6 negatively regulates IL-4 production we wanted to test if we could rescue the suppressiveness of *Bcl6-/-* nTregs by neutralizing IL-4 during the suppressor assay. Neutralizing IL-4 partly rescued the suppressive ability of *Bcl6-/-* nTregs to wild-type levels (Fig. 29B). Importantly, addition of IL-4 mitigated the suppressiveness of wild-type nTregs as expected. These results suggested that *Bcl6-/-* nTregs are less suppressive than wild-type nTregs because IL-4 is being secreted in the absence of BCL6. Thus, while IL-4 is important for increasing Foxp3 expression, the production of IL-4 by Foxp3 expressing cells inhibited their suppressive ability.

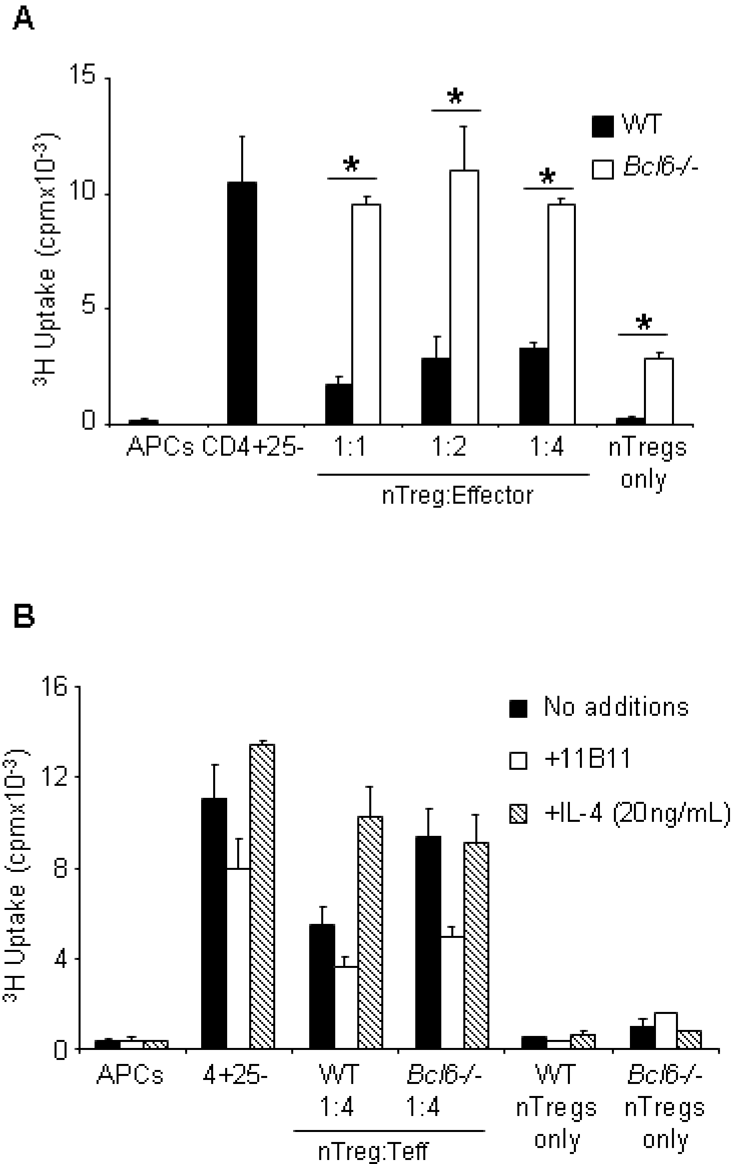

Figure 29. BCL6 is required for nTreg suppressive function. (A) CD4+25- responder cells from WT littermate-control mice were stimulated in the presence of anti-CD3 and irradiated T cell depleted splenocytes in the presence or absence (CD4+25- cells alone) of increasing numbers of nTreg cells from WT or *Bcl6-/-* mice (1:1, 1:2 and 1:4 ratio of nTreg cells:CD4+25- cells). Data was presented as cpm ± SD of triplicate samples. *, thymidine uptake by the wild-type cells is significantly different ($p<.05$) than the uptake by the Bcl6-/- cells as indicated by the lines using unpaired Student's T-test. The data are representative of 2 independent

experiments. (B) Suppression assay was prepared as in (A) except that, where indicated, the wells contained either a neutralizing antibody to IL-4 (11B11) or 20 ng/mL IL-4. Data are presented as cpm ± SD of triplicate samples.

STAT4 Structure/Function Analyses

Many studies have focused on structure/function relationships of the STAT proteins. Similar to different STAT proteins regulating a distinct subset of genes, domains within the STAT protein each have their unique functions. Two domains of interest due to their role in STAT activation and function are the N- and C-terminal domains of STAT4. We wanted to examine the role those domains play in STAT4 function and gene regulation of inflammatory cytokines.

Loss of STAT4 activation due to deletion of the N-terminal domain can be rescued by increasing intracellular Jak2 concentration

Previous results showed that the loss of the STAT4 N-terminal domain resulted in the inability of STAT4 to be activated upon IL-12 and IFN-α signaling (191, 192). Furthermore, it was shown that site-directed mutagenesis of amino acids predicted to be important for the N-terminal domain dimerization at amino acid positions D19 and L78 also resulted in the loss of STAT4 activation upon signaling (193). To further delineate the function of the N-terminal domain in activating STAT4, a series of N-terminal deletion mutant STAT4 plasmids were cotransfected into Cos7 cells, which lack endogenous STAT4 expression, with increasing concentrations of Jak2 plasmid in order to assay whether the loss of STAT4 activation upon the deletion of the N-terminal domain could be rescued with higher concentrations of Jak2 (Fig. 30A). In extracts of cells transfected with the full-length STAT4 and Δ10STAT4, there was basal phosphorylation that increased with increasing concentrations of Jak2. The Δ21STAT4 and mutants that lacked additional N-terminal residues displayed a complete loss of STAT4 phosphorylation that could be rescued to wild-type levels with increasing concentrations of Jak2. Thus, the loss of the region C-terminal to E11 resulted in

a loss of STAT4 activation. However, overexpressing the substrate for STAT4 activation, the Jak2 kinase, rescued STAT4 phosphorylation.

To further examine how Jak2 overexpression might rescue the ability of the N-terminal truncated STAT4 protein to be activated, an immunoprecipitation was performed between purified recombinant Jak2 protein complexed to agarose and either full-length STAT4 or a Δ88 STAT4 (Fig. 30B). The full-length STAT4 protein was consistently pulled down more efficiently with the Jak2 protein as compared to the Δ88 STAT4. This implied that the loss of the N-terminal domain resulted in a less efficient interaction between STAT4 and Jak2. Thus, impaired interaction with Jak2 may partially account for the complete loss of STAT4 activation seen in vivo (191, 192).

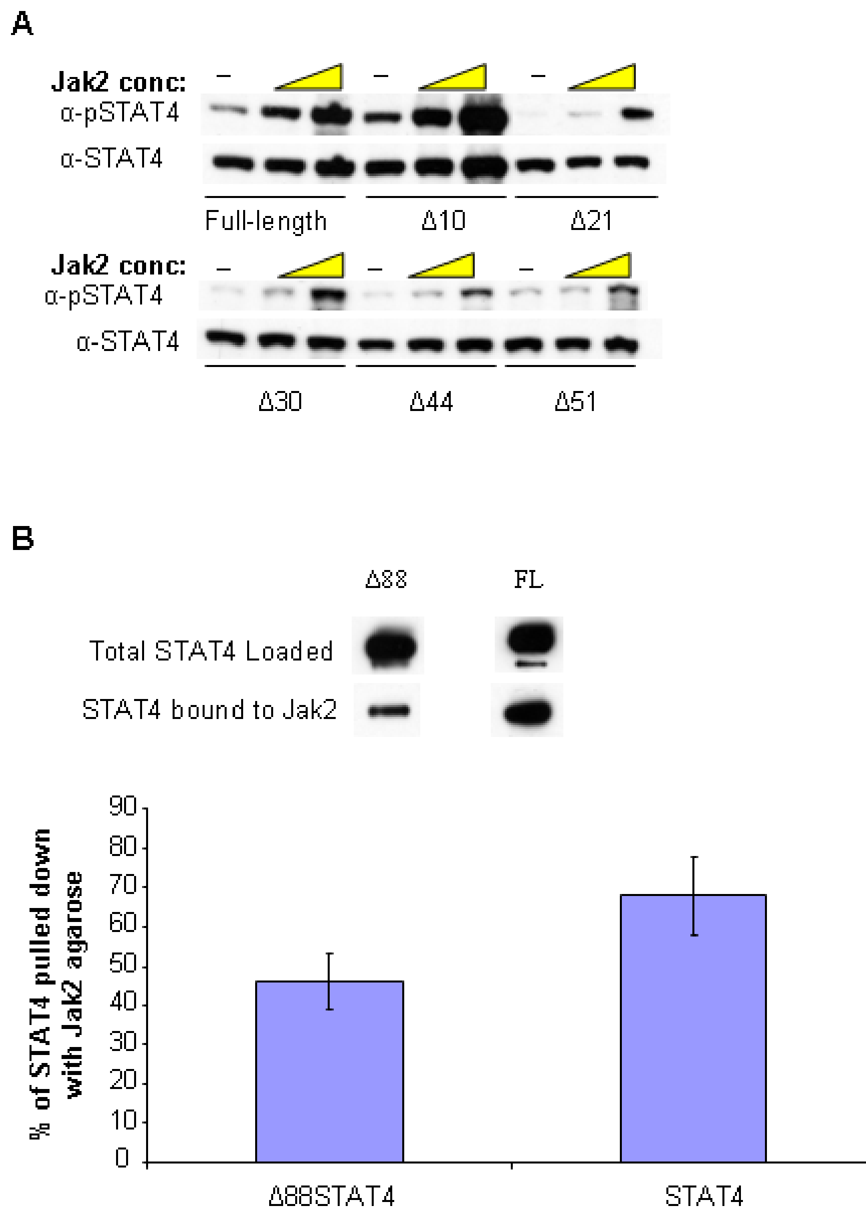

Figure 30. Loss of STAT4 activation in N-terminal deletion mutants can be rescued with increasing concentrations of intracellular Jak2. (A) Cos7 cells were transiently transfected with the indicated expression vectors (Δ#= # of amino acids deleted from N-termial domain of STAT4) and 0, 31, or 250 ng Jak2 plasmid. Cells were lysed after 48 hours and an immunoblot was performed with anti-pY STAT4 antibody or STAT4 antibody. (B) Either full-length (FL) or Δ88 STAT4 proteins were

incubated in the presence of a Jak2-Agarose complex. STAT4 bound to Jak2 was immunoprecipitated and resolved by SDS-PAGE and detected by Western blotting with anti-STAT4. STAT4 bound to Jak2 was compared to total input STAT4 by densitometry. Results are representative of 2 experiments.

Generation and characterization of STAT4 Estrogen Receptor (STAT4ERT2) constructs

Previous literature suggested that STAT4 activation was enhanced because STAT4 monomers were preassembled into nonphosphorylated dimers mediated by the N-terminal domain. We hypothesized that if we could inducibly dimerize the N-terminal deletion mutants, we could examine if dimerization is sufficient to rescue STAT4 phosphorylation to wild-type levels. In order to test the importance of nonphosphorylated dimerization in mediating STAT4 activation, we utilized an expression vector containing an N-terminal c-myc tag as well as an estrogen receptor steroid ligand binding domain responsive to 4-OH tamoxifen. We engineered either the full-length, Δ21, or the Δ124 STAT4 cDNAs on the C-terminal end of the estrogen receptor steroid ligand binding domain so that the induced dimerization occurred at the N-terminal end of the molecule, mimicking the natural nonphosphorylated dimerized STAT4. The deletion mutants corresponded to the deleted region determined to be required for STAT4 phosphorylation (Δ21) and a mutant that had the whole N-terminal region deleted (Δ124).

The ability of 4-OH tamoxifen induced dimerization was tested by cotransfecting a vector containing c-myc and the tamoxifen-responsive steroid ligand binding domain (referred to as c-mycERT2) with the STAT4ERT2 constructs, immunopreciptating STAT4, and immunoblotting with c-myc (Fig. 31). As expected, the FLSTAT4ERT2 protein did not dimerize with the CmycERT2 protein when 4-OH tamoxifen was absent (Fig. 31 lane 1). On the other hand, the addition of 4-OH tamoxifen caused both the FLSTAT4ERT2 and

Δ124STAT4ERT2 proteins to dimerize with the c-myc protein since both c-myc tagged proteins are seen after immunoprecipitating with STAT4 (Figure 31 lanes 2-4).

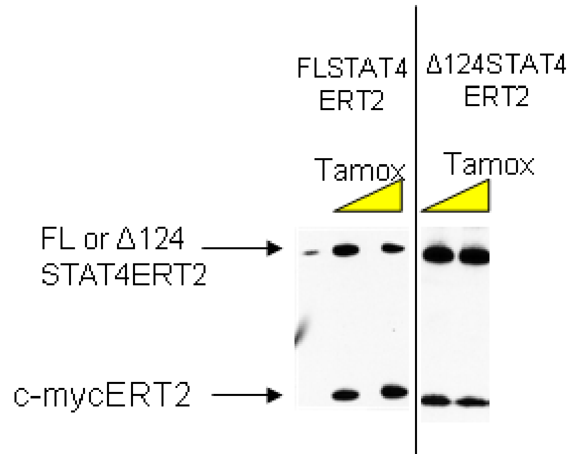

Figure 31. STAT4 estrogen receptor constructs are induced to dimerize upon addition of 4-OH tamoxifen. Cos7 cells were transfected with 1 µg of the indicated STAT4 expression vectors containing an estrogen receptor steroid ligand binding domain and a c-myc tag (pFLERT2 or pΔ124ERT2). Another plasmid that contained only the steroid ligand binding domain and the c-myc tag (pERT2) was cotransfected with the aforementioned plasmid. Ten nM or 1 µM of 4-OH tamoxifen was added to the cells for 24 hours. Cell extracts were immunoprecipitated with STAT4 antibody. Immunoblot with anti-myc antibody was performed after precipitation. Results are representative of two experiments.

Upon further testing, we observed that addition of 4-OH tamoxifen addition resulted in phosphorylation of the STAT4ERT2 mutants without addition of Jak2 (Fig. 32). Furthermore, we noticed that 10 nM 4-OH tamoxifen was sufficient to phosphorylate Δ21STAT4ERT2 and that the lowest concentration for maximal STAT4 phosphorylation in both the full-length and Δ124 mutant was 250 µM 4-OH tamoxifen (Fig. 32). The activation of STAT4 by 4-OH tamoxifen precluded assessing the importance of nonphosphorylated dimerization in activating STAT4

but suggested that the addition of the Estrogen Receptor steroid ligand binding domain resulted in a STAT4 protein that could be inducibly phosphorylated by the addition of 4-OH tamoxifen.

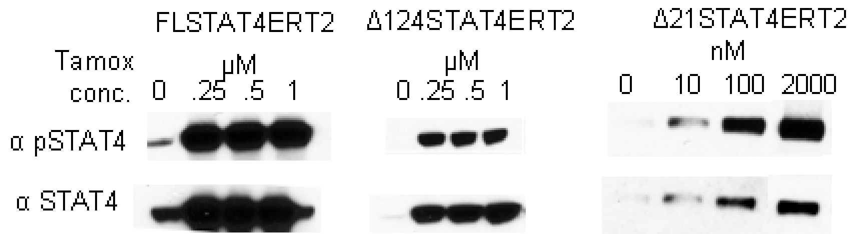

Figure 32. Induced N-terminal dimerization by 4-OH tamoxifen results in STAT4 activation. (A) Cos7 cells were transfected with the indicated expression vectors. 24 hours later, 3 different concentrations of 4-OH tamoxifen (or addition of vehicle alone) were added to the cells for 24 hours. Cell extracts were immunoblotted with anti-pYSTAT4 and anti-STAT4. (B) Cos7 cells were transiently transfected with the indicated expression vector and treated with vehicle alone or with 250 nM 4-OH tamoxifen for 24 hours. Protein extracts were made and western blotting was performed using anti-pYSTAT4 antibody or anti-STAT4.

Th1 cells expressing a C-terminal truncated STAT4 (STAT4β) secrete more TNF-α upon TCR stimulation than STAT4α-expressing Th1 cells.

While previous studies demonstrated that T cells expressing either STAT4α or STAT4β can differentiate into Th1 cells, STAT4α was more efficient than STAT4β in the induction of IFN-γ following IL-12 stimulation. To extend these findings, we examined supernatants for IFN-γ production from naïve CD4+ T cells undergoing Th1 differentiation in the presence of IL-12 (Fig. 33A). Consistent with previous literature, there was significantly less IFN-γ present in the supernatant throughout the differentiation period in STAT4β-expressing and *Stat4*-deficient cultures. Despite these differences during differentiation, anti-CD3 restimulation of the differentiated Th1 cells resulted in no significant differences in IFN-γ production between the isoforms (Fig. 33B). These results

suggested that the differences in endogenous IFN-γ production stimulated by the STAT4 isoforms during the differentiation period did not affect the process of differentiation.

Although IFN-γ levels were not different between STAT4α- and STAT4β-expressing Th1 cells, we wanted to examine the levels of other cytokines. The dependence of TNF-α production on STAT4 either in vitro or in vivo during the development of disease is not clear (62, 296). To examine STAT4-dependent TNF-α production, wild type and *Stat4-/-* naïve CD4+ T cells were cultured in Th1 priming conditions for five days. At the end of the five-day culture, the cells were stimulated with IL-12, IL-12+IL-18, anti-CD3 or PMA+ionomycin and analyzed for TNF-α and IFN-γ production. Maximal TNF-α production, as assessed by ICS, was dependent upon STAT4 (Fig. 33C). While the percentage of TNF-α positive CD4+ T cells did not differ drastically between wild type and *Stat4-/-* cells, the mean fluorescence intensity (MFI) at 4 hours and the secretion of TNF-α over a 24-hour time period showed TNF-α production significantly reduced in the absence of STAT4 (Fig. 33C and D). In contrast, TNF-α production was not detected following stimulation with IL-12, in the presence or absence of IL-18 (data not shown).

Having demonstrated the STAT4-dependence in TNF-α production, we wanted to examine the ability of the STAT4 isoforms to prime Th1 cells to secrete TNF-α. Naïve CD4+ T cells expressing either STAT4α or STAT4β were cultured under Th1 culture conditions for five days and stimulated with anti-CD3 to examine the levels of TNF-α and IL-2 using ELISA. The Th1 cells expressing STAT4β consistently secreted significantly more TNF-α compared to the CD4+ T cells expressing STAT4α while IL-2 levels between cells expressing the STAT4 isoforms were similar (Fig. 33E). These results suggested that IL-12 stimulation of STAT4β differentially programmed the developing Th1 cells to secrete more TNF-α and that this programming was specific and independent of the

concentration of IFN-γ throughout the culture period. Thus, these data suggested that STAT4 isoforms can dictate differential cytokine expression in Th1 cells.

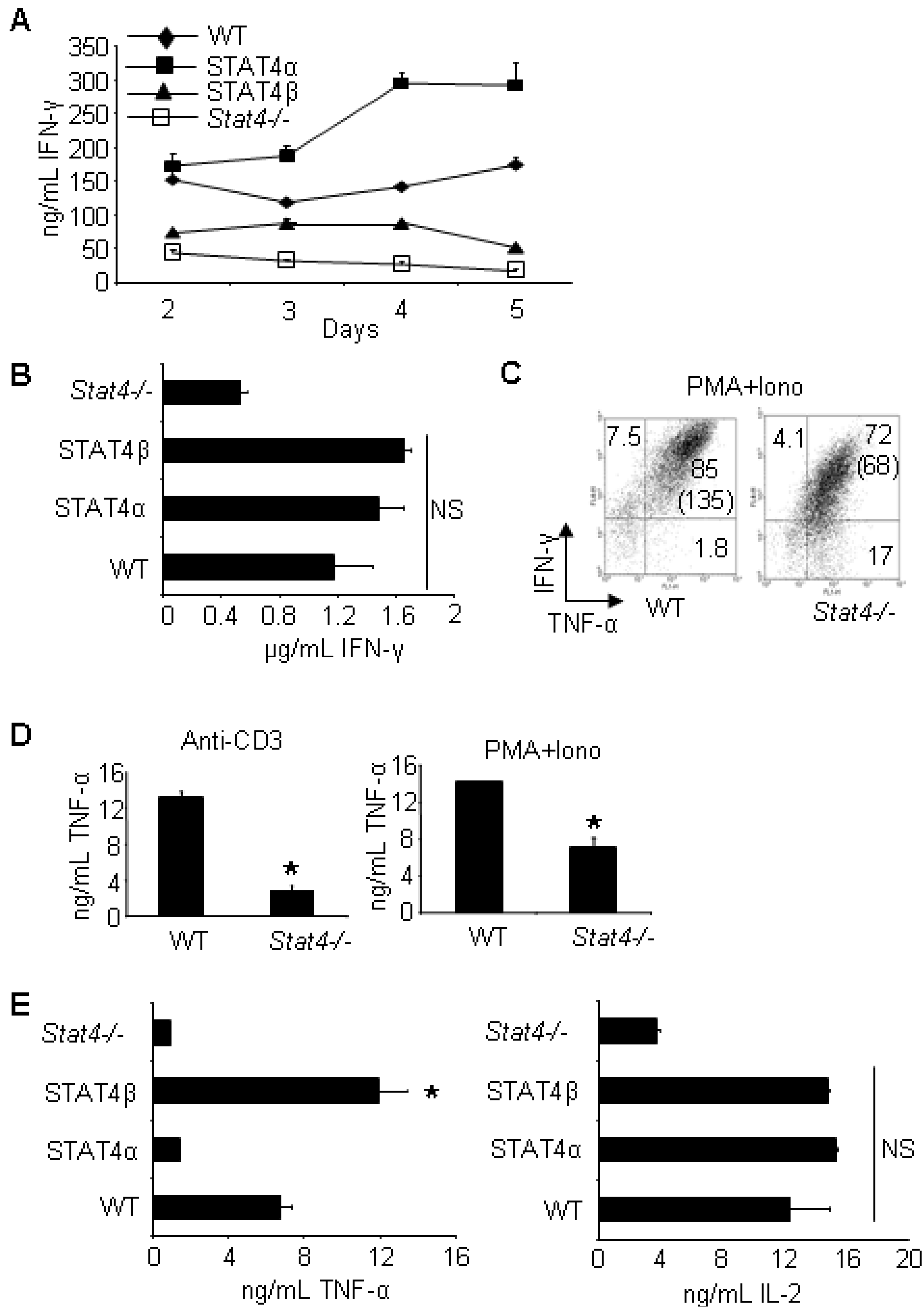

Figure 33. T cells expressing STAT4 isoforms have differential TNF-α production. (A) CD4+CD62L+ T cells from mice of the indicated genotypes were cultured under Th1 priming conditions (IL-12, anti-IL-4, α-CD3, α-CD28) with irradiated APCs (30 Gy) for five days. Every 24 hours,

supernatants of the developing Th1 cells were collected from each genotype. Cell free supernatants were analyzed for IFN-γ production using ELISA. Results are represented as mean ± SD of duplicates. (B) Cells cultured as in (A) for five days were stimulated for 24 hours and cell-free supernatants were analyzed for IFN-γ using ELISA. Results are represented as mean ± SD of duplicates and are representative of 3 experiments. (C) CD4+CD62L+ T cells were cultured as in (A) for five days. Cells were collected, washed, and stimulated with PMA and ionomycin in the presence of Golgi-Plug before intracellular staining for the indicated cytokines. Data shown are gated on CD4+ cells. Numbers represent % of cells in the respective quadrant while numbers in parentheses represent the MFI of the x-axis. Results are representative of 3 experiments. (D) Cells cultured under Th1 priming conditions for five days were stimulated in the indicated condition for 24 hours before cell-free supernatants were collected for analysis of TNF-α. Error bars represent SD of duplicates. *, significantly different ($p<0.05$) from wild-type cells. (E) Cells cultured as in (A) for five days were stimulated for 24 hours and cell-free supernatants were analyzed by ELISA for TNF-α and IL-2. Results are represented as mean ± SD from duplicates and are representative of 4 experiments. *, significantly different ($p<0.05$) from wild-type, Stat4α, and Stat4-/- Th1 cultured cells using unpaired Student's T-test.

To determine if differential activation of STAT4 contributed to the production of distinct Th cytokines, we stained developing Th1 cultures for phospho-STAT4 (pSTAT4) levels over the first three days of culture. Wild-type and STAT4β-expressing cells showed similar percentages of pSTAT4+ cells at all of the time points examined (Fig. 34A). In contrast, there was less pSTAT4α than pSTAT4β or pSTAT4 in wild type cells at all of the time points. Moreover, after five days of differentiation, IL-12 stimulation resulted in greater induction of pSTAT4 in wild type and STAT4β-expressing cells than in STAT4α-expressing cells, despite

similar levels of total STAT4 expression (Fig. 34B and C). Despite lower levels of pSTAT4α during Th1 differentiation and following IL-12 restimulation, STAT4α was still more potent than STAT4β in the acute production of IFN-γ (Fig. 34D). These data suggested that the differential activation of the isoforms in response to IL-12 contributed to differential gene expression but that the amount of activated STAT4 did not directly correlate with *Ifng* gene transcription.

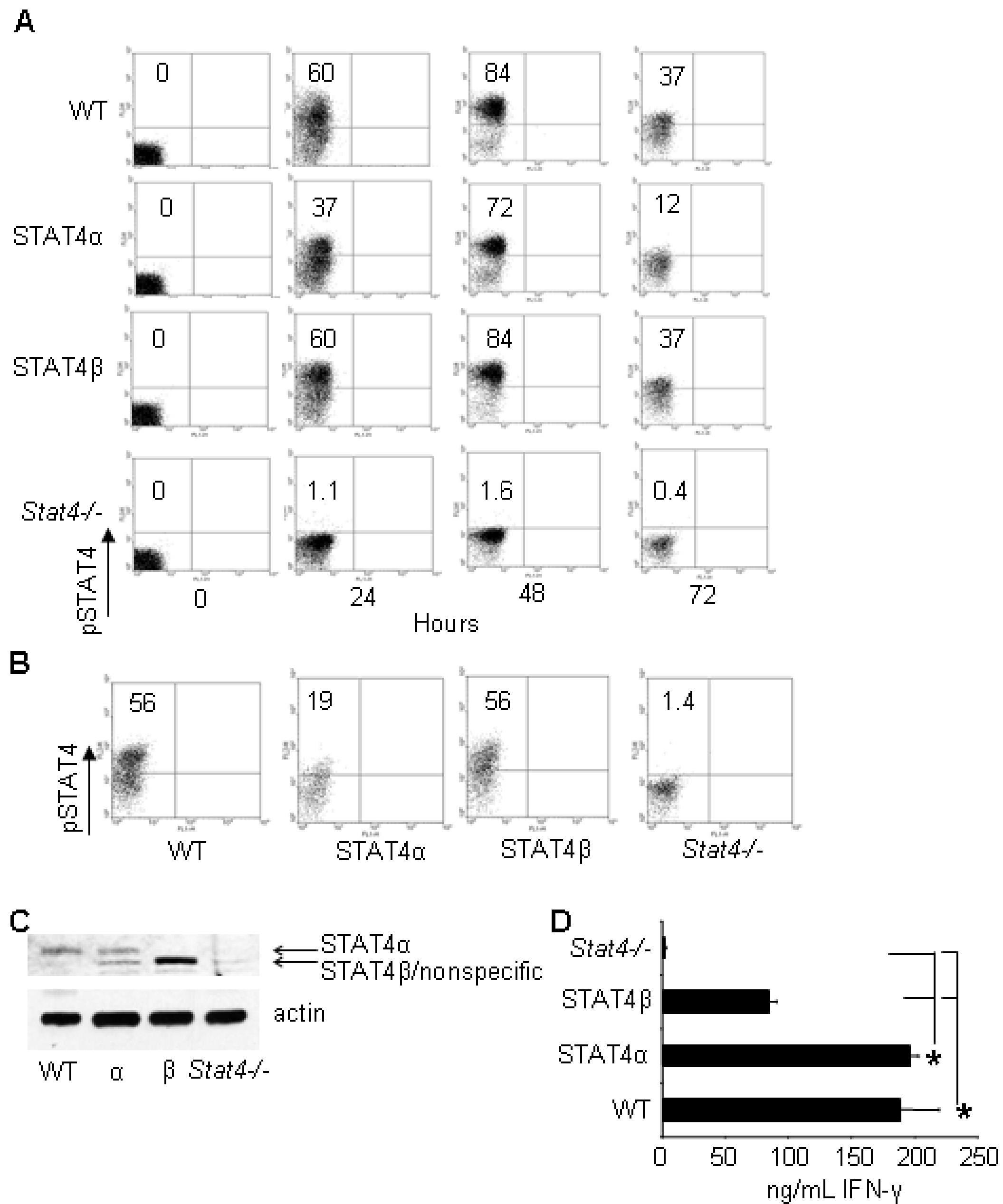

Figure 34. Activation kinetics of the STAT4 isoforms during Th1 differentiation. (A) Naïve CD4+ T cells freshly isolated (0 time point) or cultured in Th1 conditions for 24, 48 or 72 hours were collected for intracellular staining with anti-pSTAT4. Results are representative of two experiments. Numbers in quadrants represent % of pSTAT4+ T cells. (B) Cells cultured as in (A) were washed and stimulated with IL-12 for 2 hours

before staining for pSTAT4. Numbers in quadrants represent % of pSTAT4+ T cells. (C) Five-day cultured Th1 cells were lysed and immunoblotted for STAT4 and actin as a control. Symbols α and β refer to STAT4α- and STAT4β-expressing T cell lysates, respectively. (D) Th1 cells were stimulated with IL-12 and IL-18 for 24 hours and cell-free supernatants were analyzed for IFN-γ using ELISA. Results are shown as mean ± SD from duplicates. Results are representative of 2 independent experiments.

STAT4 isoforms are equally efficient in promoting Th17 differentiation

IL-23 also activates STAT4 and induces Th17 cells to secrete IL-17 (171). Since we observed a differential induction of Th1 cells to secrete TNF-α by the STAT4 isoforms, we examined the ability of Th17 cells expressing STAT4 isoforms to secrete IL-17 and TNF-α. We differentiated naïve T cells with TGF-β1, IL-6, and IL-23 for five days and then restimulated cells with anti-CD3 or PMA and ionomycin (Fig. 35A). There were no significant differences between the percentage of TNF-α positive cells in Th17 cells expressing either isoform although the percentage of TNF-α positive cells was considerably higher following PMA and ionomycin stimulation, compared to anti-CD3 (Fig. 35A). In addition, the Th17 cells expressing either isoform had similar capabilities to produce IL-17. To assess the responsiveness of the STAT4 isoforms to IL-23-induced cytokine production, we examined IL-17 levels by ELISA after 24 hours of stimulating the cells with IL-23 and IL-18 (Fig. 35B). Th17 cells expressing the STAT4α isoform secreted significantly more IL-17 than the Th17 cells expressing the STAT4β isoform. Thus, while either STAT4 isoform is sufficient for the generation of Th17 cells, activation of STAT4α by IL-23 can more efficiently induce IL-17 production than the STAT4β isoform.

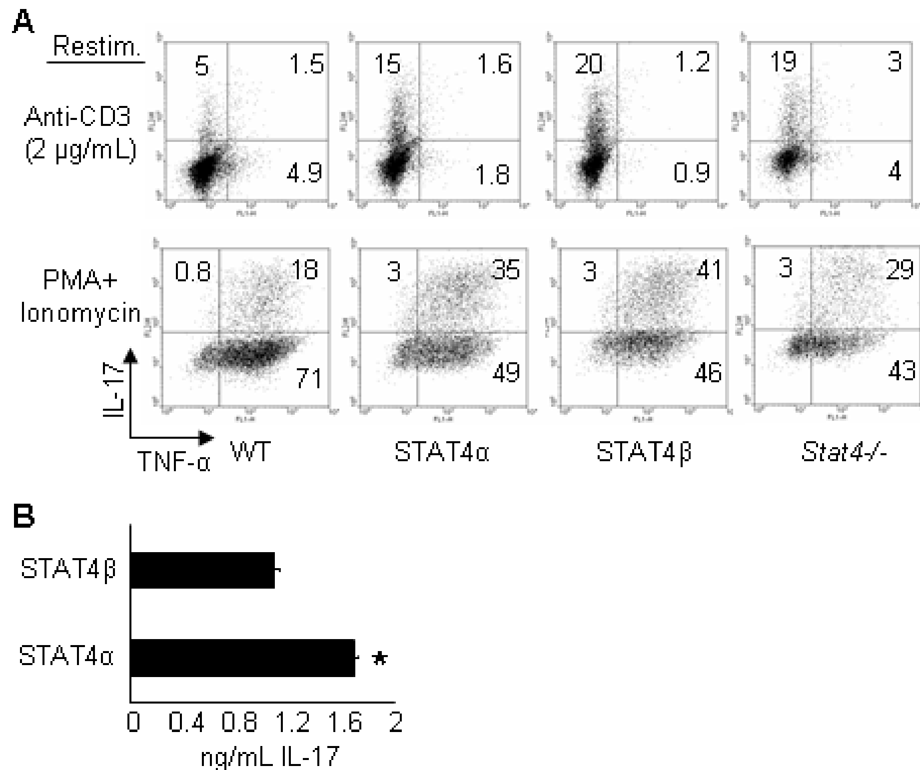

Figure 35. STAT4α and STAT4β are equally capable of inducing Th17 differentiation. (A) CD4+CD62L+ T cells were cultured in the presence of TGF-β1, IL-6, IL-23, anti-IL-4, and anti-IFN-γ for 5 days. Cells were collected, washed, and stimulated with plate-bound α-CD3 or PMA and ionomycin in the presence of Golgi-Plug before intracellular staining for the indicated cytokines. CD4+ cells were gated and the results were plotted as indicated. Numbers represent % of cells in the respective quadrant. Results are representative of 3 experiments. (B) Th17 cells as in (A) were stimulated with IL-23 and IL-18 for 24 hours and cell-free supernatants were analyzed by ELISA for IL-17A. Results are shown as mean ± SD from duplicates and are representative of 3 independent experiments. *, significantly different ($p<.05$) than STAT4β.

STAT4β promotes more severe colitis than STAT4α

Since we observed some differences in the ability of T cells expressing STAT4α or STAT4β to secrete inflammatory cytokines, we wanted to test the ability of the

T cells expressing each isoform to mediate inflammation. Therefore, we reconstituted SCID mice with CD4+CD45RB^{high} or CD4+CD45RB^{low} T cells that expressed either STAT4α or STAT4β and examined the weight loss kinetics of the mice. There was no significant difference in the kinetics of weight loss or the end point weight loss between the SCID mice reconstituted with either isoform (Fig. 36A). However, there was a significant difference between the weight loss of mice reconstituted with the CD4+CD45RB^{high} cells compared to the mice reconstituted with CD4+CD45RB^{low} cells, indicating that the CD4+CD45RB^{high} T cells expressing either isoform were sufficient to induce colitis (Fig. 36A). To determine if the differences in T cell proliferation between the Stat4 isoforms seen previously in vitro (194) resulted in differences in cell reconstitution in vivo, we determined the absolute CD4+ cell numbers in mesenteric lymph node cells and the percentage of CD4+ T cells in the splenocytes and observed no significant difference between the repopulation efficiency of the CD4+ T cells expressing either isoform (Fig. 36B and data not shown).

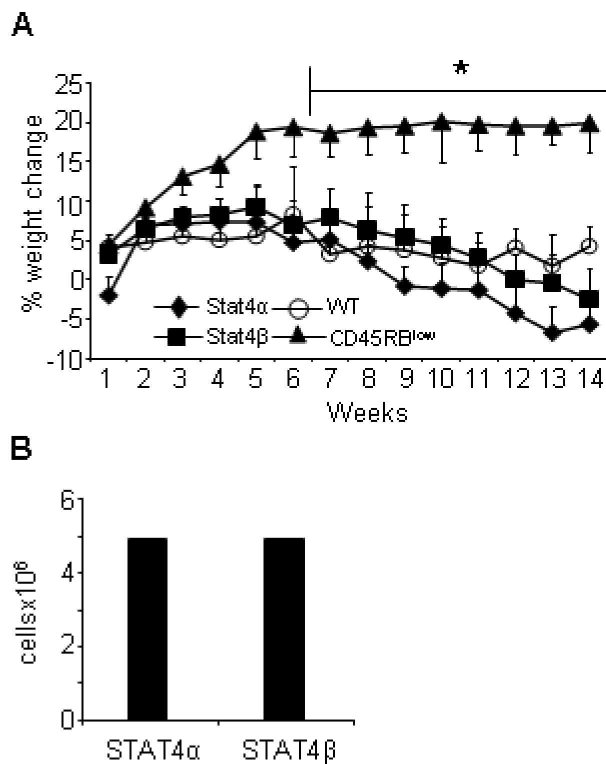

Figure 36. STAT4α and STAT4β mediate inflammatory bowel disease. (A) The change of weight over time is expressed as percent of the original

weight. Unless indicated, cells are $CD45RB^{high}$. Data represent the mean ± SEM of each group (7-10 mice per group). Mice were sacrificed 14 weeks after T cell reconstitution. *, $CD45RB^{low}$ cells are significantly different ($p<0.05$) from $CD45RB^{high}$ STAT4α or STAT4β using 2-way ANOVA and unpaired Student's T-test post-hoc. (B) Pooled mesenteric lymph node single-cell suspensions were counted and surface stained for CD4 and analyzed by FACS. Absolute cell numbers were calculated from % of CD4+ cells and live cell counts.

Although weight loss was not significantly different between the SCID mice reconstituted with either STAT4 isoform, gross examination of the colon and scoring of the slides showed that the SCID mice reconstituted with the $CD4+CD45RB^{high}$ cells expressing STAT4β had more significant mucosal inflammation than the SCID mice reconstituted with STAT4α as assessed by area and severity of the lesion (Fig. 37A-C). There was no difference in mucosal hyperplasia between the mice reconstituted with STAT4α or STAT4β-expressing T cells (Fig. 37B and C). Importantly, SCID mice reconstituted with the $CD4+CD45RB^{low}$ cells had essentially no inflammatory infiltrates into the tissues (Fig. 37B and C).

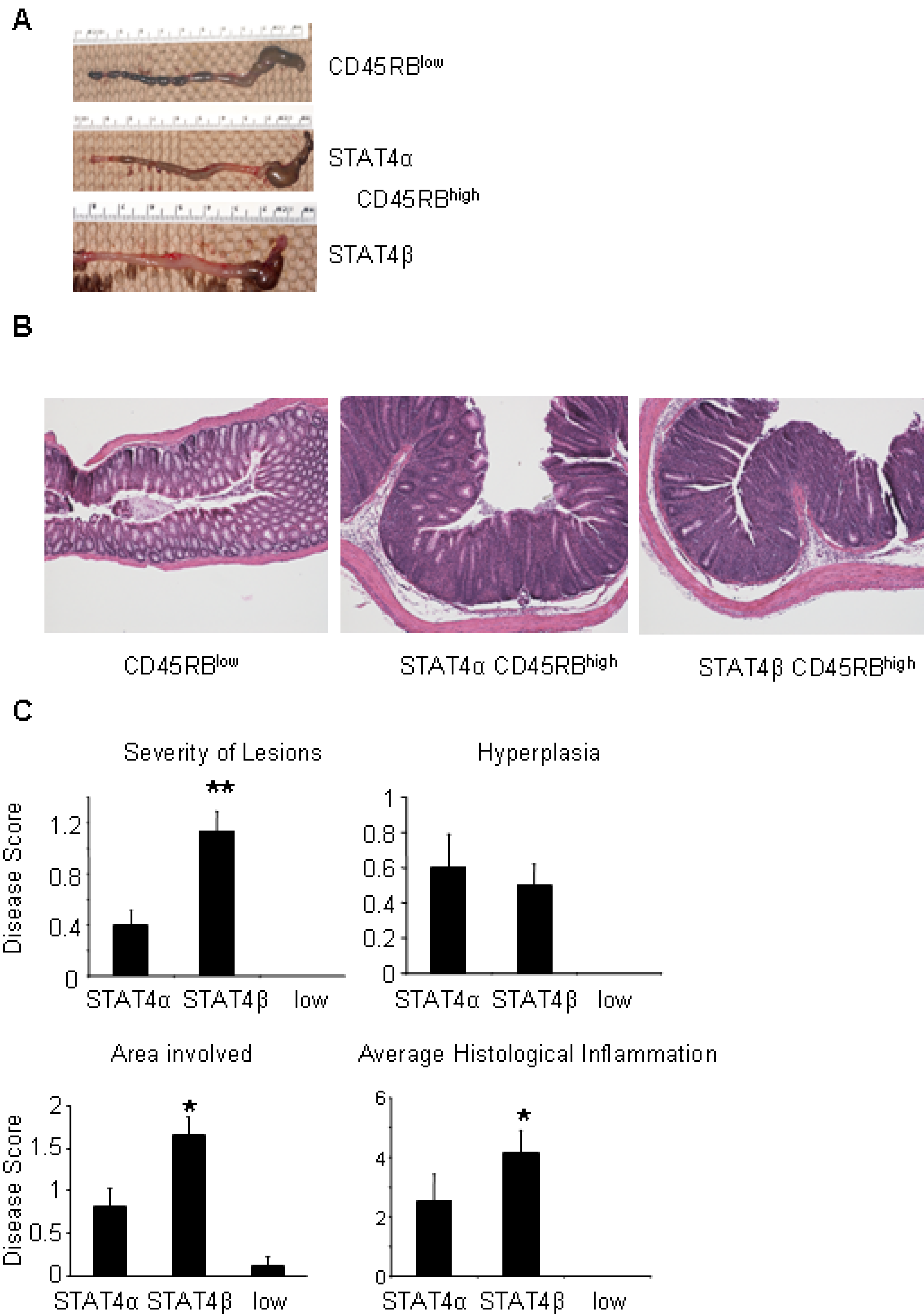

Figure 37. STAT4β mediates more severe histological inflammation than STAT4α. (A) Gross appearance of representative colon from each group as indicated. (B) Representative photomicrographs (100x) of colon from mice of the indicated group were stained with H&E. (C) The mean

histological scores ± SEM for the SCID mice reconstituted with the CD4+ T cells as indicated with STAT4α or STAT4β signifying histological scores from the SCID mice reconstituted with the $CD45RB^{high}$ subset and the low signifying histological scores from the SCID mice reconstituted with the $CD45RB^{low}$ subset. *, $p<0.05$ where STAT4β is significantly different from both STAT4α and the $CD45RB^{low}$ subset using the Mann-Whitney U-test.

STAT4β-expressing T cells from colitic mice have increased inflammatory cytokine production compared to mice reconstituted with STAT4α T cells. To examine whether the increased histological inflammation seen in the SCID mice reconstituted with the STAT4β-expressing T cells correlated with increased TNF-α production, isolated splenocytes and mesenteric lymph node cells were stimulated with anti-CD3 to assess ex vivo TNF-α production (Fig. 38A). The SCID mice reconstituted with the STAT4β-expressing T cells had significantly more TNF-α compared to the mice adoptively transferred with the STAT4α-expressing T cells upon stimulation with anti-CD3. SCID mice reconstituted with $CD4+CD45RB^{low}$ from either isoform had barely detectable TNF-α that was significantly less than the cells isolated from the SCID mice reconstituted with the $CD45RB^{high}$ subset of cells (data not shown).

To determine if the STAT4 isoforms differentially regulated other cytokines in vivo, we examined T cell produced cytokines that have been implicated in the pathogenesis of colitis, including IFN-γ, IL-6, IL-10, and IL-17 (100, 101, 297-301). Corresponding to the level of inflammation, SCID mice reconstituted with the STAT4β-expressing T cells had more inflammatory cytokine production (Fig. 38B). IFN-γ production was significantly increased from STAT4β-expressing cells compared to STAT4α-expressing cells from either spleen or mesenteric lymph nodes. IL-6 production in the spleen was also increased but not in the mesenteric lymph nodes of mice reconstituted with the STAT4β T cells. However, IL-17 did not significantly differ between SCID mice reconstituted with T cells expressing either isoform.

Since previous data (194) and data in Figures 2 and 3 show that STAT4α is more efficient than STAT4β in IL-12 or IL-23 stimulated production of IFN-γ and IL-17, we next examined the mesenteric lymph node cells from colitic mice to produce these cytokines following treatment with IL-12 and IL-18 or IL-23 and IL-18. While the IL-23 and IL-18 stimulated cells from the SCID mice reconstituted with STAT4α secreted more IL-17, similar to results from in vitro differentiated cells, there was no significant difference in the amount of IFN-γ secreted from the cells isolated from the SCID mice reconstituted with either isoform (Fig. 38C). Overall, these data indicated that the increased inflammatory disease caused by STAT4β-expressing T cells correlated with the increased inflammatory cytokine production.

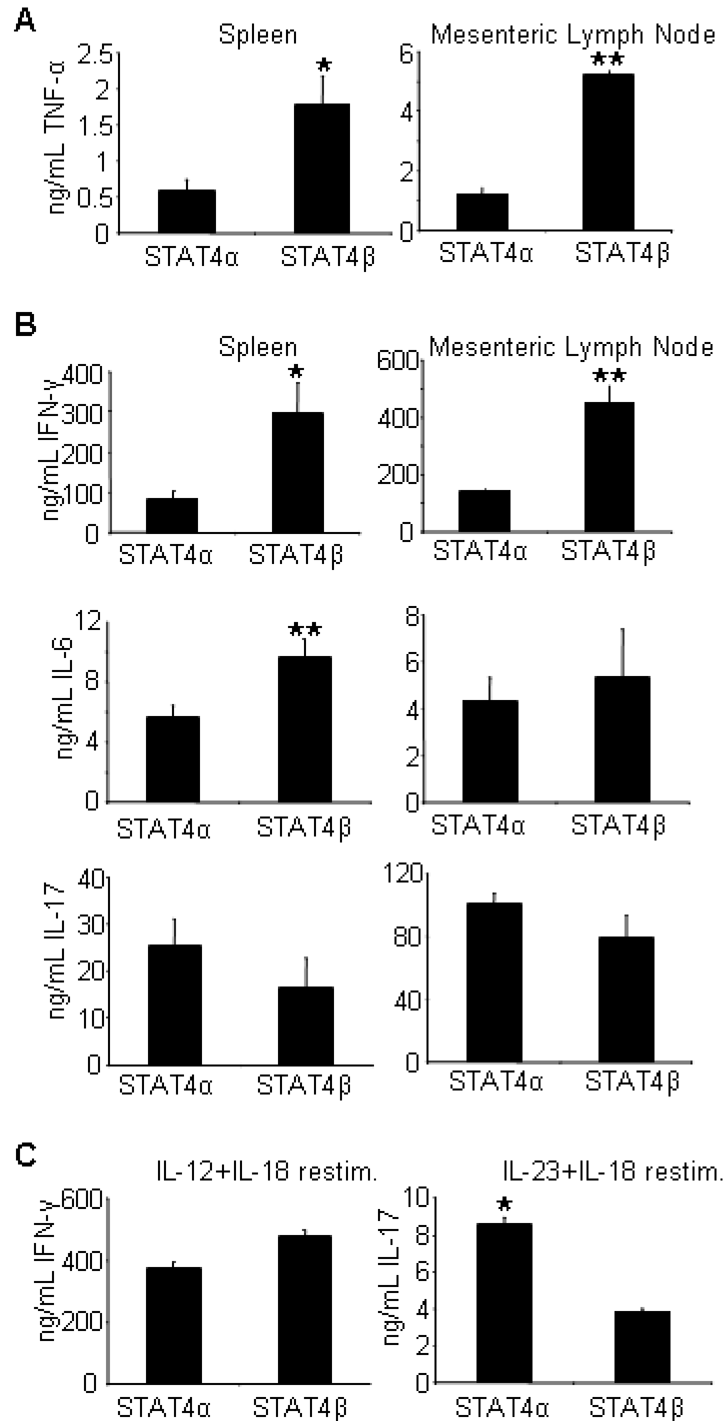

Figure 38. Cytokine production from STAT4α- and STAT4β-expressing T cells ex vivo. (A and B) Cells were isolated and stimulated as described in Materials and Methods and concentration of cytokines were determined by ELISA and are displayed as mean ± SEM (STAT4α n=9; STAT4β n=10). *, $p<0.05$, ** $p<0.02$ using Unpaired Student's T-test. (C) Cells

were isolated and stimulated as described in Materials and Methods. The concentration of cytokines were determined by ELISA and are displayed as mean ± SD of pooled mesenteric lymph nodes from the SCID mice reconstituted with the CD45RB^{high} subset of the indicated STAT4 isoform. *, $p<0.05$ using unpaired Student's T-test.

We had previously found that STAT4 downstream of IL-12 can inhibit TGF-β1-induced Foxp3 expression and aTreg development. We hypothesized that the increased inflammation seen in the SCID mice reconstituted with the STAT4β-expressing T cells was partly due to increased Foxp3 repression by STAT4β in aTreg cells. To examine this, we cultured wild-type, *Stat4-/-*, STAT4α or STAT4β-expressing T cells under aTreg or TGF-β1+IL-12 conditions and examined levels of Foxp3 expression. Both isoforms had similar abilities to repress Foxp3 (Fig. 39). This suggested that the STAT4 isoforms were equally capable of suppressing TGF-β1-induced Foxp3 expression. Therefore, STAT4 repressing Foxp3+ aTreg cells did not account for the increased inflammation observed in the SCID mice reconstituted with the STAT4β-expressing T cells.

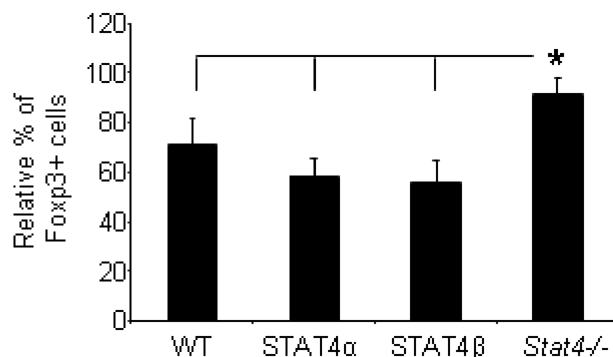

Figure 39. STAT4 isoforms downstream of IL-12 activation are equally capable of repressing TGF-β1 induced Foxp3. (A) Foxp3 expression was determined from CD4+ T cells cultured under aTreg or TGF-β1+IL-12 conditions for five days. Data are presented as % Foxp3+ cells in the TGF-β1+IL-12 condition relative to Foxp3+ cells in the aTreg conditions. Relative % of Foxp3+ cells=% of TGF-β1+IL-12 cultured Foxp3+ cells / % Foxp3+ cells in aTreg conditions. *, Foxp3 expression in *Stat4-/-* cultured

cells is significantly different ($p<.05$) than the other genotypes examined using unpaired Student's T-test. Data are averaged values over 2 independent experiments.

STAT4β-expressing T cells have an increased propensity to secrete GM-CSF

Previous literature suggested that TNF-α and GM-CSF are important in neutrophil chemotaxis to inflamed tissues (299, 302). To examine whether the increased TNF-α secretion from STAT4β-expressing T cells correlated with increased neutrophils in the lamina propria, we analyzed microscopic sections of the colon for PMN infiltration. Consistent with the increased TNF-α seen in the SCID mice reconstituted with the STAT4β isoform, there were also increased neutrophils present in the lamina propria compared to the SCID mice reconstituted with STAT4α (Fig. 40A). Since anti-TNF therapies have been shown to inhibit GM-CSF production, we next wanted to look at GM-CSF levels in the mice with colitis (299). We examined supernatants from stimulated mesenteric lymph node cell cultures to assess GM-CSF production (Fig. 40B). Consistent with the increased neutrophil infiltration, GM-CSF was significantly increased from STAT4β-expressing T cells, further supporting the ability of T cells expressing the STAT4α isoform to mediate potent inflammatory responses.

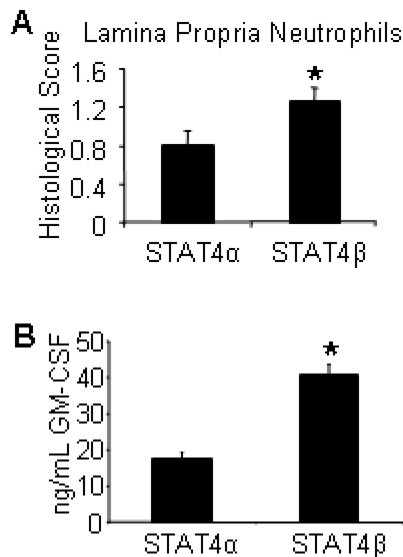

Figure 40. Increased lamina propria neutrophil infiltration correlates with increased GM-CSF levels seen in the SCID mice reconstituted with the STAT4β isoform. (A) PMN scores were determined as described in Materials and Methods. Data are presented as mean ± SEM. *, $p<0.05$ using Mann-Whitney U test. (B) Single cell suspensions from mesenteric lymph nodes were pooled from the indicated mice, stimulated with anti-CD3 for 72 hours and cell-free supernatants were analyzed using ELISA for GM-CSF. Data are presented as mean ± SD. *, $p<0.05$ using unpaired Student's T-test.

Since there was increased GM-CSF production from STAT4β-expressing cells ex vivo, we wanted to define whether this reflected an increased propensity for STAT4β-expressing T cells to produce GM-CSF or whether it was a result of the in vivo inflammatory environment. To test this, we isolated naïve T cells expressing either isoform and differentiated them in Th1 or Th17 conditions for five days and stimulated them with anti-CD3 to examine their ability to secrete GM-CSF. Production of GM-CSF in Th1 cultures was dependent upon STAT4 (Fig. 41A). Consistent with what we observed in the ex vivo stimulated cells, the STAT4β-expressing Th1 cells secreted significantly more GM-CSF than STAT4α-

expressing Th1 cells. In contrast, there was no STAT4 requirement in GM-CSF production from Th17 cells and no significant difference in the amount of GM-CSF produced by Th17 cells expressing either STAT4 isoform (Fig. 41B). In addition, no detectable GM-CSF was secreted upon acute stimulation with IL-12 or IL-23 with or without IL-18 suggesting that STAT4 does not directly induce transcription of GM-CSF (data not shown). Together these data demonstrated the increased inflammatory propensity of T cells expressing STAT4β and suggested that the increased inflammatory cytokine production by STAT4β-expressing T cells resulted in greater inflammatory disease in vivo.

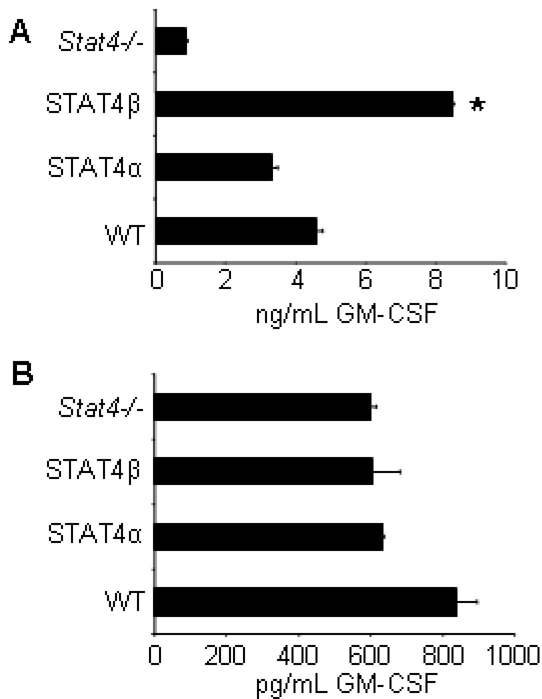

Figure 41. STAT4β Th1 cells are programmed to secrete more GM-CSF than STAT4α Th1 cells. (A) CD4+CD62L+ T cells were primed for Th1 differentiation using the same conditions as in Fig. 1. After five days, cells were stimulated for 24 hours and cell-free supernatants were analyzed by ELISA for GM-CSF. Results are represented as mean ± SD and are representative of 3 experiments. *, $p<0.05$ using unpaired Student's T-test. (B) Cells cultured under Th17 conditions as in Fig. 34 for five days were stimulated for 24 hours and analyzed by ELISA for GM-CSF

production. Results are presented as mean ± SD and are representative of two independent experiments.

In summary, we find that STAT3, STAT4, and STAT6 activation downstream of specific cytokines utilized a common mechanism to inhibit aTreg generation by inhibiting STAT5, a positive regulator of Foxp3 expression, from binding to the *Foxp3* gene. In addition, we found that a STAT4 splice isoform that lacks the C-terminal domain promoted greater inflammation in vivo than the full-length STAT4α and this inflammation correlated with STAT4 isoform-dependent expression of inflammatory cytokines, most notably TNF-α and GM-CSF. Thus, cytokine-stimulated STAT proteins orchestrate T helper cell pro- and anti-inflammatory cell phenotypes.

DISCUSSION

The development of aTreg cells is regulated by a variety of cytokines. While the ability of IL-6, IL-21 and Stat3 to mediate repression of Foxp3 and aTreg cell development is appreciated, the ability of additional Th cell differentiating cytokines to inhibit this pathway has only recently been documented (154). We demonstrate that IL-4 and IL-12 also inhibit the development of aTreg cells through activation of their requisite Stat proteins. Our data suggest that Th instructive cytokines promote chromatin remodeling of the *Foxp3* locus which subsequently limits access of Stat5 to the Foxp3 regulatory elements. We also show that STAT4β-expressing T cells mediate more severe colitis than STAT4α-expressing T cells. The mechanism, at least in part, is the increased propensity of STAT4β-expressing Th1 cells to secrete higher levels of two pathogenic cytokines TNF-α and GM-CSF compared to STAT4α-expressing Th1 cells.

Transcription factor requirements for repression of TGF-β1 induced Foxp3

STAT1

We observe only approximately 10% repression of Foxp3 by culture with IFN-γ and a reciprocal percent increase in Foxp3 expression when IFN-γ is neutralized in the culture (Fig. 2B and C). We determined that this repression is STAT1-dependent. A recent report has confirmed these findings although they find greater Foxp3 repression by IFN-γ (~40%). Wei et al. (2007) described a mechanism relying on the induction of the Th1 lineage promoting factor T-bet as the inhibitor of Foxp3 expression. This idea of lineage promoting factors repressing Foxp3 parallels our observation that RORγt transduction can repress Foxp3 in Th17 cells (Fig. 9B). Although Wei et al (2007) confirm our findings, we find that T-bet is most likely not playing a role in IL-6 induced Foxp3 repression since *Stat3*$^{CD4-/-}$ Th17 cells maintain wild-type levels of T-bet mRNA levels while Foxp3 expression is maintained (Fig. 12). Moreover, *Tbx21*-deficiency has no effect on IL-6 mediated Foxp3 repression. While lineage-determining factors are clearly important, we observed T-bet independent effects of IL-12 (Fig. 14D) suggesting that other pathways contribute to Foxp3 repression. Lineage

promoting STAT proteins are known to induce Th subset-specific factors (98, 108, 146, 149, 222, 303) though their function is not restricted to this role alone. For example, while transduced GATA-3 or T-bet can partially rescue *Stat6*- or *Stat4*-deficiency, respectively, they do not recapitulate wild-type levels of cytokine production or histone modification (222, 279, 280, 304). Thus, it seems likely that optimal Foxp3 repression involves the induction of lineage determining factors as well as STAT protein functions separate from their upregulation of T-bet, GATA-3 and RORγt.

STAT3

We uncovered some unappreciated aspects of STAT3 regulation of Foxp3 inhibition. For instance, we discovered that STAT3 is unable to repress TGF-β1-induced Foxp3 expression from the outset. This suggests that TGF-β, IL-2 and TCR activation are sufficient to induce Foxp3 while IL-6 through STAT3 dependent mechanisms has a limited timeframe with which to repress TGF-β1 induced Foxp3. Our observation that RORγt upregulation in Th17 differentiation coincides with repression of Foxp3 implies that STAT3 upregulation of RORγt may account for this limited time window. In addition, cells cultured under aTreg conditions show minimal, if any, RORγt upregulation in the first 48 hours while transducing RORγt into $Stat3^{CD4-/-}$ cells cultured in Th17 conditions repressed Foxp3. Furthermore, adding IL-6 after 48 hours into aTreg culture severely mitigates RORγt upregulation. Thus, the presence of TGF-β1 in the absence of IL-6 is sufficient to make developing aTreg cells refractory to IL-6 mediated Foxp3 repression. The mechanism, at least in part, is most likely TGF-β1 inhibition of RORγt after delayed addition of IL-6.

Another interesting finding in these studies is that not all STAT3-activating cytokines are sufficient to repress TGF-β1-induced Foxp3 expression. In fact, only IL-6 and IL-21 can repress Foxp3. This could possibly imply that alternate signaling pathways activated by these cytokines may cooperate with STAT3 activation to inhibit Foxp3. One potential signaling pathway that plays a role in

TGF-β induced Foxp3 repression and is activated by IL-6 and IL-21 is the Akt-mTOR pathway (305, 306). It has been recently found that the rapamycin-sensitive mTOR pathway is required for repression of TGF-β induced Foxp3 expression (307). It would be interesting to examine if there is an interrelationship between STAT3 and mTOR activation in inhibiting Foxp3 repression since crosstalk between mTOR signaling and STAT3 activation has been reported (308). Another possibility that could account for the difference between the cytokines is that the level and duration of the STAT3 phosphorylation downstream of the STAT3-activating cytokines differs. While we tested and confirmed that the ability of the T cells cultured in the different STAT3-activating cytokines secreted greater amounts of cytokines such as IFN-γ, IL-17 and IL-10 compared to the aTreg cultured cells after anti-CD3 restimulation, we did not directly examine the strength or duration of the STAT3 phosphorylation in the culture conditions we examined. Although we did use concentrations of the STAT3-activating cytokines that previous reports had shown substantial STAT3 phosphorylation, it is possible that the cytokines did not phosphorylate STAT3 as efficiently as IL-6 and IL-21 and this resulted in an inability to repress TGF-β1 induced Foxp3.

STAT4

STAT4 downstream of IL-12 signaling is sufficient to repress TGF-β induced Foxp3. It is interesting to note that IL-12 cannot repress Foxp3 as efficiently as IL-4 or IL-6 although it exhibits a similar capacity to induce the repressive chromatin modification H3K9me3 and decrease STAT5 binding to the *Foxp3* first intron. However, STAT5 could bind the *Foxp3* promoter to a greater extent in the TGF-β1+IL-12 conditions compared to the Th17 or TGF-β1+IL-4 conditions. Therefore, it is possible that the binding of STAT5 to the *Foxp3* promoter is a better indicator of Foxp3 expression than binding to the first intron of the *Foxp3* gene. When assessing the ability of the STAT proteins to bind the STAT consensus sequences found in the *Foxp3* promoter, STAT4 was able to bind with similar efficiency as STAT3 and STAT6 as determined by the DAPA (Fig. 22B).

Moreover, STAT4 inhibited STAT5 binding those consensus sequences with similar efficiency as STAT3 and STAT6. This suggests that STAT3, STAT4, and STAT6 can bind the STAT consensus sequences in the *Foxp3* promoter with similar affinity. Therefore, the differential STAT binding observed in the ChIP suggests that STAT3 and STAT6 are utilizing a different mechanism than differential binding in preventing access of STAT5 to the *Foxp3* gene. Multiple possibilities exist for these differences. One possible explanation is that STAT3 and STAT6 may bind other STAT sites in the *Foxp3* gene while STAT4 may not have access to those same sites or cannot bind with similar affinity. This may occur due to other factors being present in the IL-4 and IL-6 stimulated cells that cooperate with STAT6 and STAT3 to enhance their binding to the *Foxp3* gene while IL-12 and STAT4 do not induce those cooperative factors. Alternatively, it is possible that STAT3 and STAT6 can bind other regions of the *Foxp3* gene due to their abilities to bind slightly different STAT consensus sequences than STAT4. Indeed, STAT6 binds a silencer region in the *Foxp3* gene (234). It would be interesting to test if STAT3 and STAT4 can bind the same silencer region in the *Foxp3* gene. A third possibility is that the lineage determining factors the STAT proteins induce have varying effects on Foxp3 repression. It is likely that GATA-3, RORγt and T-bet can bind the *Foxp3* gene at different regions and with different affinities. This may also account for the differential abilities of STAT3, STAT4, and STAT6 to inhibit Foxp3. However, our results suggest that the induction of T-bet in Th1 promoting conditions does not solely account for Foxp3 repression since there are T-bet independent mechanisms of Foxp3 repression by STAT4 (Fig. 14D). Therefore, cooperation between STAT proteins binding the *Foxp3* gene and STAT protein induction of lineage determining factors are most likely required to maximally repress TGF-β induced Foxp3.

STAT4 was also subject to a limited timeframe with which it could repress Foxp3. It is possible that STAT4 is unable to bind the *Foxp3* locus when added back later in the culture due to limited access to the *Foxp3* gene due to chromatin

remodeling. Alternatively, it is known that TGF-β1 can induce the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 (Shp-1). Shp-1, in turn, can inhibit T-bet induction (309). Therefore, it is plausible that TGF-β1, in the absence of IL-12, can block T-bet induction and subsequent Foxp3 repression. Shp-1 can also inhibit STAT4 phosphorylation so IL-12 stimulation of STAT4 when added back later into the aTreg cultures may result in less STAT4 phosphorylation. This also could result in an inability to repress TGF-β induced Foxp3.

STAT6

The ability of IL-4 to inhibit aTreg generation is in contrast to the ability of IL-4 to increase TGF-β1 secretion (Fig. 14), increase Foxp3 expression in nTregs (Fig. 25) and to promote the expansion of antigen-specific Treg cells in vivo (232). Moreover, while STAT6VT expressing T cells have a defect in the development of aTregs (Fig. 13C), STAT6VT transgenic mice have an increased percentage of nTregs (232). Recent literature has suggested that STAT6-dependent upregulation of GATA-3 can repress Foxp3 in aTreg cells while another group showed that STAT6 can directly impact Foxp3 expression by binding a silencer element in the *Foxp3* gene (154, 233, 234). However, the mechanism by which IL-4 and STAT6 upregulate Foxp3 expression in nTregs is currently unknown. This suggests that IL-4 has opposing roles in nTregs and aTregs.

Delayed addition of IL-4 repressed TGF-β1-induced Foxp3 expression at all of the time points examined (Fig. 15B). We have shown that TGF-β1 is sufficient to inhibit RORγt upregulation and other groups have shown that TGF-β1 inhibits T-bet upregulation (Fig. 5C). Consistent with the other lineage determining factors, TGF-β1 can repress GATA-3 expression in Th2 cells. Interestingly, it has also been shown that STAT6 phosphorylation remains comparable downstream of IL-4 signaling in the presence or absence of TGF-β1 (283, 286). It was determined from our results that IL-4 maintained its ability to repress Foxp3 even when it was added 72 hours into aTreg culture conditions. This suggests that the continued

ability for IL-4 to repress Foxp3 may occur through a STAT6 dependent, GATA-3 independent mechanism of Foxp3 repression although further examination of GATA-3 inhibition by TGF-β1 in our culture conditions is required before conclusively saying that IL-4 can repress Foxp3 independent of GATA-3.

Differences in Foxp3 expression between the aTreg and nTreg subsets

While the importance of Foxp3 expression in immune homeostasis has been well characterized, it is unclear if Foxp3 expression is regulated similarly between the aTreg and nTreg subsets. Our results demonstrate that multiple STAT proteins can inhibit TGF-β1-induced Foxp3 expression. However, these same STAT proteins downstream of instructive cytokines are unable to inhibit Foxp3 expression to a similar extent in nTreg cells (Fig. 25). This differential sensitivity to STAT protein regulation in nTreg cells and the TGF-β induced Foxp3+ cells underscores the significant differences between these states (310) and suggests chromatin states may differ between aTregs and nTregs. For example, nTreg cells may have an irreversible, accessible state of chromatin at the *Foxp3* locus in nTreg cells while a reversible one exists in the early stages of TGF-β induced Foxp3 expression. Since the *Foxp3* locus is characterized by complete demethylation of CpG motifs and histone modifications within the *Foxp3* locus in nTreg cells while the *Foxp3* locus is characterized by incomplete demethylation after TGF-β induction, this theory sounds plausible (311). Moreover, it is interesting to note that aTregs show significant STAT5 binding to the first intron in the *Foxp3* gene while others have shown that nTregs do not exhibit STAT5 binding in that intron (125). It is tempting to speculate that STAT5 binding to the first intron may be crucial to the continued expression of Foxp3 in aTreg cells while it is not required in nTreg cells. These studies highlight the fact that cytokines and STAT proteins have differing effects on aTregs and nTregs.

Different roles of TGF-β1 and Foxp3 in inhibiting Th subset specific proinflammatory cytokine secretion

While TGF-β1 and Foxp3 have both been shown to repress cytokine gene expression and effector functions of T helper cells, it is unclear whether the ability of TGF-β1 to inhibit cytokine secretion in T helper cells is wholly dependent on its ability to upregulate Foxp3. The mechanism by which Foxp3 inhibits IL-2, IL-4, and IFN-γ production is by interacting with NFAT and NF-κB and blocking their ability to induce the endogenous expression of their target genes (200). TGF-β1 inhibits Th1 differentiation by attenuating the expression of IL-12Rβ2 and inhibiting the expression of T-bet (284, 285). TGF-β1 also inhibits Th2 development by inhibiting GATA-3 at the transcriptional level (283, 286). Therefore, it appears that TGF-β1 can inhibit IFN-γ and IL-4 production by mechanisms independent of Foxp3 induction. However, the induction of Foxp3 synergizes with the Foxp3-independent functions of TGF-β1 in inhibiting cytokine secretion in developing T helper cells. Our results agree with the previous data in that we observe a decreased ability of developing T helper cells cultured in Th1 or Th2 conditions in the presence of TGF-β1 to secrete IFN-γ and IL-4, respectively, despite the fact that Foxp3 expression is reduced compared to aTreg cells in those conditions. In addition, cells cultured in aTreg conditions are severely impaired in their ability to secrete IFN-γ and IL-4. This suggests that TGF-β1 and Foxp3 can synergistically inhibit proinflammatory cytokine secretion.

It is also interesting to note that nTregs are able to secrete proinflammatory cytokines when cultured in the presence of TGF-β1 and IL-6 or IL-12 despite high levels of Foxp3 expression. These results suggest that the presence of TGF-β1 and subsequent Foxp3 induction are not sufficient to inhibit proinflammatory secretion in nTregs. While we have not explored the mechanisms for these observations, it could be that nTregs are not as sensitive to TGF-β1 concentration as naïve T cells. If that is the case, then increasing TGF-β1 in the cultures should inhibit the ability of nTregs to secrete proinflammatory cytokines. In addition, the five day in vitro culture may change expression of transcription

factors or surface proteins such that the nTregs are not phenotypically the same as freshly isolated nTreg cells despite maintaining Foxp3 expression and, upon TCR stimulation, the nTregs are induced to secrete proinflammatory cytokines.

Model for STAT-dependent Foxp3 inhibition

CD4+ T cells that are activated in the presence of TGF-β1 and no other instructive cytokines efficiently develop into aTreg cells with the majority becoming Foxp3+ T cells. We have shown that these suppressive cells do not perpetuate T cell proliferation and/or proinflammatory secretion. However, if those same cells are activated in the presence of TGF-β1 and IL-4, IL-6, or IL-12, then aTreg development is suppressed and, instead, T cells develop that are non-suppressive and are capable of secreting Th2, Th17, and Th1 cytokines, respectively (Fig. 41 and (274)). Thus, it is plausible that this is another checkpoint for keeping the initiation of immune responses under control. Theoretically, IL-4, IL-6, or IL-12 should only be present in the microenvironment if the immune system has recognized non-self and has become activated. In this instance, it is important to turn off aTreg cell development and initiate pro-inflammatory T helper cells to control the insult to the immune system (Fig. 42).

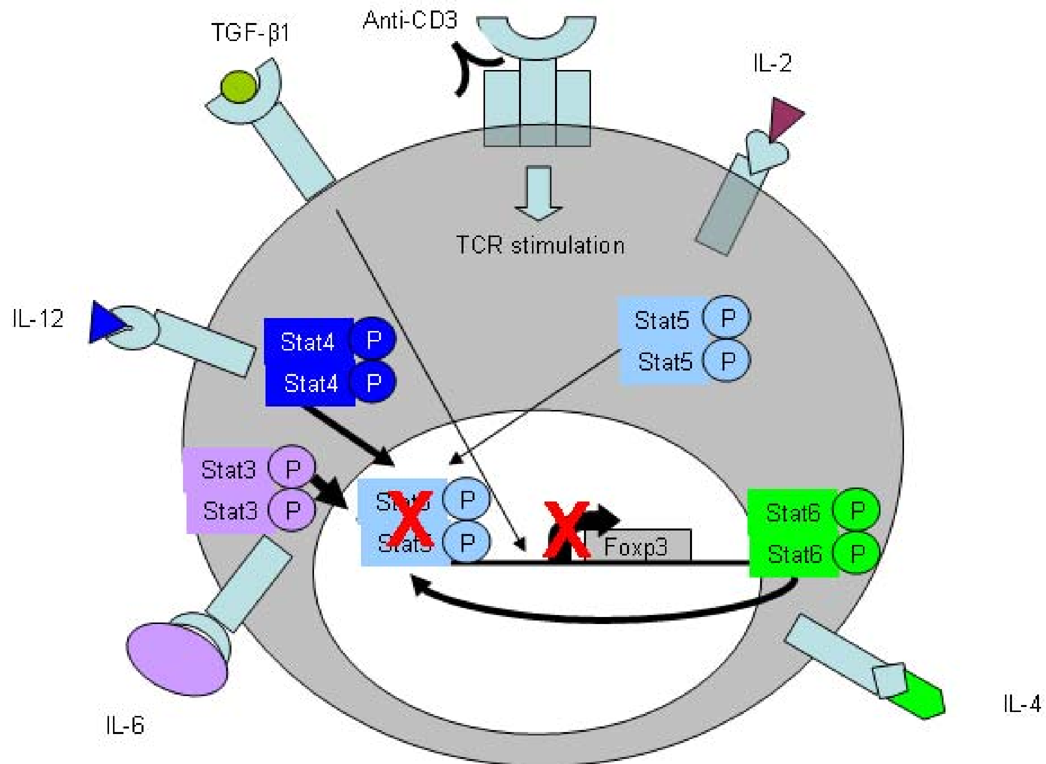

Figure 42. Model for STAT3, STAT4, and STAT6 inhibiting Foxp3 expression and aTreg development. Schematic cell representing the positive and negative STAT regulators of TGF-β1-induced Foxp3 expression. In the absence of the instructive cytokines IL-4, IL-6 and IL-12, TGF-β1 and IL-2 can induce naïve T cells that have been recently activated to become Foxp3+ aTreg cells. This is most likely beneficial to the host because, in the absence of acute phase proteins (IL-6 being one of them), the T cell is most likely reacting to self-protein and differentiation into an effector Th subset (Th1, Th2, Th17) would be detrimental to the host. However, when there is a proinflammatory cytokine milieu, STAT3, STAT4, or STAT6 can be activated downstream of IL-6, IL-12, or IL-4, respectively, and inhibit Foxp3 expression by preventing STAT5 access to

the *Foxp3* gene. In this way, inflammation mediated by Th effector cells can ensue in an attempt to neutralize the offending agent.

In addition, the downstream STAT proteins activated by the instructive cytokines help determine the resultant developmental pathway to either be pro- or anti-inflammatory. Despite promiscuity in interleukin-mediated STAT activation, the STAT protein primarily activated by the cytokine milieu dictates whether Foxp3 expression is repressed or enhanced. Importantly, we have implicated a large array of STAT proteins that can limit aTreg development by inhibiting STAT5 from mediating positive chromatin remodeling at the *Foxp3* locus. This knowledge has broad implications for Th cell development and Th cell orchestration of pro- and anti-inflammatory immune responses. Ultimately, it is clinically important to determine whether aTregs ever become completely refractory to this STAT-dependent inhibition of Foxp3 in vivo. Thus, future work will attempt to define the relevance of STAT proteins inhibiting TGF-β induced Foxp3 expression in vivo.

The STAT4 N-terminal domain as an attractive target for pharmacologic targeting

It has been well characterized that the N-terminal domain of STAT4 is required for the tetramerization of two activated STAT4 dimers (173). It was subsequently discovered that the N-terminal domain of STAT4 is also required for STAT4 tyrosine phosphorylation and the formation of nonphosphorylated dimers before cytokine activation (191-193). Theoretically, inhibiting the N-terminal domain could inhibit STAT4 activation. If one could determine if different portions of the N-terminal domain mediate tyrosine phosphorylation, nonphosphorylated dimerization, and tetramerization, then one could rationally design small molecule inhibitors to the N-terminal domain that could inhibit specific STAT4 functions, namely STAT4 activation and/or STAT4 tetramerization. These inhibitors have the therapeutic potential to downmodulate the inflammation associated with a variety of Th1 mediated autoimmune diseases.

The importance of STAT4 in inflammatory bowel disease (IBD)

The activation of STAT4 by IL-12 in naïve CD4+ T cells is essential for their ability to develop into Th1 cells, characterized by their secretion of IFN-γ but not IL-4 or IL-17 upon TCR stimulation (22, 312). In addition to IFN-γ, Th1 cells also preferentially secrete other proinflammatory cytokines including TNF-α, GM-CSF and IL-2 (22). While the STAT4-dependence of IFN-γ gene expression has been well characterized (151, 313-315), STAT4-dependent regulation of other Th1 and Th17 proinflammatory cytokines, such as TNF-α and GM-CSF is less well-defined (62, 296, 316, 317). The requirement for STAT4-dependent cytokine regulation in the development of inflammatory immune responses including EAE, arthritis, and IBD highlights the critical role STAT4 plays in autoimmune diseases (51). With regard to colitis, T cells from *Stat4*-deficient mice developed significantly milder inflammation of the colon compared to wild type mice (62). Furthermore, mice that constitutively express STAT4 developed chronic transmural colitis characterized by massive influxes of CD4+ T cells of the Th1 phenotype (318). In humans, there is evidence that STAT4 is also a pathogenic factor in IBD since STAT4 is constitutively activated in patients with ulcerative colitis and IL-12Rβ2 is markedly upregulated with increased STAT4 activation in patients with Crohn's Disease (297, 319). Higher levels of the instructive cytokines IL-12 and IL-23 and the Th1 and Th17 produced cytokines IFN-γ, IL-17, IL-21, IL-6, and GM-CSF correlate with more severe pathologies in these diseases both in humans and in mice (100, 101, 297-301). In addition, TNF-α is a pathogenic factor in Crohn's Disease and anti-TNF-α therapies have shown impressive clinical efficacy in these patients (320, 321).

Role of the STAT4 C-terminal domain in mediating the pathogenesis of colitis

IBD consists of two chronic, inflammatory diseases of the gastrointestinal tract, Crohn's Disease and Ulcerative Colitis, where CD4+ Tcells play an important role in the dysregulated immune response. Crohn's Disease is typically associated with a Th1 and Th17 mediated response while Ulcerative Colitis is associated

with a Th2 response (322, 323). In genetically susceptible individuals, it is thought that CD4+ T cells activated by environmental antigens and enteric bacteria secrete proinflammatory cytokines and stimulate macrophages within the lamina propria to release a variety of soluble proinflammatory mediators. These mediators recruit leukocytes and stimulate the release of cytokines that damage the epithelial cells and the mucosal tissues creating the inflammation that characterize these disorders. To date, the most effective therapy has been aminosalicylates, sulfasalazine, corticosteroids and anti-TNF-α therapy, all of which either limit the production or activity of proinflammatory cytokines secreted by the leukocytes (324). While STAT4 is well known as an important regulator of inflammatory responses, the abilities of the STAT4 isoforms, STAT4α and STAT4β to mediate inflammatory disease has not been well characterized. As STAT4 has been implicated as a pathogenic factor in Th1 and Th17-mediated autoinflammatory diseases, including IBD (319), we chose an IBD model system where colitis is induced in SCID mice upon reconstitution with CD4+CD45RB^{high} T cells to test the roles of STAT4 isoforms in disease. This model system has the advantage of being able to directly test the ability of T cells expressing the STAT4 isoforms to mediate pathogenesis with minimal manipulation after reconstitution.

Many cytokines seem to play an important role in the development of colitis in this model of IBD. That TNF-α production plays an essential, non-redundant role in the pathogenesis of colitis in this disease model (325) is important as that is one of the cytokines that we observe to be differentially regulated by STAT4 isoforms. IFN-γ was also produced from STAT4β cells at higher levels ex vivo and has a role in disease development. While IL-17 is also critical for disease development, it was produced equally by STAT4α- and STAT4β-expressing T cells suggesting that the levels induced by the STAT4 isoforms were sufficient for the establishment of disease. Similarly, we did not observe any difference in the potential of STAT4α or STAT4β-expressing T cells to differentiate into adaptive T regulatory cells in vitro (data not shown). The differential effects of STAT4

isoforms on TNF-α and GM-CSF is interesting as there are some studies highlighting the cross-regulation of these two cytokines (326, 327). Importantly, infliximab (anti-TNF-α) treatment causes a concomitant decrease in both TNF-α and GM-CSF which is thought to contribute to apoptosis of PMNs and lead to decreased inflammation (299). It is possible that the differential expression of the STAT4 isoforms promote Th1 heterogeneity independent of IFN-γ production, due to the ability of STAT4β to enhance the secretion of TNF-α and GM-CSF.

The in vitro and in vivo data were divergent with regard to IFN-γ production upon anti-CD3 stimulation. While the IFN-γ levels were similar in the in vitro differentiated Th1 cells, there was significantly more IFN-γ in the ex vivo stimulated cells of the SCID mice reconstituted with the STAT4β isoform. This was not due to more T cells being present in the splenocytes or mesenteric lymph nodes as the percentage of CD4+ cells in those organs was not significantly different between the SCID mice reconstituted with either isoform (Fig. 4B and data not shown). It is possible that, given the ability of STAT4β to promote IL-12-stimulated proliferation to a greater extent than STAT4α (194) that there might be more differentiated Th1 cells expressing STAT4β in vivo. This would also explain the results with IL-12 and IL-18 stimulation where STAT4β-expressing cells were less responsive following in vitro stimulation, but produced similar levels of IFN-γ to STAT4α-expressing cells when stimulated with cytokine ex vivo. More Th1 cells, even if they produced less cytokine per cell, would generate the result observed. It is also possible that the increased IFN-γ is produced by accessory cells in the ex vivo cultures that might be differentially stimulated by interactions with STAT4α- or STAT4β-expressing cells.

Why the lack of a C-terminal transactivation domain mediates more severe inflammatory disease is still unknown although it may partly relate to the ability of STAT4β to stay phosphorylated longer than STAT4α. Possible reasons for this increased phosphorylation may relate to the structure of STAT4β. The lack of a C-terminal domain may cause conformational changes such that it inhibits

access to a phosphatase compared to STAT4α. Another possibility is that it may have differential abilities promote the upregulation of SOCS proteins. SOCS proteins can bind the IL-12R by virtue of their SH2 domains and can competitively inhibit STAT4 activation. If STAT4β-expressing T cells did not upregulate SOCS-3 to the same extent as STAT4α-expressing T cells, then that would result in higher levels of phosphorylated STAT4β. Alternatively, STAT4β may be unable to bind the SOCS promoter and upregulate its transcription thereby maintaining STAT4β phosphorylation. While the phosphorylation of the serine residue in the C-terminal domain of STAT4 is important for full transcriptional activity, it seems that STAT4β is not affected in mediating inflammatory cytokine production in vivo. These data suggest that the C-terminal domain may be inhibitory to the ability of STAT4 to transcribe genes unless the serine is phosphorylated. Thus, the splicing of the STAT4 RNA to delete the C-terminal 44 amino acids, which includes the serine, may aid STAT4β in the transcription of certain genes such as TNF-α and GM-CSF.

Comparison of the STATβ isoforms

STATβ isoforms have been characterized for STAT1, STAT3, STAT4 and STAT5. The β isoforms have been characterized as having attenuated functional properties compared to the full-length STAT proteins. The STAT1β isoform is unable to stimulate transcription as efficiently as STAT1α due to its inability to recruit the histone acetyltransferases p300/CBP (328). The STAT3β isoform also seems to function as a negative regulator of transcription (329). However, it appears that STAT3β is required for some functions since *Stat3β-/-* mice show decreased STAT3 activity overall and were less able to recover from endotoxic shock. *Stat3β*-deficient mice also demonstrated a hyperresponsiveness of a subset of endotoxin inducible genes in the liver (330). These findings indicate an important role for STAT3β in controlling systemic inflammation. STAT5β is derived from incompletely spliced Stat5a and Stat5b transcripts (331). Interestingly, the tyrosine phosphorylated carboxy-truncated forms were inherently more stable than the full-length proteins. Overexpression of the

shortened STAT5a isoform inhibited transcription of genes normally regulated by the full-length STAT5a gene suggesting that STAT5β is a dominant negative isoform like STAT1 and STAT3 (331). In certain circumstances, the STAT4β isoform behaves similarly to the other STATβ isoforms such as being unable to induce IFN-γ production as efficiently as STAT4α upon IL-12 stimulation. However, STAT4β is different than the other STATβ isoforms because it mediates increased inflammatory disease in vivo compared to STAT4α due to its differential regulation of proinflammatory cytokines. Thus, STAT4β is unique compared to the other STATβ isoforms due to its enhanced role in mediating inflammation.

Potential role for STAT4β as a pathogenic factor in auto-inflammatory diseases

There is potentially exciting clinical relevance to this study. The understanding of STAT4 isoform regulation in vitro or in vivo is unknown. For other human STAT isoforms, including STAT3 and STAT5, differentiation signals like G-CSF can induce the β isoform in myeloid cells (332, 333). In human T cell lines, both STAT4α and STAT4β are detectable, though it is not known if there are signals that might regulate splicing between the isoforms. It is also unclear whether an individual T cell expresses both isoforms or if one isoform is preferentially expressed. It will be important to ascertain the relative amounts of STAT4 isoforms in humans and whether an increase in the STAT4β isoform has any relevance to severity of disease or susceptibility to disease in human patients. It is plausible that there could be an association with an increase in the STAT4β isoform and Th1-mediated autoinflammatory diseases like colitis.

The importance of STAT4 in human disease has been demonstrated both by the requirement for STAT4 in human IL-12 signaling (334) and the association of STAT4 single nucleotide polymorphisms (SNPs) with autoimmune diseases (68-70, 335). A further understanding of the signals that regulate STAT4 mRNA splicing and the mechanisms through which STAT4 isoforms result in distinct

effector phenotypes will be important in characterizing progression in human disease and may provide additional targets for the treatment of disease.

Summary

In summary, Th cell subsets are essential for normal immune homeostasis. The indispensable role STAT proteins play in the induction and maintenance of Th cell subsets are highlighted in the immune sequalae found in STAT-deficient mice as well as the symptoms of patients who have dysfunctional STAT proteins. Thus, STAT proteins play specific, nonredundant roles in Th cell subset differentiation and normal immune homeostasis. Moreover, activation of STAT isoforms add another layer of control to the cytokine secretion profile of Th cell subsets.

Despite STAT proteins binding similar consensus DNA sequences, individual STAT proteins are most widely appreciated as promoting specific Th cell differentiation pathways. This implies that complex cooperative networks of STAT proteins, cofactors and other transcription factors contribute to the ability of specific STAT proteins to activate target genes required for the development of Th cell subsets. There are a variety of STAT mediated controls of Th cell differentiation. Not only can STAT activation induce specific Th cell subsets through epigenetics and regulation of surface receptors and transcription factors, the same STAT proteins also play essential roles in inhibiting transcription factors important for other Th cell subsets through direct and indirect mechanisms (Fig. 43).

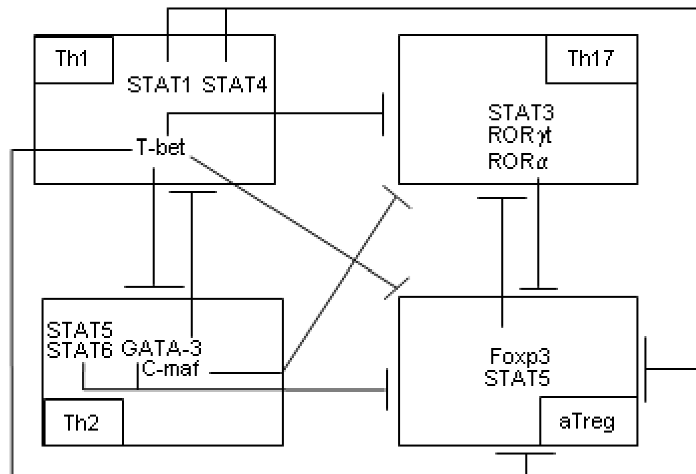

Figure 43. Summary of T helper cell subset specific transcription factors crossregulating the differentiation of other Th subsets. Each box represents the Th subset and within the box are the transcription factors important for the respective Th subset. Lines represent known inhibitory roles of the transcription factors in cross-regulating an opposing Th subset.

In addition, there is a hierarchy of certain Th cell subsets dominantly crossregulating other Th cell subsets. For example, Th1 and Th2 cells can inhibit the development of aTreg, Th17 cells, and their opposing subset. In contrast, Th17 cells can only inhibit aTreg development but not Th1 or Th2 development. Likewise, aTregs, during development, are sensitive to inhibition by the other subsets but can only crossregulate Th17 development. However, once these cells are fully differentiated, both aTreg and Th17 cells seem more resistant to inhibition by the other subsets and, in the case of aTregs, can suppress the proliferation of other effector Th cells (128, 134). In addition, Foxp3 expression in nTregs is relatively impervious to inhibition by STAT proteins and nTregs can inhibit the proliferation of the other effector Th cell subsets.

Ultimately, the in vivo cytokine milieu is critical in dictating Th cell development and immune response. STAT protein activation downstream of cytokines, DNA binding of STAT proteins, splice isoform regulation, physical interactions of transcription factors and chromatin remodeling all contribute to sculpting a repertoire of cytokines within a developing Th cell. Once differentiated, the effector Th cell can unleash its cytokines upon stimulation in an attempt to eradicate the pathogen. Regulatory T cells, on the other hand, work to control inflammation to minimize harm to the host. STAT protein regulation is a central component to this controlled process of Th cell differentiation and normal immune homeostasis.

Intrinsic to the process of inflammation is a mechanism by which STAT proteins within Th cells can selectively inhibit the development of cells that would impede inflammation and the resultant attempts of Th cells to eradicate a pathogen. More specifically, STAT proteins that are activated by instructive cytokines such as IL-4, IL-6 and IL-12 serve simultaneous roles of promoting proinflammatory cytokine secretion while inhibiting the anti-inflammatory transcription factor Foxp3 from being expressed in developing Th cells. Furthermore, STAT4 isoforms have differential abilities to regulate the induction of proinflammatory cytokines further refining the ability of Th cell subsets to mediate an immune response. As additional factors that impact this process are identified, the basis of specificity in STAT-dependent lineage determination and inflammatory cytokine production can be more clearly defined.

FUTURE DIRECTIONS

Defining the relative importance of inhibiting STAT5 from binding to the *Foxp3* gene and lineage determining transcription factors in repressing Foxp3

According to our results, IL-4, IL-6, and IL-12 can inhibit TGF-β1-induced Foxp3 by inhibiting access of STAT5 to the *Foxp3* locus. However, it is still unclear whether the repression by the STAT proteins is direct or indirect. We know from previous literature that lineage determining factors are also important in mediating Foxp3 repression (154, 233). To determine the relative importance of the lineage determining factors in repressing Foxp3, we could examine the ability of *Rorc-/-* to repress Foxp3 in Th17 conditions and *Gata3-/-* T cells to repress Foxp3 in TGF-β1+IL-4 conditions. While we observed T-bet independent effects of STAT4 on TGF-β induced Foxp3 repression, it is important to determine the roles GATA-3, downstream of IL-4, and RORγt, downstream of IL-6, play in Foxp3 repression.

To determine the relative importance of prevented access of STAT5 to the *Foxp3* locus, we could utilize *Stat5a/b-/-* CD4+ T cells since they do not completely lose their ability to express Foxp3 post-TGF-β1 stimulation compared to wild-type T cells (66% reduction). We could determine Foxp3 levels of Stat5a/b-/- T cells cultured under aTreg, TGF-β+IL-4, Th17, and TGF-β+IL-12 conditions for five days. If the prevention of STAT5 from binding the Foxp3 locus is the major mechanism by which STAT3, STAT4, and STAT6 use to inhibit Foxp3 expression, then we would expect very little, if any, diminution of Foxp3+ cells in the *Stat5a/b-/-* T cells. Based on these two experiments, we could begin to dissect the relative importance of lineage determining factors (T-bet, GATA-3, and RORγt or RORα) versus inhibiting STAT5 from binding to the *Foxp3* locus.

Characterizing STAT binding sites in the *Foxp3* gene

Based on our results, STAT proteins are important in regulating TGF-β1-induced Foxp3 regulation. Furthermore, STAT binding sites in the Foxp3 promoter are beginning to be characterized. Some STAT binding sites are present in silencer regions while others are present in the promoter and first intron (125, 234). To further identify evolutionarily conserved signaling pathways, we could perform a phylogenetic footprinting analysis of the STAT consensus binding site of $TTCN_{3-4}GAA$. Since functional STAT regulatory regions are likely to be conserved among diverse species, we would only consider those that had 100% homology at STAT consensus sites between humans, chimpanzees, mice, and rats. The combination of these species confers good specificity in discriminating important STAT regulatory regions (336). Using this analysis, we could culture T cells in TGF-β1 in the presence or absence of the instructive cytokines for 72 hours and perform ChIP assays on the regions identified for STAT binding. Then, we could perform analyses at these sites looking at histone modifications such as acetylation and methylation to get an impression on whether the chromatin is being remodeled positively (histone acetylation) or negatively (histone methylation at H3K9, H3K27, or H4K20) with regard to accessible chromatin. Furthermore, we could examine differences between nTregs and aTregs in chromatin modifications at the STAT regulatory regions to further define mechanisms by which STAT3, STAT4 and STAT6 can inhibit Foxp3 expression in aTreg cells but not in nTregs. Theoretically, STAT binding sites that bind STAT5 would primarily be associated with chromatin modifications that enable permissive transcription of the *Foxp3* gene. If there were STAT binding sites that were permissive to binding early in aTreg differentiation (<48 hours after TCR activation) but were associated with repressive chromatin modifications later in aTreg cells, then those sites would most likely be associated with STAT proteins negatively regulating Foxp3 expression.

Determining the mechanism by which TGF-β induced Foxp3 is refractory to inhibition after delayed addition of IL-6 and IL-12

We observed that TGF-β induced Foxp3 expression was refractory to IL-6 and IL-12 mediated repression while IL-4 could still repress Foxp3 if those cytokines were added 48 hours into aTreg culture. In order to begin to determine the mechanism for this observation, it would be important to ascertain whether T-bet and GATA-3 were differentially regulated by TGF-β1 48 hours into aTreg culture. If T-bet was suppressed similar to RORγt in aTreg conditions while GATA-3 maintained high levels of expression, then one could infer that IL-4 maintains the ability to repress Foxp3 due to maintenance of GATA-3 expression in the presence of TGF-β1. If T-bet and GATA-3 were suppressed like RORγt, then the refractoriness of aTreg cells to IL-6 and IL-12 mediated Foxp3 repression would not likely be due to lineage determining factors. If that were the case, we could then examine if the STAT proteins can still bind the *Foxp3* gene when IL-6 and IL-12 are added back later into culture. It is possible the STAT consensus DNA sequences that STAT3 and STAT4 bind in the *Foxp3* gene are no longer accessible or are occupied by STAT5 such that STAT3 and STAT4 cannot bind to the *Foxp3* gene.

Since STAT proteins need to be present early after TCR activation to inhibit Foxp3, this suggests potential cooperativity between TCR associated transcription factors and STAT proteins. Therefore, we could examine if there are binding sites for TCR associated transcription factors like NFAT, AP-1 or NFκB near STAT binding sites. If we found any, we could examine if STAT activation at different time points during the culture showed differential binding to those sites. If we observed differential binding, then we could directly examine if there is cooperativity between STAT proteins and NFAT or NFκB by adding pharmacological inhibitors to NFAT or NFκB and examine if STAT binding is altered at those sites.

Defining the specific roles of TGF-β1 and Foxp3 inhibiting Th subset specific proinflammatory cytokine secretion

To more clearly dissect the roles TGF-β1 and Foxp3 play in inhibiting proinflammatory cytokine secretion, we could culture CD4+ T cells from scurfy and wild-type mice in Th1, Th2, TGF-β1+IL-4 and TGF-β1+IL-12 for five days and restimulate the cells with anti-CD3. We then could compare IFN-γ expression levels by ELISA in Th1 and TGF-β1+IL-12 conditioned cells. Likewise, we could test IL-4 expression levels in Th2 and TGF-β1+IL-4 conditioned cells. This would allow us to observe the degree with which TGF-β1 suppressed the secretion of those cytokines in the absence of Foxp3. Depending on the degree with which TGF-β1 suppressed the secretion of IL-4 and IFN-γ in the absence of Foxp3, we could more clearly determine the relative importance of TGF-β1 induced Foxp3 in inhibiting effector cytokine production.

Potential ways to test the physiological relevance of STAT inhibition of Foxp3 expression and aTreg development in vivo

Adaptive Tregs are sufficient to inhibit inflammatory disease in vivo (128, 129). However, it has been difficult to directly assess the physiological relevance of the ability of STAT3, STAT4 and STAT6 to inhibit Foxp3 and aTreg development. For one thing, it is currently impossible to differentiate nTregs from aTregs. Therefore, one cannot assess if the protective effects of Foxp3 expressing cells are due to aTregs or nTregs. The most direct way to examine the in vivo role of STAT protein inhibition of Foxp3 in Th cells would be to induce an inflammatory disease like asthma in a *Stat4-/-/Stat6-/-/Stat3*$^{CD4-/-}$ mouse and compare the disease pathogenesis and numbers of Foxp3 expressing cells within lung tissues to wild-type mice. If our in vitro results translate in vivo, then we would expect protection from asthma in the *Stat4-/-/Stat6-/-/Stat3*$^{CD4-/-}$ mice with a concomitant increase of Foxp3-expressing cells compared to wild-type mice. However, obtaining such a mouse, although possible, is not feasible. A more indirect but realistic way to test the importance of STAT4 in regulating Treg function and development in vivo is examining asthma pathogenesis and Foxp3-expressing T

cells in a *Stat4-/-* and wild-type mice. During pilot studies in our lab, *Stat4-/-* mice were protected from asthma. However, we did not quantitate the numbers of Foxp3-expressing T cells within the lungs of the mice. We found that STAT4 downstream of IL-12 signaling does not inhibit Foxp3 in nTregs but does inhibit Foxp3 in aTreg cells. If we found increased Foxp3+ T cells in the lungs of *Stat4-/-* mice then we could indirectly attribute the increased Treg cells in the *Stat4-/-* mice being due to an inability of IL-12 to inhibit Foxp3 due to the absence of STAT4. A similar experiment using *Stat6-/-* and wild-type mice could be done to examine if Foxp3 expressing T cells were significantly different in the absence of STAT6. These experiments would help to define the physiological relevance of STAT protein inhibition of TGF-β1-induced Foxp3 in vivo.

Determining the functional relevance of STAT4 tetramerization

Since loss of the N-terminal domain prevents STAT4 from being phosphorylated, studying the importance of STAT4 tetramerization in inducing Th1 genes downstream of IL-12 signaling has been difficult. However, we have developed STAT4 N-terminal deletion mutants that can be inducibly phosphorylated upon the addition of 4-OH tamoxifen. Therefore, we could subclone STAT4ERT2 and Δ124STAT4ERT2 into retroviral vectors and transduce STAT4ERT2 and Δ124STAT4ERT2 into primary Th cells. Then, we could add 4-OH tamoxifen to those cells to activate STAT4. After a five-day culture, we could examine Th1 products such as IL-18Rα, CD25, CXCR3, IL-12Rβ2, CCR5, and IFN-γ. Alternatively, *Stat4-/-* T cells could be transduced with virus in order to exclude the possibility of endogenous STAT4 playing a role in gene induction. In this way, we could determine if the deletion of the N-terminal domain affected gene induction by STAT4 proteins.

Determining the role of STAT4 in inducing TNF-α and GM-CSF upregulation

With regard to the C-terminal domain studies, it is unclear if the STAT4 requirement for CD4+ Th1 cells to secrete TNF-α and GM-CSF is direct or indirect. To examine if STAT4 directly regulates TNF-α and GM-CSF, we could

perform phylogenetic footprinting assays looking for evolutionarily conserved STAT4 consensus sequences within the *Tnf* and *Csf2* genes. Then, we could perform ChIP assays on *Stat4-/-* and wild-type CD4+ T cells to directly determine STAT4 binding to those sites. Subsequently, we could examine the histone modifications at those sites to determine the openness of the chromatin for STAT binding to the *Tnf* and *Csf2* genes.

To examine if the STAT4-dependence is indirect, we could examine other plausible STAT4 dependent Th1 genes that are important for TNF-α and GM-CSF production. One possibility includes T cell costimulatory molecules that are upregulated by STAT4 and are important in TNF-α regulation including CD40L and LIGHT. We could examine costimulatory molecule upregulation between *Stat4-/-*, STAT4α, and STAT4β Th1 cells and examine if the costimulatory molecules are differentially regulated between STAT4α and STAT4β cells. In order to determine if those costimulatory molecules are playing a role in TNF-α and GM-CSF production, we could add neutralizing antibodies to the Th1 cultures and assess if TNF-α and GM-CSF production are altered upon anti-CD3 restimulation compared to cultures that did not receive neutralizing antibodies.

Determining the role of STAT4β in pediatric patients afflicted with colitis

Since STAT4β can mediate more enhanced inflammation in a murine model of colitis than STAT4α-expressing T cells, we would like to determine if STAT4 isoforms are differentially expressed by patients with colitis compared to normal healthy control patients. Furthermore, we would like to experimentally examine if STAT4β enhancement correlates with an increased propensity of the T cells from those patients to secrete TNF-α and GM-CSF. To determine this, we have designed primers that differentially recognize STAT4α or STAT4β cDNA. Utilizing these primers, we could perform PCR on cDNA isolated from the patient's peripheral blood mononuclear cells (PBMC) with or without colitis to examine if STAT4α:STAT4β ratios are different from normal healthy patient controls. In parallel, we could characterize the inflammatory cytokine production

from the T cells of those patients to see if increased STAT4α:STAT4β ratios correlates with increased inflammatory cytokine production (IFN-γ, TNF-α, and GM-CSF).

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CURRICULUM VITAE

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Education

2002	**B.S.** Biological Sciences, University of Notre Dame	Notre Dame, IN
2008	**Ph.D.** Department of Microbiology and Immunology, Indiana University	Indianapolis, IN

Honors, Awards, and Fellowships

2001	National Science Foundation Research Fellowship
2001	Inducted into Alpha Epsilon Delta
2002	Lawrence H. Baldinger Award-given to the Senior student in pre-professional studies who excelled in scholarship, leadership, and character
2002	Outstanding Biological Scientist Award-given to five Senior students who demonstrated the most promise in the Biological Sciences as evidenced by both academic performance and research participation
2002	Inducted and current member of Phi Beta Kappa
2002-2004	Recipient of Lilly Foundation Indiana Medical Scientist Training Program grant
2004	Awarded fellowship on Immunology and Infectious Disease T32 training grant
2005-2008	Dean-Appointed member of Teacher Learner Advocacy Committee for the Indiana University School of Medicine
2007	Young Scientist Travel Grant. 13th International Congress of Immunology 2007. Rio de Janeiro, Brazil
2007	Travel Fellowship. IUPUI
2008	IU School of Medicine Graduate Senior Award
2008	Lois Kinzer Endowed Fellowship-given to student who exemplifies scholarship and character

Abstracts Presented and Conferences Attended

2004	Autumn Immunology Conference-"Requirement for the N-Terminal Domain of STAT4 in IL-12 Stimulated Activation." Chicago, IL
2005	Autumn Immunology Conference-"Distinct Requirements for the N-Terminal Domain of STAT4 in Tyrosine Phosphorylation and DNA Bound Tetramerization." Chicago, IL
2006	Autumn Immunology Conference-"Differing Pathogenic Roles of STAT4 Isoforms in a Murine Model of Colitis." Chicago, IL

2007 International Immunological Congress-"Cytokines instructive in T helper cell development inhibit Foxp3 expression and adaptive T regulatory cell development." Rio De Janeiro, Brazil

2007 Keystone Symposia: Jaks, Stats, and Immunity-" Differing Pathogenic Roles of STAT4 Isoforms in a Murine Model of Colitis." Steamboat, CO

Publications

2007 Kaplan MH, Sehra S, Chang HC, O'Malley JT, Mathur AN, Bruns HA. Constitutively active Stat6 predisposes towards a lymphoproliferative disorder. *Blood*. 2007 Dec 15;110(13):4367-9.

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O'Malley JT and Kaplan MH. STAT protein regulation of T cell phenotypes. Chapter. Jak-Stat Pathway in Disease. Landes Publishing. *In press.*

Papers submitted

O'Malley JT, Sehra S, Thieu VT, Yu Q, Chang H-C, Stritesky GL, Nguyen ET, Mathur AN, Levy DE and Kaplan MH. STAT4 limits the development of adaptive Treg cells. Submitted.

Good SR, Thieu VT, Mathur AN, O'Malley JT, Perumal NB and Kaplan MH. Temporal induction patterns of STAT4 target genes defines potential for Th1 lineage-specific programming. Submitted.