THE DEVELOPMENT AND COMMITMENT OF T HELPER SUBSETS

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DEDICATION

I would like to dedicate this thesis to my family.

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ABSTRACT

Gretta L. Stritesky

THE DEVELOPMENT AND COMMITTEMENT OF T HELPER SUBSETS

T helper cells play a crucial role in providing protection against a wide variety of pathogens. The differentiation and effector function of T helper cell subsets is dependent on cytokine activation of Signal Transducer and Activator of Transcription (STAT) family members. The development of Th17 cells, which are important for immunity to fungi and extracellular bacteria, relies on STAT3. We show that IL-23 in combination with IL-1β promotes maintenance of the Th17 phenotype following multiple rounds of stimulation. However, IL-23 does not promote commitment of Th17 cells, and when Th17 cells are cultured with IL-12 or IL-4 they switch to a Th1 and Th2 phenotype, respectively. The maintenance of the Th17 phenotype by IL-23 also requires STAT4. STAT4-deficient memory cells cultured with IL-23 have reduced IL-17 production following stimulation with either anti-CD3 or IL-18+IL-23 stimulation compared to wild type memory cells. Furthermore, STAT4-deficient mice have impaired *in vivo* Th17 development following immunization with ovalbumin. This challenges a one-STAT/one-subset paradigm and suggests that multiple STAT proteins can contribute to a single phenotype. To test this further we examined whether STAT3 is required for the development of Th2 cells, a subset known to depend upon the IL-4-induced

activation of STAT6 for immunity to parasites and promoting allergic inflammation. We demonstrate that in the absence of STAT3, the expression of Th2-associated cytokines and transcription factors is dramatically reduced. STAT3 is also required for *in vivo* development of Th2 cells. Moreover, allergic inflammation is diminished in mice that have T cells lacking expression of STAT3. STAT3 does not affect STAT6 activation, but does impact how STAT6 functions in binding target genes. Thus, multiple STAT proteins can cooperate in promoting the development of specific T helper subsets.

Mark H. Kaplan, Ph.D.-Chair

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LIST OF ABBREVIATIONS

Ag	Antigen
AhR	Aryl hydrocarbon receptor
AITL	Angioimmunoblastic T cell lymphoma
Alum	Aluminum hydroxide
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BATF	Basic leucine zipper transcription factor ATF-like
Bcl-6	B-cell lymphoma 6 protein
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
CIA	Collagen induced arthritis
CTL	Cytotoxic T lymphocyte
CVID	Common variable immunodeficiency
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis
ERM	Ets related molecule
Foxp3	Forkhead box protein 3
GATA-3	GATA binding protein 3
Gfi	Growth factor independence
Hlx	H2.0-like homeobox-1

ICS	Intracellular cytokine staining
ICOS	Inducible T-cell co-stimulator
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-4R	IL-4 receptor
IL-6R	IL-6 receptor
IL-21R	IL-21 receptor
IL-23R	IL-23 receptor
IPEX	Immunodeficiency, polyendocrinopathy enteropathy, and X-linked syndrome
iTregs	Inducible T regulatory cells
JAK	Janus family tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
MHC	Major histocompatibility complex
NFAT	Nuclear factor of activated T cells
NK	Natural killer
nTregs	Natural T regulatory cells
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed death 1
PRR	Pattern recognition receptor
pSTAT	Phospho-STAT
ROR	Retinoic-acid-related orphan receptor

RORC	Gene name for RORγt
RT-PCR	Real-time polymerase chain reaction
SH2	Src homology 2
SLE	Systemic lupus erythematosus
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
TCR	T cell receptor
Tfh	T follicular helper
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
Th9	T helper type 9
Th17	T helper type 17
TGF	Transforming growth factor
WT	Wild type
α-CD3	anti-CD3
α-CD28	anti-CD28
α-IL-6	anti-IL-6
α-IL-21	anti-IL-21

INTRODUCTION

Innate and adaptive immune responses

Humans are threatened by invading pathogens on a daily basis. However, we are usually unaware of this due to the sophisticated protection provided by our immune system. The immune system is composed of two subdivisions important for host protection; the innate and adaptive immune systems. The innate immune system plays an important role in fast acting clearance of pathogens. Following infection, the innate immune response does not provide long lasting immunity. Conversely, the adaptive immune response is specific for particular pathogens or components of pathogens. The adaptive immune system plays a critical role in protection against subsequent infections due to its ability to provide long-lasting protective immunity. Collectively, the innate and adaptive immune systems provide a comprehensive defense against most pathogens the host may encounter.

The ability to recognize a wide variety of pathogens allows the innate immune system to combat a diverse repertoire of pathogens. The innate immune system employs several mechanisms that help avoid or destroy pathogens. The first line of defense provided by the innate immune system includes both physical and chemical barriers of the body. The skin and linings of the gastrointestinal tract and respiratory tract provide a physical barrier that blocks the entrance of many infectious organisms. Working in concert with the physical barriers, chemical barriers such as sweat, digestive enzymes, defensins, gut flora, surfactant,

mucus, saliva, and tears provide an additional line of defense. Even though the physical and chemical barriers are successful in providing protection against most invaders, some pathogens break the barriers and initiate an infection. The innate immune system provides further protection upon breach of the host barriers through the actions of the innate immune cells. The basic function of the innate immune system is to opsonize or phagocytose pathogens, activate the complement cascade, and/or initiate the pro-inflammatory response.

In order to carry out these functions the innate immune system is armed with many highly specialized cell types including granulocytes (neutrophils, eosinophils, basophils, and mast cells) Natural Killer (NK) cells, γδ T cells, NKT cells, and phagocytic cells (neutrophils, dendritic cells, and macrophages). Granulocytes are one of the first responders to infection. They express several chemokine and cytokine receptors that help them quickly migrate to the site of infection. Granulocytes, as their name implies, secrete granules that are anti-microbial as well as cytokines that aide in the recruitment and activation of other immune cells (Nathan, 2006).

NK cells play an important role in tumor rejection and killing of virally infected cells. NK cells express several surface receptors that recognize target cells that do not express inhibitory ligands. Upon encountering cells lacking these ligands, NK cells become activated and release cytotoxic granules consisting of perforin and granzyme, which can kill tumor or infected cells. NK cells also release large

amounts of IFN-γ and IL-4 which in turn activate other immune cells (Lodoen and Lanier, 2005; Raulet and Guerra, 2009).

γδ T cells represent a small subset of T cells that express a T cell receptor (TCR) composed of the γ chain and the δ chain. The expression of the γδ TCR makes these T cells distinct from the conventional T cells that express a TCR composed of an α and β chain. γδ T cells have restricted TCR diversity and it is thought that antigen processing and presentation is not necessary for antigen recognition. The TCR of γδ T cells can function as a pattern recognition receptor and therefore can recognize and respond to microbes directly. γδ T cells have many functions that are not restricted to innate immunity, but rather are also thought to be important in adaptive immunity (Born et al., 2006).

Like γδ T cells, the characterization of NKT cells as innate versus adaptive immune cells is not very clear. NKT cells are a lineage of T cells that express NK cell markers. The T cell receptors expressed on NKT cells are very limited and are thought to play an important role in recognizing lipids. The recognition of lipids by NKT cells requires the antigen-presenting molecule CD1. After lipid recognition, NKT cells secrete cytokines, such as IL-4 and IFN-γ, which help guide the adaptive immune response (Bendelac et al., 2007).

Perhaps the most critical cell in innate immunity is the phagocyte. Phagocytes are able to discriminate between pathogen and host surface molecules and upon

recognition, phagocytes engulf the pathogen. Once the pathogen is internalized, phagocytes utilize several mechanisms to kill the pathogen. The intracellular compartments of the phagocyte contain antimicrobial enzymes, proteins and peptides that can fuse with the pathogen-containing compartment and lead to killing of the pathogen. Other mechanisms of pathogen killing utilized by phagocytes include the production of nitric oxide, superoxide anion, and hydrogen peroxide, which are all lethal to bacteria. The other role of phagocytes in innate immunity lies at the crucial interface of the innate and adaptive immune responses. After ingestion by the phagocyte, pathogens are broken down into small peptides, which are then loaded onto major histocompatibility complex (MHC) molecules. This MHC-antigen complex is then transported to the cell surface where it can activate lymphocytes and initiate the adaptive immune response (Blander, 2008; Guermonprez et al., 2002; Jutras and Desjardins, 2005).

It is critical for the innate immune system to be capable of distinguishing foreign pathogens from host molecules. Innate immune cells express a variety of receptors that allow them to recognize many components of bacteria, viruses, fungi, and parasites or pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs is through pattern recognition receptors (PRRs) which innate immune cells express at high levels. Surface pattern recognition receptors that recognize PAMPs include toll like receptors and the mannose receptor (Apostolopoulos and McKenzie, 2001; Beutler et al., 2006; Takeda et

al., 2003). There are also PRR family members, NOD-like receptors and RNA helicases, that are cytoplasmic and therefore recognize intracellular pathogens (Inohara et al., 2005; Thompson and Locarnini, 2007). Upon PRR engagement, innate cells secrete several cytokines and chemokines, up-regulate co-stimulatory molecules, and process and present antigen to adaptive immune cells. These processes are important for the clearance of the pathogen through the activation and recruitment of adaptive immune cells (Blander, 2008).

The primary cell type important for carrying out the adaptive immune response is the lymphocyte. The major types of lymphocytes are B and T cells. The prominent function of B cells in the adaptive immune response is to produce antibodies. B cells express a B cell receptor on the cell surface, which recognizes a specific native antigen. Upon engagement of the B cell receptor (and in most cases additional help from CD4+ T cells is required) the B cell differentiates into a short-lived plasma cell. Plasma cells produce and secrete large amounts of antibody. A small percentage of plasma cells become memory B cells, which are important in subsequent infections. T cells are the other important lymphocyte type in the adaptive immune response. T cells are further differentiated based on the surface expression of CD8 or CD4. CD8 cells T cells, also called cytotoxic T lymphocytes (CTLs), are important for protection and clearance of intracellular pathogens and tumor surveillance due to their cytotoxic potential (Harty et al., 2000). CD4 T cells are referred to as “T helper cells” (Th).

Like their name suggests, T helper cells provide protection to the host by helping or directing other cells to carry out effector functions.

JAK-STAT pathway

Cytokines play a critical role in T helper cell differentiation, the process through which antigen naïve CD4 T cells acquire effector phenotypes. They mediate their actions primarily through the activation of the Janus family tyrosine kinases (JAKs)/Signal Transducer and Activator of Transcription (STAT) pathway. The Janus kinase family consists of 4 family members, JAK1, JAK2, JAK3, and TYK2. JAKs associate and bind to receptors. Upon cytokine binding to the receptor, JAKs become activated and phosphorylate specific tyrosine residues on the intracellular portion of the receptor. The specific tyrosine residues phosphorylated by JAKs provide docking sites for the STAT proteins. The STAT family of transcription factors includes STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. The STAT family members are all capable of carrying out 6 specific functions: bind phosphorylated tyrosines on the intracellular portion of the appropriate receptor, become phosphorylated on their conserved tyrosine residue, hetero- or homo-dimerize, translocate to the nucleus, bind DNA, and modulate gene expression. The first function of STAT proteins occurs when STAT proteins are recruited to the phosphorylated tyrosine residues on the receptor through their conserved src homology 2 (SH2) domains. After docking to the receptor, a conserved tyrosine residue on the STAT protein becomes phosphorylated. The phosphorylated tyrosine residue allows STAT family

members to form homo or heterodimers through the interaction of the SH2 domain and the phosphorylated tyrosine present on both proteins. Dimerization of the STAT proteins promotes translocation to the nucleus where they bind and activate lineage specific genes (Figure 1). Thus, cytokine induced STATs allow appropriate expression of genes important for differentiation and commitment to a specific lineage. Each cytokine receptor is associated with specific JAK and STAT family members. Therefore, each T helper cell lineage is associated with specific STAT family members and their differentiation and effector function is dependent on them (Leonard and O'Shea, 1998).

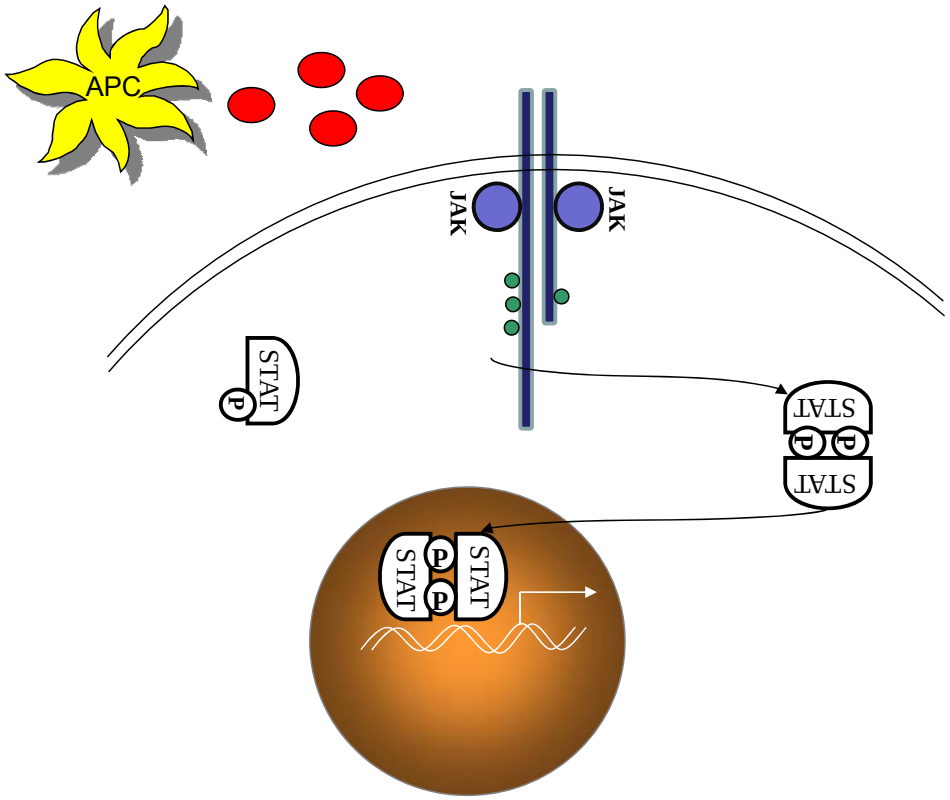

Figure 1. Activation of STAT proteins following cytokine stimulation. Cytokines in the microenvironment (often secreted by antigen presenting cells) bind to their specific receptor on the T cell. Receptor engagement activates

JAKs which phosphorylate specific tyrosine residues on the intracellular portion of the receptor. Following phosphorylation of the receptor, STATs are recruited and bind to the receptor where they become phosphorylated. Phosphorylated STATs dimerize and translocate to the nucleus where they mediated gene expression.

T helper subsets

T helper cell activation and differentiation requires 3 distinct signals from the antigen-presenting cell. The first signal is the recognition of the peptide-MHC molecule presented by the antigen-presenting cell. The second signal is termed co-stimulation or verification. The APC up-regulates ligands that engage receptors on the T cell leading to further stimulation and activation. The final signal is transduced through the secretion of cytokines that skew the cells toward a specific cell lineage. Depending on the strength of signal and cytokines present during the APC-T cell interaction, the T helper cell will become activated, proliferate, and differentiate into an effector cell. Each T helper cell lineage expresses specific transcription factors and cytokines important for associated effector functions. Originally T helper subsets were defined as either Th1 or Th2 (Mosmann et al., 1986). However, recently many new distinct T helper subsets have been added to the T helper cell family. Each T helper subset differentiates in the presence of specific cytokines and thus specific STAT family members are required. Once differentiated, T helper cells express lineage specific transcription factors and cytokines that play a crucial role in clearance of pathogens (Figure 2).

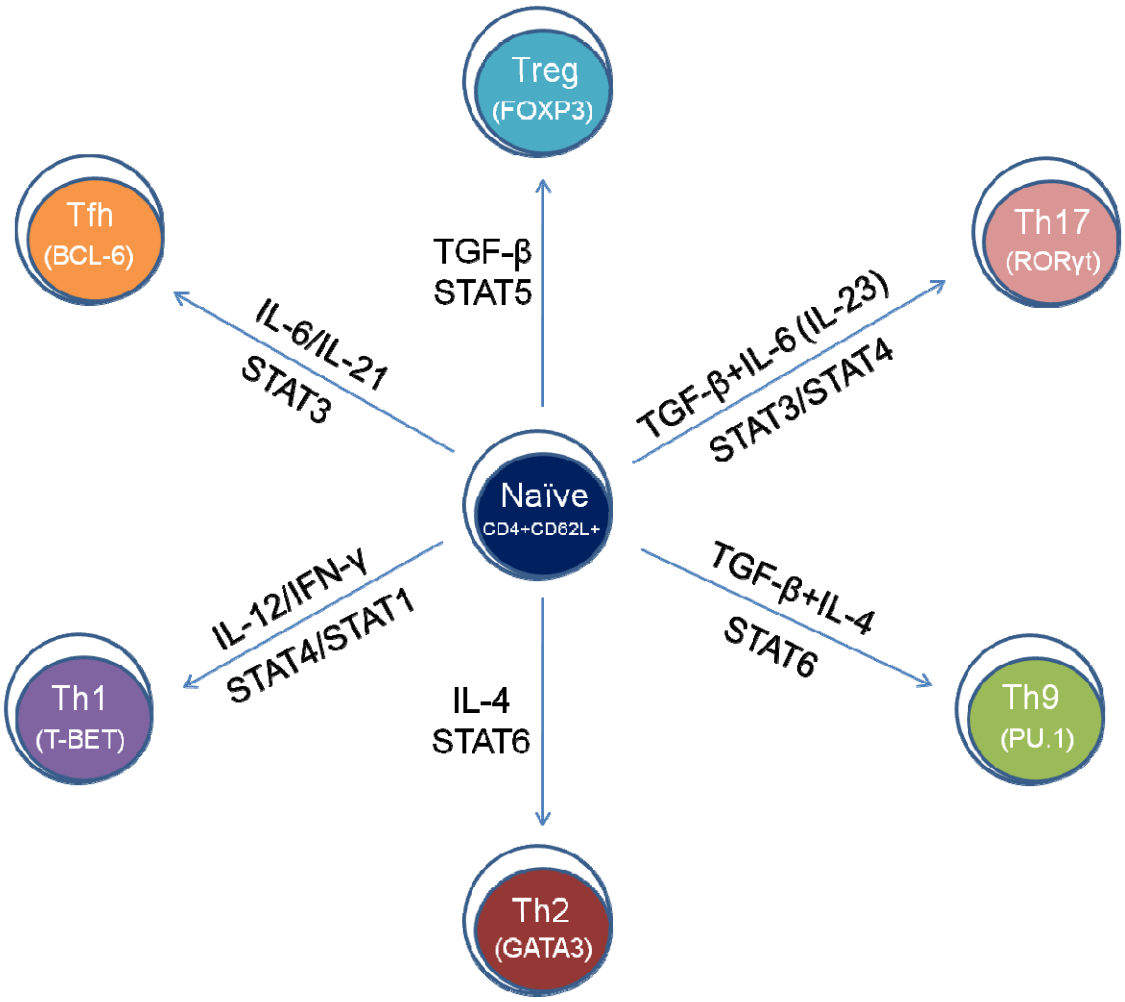

Figure 2. Differentiation of T helper cell subsets from naïve precursors. Naïve T cells differentiate into several distinct subsets based on the signals received during their interaction with APCs.

Th1 cells

Naïve CD4+ T cells activated in the presence of IL-12 and anti-IL-4 differentiate into Th1 cells (Hsieh et al., 1993; Manetti et al., 1993). Th1 cells are a unique subset of T helper cells characterized by the production of IFN-γ and lymphotoxin-α. IFN-γ is important for the activation of macrophages and can increase their microbicidal activity (Suzuki et al., 1988). Immunity against intracellular bacteria, protozoa, and fungi is dependent on Th1 effector function

(Fieschi et al., 2004; Fieschi et al., 2003; Hsieh et al., 1993). The differentiation of Th1 cells requires several cytokines, transcription factors and receptors.

Importance of STAT family members in Th1 development

Th1 cells differentiate in the presence of IL-12 and differentiation is further enhanced by IFN-γ. IL-12 and IFN-γ signal through STAT family members STAT4 and STAT1, respectively. The combination of the cytokines IL-12 and IL-18 can also synergize to promote IFN-γ production. The synergistic effects of IL-12 and IL-18, like IL-12 stimulation alone, are STAT4 dependent (Barbulescu et al., 1998; Ouyang et al., 1999; Yang et al., 1999). Since the differentiation of Th1 cells is dependent on both STAT1 and STAT4, mice deficient in either STAT4 or STAT1 have impaired Th1 differentiation (Afkarian et al., 2002; Kaplan et al., 1996b; Lighvani et al., 2001; Thierfelder et al., 1996). Additionally, STAT1 and STAT4-deficient mice have increased susceptibility to both bacterial and viral infections illustrating their importance in Th1 development and effector function (Cai et al., 2000; Kaplan, 2005; Meraz et al., 1996; Tarleton et al., 2000).

Additional transcription factors required for Th1 differentiation

Along with the STAT family members STAT4 and STAT1, there are several other transcription factors important in the development of Th1 cells. T-box transcription factor family member T-bet (*Tbx21*) has been identified as the "master regulator" of Th1 differentiation. T-bet expression is induced upon TCR stimulation and is downstream of IFN-γ/STAT1 (Afkarian et al., 2002; Lighvani et

al., 2001). The expression of T-bet was originally thought to be independent of STAT4, however T-bet expression is significantly reduced in *Stat4-/-* Th1 cells. Moreover, it is clear that T-bet requires STAT4 for complete IL-12 induced Th1 differentiation (Mullen et al., 2001; Thieu et al., 2008). T-bet is expressed specifically in Th1, but not Th2 cells. However, over-expression of T-bet in Th2 cells using retro-viral transduction can induce the expression of IFN-γ (Mullen et al., 2002; Szabo et al., 2000). In addition to inducing IFN-γ expression, T-bet also induces the expression of IL-12Rβ2. Increased expression of IL-12Rβ2 on developing Th1 cells leads to increased IFN-γ production and Th1 lineage commitment (Afkarian et al., 2002; Mullen et al., 2001). The importance of T-bet in Th1 differentiation and effector function can be seen using T-bet-deficient mice. *In vitro*, T-bet-deficient CD4 T cells cultured under Th1 skewing conditions have significantly impaired IFN-γ production. Furthermore, *in vivo,* T-bet-deficient mice failed to mount a Th1 response following infection or antigen immunization (Szabo et al., 2002). T-bet is also important for the expression of the transcription factor H2.0-like homeobox-1 (*Hlx)*. *Hlx* is a homeobox gene that is specifically expressed in Th1 cells and can synergize with T-bet to promote IFN-γ production and increased IL-12Rβ2 expression (Martins et al., 2005; Mullen et al., 2002). Other transcription factors including ERM and Runx3 are highly expressed in Th1 cells. ERM is induced by IL-12 in a STAT4 dependent manner, however the role of ERM in Th1 development is still not completely understood (Ouyang et al., 1999). Runx3 has the ability to both repress Th2 development and augment IFN-γ production. Runx3 repression of Th2

development is mediated through binding of the IL-4 silencer in a complex with T-bet and attenuating GATA3 transcriptional activity (Djuretic et al., 2007; Kohu et al., 2009). The expression and cooperation of all Th1 associated transcription factors play an important role in promoting Th1 development and repressing other lineages specific factors.

The role of Th1 cells in human diseases

The protection Th1 cells provide against mycobacterial species is further illustrated by the increased susceptibility to these infections in patients who have mutations in IFNGR1, IFNGR2, STAT1, IL12RB1, and IL12B (Filipe-Santos et al., 2006; Zhang et al., 2008). Additionally, polymorphisms in the Th1 master regulator, TBX21 (T-bet) are associated with increased incidence of asthma and airway hyperresponsiveness, suggesting a switch in the Th1-Th2 balance (Raby et al., 2006). It is important to keep in mind that many of the Th1 associated genes mutated in these patients are also required for IFN-γ production from innate cells including NK cells. Delineating the role the mutations play in Th1 cells versus innate cells in these patients will need further study. Additionally, Th1 cells are important in the anti-tumor response. CD4 T cells from lymphoma patients who underwent autologous stem cell transplantations are deficient in STAT4 expression and do not respond to IL-12 immunotherapy. This acquired decreased expression of STAT4 in CD4 T cells from these patients' results in the inability of the cells to differentiate into Th1 cells. Reconstitution with STAT4 rescues the Th1 defect in the patients' cells demonstrated by the production of

IFN-γ. These data suggest that the treatment given to lymphoma patients may affect their ability to respond to systemic IL-12 therapy. Therefore, it is important to elucidate the mechanism leading to reduced STAT4 expression in patient CD4 T cells following transplantation before therapies such as IL-12 are used (Chang et al., 2009; Robertson et al., 2005).

Role of Th1 cells in mouse models of disease

It is evident that Th1 responses are required for clearance of intracellular bacteria and parasites, however inappropriate Th1 responses contribute to autoimmunity. It was originally shown that Th1 cells were required for and were the predominant pathogenic cell type in mouse models of autoimmune diseases including experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveitis (EAU), and collagen induced arthritis (CIA) (Caspi et al., 1996; Germann et al., 1996; Merrill et al., 1992; Pettinelli and McFarlin, 1981; Saoudi et al., 1993; Tarrant et al., 1998). These data contradicted the studies that showed targeting IFN-γ or the p35 subunit of IL-12 did not protect mice from developing EAE or CIA, or EAU. Furthermore, neutralization of IFN-γ or IL-12 or mice deficient in IFN-γR had exacerbated EAE and CIA (Billiau et al., 1988; Ferber et al., 1996; Gran et al., 2002; Jones et al., 1997; Vermeire et al., 1997). The recent discovery of Th17 cells (discussed below) and the role they play in autoimmunity has helped clarify the conflicting results. Even though the contribution of Th1 versus Th17 is not completely clear, increasing evidence suggests that both Th1 and Th17 cells contribute to the pathogenesis of most autoimmune diseases.

Th2 cells

The differentiation of Th2 cells occurs when a naïve T cell is activated by antigen in the presence of IL-4. Th2 cells secrete IL-4, IL-5, and IL-13. IL-4 production leads to a positive feedback loop and increased Th2 differentiation, and is also important in B-cell class switching to IgE (Kopf et al., 1993; Le Gros et al., 1990; Swain et al., 1990). IL-5 is necessary for the recruitment of eosinophils (Coffman et al., 1989) whereas the induction of airway hypersensitivity and expulsion of worms are two functions of IL-13 (Urban et al., 1998; Wynn, 2003). Collectively, Th2 cytokines are important for immunity against extracellular parasites and for providing B cell help leading to antibody production (Zhu and Paul, 2008). IL-4 is required for Th2 differentiation, however the initial source of IL-4 is not completely clear. The IL-4 receptor is composed of the common cytokine receptor gamma subunit and the IL-4Rα chain. IL-4R is expressed at low levels on the surface of naïve CD4 T cells, but upon antigen stimulation IL-4R surface expression is increased. IL-4 binding to its receptor leads to the activation of STAT6 and Th2 specific genes (Nelms et al., 1999).

Importance of STAT family members in Th2 development

Because of its role in IL-4 signaling, STAT6 was initially shown to be required for Th2 development, however in some *in vivo* Th2 models STAT6 is dispensable for Th2 differentiation (Kaplan et al., 1996a; Paul and Zhu, 2010; Shimoda et al., 1996; Takeda et al., 1996). Moreover, STAT6 is required for the expression of the Th2 transcription factors *Gata3* and *Gfi1* as well as Th2 cytokine genes and

has been shown to bind directly to a subset of these loci (Lee and Rao, 2004; Sherman et al., 2002; Zhu et al., 2002). The importance of the IL-4-STAT6 pathway in Th2 differentiation and effector function has been illustrated by use of mice with impaired IL-4-STAT6 signaling. *Stat6-/-* mice or mice injected with neutralizing IL-4 antibodies have an impaired Th2 response following nematode infection (Else et al., 1994; Takeda et al., 1996). Conversely, CD4 T cells from transgenic mice expressing constitutively active STAT6 (STAT6VT) have a predisposition towards the Th2 lineage even in non-skewing conditions (Bruns et al., 2003; Sehra et al., 2008). In addition to IL-4-STAT6 signaling, IL-2 induced STAT5 activation is also necessary for optimal Th2 differentiation. The role of IL-2-STAT5 signaling in Th2 cells is both through direct effects on Th2 cytokines, as well as induction of Th2 gene expression. STAT5 is required for *Il4* chromatin accessibility, *Il4ra* expression, and the induction of *Socs3* which extinguishes Th1 differentiation (Cote-Sierra et al., 2004; Kagami et al., 2001; Takatori et al., 2005b). Disruption of the IL-2-STAT5 pathway by the neutralization of IL-2 or STAT5a-deficiency in Th2 cultures impaired *Il4* expression (Takatori et al., 2005a; Zhu et al., 2003). Conversely, the expression of constitutively active STAT5a induced, albeit limited, Th2 differentiation and cytokine production even in the absence of STAT6 (Zhu et al., 2003). Therefore, both STAT6 and STAT5 are required for the induction of Th2 specific genes leading to Th2 development.

Additional transcription factors required for Th2 differentiation

In addition to STAT6 and STAT5 there are a number of transcription factors associated with establishing the Th2 phenotype. IL-4 activation of STAT6 induces expression of GATA3 which is considered to be the master regulator of Th2 cells (Ouyang et al., 2000). GATA3 directly binds to the promoter regions of IL-5 and IL-13 and the enhancer regions of IL-4, and is therefore important in Th2 cytokine expression (Agarwal et al., 2000; Kishikawa et al., 2001; Siegel et al., 1995; Yamashita et al., 2002; Zhang et al., 1998). Retroviral expression of GATA3 in Th1 cells promotes IL-4 production and the up-regulation of GATA3 itself (Ouyang et al., 2000; Ouyang et al., 1998). Alternatively, mice deficient in GATA3 or expressing a dominant-negative form of GATA3 have impaired Th2 development both *in vitro* and *in vivo* (Pai et al., 2004; Zhang et al., 1999a; Zhu et al., 2004). In addition to GATA3, c-maf plays a critical role in Th2 development. The expression of c-maf is induced during Th2 differentiation by IL-6 and not IL-4 (Yang et al., 2005). c-Maf is important for the expression of IL-4, but not IL-5, or IL-13 in Th2 cells. c-Maf can directly bind and transactivate the IL-4 promoter and when it is expressed ectopically can induce IL-4 production (Ho et al., 1996). In agreement with the role of c-maf in promoting IL-4 production, c-maf-deficient mice have decreased IL-4 production (Kim et al., 1999). The transcription factor IFN regulatory factor 4 (IRF4), which is up-regulated following TCR stimulation, is also important in Th2 differentiation (Lohoff et al., 2002; Matsuyama et al., 1995; Rengarajan et al., 2002). Th2 cells lacking IRF4 have diminished IL-4 production. Since over-expression of GATA3

can rescue IL-4 production from IRF4-deficient cells, it has been suggested that one function of IRF4 might be to up-regulate GATA3 expression (Lohoff et al., 2002). Furthermore, IRF4 might be directly involved in the regulation of IL-4 expression by cooperating with NFATc2 and c-maf (Rengarajan et al., 2002). The activation of STAT6 by IL-4 induces expression of Growth factor independent 1 (*Gfi-1*). *Gfi-1* is preferentially expressed in Th2 cells and is important for the expansion of cells expressing GATA3 (Zhu et al., 2002; Zhu et al., 2006). Another transcription factor expressed by Th2 cells is JunB. JunB plays an important role in the induction of IL-4 production by collaborating with c-maf to bind and activate the *Il4* promoter (Li et al., 1999). Additionally, the expression of the NFAT family members, NFATc1 and NFATc2, is augmented by co-stimulatory signals and IL-6, respectively. NFATc1 and NFATc2 activation and their cooperation with other Th2 transcription factors leads to increased IL-4 production and Th2 differentiation (Diehl et al., 2002; So et al., 2006). It has recently been shown that the transcription factor Dec2 is induced throughout Th2 differentiation. Mice deficient in Dec2 have an impaired Th2 response both in an asthma model and a parasite infection model (Yang et al., 2009). The over-expression of Dec2 in CD4 T cells or transgenic mice expressing Dec2 have increased Th2 development (Yang et al., 2009). The mechanism of Dec2 in mediating increased Th2 development is thought to be partially through the induction of IL-2Rα (Liu et al., 2009). BATF is a transcription factor important in Th17 development. However, BATF is also required for Th2 development and mice deficient in BATF have reduced IL-4 production and *Gata3* expression (Betz

et al., 2010). The expression of all of these transcription factors is necessary for optimal Th2 development.

The role of Th2 cells in human diseases

Non-protective Th2 responses contribute to asthma, allergy and other allergic diseases. Polymorphisms in a variety of Th2 genes including the human *Il13* promoter and *Il4* locus have been directly linked to susceptibility of allergic disease (Ono, 2000; Wills-Karp, 2000). Additionally, gain of function mutations in the human IL-4Rα gene, which is required for Th2 differentiation, are associated with atopic asthma, *Stat6* activation, and elevated IgE production as well as atopic dermatitis (Hershey et al., 1997; Mitsuyasu et al., 1998; Mitsuyasu et al., 1999). Conversely, patients with GATA3 haploinsufficiency have reduced Th2 cell numbers and their T cells have diminished ability to differentiate into Th2 cells *in vitro*. These individuals also suffer from hypoparathyroidism, sensorineural deafness, and renal dysplasia syndrome (Skapenko et al., 2004; Van Esch et al., 2000). Furthermore, polymorphisms in GATA3 observed in Finnish populations have been shown to be associated with increased IgE and susceptibility to asthma (Pykalainen et al., 2005).

Role of Th2 cells in mouse models of disease

Various mouse models have been established to determine the role of Th2 cells in the development of allergic disease. It is evident that both IL-4 and STAT6 are important in driving Th2 mediated allergic disease. Transgenic mice expressing

IL-4 or constitutively active STAT6 are characterized by the development of spontaneous allergic inflammation (Sehra et al., 2008; Tepper et al., 1990). Conversely, the development of allergic disease is dependent on IL-4 as allergic inflammation is diminished in mice deficient in IL-4 or STAT6 (Akimoto et al., 1998; Brusselle et al., 1995; Kuperman et al., 1998; Sehra et al., 2008). Futhermore, mice expressing the dominant-negative form of GATA3 are protected from the induction of airway hypersensitivity (Zhang et al., 1999a). These reports take together highlight the importance of Th2 cells in the development and pathogenesis of allergic inflammatory diseases.

Th17 cells

Within the past 10 years a new T helper subset, Th17, has been discovered and studied intensely. Th17 cells are pro-inflammatory cells that secrete IL-17A, IL-17F, IL-21 and IL-22 (Bettelli et al., 2007a; Cua and Tato, 2010). IL-17A activates other cell types and results in the production of pro-inflammatory cytokines (Yao et al., 1995). Both IL-17 family members, IL-17A and IL-17F, can recruit and activate neutrophils, whereas IL-22 promotes skin inflammation and is also important for hepatocyte protection during acute liver injury (Zenewicz et al., 2007; Zheng et al., 2007a).

Th17 cells play an important role in the adaptive immune response by providing immunity to several extracellular bacteria and fungi. More specifically, Th17 cells are required for host defense against infections with Klebsiella, Citrobacter,

Bacteroides, and Candida (Happel et al., 2005; Huang et al., 2004; Mangan et al., 2006; Ye et al., 2001). The initial characterizations of Th17 cells showed that Th17 cells differentiate when cultured with IL-23, although only a small percentage of cells within these cultures were IL-17-positive (Harrington et al., 2005; Park et al., 2005). This resulted from the inability of IL-23 to prime naive cells to become Th17 cells, largely due to the lack of IL-23R on naïve CD4 T cells (Zhou et al., 2007). Subsequently, it was shown that Th17 cells differentiate de novo in the presence of TGF-β+IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a). Furthermore, the cytokine IL-21, a cytokine also secreted by Th17 cells, in combination with TGF-β can promote Th17 differentiation (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007).

In addition to TGF-β, IL-6, and IL-21, several cytokines are important in the development and repression of Th17 cells. The Th1 cytokine IFN-γ and Th2 cytokine IL-4 both negatively regulate Th17 development, and neutralization of these cytokines is necessary for *in vitro* Th17 differentiation (Harrington et al., 2005; Park et al., 2005). The IL-12 family member IL-27 is not only important in promoting IFN-γ production, but is also a negative regulator of Th17 development. Inhibition of Th17 development by IL-27 requires STAT1, however the direct mechanism of repression is still unclear (Batten et al., 2006; Stumhofer et al., 2006). In addition, IL-2 is important in Treg development and consistent with the reciprocal development of Th17 and Treg cells, IL-2 inhibits Th17 development. Th17 cells differentiated in the presence of IL-2 have reduced

numbers of IL-17 secreting cells. Conversely, cells deficient in IL-2 or the downstream transcription factor of IL-2, STAT5, display enhanced Th17 differentiation. This same effect can be observed when Th17 cells are cultured in the presence of IL-2 neutralizing antibodies (Laurence et al., 2007). In contrast, the cytokine IL-1β promotes Th17 development. IL-1β augments Th17 differentiation and enhances IL-23 responsiveness (Cho et al., 2006; Cua et al., 2003; Veldhoen et al., 2006a). Furthermore, the importance of IL-1β in the Th17 lineage is illustrated by mice deficient in IL-1RI, as mice deficient in IL-1 signaling are protected from the development of Th17 mediated disease autoimmune encephalomyelitis (Sutton et al., 2006).

IL-23 is required for the *in vivo* function of Th17 cells. Although some Th17 cells can develop in the absence of IL-23, mice deficient in IL-23p19 have a greatly diminished ability to mediate inflammation (Bettelli et al., 2007a; Weaver et al., 2007). In short-term cultures, IL-23 maintains a population of IL-17-secreting CD4 T cells, which has been interpreted as a function of promoting Th17 cell expansion or survival (Veldhoen et al., 2006a). Additionally, IL-23 has also been shown to maintain a pathogenic Th17 phenotype compared with cells cultured in TGF-β and IL-6 due to an inability of IL-23 to induce IL-10 production (McGeachy et al., 2007).

Importance of STAT family members in Th17 development

Many cytokines important for the differentiation and maintenance of Th17 cells, such as IL-6, IL-21, and IL-23, signal through STAT3. Thus, STAT3 is required for the differentiation and effector function of Th17 cells (Mathur et al., 2007; Yang et al., 2007). STAT3-deficient Th17 cells fail to produce IL-17 and induce transcription factors RORγt and RORα, which are important for Th17 development (Mathur et al., 2007; Yang et al., 2007; Yang et al., 2008b). Human mutations in STAT3 also results in the loss of IL-17 producing CD4 T cells (Ma et al., 2008; Milner et al., 2008). STAT3 binds directly to the *Il17* and *Il21* genes and is required for the expression of IL-23R, RORγt and RORα (Chen et al., 2006b; Mathur et al., 2007; Wei et al., 2007; Yang et al., 2007; Yang et al., 2008b). Th17 cells and T regulatory cells are thought to have a reciprocal relationship. IL-6 plays a critical role in the switch from Tregs to Th17 cells (Bettelli et al., 2006). Therefore, an additional role of IL-6 induced STAT3 is to down regulate Foxp3 expression. The down regulation of Foxp3 leads to Th17 development and the repression of Treg development (O'Malley et al., 2009; Yang et al., 2007; Yao et al., 2007). IL-23 is required for Th17 differentiation *in vivo* and the maintenance of Th17 cells *in vitro*. IL-23 in Th17 cells signals through both STAT3 and STAT4. STAT4 is partially required for IL-17 production from IL-23 cultured cells and is completely required for IL-17 production from Th17 cells stimulated with IL-23 and IL-18 (Mathur et al., 2007). Thus, STAT4 is also required for the IL-23 driven Th17 response. This is in agreement with

studies showing that STAT4-deficient mice are completely protected from the Th17 mediated disease EAE (Chitnis et al., 2001).

Additional transcription factors required for Th17 differentiation

In addition to STAT3 and STAT4, many other transcription factors contribute to Th17 development and effector function. Like T-bet in Th1 and Gata3 in Th2 cells, RORγt is thought to be the Th17 master regulator. RORγt expression is dependent on STAT3 activation (Ivanov et al., 2006) and mice deficient in RORγt have dramatically impaired IL-17 production and are protected from EAE (Ivanov et al., 2006). Subsequently it was shown that RORα is also induced by TGF-β+IL-6 and promotes Th17 differentiation through direct activation of *Il17*. The expression of RORα is also dependent on STAT3. Mice deficient in both RORα and RORγt lack IL-17 production and are completely protected from EAE (Yang et al., 2008b). The transcription factor IκBζ cooperates with both RORγt and RORα to promote Th17 development by binding and activating *Il17a*. Furthermore, IκBζ itself is required for Th17 differentiation and mice deficient in IκBζ have impaired Th17 development and are resistant to EAE (Okamoto et al.). The Th2 associated transcription factor IRF4 is also required for Th17 development. IRF4 is important in the IL-21-mediated autocrine response and in early Th17 differentiation, although the mechanisms are not well understood (Chung et al., 2009; Huber et al., 2008). However, it is known that IRF4-deficient mice have impaired Th17 differentiation and have increased resistance to EAE (Brustle et al., 2007). Another Th2 associated transcription factor, c-maf, is

expressed in Th17 cells. c-Maf expression is induced by TGF-β+IL-6 and is required for IL-10 production from Th17 cells (Xu et al., 2009). Moreover, c-maf can directly bind and activate the promoter of the Th17 cyotkine IL-21 (Hiramatsu et al., 2010). BATF is a basic leucine zipper transcription factor that is expressed in Th1, Th2 and Th17 cells (Schraml et al., 2009). BATF is required for both *in vitro* and *in vivo* Th17 development (Betz et al., 2010; Schraml et al., 2009). BATF-deficient mice have decreased IL-17 production and increased Treg cell numbers and are therefore protected from the development of EAE (Schraml et al., 2009). One function of BATF is to synergize with RORγt at the *Il17* promoter to induce expression, however the exact mechanism of BATF in Th17 development is not completely understood (Schraml et al., 2009). The transcription factor BCL6 is important for the repression of Th2 development and is required for Tfh differentiation. (Johnston et al., 2009; Kusam et al., 2003; Nurieva et al., 2009; Yu et al., 2009). Additionally it has been shown that BCL6 is important in Th17 development. *In vitro*, BCL6-deficient Th17 differentiation is defective resulting in reduced IL-17 production. However, BCL6-deficient Th17 development is similar to wild type when purified naïve T cells are used, blocking IL-4 throughout Th17 culture, or increased doses of TGF-β are used in culture. Retroviral expression of BCL6 leads to increased Th17 development (Mondal et al., 2010). Conversely, BCL6-deficient memory cells have increased IL-17 production compared to wild type controls. The increase in Th17 development *in vivo* differentiated memory cells could be due to increased Th17 promoting cytokines from BCL6 deficient macrophages. Therefore, Th17 development may

require BCL6, however the role of BCL6 in Th17 development may be more important in the macrophage lineage (Mondal et al., 2010). Aryl hydrocarbon receptor (AhR) ligands are found in T cell culture medium and in the environment (Veldhoen et al., 2009). Engagement of the AhR and various ligands induces Th17 development and represses Treg development (Quintana et al., 2008). AhR-deficient T cells have impaired Th17 differentiation due to the activation of STAT1 and STAT5 which are both negative regulators of Th17 development (Kimura et al., 2008). Furthermore, the addition of AhR ligands during the onset of EAE increases disease severity and pathology (Veldhoen et al., 2008a). These data provide a solid link between the environment and autoimmunity.

Role of Th17 cells in human diseases

The importance of Th17 cells is illustrated by humans who have mutations or polymorphisms in genes important in the Th17 pathway. Patients who have dominant-negative mutations in STAT3 suffer from hyper-IgE syndrome. T cells from hyper-IgE syndrome patients fail to develop into Th17 cells. Some abnormalities associated with these patients include increased susceptibility to *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Candida albicans* infections (Buckley, 2001; Holland et al., 2007; Ma et al., 2008; Milner et al., 2008; Minegishi et al., 2007). There is reasonable overlap of susceptibility to infections between patients with mutated STAT3 and mice deficient in Th17 cells, however the link between hyper-IgE syndrome and Th17 is still not apparent. Conversely, polymorphisms in the p40 subunit of IL-23 or the IL-23R is

associated with the increased risk of another Th17 mediated disease, psoriasis (Cargill et al., 2007). Mutations in the IL-23R are also associated with inflammatory bowl diseases such as Crohn's disease (Duerr et al., 2006). Furthermore, in humans the expression of IL-17 is directly linked to multiple sclerosis, rheumatoid arthritis, and psoriasis (Aarvak et al., 1999; Lock et al., 2002; Matusevicius et al., 1999; Teunissen et al., 1998). Additionally, the expression of IL-23p19, which induces IL-17 production, is increased in psoriatic lesions and inflamed tissues from patients with Crohn's disease and ulcerative colitis (Lee et al., 2004; Schmidt et al., 2005; Stallmach et al., 2004). These studies taken together provide a clear relationship between autoimmune diseases and Th17 cells.

Role of Th17 cells in mouse models of disease

Th17 cells have become of great interest due to the key role they play in autoimmune diseases. Originally Th1 cells were thought to be the main mediator of autoimmune diseases, however following the description of Th17 cells and subsequent analysis, it has become evident that Th17 cells are indispensible for most autoimmune diseases. Many mouse models reflecting human autoimmune diseases have been utilized in order to study the effector cells and cytokines important in disease development. Studies using mouse models of autoimmunity have shown the importance of the Th17 pathway in autoimmunity. For example, *Il17*-/- mice have reduced collagen induced arthritis and development of EAE is delayed with decreased severity (Komiyama et al., 2006; Nakae et al., 2003a).

In agreement with IL-17-deficient studies, the transfer of IL-17 producing Th17 cells to wild type mice were more potent in inducing EAE compared to the transfer of Th1 cells (Langrish et al., 2005). Along with IL-17, other factors important in Th17 differentiation have significant effects on the development of autoimmune diseases. IL-23 is required for the development of EAE, and therefore IL-23p19-deficient mice are resistant to EAE (Cua et al., 2003; Murphy et al., 2003). In addition, wild type mice with EAE that were treated with anti-p19 were protected from disease relapse and had reduced levels of IL-17 in their serum (Chen et al., 2006a). As TGF-β is required for Th17 differentiation, mice that express a dominant negative mutant of the TGF-βR in CD4 T cells lack Th17 cells and are completely resistant to EAE development (Veldhoen et al., 2006b). Mice deficient in IL-6, which promotes Th17 differentiation in combination with TGF-β, do not develop Th17 cells and like TGF-βR mutant mice are protected from EAE development (Bettelli et al., 2006). Although the exact role of IL-1β in Th17 differentiation and effector function is not clear, there is evidence suggesting that it is critical in Th17-mediated autoimmunity. Mice deficient in the natural IL-1 receptor antagonist develop spontaneous arthritis through an IL-17-dependent mechanism (Nakae et al., 2003b). Alternatively, incidence of EAE in mice deficient in IL-1R1 is significantly lower than that observed in wild type mice which correlates with a reduction in antigen specific IL-17 producing cells (Sutton et al., 2006). Taken together, these data indicate that the IL-23-Th17 pathway is critical in the development of autoimmune diseases.

T regulatory cells (Tregs)

The immune system has a specialized subset of T cells, T regulatory cells, that play an important role in down modulating the immune response and providing self-tolerance. Naturally occurring Tregs (nTregs) emigrate from the thymus expressing the transcription factor Foxp3 and have suppressive functions (Fontenot et al., 2005). T regulatory cells can also differentiate *in vitro* from naïve CD4 T cells in the presence of TGF-β (Chen et al., 2003; Fantini et al., 2004; Fu et al., 2004; Sakaguchi et al., 2008; Zheng et al., 2004). Differentiation of Tregs can be enhanced in the presence of retinoic acid, which at the same time blocks Th17 differentiation (Benson et al., 2007; Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007). Naïve cells differentiated in the presence of TGF-β with or without retinoic acid are referred to as induced Tregs (iTregs) and share many of the *in vitro* and *in vivo* characteristics of nTregs (DiPaolo et al., 2007). The differentiation of naïve CD4 T cells to iTregs results in cells that are unresponsive to TCR stimulation, lack production and secretion of T effector cytokines, and produce the immunosuppressive cytokine TGF-β. In co-cultures, iTregs can inhibit proliferation of CD4+CD25- T cells in response to anti-CD3 stimulation. *In vivo*, iTreg transfer can suppress T cell proliferation in response to OVA peptide following immunization. Furthermore, in an asthma model using house dust mite, co-transfer of iTregs prevents allergic pathogenesis in the lungs (Chen et al., 2003). At this time there is not a way to distinguish iTregs from nTregs and the existence of endogenous iTregs *in vivo* is still controversial.

Importance of STAT family members in Treg cell development

In addition to TGF-β, iTreg development and effector functions require STAT5 activation by IL-2 (Burchill et al., 2007; Davidson et al., 2007). IL-2 is critical for the differentiation and survival of iTreg cells. Furthermore, iTreg cell responsiveness to IL-2 can be attributed to high surface expression of CD25 (IL-2Rα), which is expressed higher on iTreg cells than all other T helper cell lineages. The mechanism of how IL-2 induced STAT5 promotes iTreg development is still not clear. However, STAT5 may be important for the direct induction of Foxp3 expression (Burchill et al., 2007; Yao et al., 2007).

Additional transcription factors required for Treg cell differentiation

Foxp3 is thought to be the critical transcription factor for iTreg cell function. Foxp3 directly binds several genes encoding signal transduction molecules, transcription factors, cytokines, cell-surface molecules, and enzymes for cell metabolism (Marson et al., 2007; Zheng et al., 2007b). It is known that Foxp3 acts as a transcriptional activator and repressor, however more detailed studies need to be performed to further examine the requirement and exact function of Foxp3 in iTreg cells. The induction of Foxp3 is dependent on TGF-β signaling. TGF-β activation of Smad3 and TCR activation of NFAT cooperate in remodeling and expression of Foxp3 (Tone et al., 2008). In addition to STAT5 and Foxp3, the Runx family member, Runx1, is required for Treg cell development. Runx1 interacts with Foxp3 and is required for both the expression of Foxp3 and

suppressive function of Treg cells (Egawa et al., 2007; Kitoh et al., 2009; Ono et al., 2007; Rudra et al., 2009).

Role of Treg cells in human diseases

Abnormalities in Treg differentiation due to mutations in FOXP3 in humans results in immunodeficiency, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX). Patients with mutated FOXP3 suffer from autoimmune symptoms such as insulin-dependent diabetes, hypothyroidism, anemia, and neutropenia (Bennett et al., 2001; Wildin et al., 2001). Since IL-2 is critical for Treg development and function, patients with IL2RA mutations have syndromes resembling IPEX (Caudy et al., 2007). It has also been suggested that Treg cells are increased in tumor tissues and in the blood of cancer patients. Furthermore, Treg cells may inhibit anti-tumor immunity and induce immune tolerance to tumors in several different cancers (Wang and Wang, 2007). Therefore, Treg cells may contribute to the progression of cancer and tumor growth.

Role of Treg cells in mouse models of disease

Mutation of Foxp3 in mice results in diseases similar to human patients with FOXP3 mutations (Brunkow et al., 2001; Patel, 2001). Mice deficient in IL-2, IL-2Rα, or IL-2Rβ have significantly reduced Treg cells, which correlates with the development of autoimmune diseases (Malek et al., 2002; Papiernik et al., 1998; Thornton and Shevach, 1998).

Tfh cells

A central function of T helper cells is providing B cell help leading to antibody production and class switching. Although Th2 cells can direct class switching in B cells, a specific subset of T helper cells termed T follicular helper (Tfh) cells specialize in providing B cell help within the lymphoid tissues (King et al., 2008). Tfh cells differentiate in the presence of IL-6 or IL-21. Initially both IL-6 and IL-21 were thought to be required for Tfh cell differentiation and function and mice deficient in either IL-21 or IL-6 have significantly reduced Tfh cells (Chtanova et al., 2004; Nurieva et al., 2008). Tfh cells are characterized by the expression of chemokine receptor CXCR5, high expression of inducible co-stimulator (ICOS), and IL-21 production (Breitfeld et al., 2000; Chtanova et al., 2004; Hutloff et al., 1999; Mackay, 2000; Schaerli et al., 2000). The interaction of B cells and Tfh cells occurs in the B cell follicles. Therefore, Tfh cells express CXCR5 which allows them to localize to the B cell follicles where the CXCR5 ligand CXCL13 is expressed (King et al., 2008). ICOS is a co-stimulatory molecule expressed by most T helper cell lineages, but expression on Tfh cells is very high. Mice deficient in ICOS lack Tfh cells and therefore have defective germinal center formation following exposure to a T-cell dependent antigen (Akiba et al., 2005; Bossaller et al., 2006; Dong et al., 2001; Tafuri et al., 2001). ICOS expression is also required for optimal IL-21 production by Tfh cells (Vogelzang et al., 2008). IL-21 is produced by both Th17 and Th2 cells, but production by Tfh cells is significantly greater (Chtanova et al., 2004). IL-21 is an autocrine growth factor and is a potential explanation why IL-21-deficient mice have significantly reduced

Tfh cell number (Nurieva et al., 2008; Vogelzang et al., 2008). IL-21 signaling also leads to increased CXCR5 expression on Tfh cells which is important for migration (Vogelzang et al., 2008).

Importance of STAT family members in Tfh development

Both cytokines important for Tfh cell development, IL-6 and IL-21, signal through STAT3. Consistent with the requirement for STAT3 in both IL-6 and IL-21 signaling, STAT3-deficient mice have a significantly reduced number of Tfh cells (Nurieva et al., 2008). Moreover, STAT3-deficent mice have a defect in germinal center B cell formation and antibody response (Nurieva et al., 2008).

Additional transcription factors required for Tfh differentiation

Recently it has been discovered that the transcriptional repressor B-cell lymphoma 6 protein (Bcl-6) is required for Tfh cell development and commitment (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Bcl-6 is necessary and sufficient for expression of Tfh cell associated molecules CXCR5, PD-1, IL-6R, and IL-21R (Johnston et al., 2009; Nurieva et al., 2009). In support of the importance of Bcl-6 in Tfh cell differentiation, studies where a known Bcl-6 repressor Blimp-1 is retrovirally expressed, Tfh cell differentiation was blocked and this coincided with reduced expression of factors associated with Tfh cells (Johnston et al., 2009). A more recent report shows that the up-regulation of Bcl-6 and therefore differentiation of Tfh cells was independent of IL-6 and IL-21 and the factor or factors involved in the induction of Bcl-6 expression *in vivo* have not

been elucidated (Poholek et al., 2010). The transcription factor BATF is also important for Tfh cell development and function. Mice deficient in BATF have reduced numbers of Tfh cells (CCR5+). Furthermore, the Tfh cells from BATF-deficient mice have impaired chemotaxis (Betz et al., 2010).

Role of Tfh cells in human diseases

The critical role of Tfh cells in B cell antibody production is exemplified in patients lacking Tfh cell associated molecule ICOS. Patients who are deficient in ICOS have decreased numbers of CXCR5+CD4+ T cells. Many of the patients suffer from common variable immunodeficiency (CVID). CVID patients lack germinal cell formation and consequently have a significant reduction in isotype-switched memory B cells (Bossaller et al., 2006; Grimbacher et al., 2003). There is also substantial evidence showing that Tfh cells are the cells driving the auto-antibody production in diseases such as systemic lupus erythematosus (SLE) (Pugh-Bernard et al., 2001). Furthermore, patients with SLE have increased frequency of CD4+ICOS+ T cells in their blood (Hutloff et al., 2004). Additionally, Tfh cells may also contribute to the development of lymphomas. Malignant T cells from patients with angioimmunoblastic T cell lymphoma (AITL) share many characteristics of human Tfh cells including the expression of Bcl-6 and CXCR5. It has been proposed that these cells may be responsible for increased recruitment of B cells to follicles leading to aberrant activation and antibody production (de Leval et al., 2007; de Leval et al., 2001; Krenacs et al., 2006; Ree et al., 1999).

Role of Tfh cells in mouse models of disease

Human disease can be recapitulated in several mouse models of SLE. One mouse model of lupus is the sanroque mouse. Sanroque mice have a missense mutation in the gene Roquin encoding an ICOS repressor. These mice develop lupus which correlated with increased number of Tfh cells and increased IL-21 production (Vinuesa et al., 2005). Other lupus mouse models had similar results showing increased Tfh cells and factors in mice with lupus (Ozaki et al., 2004; Subramanian et al., 2006). Moreover, blocking ICOS interactions or IL-21 signaling can delay and reduce disease progression of SLE (Herber et al., 2007; Iwai et al., 2003).

Th9 cells

The most recently discovered subset of CD4 T helper cells is Th9. *In vitro* Th9 cells differentiate de novo with TGF-β+IL-4 or by culturing Th2 differentiated cells in the presence of TGF-β (Dardalhon et al., 2008; Veldhoen et al., 2008b). The secretion of IL-9 and not IL-4, IL-5, and IL-13 make Th9 cells distinct from Th2 cells. IL-9 was initially identified as a cytokine that promoted T helper cell growth and proliferation (Uyttenhove et al., 1988; Van Snick et al., 1989). In addition to IL-9 promoting T helper cell growth and proliferation, IL-9 stimulates mucin expression in epithelial cells and is an important factor in mast cell growth and survival (Hultner et al., 1990; Longphre et al., 1999; Renauld et al., 1995). Before the discovery of Th9 cells, IL-9 was characterized as a Th2 cytokine important for immunity to helminth infections (Faulkner et al., 1998; Khan et al.,

2003). Recently the role of Th9 cells in worm infections was demonstrated with the use of mice expressing a dominant-negative form of the TGF-βR. Wild type mice infected with *Trichuris muris* exhibited a protective immune response, which correlated with the accumulation of mast cells and subsequent expulsion of the worm. Conversely, transgenic mice expressing the dominant-negative form of the TGF-βR failed to mount a protective immune response. The defective immune response correlated with low IL-9 expression and therefore decreased mast cell recruitment and/or survival (Veldhoen et al., 2008b). These results suggest that specifically Th9 and not Th2 cells are required for immunity to worm infection.

Importance of STAT family members in Th9 development

Differentiation of Th9 cells requires both TGF-β and IL-4. IL-4 by itself induces Th2 differentiation through the activation of STAT6, so as expected STAT6 is also required for TGF-β+IL-4 mediated Th9 differentiation (Dardalhon et al., 2008). The requirement for other Th2 associated STAT family members such as STAT5 and STAT3 in Th9 differentiation and effector function have not been examined carefully.

Additional transcription factors required for Th9 differentiation

Th9 cell development requires the Th2 associated transcription factor GATA3. In Th2 cells GATA3 is important for the expression of IL-4, IL-5 and IL-13, however Th9 cells repress the production of these cytokines and produce IL-9.

Furthermore, differentiated Th9 cells do not express GATA3 suggesting that GATA3 is important for the initial differentiation of Th9 cells but is not required for effector function (Dardalhon et al., 2008; Veldhoen et al., 2008b). Recently the transcription factor PU.1 was shown to be expressed predominantly in Th9 cells. PU.1 was previously shown to be important in negatively regulating Th2 cytokine production (Chang et al., 2005). Distinct from GATA3, PU.1 is expressed in fully differentiated Th9 cells and is important for Th9 effector function (Chang et al., 2010). Ectopic expression of PU.1 in Th2 or Th9 differentiating cells induced IL-9 production. *In vivo*, PU.1 expression is required for a mouse model of allergic inflammation. These data clearly show a role for PU.1 in Th9 differentiation and effector function (Chang et al., 2010). Nevertheless, PU.1-deficient Th9 cells still have the ability to secrete low levels of IL-9 suggesting other transcription factors may contribute to Th9 development.

Role of Th9 cells in human diseases

IL-9, the hallmark cytokine produced by Th9 cells, has been linked to allergic disease in humans. Genetics studies have linked the regions of DNA containing IL-9 and IL-9R to susceptibility to allergic disease (Doull et al., 1996; Holroyd et al., 1998). Subsequently, it was shown that the expression of IL-9 in asthmatic lungs is significantly increased compared to healthy controls (Erpenbeck et al., 2003b; Shimbara et al., 2000). Additional studies have observed increased IL-9 in the bronchiolar alveolar lavage (BAL) of asthmatic patients following allergen challenge and increased IL-9 mRNA+ cells in the airway of asthmatics compared

to control patients (Erpenbeck et al., 2003a; Shimbara et al., 2000; Ying et al., 2002). Collectively, these reports have clear evidence linking IL-9 thus Th9 to allergic disease.

Role of Th9 cells in mouse models of disease

Similar to what is observed in humans, IL-9 is important for several allergic disease models in mice. Transgenic mice expressing IL-9 have increased airway hyper-responsiveness, elevated IgE, and eosinophilia following antigen challenges (Forbes et al., 2008; McLane et al., 1998; Temann et al., 1998; Temann et al., 2002). Conversely, IL-9-deficient mice are not resistant to allergic disease, however blocking IL-9 prior to challenge resulted in decreased airway hyper-responsiveness and reduced eosinophils in the BAL (Cheng et al., 2002; McMillan et al., 2002). The transcription factor PU.1 is required for Th9 development and therefore, mice that have T cells deficient in PU.1 have significantly less IL-9 expression and have attenuated allergic pulmonary inflammation compared to wild type mice (Chang et al., 2010). These data provide additional evidence that Th9 cells are important in allergic inflammation.

	Protective	Pathogenic
Th1 (T-BET)	Immunity to protozoa – Leishmania Immunity to fungi - Candida Immunity to bacteria - Mycobacteria	Autoimmunity – Multiple sclerosis, arthritis, colitis
Th2 (GATA3)	Immunity to helminthic parasites - Nippostrongylus, Schistosoma Immunity to viruses - measles Immunity to bacteria - Borrelia	Allergy and atopic responses
Th17 (RORγt)	Immunity to bacteria- Klebsiella, Bacteroides, Citrobacter Immunity to fungi- candida	Autoimmunity- Multiple sclerosis, arthritis, colitis, psoriasis
Tfh (BCL-6)	Provide B cell help leading to antibody production	Autoimmunity- systemic lupus erythematosus
Th9 (PU.1)	Immunity to helminthic parasites- Trichuris	Allergy and atopic responses
Treg (FOXP3)	Tolerance to self Immune suppression	Cancer

Figure 3. T helper subsets protective and pathogenic functions. Each T helper subset is required for specific protective responses, however inappropriate responses of each T helper subset can be harmful to the host.

Plasticity of T helper subsets

Each T helper subset has a specific cytokine repertoire and transcription factor network that makes them distinct from all other subsets. Originally what defined a T helper subset was the expression of unique cytokines and transcription factors and commitment to that specific lineage. The initial clear lines drawn between T helper subsets are now arrows signifying their plasticity.

Th1/Th2 cells

Early studies showed that fully differentiated Th1 and Th2 cells cannot convert to the opposing subset (Murphy et al., 1996). However Th1 or Th2 cells primed for one week are not committed to their respective lineages and when primed for an additional week in the opposing cytokines can convert from Th1 to Th2 or Th2 to Th1 (Murphy et al., 1996). *In vivo* model of lymphocytic choriomeningitis virus (LCMV) infection illustrates Th2 cell plasticity. Virus specific cells that were differentiated for 2-3 weeks *in vitro* under Th2 conditions were transferred into mice that were subsequently infected with LCMV. Following infection, Th2 transferred cells up-regulated Th1 transcription factor T-bet and acquired IFN-γ producing abilities. This switch from Th2 to Th1 can also be observed *in vitro* when antigen specific Th2 cells are cultured with IL-12, IFN-γ, and type I interferons (Hegazy et al., 2010). Besides the ability to switch to Th1, Th2 cells can convert to Th9 cells when cultured with TGF-β. In the presence of TGF-β Th2 cells repress Th2 cytokines and stimulate IL-9 production (Dardalhon et al., 2008). Furthermore, Th2 memory cells can be re-differentiated into Foxp3 expressing cells. The re-differentiated cells behave like Treg cells and can actively suppress Th2 mediated allergic inflammation (Kim et al., 2010). Additionally, reports have suggested that Th2 cells have the ability to convert to Tfh cells following helminth infections. Infection with the enteric nematode *Heligmosomoides polygyrus* led to development of T cells in the B cell follicles that expressed the Th2 cytokine IL-4 and the Tfh cell phenotypic markers CXCR5, ICOS, PD-1, IL-21 and BCL-6 (King and Mohrs, 2009). Additional

evidence of Th2 to Tfh cell conversion was provided by Zaretsky et al. Zaretsky et al. transferred CXCR5 and PD-1 negative cells that expressed IL-4 into naïve mice. After transfer, the mice were challenged with parasite eggs. *Ex vivo* analysis of the transferred cells showed a conversion from the Th2 to Tfh cell phenotype (Zaretsky et al., 2009). However, the conversion from Th2 to the Tfh lineage is still controversial. It is possible that there are several subsets of Tfh cells, one of which that produces IL-4. In agreement with this notion, it has been shown that Tfh cells secreting IL-4 were functionally distinct from Th2 cells found in peripheral tissues (Reinhardt et al., 2009). Additional studies will have to be performed in order to clarify the relationship between Th2 and Tfh cells.

Th17 cells

Th17 cell instability has been reported both *in vitro* and *in vivo* by several groups. IL-23 cultured T cells lose the ability to produce IL-17 following multiple TCR stimulations and convert to a Th1-like cell. The conversion from IL-17 secreting to IFN-γ secreting phenotype is dependent on T-bet (Mathur et al., 2006). Furthermore, cells cultured under Th17 skewing conditions for up to 3 weeks are not committed to the Th17 phenotype and following IL-12 or IL-4 stimulation can convert to Th1 or Th2-like phenotype (Lee et al., 2009; Lexberg et al., 2008; Stritesky et al., 2008). Many *in vivo* studies recapitulate the instability of Th17 cells observed *in vitro.* Adoptively transferred Th17 cells in several different mouse models showed the conversion of Th17 cells to a Th1 phenotype

(Bending et al., 2009; Lee et al., 2009; Martin-Orozco et al., 2009; Muranski et al., 2008).

Treg cells

T regulatory cells can convert to an IL-17 secreting phenotype when exposed to several different stimuli including IL-6 (Osorio et al., 2008; Radhakrishnan et al., 2008; Xu et al., 2007; Yang et al., 2008a). Additionally, Tregs can be re-programmed to express T-bet and produce IFN-γ following Th1 *in vitro* stimulation (Wei et al., 2009). Futhermore,the conversion of Foxp3+ Tregs to Tfh cells can be observed in Peyer's patches following interactions with B cells (Tsuji et al., 2009).

Commitment to a specific subset is achieved in part through the repression of the other T helper cell lineages. The repression of other lineages can be accomplished by many different mechanisms. Committed Th1 cells repress Th2 development by disrupting IL-4 signaling (Huang and Paul, 1998). Whereas, Th2 cells block Th1 development by the selective loss of IL-12R and STAT4 expression (Szabo et al., 1995). Therefore T helper plasticity can be limited by repression of specific cytokine receptors and transcription factors. Additionally, plasticity of the T helper subsets can be inhibited through epigenetic repression of lineage specific factors of opposing lineages. Therefore, the flexibility of T helper cells may reflect the promiscuous epigenetic status of loci of lineage specific factors. Supporting this idea, it has been observed that T helper cells

that are competent to assume other T helper cell phenotypes have non-repressive marks on genes associated with opposing lineages (Wei et al., 2009).

The plasticity observed in many of the T helper cell subsets reflects the importance of the epigenetic status and expression of cytokine receptors and transcription factors of opposing lineages. However, the lack of terminal differentiation and the ability to re-program may give T helper cells the flexibility to shape their effector response to be more efficient.

Each T helper cell subset relies on a specific STAT family member for differentiation and effector function. However, many T helper subsets require several STAT family members for optimal differentiation. For example, Th17 cell development and effector function requires signals from both STAT3 and STAT4 whereas Th2 cells require signals from both STAT6 and STAT5. The activation of each STAT family member provides a unique signal that helps promote the development of a specific T helper lineage. However, the mechanism that allows naïve T helper cells to of interpret multiple STAT signals is still unclear.

Research goals

Our overall goals of this research were to gain an understanding of T helper cell commitment and determine the role of different STAT family members in the development and effector function of T helper subsets. Specifically, we have two basic goals of our research. The first goal is to determine the commitment of Th17 cells and the role of IL-23 plays in the Th17 lineage. Our second goal is to further elucidate the STAT family members important for Th17 and Th2 development and effector function.

Th17 cells are of great interest due to their proposed pathogenic role in several autoimmune diseases. *In vivo* studies have shown that IL-23 clearly plays a role in Th17 development. However, the exact mechanism of action of IL-23 is not clear. In order to determine the role of IL-23 in the Th17 lineage we will utilize several *in vitro* experiments. IL-23 has been proposed as a potential therapeutic target, therefore these experiments will be critical in understanding the Th17-IL-23 relationship. Additionally, stability of each T helper subset has become an area of increasing interest. Since the differentiation of Th17 cells yields only a small fraction IL-17 producing cells the stability of Th17 cells has been difficult to test. Using a unique IL-17 cytokine capture assay we aim to determine the stability of Th17 cells using a pure population of IL-17 secreting cells.

Many T helper subsets require more than one STAT family member for optimal differentiation. The known STAT requirements for each T helper lineage have

been highlighted above. However, the importance of STAT4 in the Th17 lineage is still not clear. Since IL-23 signals through STAT4 and our data highlights the importance of IL-23 in the Th17 lineage, we wanted to determine the role of STAT4 in the Th17 lineage.

Th2 development requires both STAT6 and STAT5. Even though several cytokines important in Th2 development activate STAT3, the requirement of STAT3 in the Th2 lineage has not been carefully examined. Furthermore, the role of STAT3 expression in T cells in Th2 mediated allergic inflammation has not been defined. Using *in vivo* models we want to determine if STAT3 is required for allergic inflammatory disease.

MATERIALS AND METHODS

Mice

The generation of *Stat3*fl/fl mice with a CD4-Cre (*Stat3*$^{CD4-/-}$) transgene were previously described (Chiarle et al., 2005; Raz et al., 1999). Wild type (WT) mice were from Harlan Sprague Dawley on a C57BL/6 background. Stat3fl/fl mice were backcrossed to a C57BL/6 genetic background and the presence of the CD4-Cre transgene deletes exons 16-21 in T cells (Raz et al., 1999). WT mice in experiments using *Stat3*$^{CD4-/-}$ mice were Cre-negative littermates. The generation of STAT6VT transgenic mice was previously described (Bruns et al., 2003). Transgene positive founders (CD2:STAT6VT), where the human *Stat6* gene with V547 and T548 mutated to alanine is under transcriptional control of the CD2 locus control region, were backcrossed to C57BL/6 mice (Harlan Breeders, Indianapolis, IN). *Stat3*$^{CD4-/-}$ mice with a conditional deletion of *Stat3* in T cells were mated to STAT6VT transgenic mice to generate STAT6VT transgenic mice with a conditional deletion of *Stat3* in T cells (STAT6VTx*Stat3*$^{CD4-/-}$). *Stat4-/-, Stat6-/-, Tbx21-/-,* STAT4α, and STAT4β mice used were all previously described (Hoey et al., 2003; Kaplan et al., 1996a; Kaplan et al., 1996b; Szabo et al., 2002). All mice were maintained in specific pathogen-free conditions and experiments approved by the Indiana University Institutional Animal Care and Use Committee.

Quantitative RT-PCR

Total RNA was isolated from either un-stimulated or anti-CD3 (2μg/ml) re-stimulated cells using Trizol and reverse transcribed according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). cDNA (2 μl) was added to 0.5 μl primer (inventoried FAM-labeled TaqMan® Gene Expression Assays, Applied Biosystems), and 5 μl Taqman Fast Universal PCR Master Mix (Applied Biosystems). DEPC H_20 was added to a final reaction volume of 10 μl. Samples were analyzed in duplicate, mixed in 96-well optical reaction plates, and capped with optical reaction caps (Applied Biosystems). Quantitative PCR was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). RNA was normalized to expression levels of β2-microglobulin and relative expression was calculated using the -ΔΔCt method.

Murine T helper cell differentiation

Naïve $CD4^+CD62L^+$ T cells were purified from spleens using magnetic isolation according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Naïve $CD4^+$ T cells ($1x10^6$ cells/ml of complete RPMI-1640 medium) were cultured with plate bound anti-CD3 (4 μg/ml 145–2C11) and soluble anti-CD28 (1 μg/ml; BD Pharmingen) monoclonal antibodies under Th2 (IL-4 at 10 ng/ml (PeproTech, Rocky Hill, NJ) and anti-IFN-γ monoclonal antibody at 10 μg/ml), Th1 (anti-IL-4 (10 μg/ml 11B11) and IL-12 (5 ng/ml; R&D Systems), or Th17 (anti-IFN-γ, anti-IL-4, TGF-β1 (2 ng/ml; R&D Systems), IL-6 (100 ng/ml; PeproTech) where noted IL-1β (10 ng/ml; eBioscience), and IL-23 (10 ng/ml; eBioscience). In neutralizing

experiments α-IL-6 (BD Pharmingen), α-IL-21 (R&D Systems), α-rat IgG (BD Pharmingen), or α-goat IgG (R&D Systems) were used at a concentration of 10 μg/ml. Cells were expanded on day three after stimulation by adding half the dose of the original cytokines in fresh medium. After 5 days of culture, differentiated cells were re-stimulated with plate bound anti-CD3 at 4 μg/ml for 1 or 3 days, and the cell-free supernatant was collected after centrifugation and stored at -20°C until use. In some experiments, cells underwent additional rounds of stimulation. For the subsequent rounds of stimulation, cells were re-plated ($0.5x10^6$ cells/ml) and stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) in the presence of CD4-depleted irradiated splenocytes (1:5). Cells were stimulated and maintained with the same cytokine and neutralizing antibody concentrations as used in the first five days.

Intracellular c-MAF staining

T cells were collected and fixed with formaldehyde (final concentration of 1.5%) for 10 minutes at room temperature. Following fixation cells were permeabilized for 15 minutes at 4°C with 100% methanol. Cells were then stained with anti-c-MAF (Santa Cruz) for 15 minutes followed by anti-IgG alexa647 for 15 minutes at room temperature.

Retroviral transduction

Bicistronic retroviral vectors encoding mouse GATA3 or IRF4 and human CD4 (Ahyi et al., 2009; Chang et al., 2005), STAT6VT or c-MAF (kindly provided by

Prof. I.-Cheng Ho) and EGFP, or STAT3C as described (Mathur et al., 2007) subcloned into the bicistronic retroviral vector containing the marker Thy1.1 and Thy1.1 empty vector (kindly provided by Shreevrat Goenka) were used to generate virus. Retroviral supernatants were generated by transfecting a Phoenix-Eco packaging cell line in chloroquine containing DMEM media with 15 µg of purified plasmid by calcium phosphate precipitation. Cells were cultured at 37°C and one day after transfection, chloroquine containing DMEM media was replaced with fresh DMEM media. After 1 more day, the supernatants containing retrovirus were collected, filtered through a 0.45 µm filter and stored at -80°C.

After 2 days of differentiation, T helper cells were transduced by spinning at 2000 RPM at room temperature for 1 hour with 4 ml of retroviral supernatant containing 4 µg/ml polybrene. For double transductions, 2 ml of each virus were mixed together for the spin infection. Following the spin infection the retroviral supernatant was replaced with the original conditioned media. On day 5 transduced cells (EGFP, Thy1.1, or hCD4 positive cells) were sorted by flow cytometry before cytokine production and gene expression analyses.

Phospho-STAT protein analysis

T cells were collected and fixed with 1.5% (final concentration) formaldehyde for 10 minutes at room temperature. Following fixation cells were permeabilized for 15 minutes at 4°C with 100% methanol. Cells were then stained for phospho-

STAT3 or phospho-STAT6 (BD Pharmingen) for 30 minutes at room temperature. Cells were analyzed using flow cytometry.

Sensitization and challenge protocol

WT and *Stat3*$^{CD4-/-}$ mice were sensitized by two intra-peritoneal (i.p.) injections of 20 µg OVA (Sigma) adsorbed with 2 mg alum (Sigma) on days 0 and 7 of the protocol. The mice were challenged intra-nasally with OVA (100 µg) from days 14-18. Forty-eight hours after the last intranasal challenge, mice were sacrificed by intra-peritoneal injection of pentobarbital (5 mg per mouse). Splenocytes from the mice were stimulated with OVA (100 µg/ml) for 72h and Th2 cytokines in cell-free supernatants were assessed using ELISA. Paraffin-embedded sections were stained with hematoxylin and eosin for evaluation of the infiltration of inflammatory cells by light microscopy.

Bronchoalveolar lavage (BAL)

BAL was performed by cannulating the trachea and lavaging the lungs 3X with 1ml PBS. The cells recovered in BAL fluid were counted with a hemocytometer. Eosinophils (CD3-B220-CCR3+) in the BAL fluid were distinguished by cell size and by expression of CCR3 by flow cytometry as described (van Rijt et al., 2004).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as previously described (Yu et al., 2007). Similar numbers of cells between samples were used in each experiment. In brief, cells

were cross linked using 1% formaldehyde, washed with PBS, re-suspended in cell lysis buffer and incubated on ice for 10 minutes. The nuclei were then lysed using Nuclear Lysis Buffer followed by shearing of genomic DNA. Cell lysates were diluted with ChIP dilution buffer and pre-cleared with salmon sperm DNA, BSA and protein A agarose bead slurry (50%) at 4°C for 1 hour. The supernatant was incubated overnight at 4°C with 5 µg antibody (anti-Stat3 sc-482x, anti-Stat6 sc-1698, Normal Rabbit serum (Santa Cruz)). The immunocomplex was precipitated with protein A agarose beads at 4°C for 1 hour and the supernatant from IgG control was used as input material. The beads were washed consecutively with low salt wash buffer, high salt wash buffer, LiCl wash buffer, and twice in TE buffer. Bound DNA was eluted from the beads twice with elution buffer by vortexing at room temperature for 10 minutes and then incubating at 37°C for 10 minutes. The supernatant was collected, supplemented with 2 mM EDTA, 20 mM Tris-Cl, 10 mg/ml Proteinase K and incubated at 42°C. DNA crosslinks were reversed by incubating the precipitates overnight at 65°C. DNA was purified by phenol/chloroform extraction and ethanol precipitation, and was resuspended in H_2O. Quantification of ChIP assay was done using site-specific SYBR Green primers (Table 1) using ABI PRISM7500. To quantify immunoprecipitated DNA, a standard curve was generated from serial dilutions of Input DNA. To calculate ChIP results as a percentage of input, the amount of the immunoprecipitated DNA from the IgG was subtracted from the amount of the immunoprecipitated DNA from the

specific antibody ChIP followed by normalizing against the amount of the input DNA.

Primers	Sequence
Gata3 FOR Gata3 REV	5' CGTATATGATGAGTCTTCTCTGGGACTTG 3' 5'AAATCTCAGAACACACACATTTCCAGG 3'
cMaf FOR cMaf REV	5' AACAGTGTTGGCTTTGTCTACTATGGGAT 3' 5' GTAGGCGGTGCTCTGATATATTGCTGTT 3'
Irf4 FOR Irf4 REV	5'GACCTCCTTACTGACTGTAAGTTGTGG 3' 5' TTCCATTGTCAGAGCCCTGGTAGTAAAC 3'
Il17f FOR Il17f REV	5'GATCTGGTCCATTTTCCGTCATCTCCC 3' 5'GACCTCCTTACTGACTGTAAGTTGTGG 3'
Il24 FOR Il24 REV	5' TCTTGAAACTGTCACCAGCTGCAAAGT 3' 5' TCAGTCTAGGTAGCCACTCCATTCTATGTCTA 3'
Il21 FOR Il21 REV	5' TGCCGCTGCTTTACTCATTG 3' 5' GCACCGTCAGCTTTCAGAGA 3'

Table 1. Primer sequences used for ChIP.

Detection of cytokines using ELISA

To generate cell free supernatants for analysis, T cells differentiated for 5 days were washed and stimulated ($1x10^6$ CD4+ T cells/ml) with plate bound anti-CD3 (4 µg/ml) for 24 hours or freshly isolated CD4+ T cells ($1x10^6$ CD4+ T cells/ml) were stimulated with plate bound anti-CD3 (4 µg/ml) for 48-72 hrs. To test for cytokine secretion, 2 µg/ml of α-IFN-γ, α-IL-17, α-IL-21, α-IL-22, α-IL-4, α-IL-5, α-IL-13, and α-IL-10 capture antibodies (BD Pharmingen, eBioscience) were dissolved in 0.1 M $NaHCO_3$ (pH 9) or and 50 µl/well was 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). 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 following the addition of Sigma 104 phosphatase substrate (5 mg/ml; Sigma) dissolved in ELISA substrate buffer (10% diethanoloamine, 0.05 mM $MgCl_2$, 0.02% NaN_3, pH 9.8) by measuring the absorbance at 415 nm (BIO-RAD microplate reader model 550).

Th17 enrichment

Th17 cells were activated with plate-bound anti-CD3 (4 µg/ml). After 4 h, Th17 cells were labeled with 75 µl of previously crosslinked (Controlled Protein-Protein Crosslinking Kit; Pierce) anti-CD45 (clone 30-F11; BD Pharmingen) and anti-IL17 (clone Tc11–18H10; BD Pharmingen) Abs (0.2 mg/ml) for 5 min on ice. Labeled cells were then diluted in pre-warmed complete RPMI 1640 to a concentration of 10^5 cells/ml and rotated for 1 h at 37°C. After capture, the Th17 cells were stained with 100 µl of biotin-labeled anti-IL-17 (0.2 mg/ml; clone Tc11–8H4.1; BD

Pharmingen) for 15 min before washing and incubating 10 min with Streptavidin-PE (BD Pharmingen). IL-17 captured cells were sorted using a FACS Aria cell sorter (BD Biosciences).

Intracellular cytokine staining

Differentiated cells (0.25-1x10^6) were stimulated with phorbol-myristate acetate (PMA) (50 ng/mL; Sigma) and ionomycin (750 ng/ml; Sigma) for 4-6 hours in the presence of GolgiPlug (BD Biosciences) or monensin for the last 2-3 hours. Cells were fixed for 10 minutes at room temperature with formaldehyde at a final concentration of 2%, stained for surface markers as described below, and permeabilized by washing twice with FACS/ELISA buffer plus 0.1% saponin. Cells were stained for IL-17, IL-4, IL-5, IFN-γ (eBioscience) using fluorescently conjugated antibodies for 30 minutes at 4°C. Cells were washed one time in FACS/ELISA buffer plus 0.1% saponin. Samples were analyzed by flow cytometry using FACS-Caliber machines and data were analyzed using WinMDI software.

Cell surface staining

Total splenocytes or isolated T cells (0.25-1x10^6) were placed in 12x75 mm flow cytometry tubes. Following centrifugation at 1,500 rpm for 5 minutes at 4°C, cells were washed once in FACS/ELISA buffer (2% BSA, 0.01% NaN_3 in PBS) and re-suspended with formaldehyde at a final concentration of 2% and a total volume of 100 μl. Cells were incubated for 10 minutes at room temperature and washed

twice with FACS/ELISA buffer. Cells were washed with FACS/ELISA buffer two times. Cells were then surface stained for CD25 and IL-4Rα using fluorochrome conjugated antibodies (BD Pharmigen) for 30 minutes at 4°C. Cells were washed one time in 2 mL of FACS/ELISA buffer and permeabilized for intracellular staining or analyzed by flow cytometry on FACS-Calibur machines (BD Biosciences). In each sample 10,000-20,000 events were collected and data were analyzed using WinMDI software.

Generation of whole cell protein lysates

T cells were collected and lysed in 30 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 Orthovandate, β-glycerol, DTT, and protease inhibitors (aprotinin, leupeptin, pepstatin A, iodoacetamide, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), benzamidine). Cells were then incubated on ice for 10-15 minutes and centrifuged (14,000 rpm) at 4°C. Supernatant was transferred to a new pre-chilled tube and stored at -80°C until used.

SDS-PAGE and Western blot

Protein (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). Proteins were denatured by boiling for 5 minutes. Samples and Precision Blue plus marker (Bio-Rad) were loaded and electrophoresed on a 10 well pre-cast 4-12% gradient Bis-Tris polyacrylamide gel (Invitrogen). The gel was run at 150 V

for 1-2 hours. Proteins from gel were transferred to a nitrocellulose membrane (Schleicher and Schuell) at 250 mAmps overnight at 4°C. Following transfer, the membrane was blocked in 1X TBST (Tris-Base, NaCl, Tween-20) supplemented with 5% nonfat powdered milk for one hour while rotating.

After blocking, membranes were immunoblotted using primary antibodies to detect phospho-STAT4 (Zymed), Stat4 (Santa Cruz), or β-actin (Calbiochem, San Diego, CA). Primary antibody was diluted in 1X TBST with 5% nonfat milk (according to manufacturer's recommendations) and incubated with membrane for at least 2 hours at room temperature or 4°C overnight. The membrane was then washed for 5 minutes 3 times with TBST. Secondary antibody diluted in TBST and 5% nonfat milk was added to the membrane for 45 minutes. Following incubation, membrane was washed with TBST 4 times (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.

RESULTS
PART I: Role of IL-23 in Th17 stability

Th17 differentiation is dependent on STAT3

The differentiation of inducible T regulatory cells occurs when naïve cells are activated in the presence of TGF-β. However, cells cultured with TGF-β+IL-6 differentiate into Th17. Therefore, the presence or absence of IL-6 is thought to be the critical switch to determine T regulatory cell versus Th17 cell fate. IL-6 signals predominantly through STAT3. In order to determine if STAT3 is required for Th17 development we differentiated both WT and STAT3-deficient naïve CD4 T cells with various concentrations of TGF-β or TGF-β+IL-6. As expected, naïve cells from both wild type and STAT3-deficent mice differentiated into iTregs when cultured with TGF-β (Figure 4). Wild type cells cultured with TGF-β+IL-6 repressed Foxp3 expression and produced IL-17. The number of IL-17 positive cells was increased when cells were cultured with increasing doses of IL-6 and/or TGF-β (Figure 4). Conversely, STAT3-deficient cells cultured with TGF-β+IL-6 failed to induce IL-17 production and a large population of cells remained Foxp3 positive (Figure 4). Therefore, IL-6 induced STAT3 activation is required for both IL-17 production and repression of Foxp3 expression.

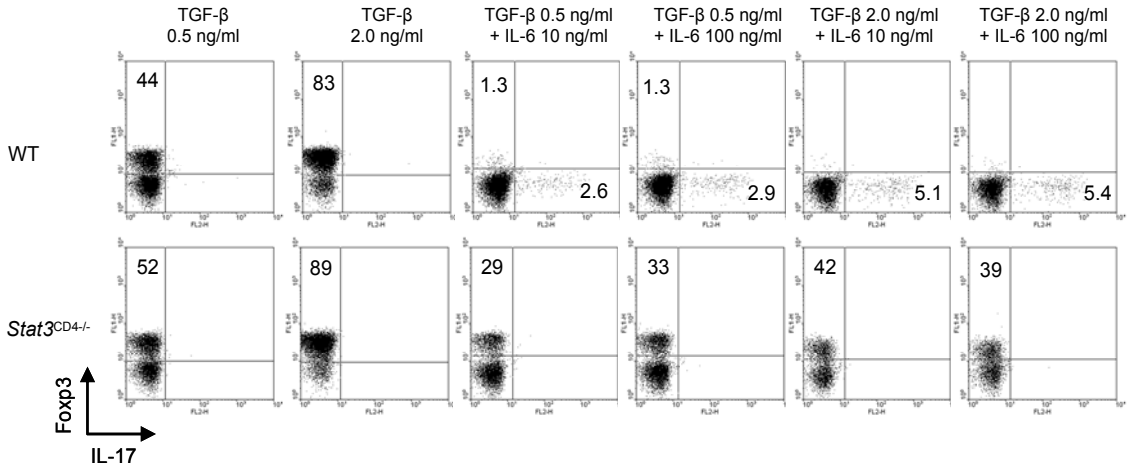

Figure 4. STAT3 is required for Th17 differentiation. Naïve CD4 T cells from WT and *Stat3*$^{CD4-/-}$ mice were activated with various concentrations of TGF-β or TGF-β +IL-6 for 5 days. On day 5 cells were re-stimulated with PMA+Iono and intracellularly stained for Foxp3 and IL-17. Data are representative of 2 independent experiments.

IL-23 maintains IL-17 secretion without affecting Th17 cell proliferation or expansion

Although the requirement for IL-23 in the function of Th17 cells *in vivo* is established, the precise role of this cytokine in affecting the Th17 phenotype is unclear. Among other functions, IL-23 was proposed to act as a Th17 cell proliferation or survival factor. To directly test these functions, we developed a cytokine capture assay for IL-17-secreting cells to compare IL-23 functions in enriched IL-17-high and low secretor populations. Naive CD4 T cells were cultured with TGF-β1+IL-6+IL-1β for 5 days before stimulation with anti-CD3 and selection of IL-17-high and low cells by cell sorting (Figure 5A). Following sorting there was a 10-12-fold enrichment for IL-17 secreting cells in the IL-17-high population (Figure 5A). The separation of cells into distinct populations was

confirmed by demonstrating segregated expression of *Il23r* and *Rorc* and production of IL-17 and IL-22 while IFN-γ production was indistinguishable between the two populations (Figure 5B and C). Intracellular staining for IFN-γ in these populations demonstrated less than 0.5% in any population.

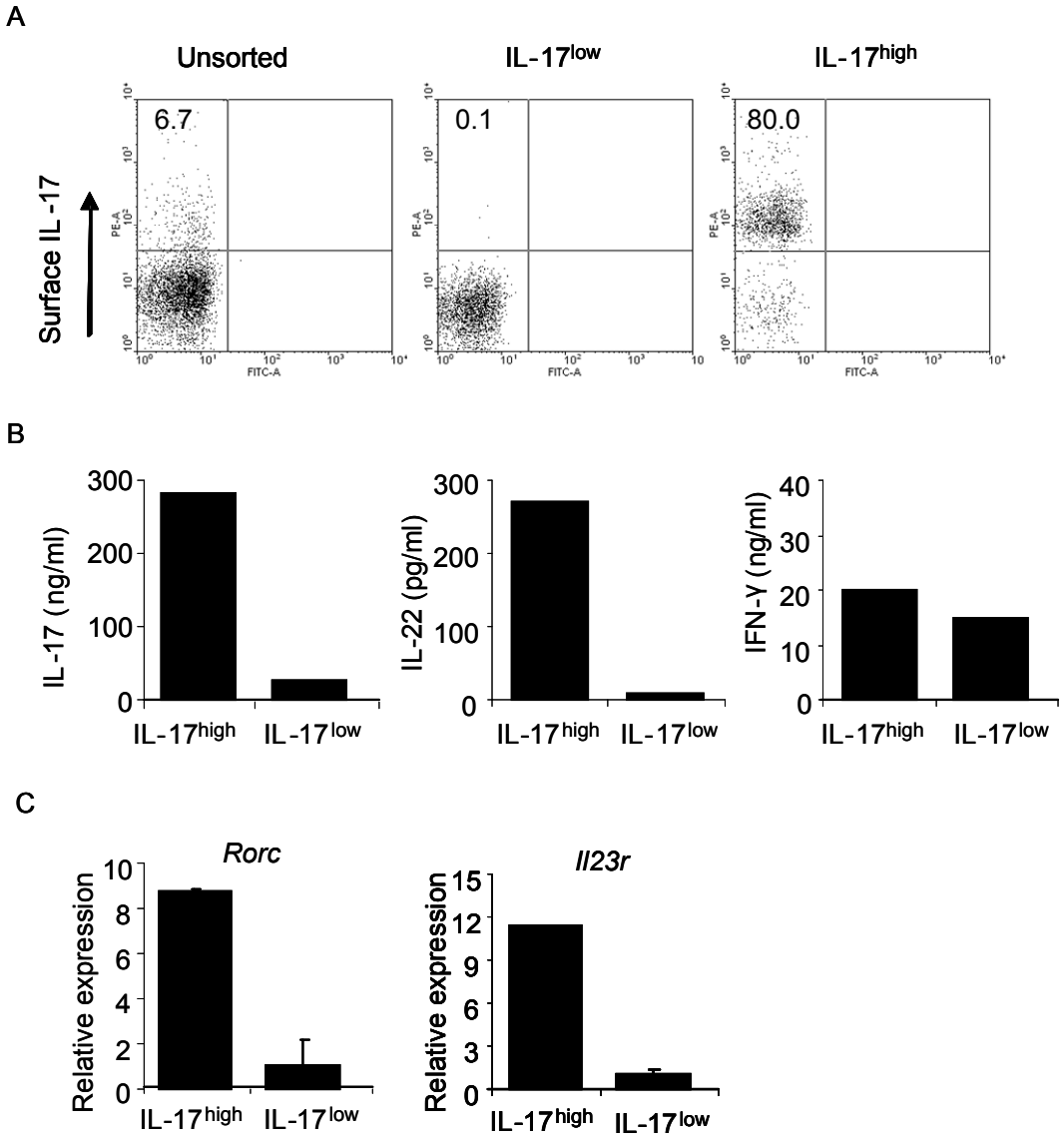

Figure 5. Cytokine selection of IL-17+ Th17 cells. (A) Naive $CD4^+$ T cells were activated, cultured in TGF-β plus IL-6 plus IL-1β and blocking antibodies (anti-IFN-γ and anti-IL-4) for 5 days and activated before surface staining for IL-17 using cytokine capture. Cells were then sorted into IL-17-high and low

populations. (B) Supernatants from IL-17-high and -low cells stimulated with anti-CD3 were tested for cytokines using ELISA. (C) RNA was isolated from cells treated in B and gene expression was assessed using real-time PCR. Data are representative of 2 experiments.

One of the proposed functions of IL-23 is promoting the proliferation or expansion of Th17 cells. To test this directly, naive CD4 T cells were cultured with TGF-β+IL-6+IL-1β for 5 days, separated based on IL-17 production and labeled with CFSE (Figure 6A). CFSE-labeled IL-17-high and low populations were cultured in blocking Abs (anti-IFN-γ and anti-IL-4) alone or blocking antibodies plus IL-23 for 24 or 48 hours to assess proliferation. Although IL-17-high cells had an intrinsically higher rate of proliferation than IL-17-low cells, there was not a significant difference in proliferation between cultures incubated with or without IL-23 (Figure 6B and C), suggesting that IL-23 does not promote a robust proliferative response.

IL-23 was also proposed to affect Th17 survival. However, in examining the overall cell growth in IL-17-high and low cells, we observed the increased proliferative capacity of IL-17-high cells that resulted in a 2-3-fold increase in cell number compared with IL-17-low cells, but only minor effects of IL-23 (Figure 6D). Similarly, IL-17-high and low cells cultured in the presence or absence of IL-23 had similar percentages of Annexin V+ cells after 2 or 4 days of culture with little effect of IL-23 culture (Figure 6E).

In contrast, we did note that in IL-17-high populations cultured with IL-23, a higher percentage of cells, with a higher intensity of IL-17 staining, was maintained compared with cells cultured in the absence of IL-23 (Figure 6B). The effects of IL-23 on maintaining the IL-17-secreting phenotype were even more dramatic when cells were activated with anti-CD3. After 2 days of activation, IL-17-high cells cultured in IL-23 still had >60% IL-17+ cells, while cultures incubated in the absence of IL-23 has <30% IL-17+ cells (Figure 6F). This effect was also observed in the IL-17-low cultures where IL-17+ cells comprised <10% of the population when cultured in the absence of IL-23 (Figure 6F). These data suggest that IL-23 maintains the IL-17-secreting phenotype without detectable effects on Th17 cell proliferation or expansion.

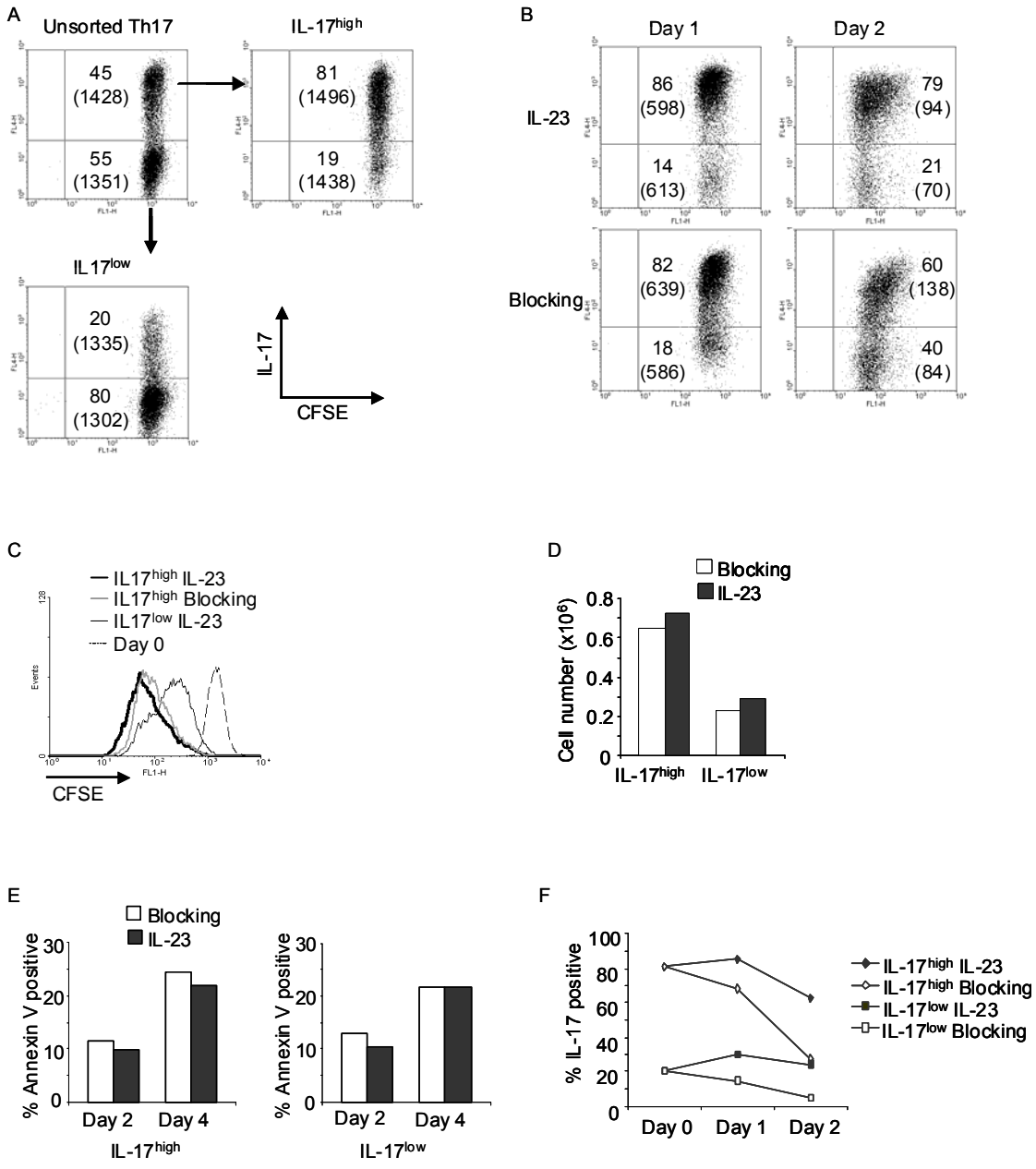

Figure 6. IL-23 maintains the IL-17-secreting phenotype without affecting cell expansion or survival. (A) Naive CD4$^+$ T cells were activated and cultured in TGF-β plus IL-6 plus IL-1β and blocking antibodies (anti-IFN-γ and anti-IL-4) for 5 days before sorting into IL-17-high and low populations. Cells were then labeled with CFSE and for intracellular IL-17 following stimulation with PMA plus ionomycin. Numbers indicate percent of cells in each quadrant and bracketed numbers indicate CFSE mean fluorescence intensity. (B) IL-17-high cells were cultured with IL-23 and blocking antibodies or blocking antibodies alone for the indicated times before cells were stimulated and stained for intracellular IL-17. Numbers indicate percent of cells in each quadrant and bracketed numbers indicate CFSE mean fluorescence intensity. (C) IL-17-high or -low CFSE-stained

cells prepared as in B were cultured for 2 days with blocking antibodies in the presence or absence of IL-23 as indicated. CFSE staining is shown from freshly stained cells (day 0) for comparison. (D), IL-17-high and -low cells cultured as in B were counted after 48 h. (E) IL-17-high and -low cells were cultured as in B and were analyzed for Annexin V staining after 2 or 4 days of culture in the presence or absence of IL-23. (F) IL-17-high and-low cells were stimulated with anti-CD3 and cultured with blocking antibodies with or without IL-23. Cells were stimulated with PMA plus ionomycin for 4 h and stained for intracellular IL-17. Data are representative of 2 experiments.

IL-23 maintains the Th17 phenotype in long-term cultures

To test the ability of IL-23 to maintain the Th17 phenotype over long term culture, we cultured naive CD4 T cells for 1 week with TGF-β plus IL-6 plus IL-1β, the latter which we found amplifies IL-23 responsiveness *in vitro* (Veldhoen et al., 2006a), and then for subsequent rounds of stimulation with either blocking antibodies alone or IL-23 with blocking antibodies. Despite a high level of IL-17 in the initial cultures, IL-23 was only partially effective in attenuating the decrease of IL-17 production from T cells following subsequent rounds of stimulation, compared with blocking antibodies alone (Figure 7A). IL-23 was effective in limiting IFN-γ production from these cultures suggesting that the decrease in IL-17 production observed over multiple rounds of stimulation is not due to coincident increases in IFN-γ production or increases in the percentage of cells that are IFN-γ positive.

Given the similarity in autoimmune disease phenotype between IL-1RI- and IL-23p19-deficient mice, and that IL-1 enhances IL-23 responsiveness (Cho et al., 2006; Cua et al., 2003; Sutton et al., 2006), we next tested the ability of IL-1β to

cooperate with IL-23 in long term cultures. Naive CD4 T cells were cultured as in Figure 5A for the first week and then cultured for subsequent rounds of stimulation with IL-23, IL-1β, or a combination of IL-23 and IL-1β. Although IL-1β was no more effective than IL-23 in attenuating the loss of IL-17 secretion over multiple rounds of stimulation, the combination of IL-23 and IL-1β was able to maintain a high level of IL-17 secretion over three rounds of stimulation (Figure 7B). There were not dramatic differences in the growth or survival among these cultures over several rounds of culture, suggesting that the effects of these cytokines are not on survival or expansion, but rather on maintaining the phenotype of the cells. The combination of IL-23 and IL-1β was similarly capable of maintaining higher levels of IL-21 and IL-22 secretion than IL-23 alone, although there were decreases in these cytokines over rounds of stimulation (Figure 7B).

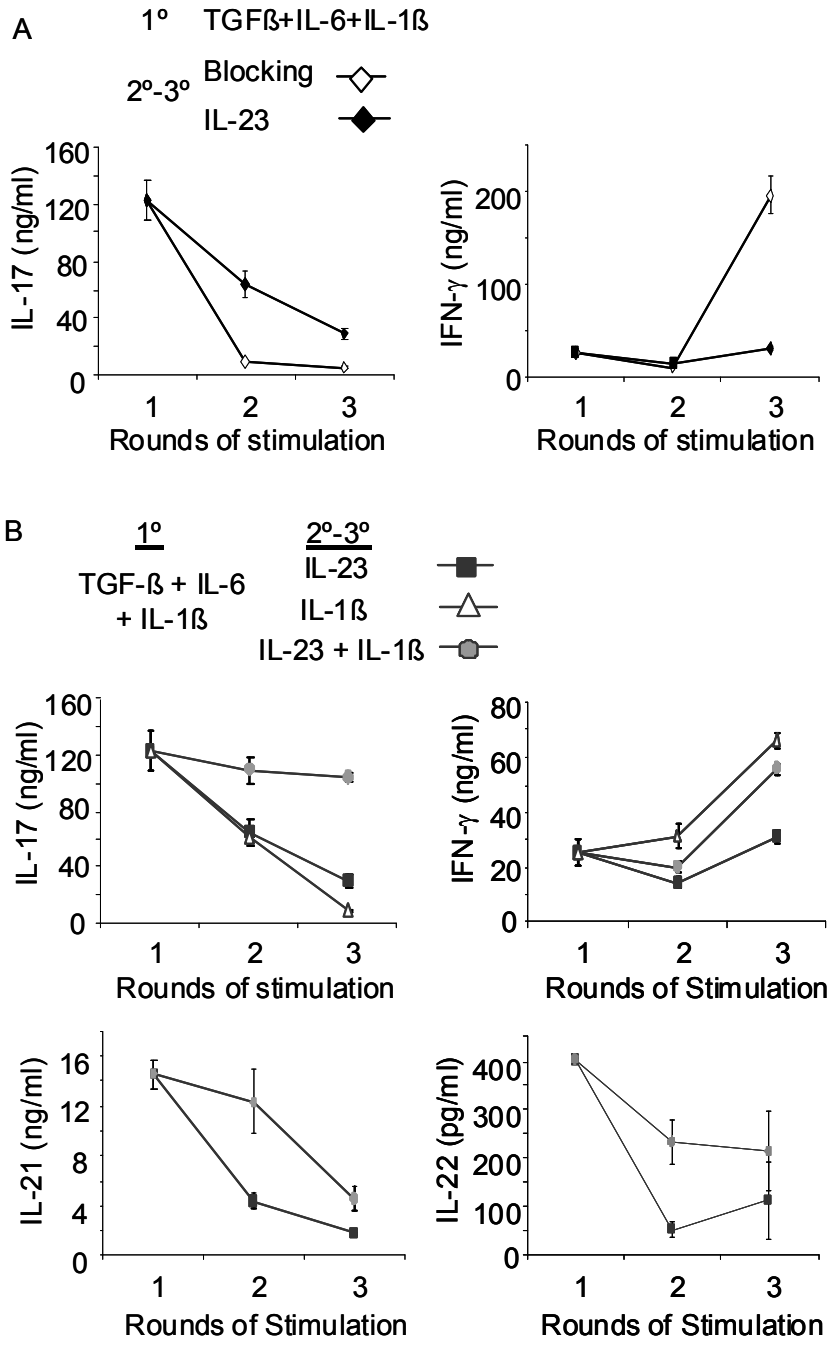

Figure 7. IL-1β increases IL-23 stimulated maintenance of the Th17 phenotype. (A). Naive T cells were activated, primed with TGF-β plus IL-6 plus IL-1β for the first round and cultured for two additional rounds of stimulation in the presence of blocking antibodies with or without IL-23. After each round of culture cells were stimulated and cell-free supernatants were tested for cytokine production using ELISA. (B) Naive T cells were activated and primed as in A and cultured for two additional rounds of stimulation with IL-23, IL-1β, or IL-23 plus IL-

1β as indicated. Cytokine production was measured using ELISA. Data are representative of 2 experiments.

To define the mechanism for the ability of IL-1β to augment IL-23 function we first analyzed IL-23R expression in cells cultured with IL-23, IL-1β, or both cytokines for the second and third rounds of stimulation. Although culture with IL-23 maintained or enhanced *Il23r* mRNA expression, there was no increased expression in cells cultured with IL-1β alone or with both cytokines (Figure 8A). We then examined IL-23 signaling using flow cytometry to assess levels of phospho-STAT3 following acute stimulation of cultures incubated for three rounds in IL-23, IL-1β, or both cytokines. IL-23 stimulated STAT3 phosphorylation in cells from each of the conditions with insignificant differences in the phospho-STAT3 levels among the conditions, suggesting that the effect of IL-1β was not altering IL-23 signaling (Figure 8B). To test whether IL-1β had altered the *Il17* gene to make it more responsive to IL-23, we took advantage of an assay we previously described for the acute stimulation of IL-17 production by a combination of IL-23 and IL-18 (Mathur et al., 2007). Naive CD4 T cells primed with TGF-β plus IL-6 plus IL-1β for the first round of stimulation and cultured in IL-23 or IL-23 plus IL-1β for two rounds of stimulation were re-stimulated with IL-23 plus IL-18. Cells that were cultured with IL-23 plus IL-1β generated higher amounts of IL-17 than cells cultured in IL-23 alone in response to IL-23 plus IL-18 (Figure 8C). Because analysis of *Il23r* expression in these cultures (Figure 8A) showed only minor differences in expression, with slightly lower levels in the IL-

23 plus IL-1β cultured cells, it suggests that culture in IL-23 plus IL-1β is directly affecting the responsiveness of the *Il17* locus, although we did not find differences in the level of total histone acetylation between cells that were cultured with or without IL-1β. IL-1β may enhance IL-23 function by activating cooperative transcription factors, or through indirect mechanisms including the ability to limit the inhibitory effects of IL-2 on Th17 development (Kryczek et al., 2007).

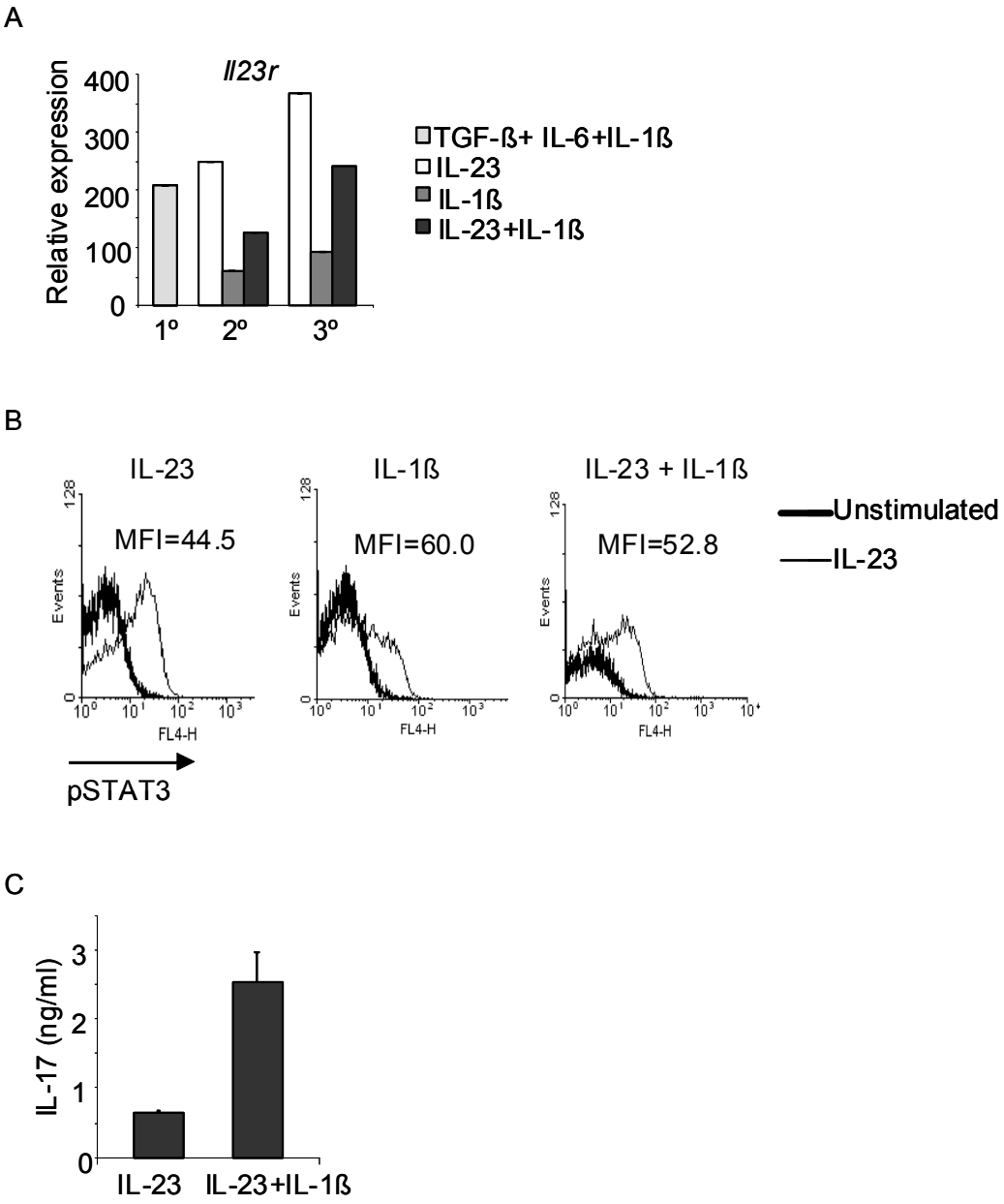

Figure 8. IL-1β increases responsiveness of the IL-17 locus. (A) Naive T cells were activated and primed with TGF-β plus IL-6 plus IL-1β for the first round and cultured for two additional rounds of stimulation with IL-23, IL-1β, or IL-23 plus IL-1β as indicated. At the end of the first, second, and third round of stimulation RNA was isolated from cells stimulated with anti-CD3 for 4 h. Quantitative PCR of *Il23r* expression is shown as relative to Th1 cultures after the third round of stimulation. (B) Cells cultured and stimulated as in A for three rounds were stimulated with IL-23 for 30 min before intracellular staining for phospho-STAT3. (C) Naive cells primed and cultured as in A were stimulated

with IL-18 and IL-23 for 24 h. Cell-free supernatants were measured for IL-17 using ELISA. Data are representative of 2 experiments.

IL-23 does not mediate commitment to the Th17 lineage

Because IL-23 was capable of maintaining the IL-17-secreting phenotype, it allowed us to determine whether IL-23 mediated commitment to the Th17 lineage, with commitment being defined as the ability of cells to maintain the IL-17-secreting phenotype in the presence of cytokines promoting the development of other subsets. Th1 and Th2 cells are committed to their respective lineages after three rounds of stimulation (Murphy et al., 1996). Naive CD4 T cells were primed with TGF-β plus IL-6 plus IL-1β for the first round, cultured for two rounds in IL-23 plus IL-1β, and then either maintained in IL-23 plus IL-1β or switched to IL-12 or IL-4 containing medium for the fourth round of stimulation before stimulation with anti-CD3 to assess cytokine production. Although cells cultured for the fourth round in IL-23 plus IL-1β maintained the ability to produce IL-17, cells switched to Th1 or Th2 promoting conditions showed diminished IL-17 production and the induction of IFN-γ and IL-4, respectively (Figure 9A). Similar results were generated using cultures derived from C57BL/6 or BALB/c mice. The levels of IL-4 induced following switching into Th2 conditions were lower than seen from cells cultured for 4 weeks under Th2 conditions, though the levels of IFN-γ secreted by Th17 cultures switched to Th1 conditions were comparable to long term Th1 cultures (Figure 9A and B). Expression of other Th17 genes including *Il17f*, *Il23r*, *Il22*, and *Rorc* were also diminished in cultures switched to

Th1 or Th2 conditions (Figure 9C and D). In contrast, naive CD4 T cells primed with TGF-β plus IL-6 plus IL-1β for the first round, cultured for two rounds in IL-23 plus IL-1β, and subsequently switched to culture conditions that promote Treg development were unable to develop into Foxp3-expressing cells (Figure 9E). Culture of cells with TGF-β plus IL-2 increased the percentage of IL-17+ cells. Thus, Th17 cells cultured with IL-23 can adopt some, but not all CD4 T cell lineages.

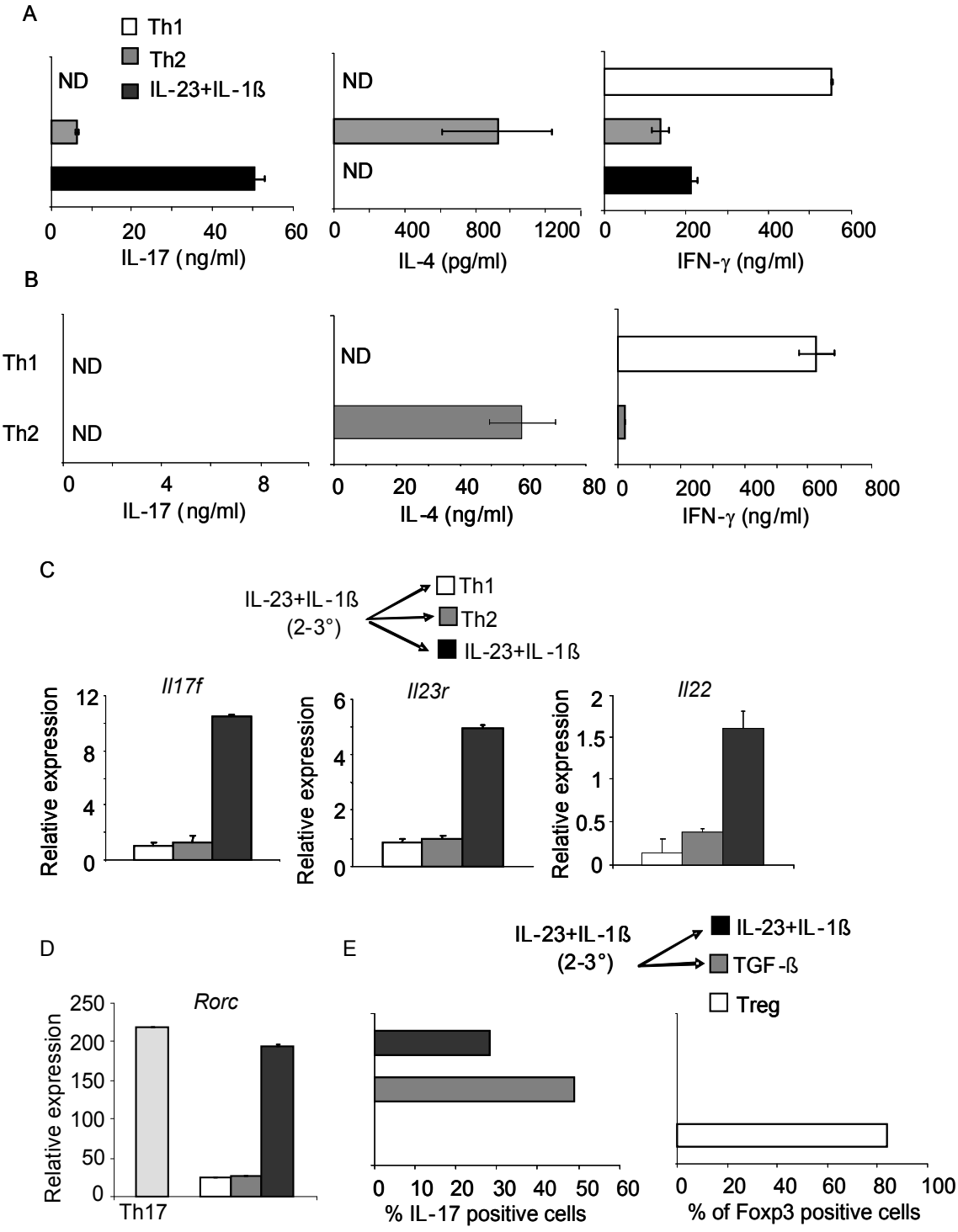

Figure 9. IL-23 does not program commitment to the Th17 lineage. (A) Naive T cells were activated, primed with TGF-β plus IL-6 plus IL-1β for the first round followed by two rounds of stimulation in IL-23 plus IL-1β, were cultured for an additional round of stimulation in Th1 or Th2 conditions, or IL-23 plus IL-1β. Supernatants from anti-CD3 stimulated cells were tested for cytokine production using ELISA. (B) Naive $CD4^+$ T cells were activated and cultured under Th1 or

Th2 priming conditions for four rounds of stimulation. At the end of the fourth round of stimulation, supernatants from anti-CD3 stimulated cells were tested for cytokine production using ELISA. (C) Cells stimulated and cultured as in A were stimulated with anti-CD3 for 4 h and RNA was isolated for quantitative PCR. (D) Cells were stimulated and cultured for three rounds as in A. After 3 days of culture in Th1, Th2, or IL-23 plus IL-1β conditions, RNA was isolated from control or switched cultures for quantitative PCR. (E) Naive T cells were activated, primed with TGF-β plus IL-6 plus IL-1β for the first round followed by two rounds of stimulation in IL-23 plus IL-1β, were cultured for an additional round of stimulation in IL-23 plus IL-1β or TGF-β plus IL-2. The percentages of cells positive for Foxp3 or IL-17 intracellular staining are indicated with cells cultured for 1 wk in TGF-β plus IL-2 shown as a control for Foxp3 expression.
ND= not detected. Data are representative of 2-3 experiments.

Although T cells cultured for three rounds under these Th17 conditions produce large amounts of IL-17, there is still some production of IFN-γ, and it remained possible that a contaminating population of cells was expanding and overtaking the Th17 cells upon switching cultures to Th1 or Th2 conditions. To eliminate this possibility, we used the cytokine capture protocol to isolate IL-17-high cells from Th17 cultures after three rounds of stimulation (Figure 10A) and then maintained the cultures with IL-23 plus IL-1β or switched to conditions promoting Th1 or Th2 development. As observed with un-separated Th17 cultures, IL-17-high cells maintained their phenotype with continued culture in IL-23 plus IL-1β, but showed decreased IL-17 and IL-22 production, and increased IFN-γ and IL-4 production in Th1 and Th2 conditions, respectively (Figure 10B and C). Thus, IL-17-high cells are not stable secretors of IL-17 and upon exposure to conditions promoting the development of other Th subsets, they acquire new cytokine secreting characteristics.

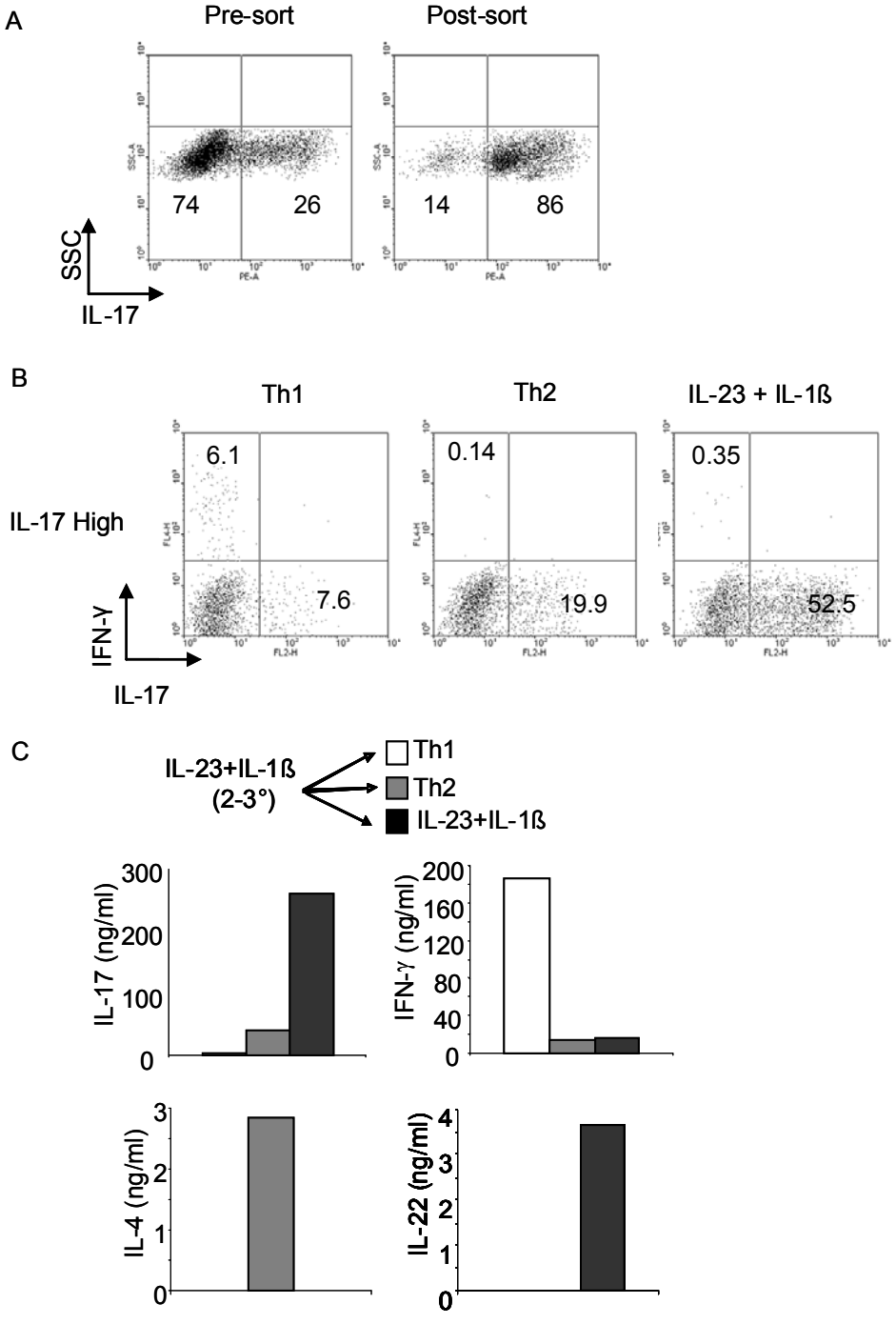

Figure 10. Sorted IL-17 high cells are not committed to the IL-17 secreting phenotype. (A) Naive CD4$^+$ T cells were primed and cultured as in 5A, and after the third round of culture, cells were enriched for IL-17-secreting cells by cytokine selection. Surface staining for IL-17 is shown pre- and post sort. (B) IL-17-high cells from A were cultured in Th1, Th2, or IL-23 plus IL-1β for an additional round of stimulation. Cells were stimulated for 4 h and stained for intracellular IL-17

and IFN-γ. (C) Supernatants from anti-CD3 stimulated cells cultured as in B were tested for cytokine production using ELISA. Data are representative of 2 experiments.

We then defined if the switch from Th17 to Th1 or Th2 was characterized by induction of the standard pathways and lineage determining factors. IL-4 signaling is qualitatively altered in Th1 cells through mechanisms that are still not clear but do not involve altered STAT6 activation (Huang and Paul, 1998), and IL-4 was able to activate STAT6 in cells cultured under Th1, Th2, or Th17 conditions (Figure 11A). During Th2 development, IL-12 signaling is extinguished, contributing to commitment in the Th2 lineage (Szabo et al., 1997). However, while *Il12rb2* expression was greatly decreased in cells cultured for three rounds in Th2 conditions, cells cultured in IL-23 plus IL-1β demonstrated expression of *Il12rb2* similar to Th1 cells (Figure 11B). To determine whether IL-12 signaling was functional, we examined STAT4 expression and IL-12-induced phosphorylation of STAT4 in Th1, Th2, and Th17 cultures after three rounds of stimulation. Although STAT4 expression is reduced and IL-12-induced STAT4 phosphorylation is eliminated in Th2 cultures, normal expression of STAT4 was retained in Th17 cultures. IL-12-induced STAT4 activation was only modestly diminished in IL-23 plus IL-1β and IL-23 cultured cells compared with Th1 cultures (Figure 11C). Despite the reduction in IL-12-induced STAT4, IL-12 and IL-4 were still able to promote an increase in *Tbx21* and *Gata3* expression in Th17 cultures switched to Th1 or Th2 conditions, respectively (Figure 11D).

Thus, Th17 cells, even after long-term culture, are competent to assume a Th1 or Th2 phenotype.

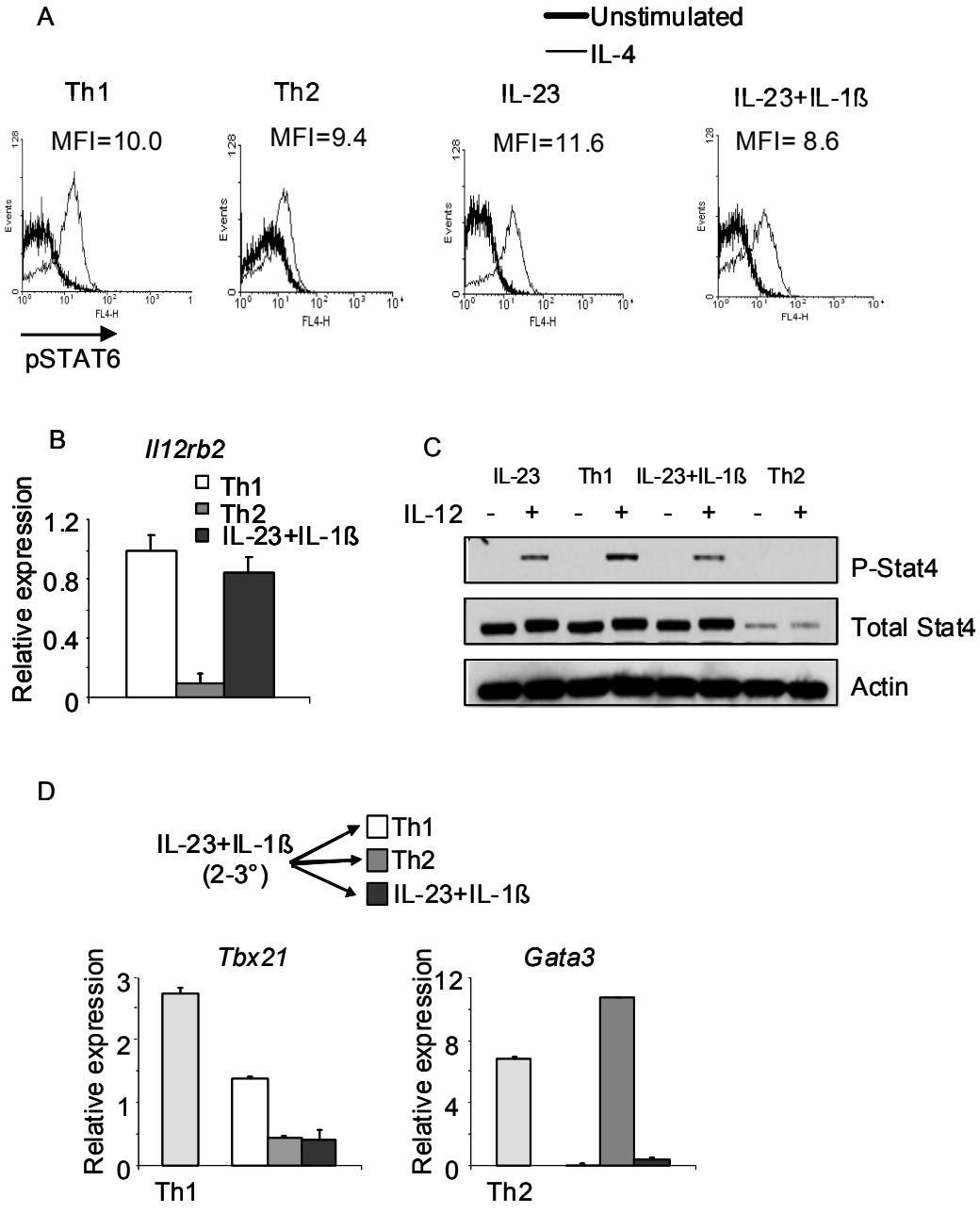

Figure 11. Signals promoting Th1 or Th2 development are intact in Th17 cultures. (A) Cells stimulated and cultured in Th17, Th1, or Th2 conditions were incubated with IL-4 for 30 min and stained for phospho-STAT6. (B) RNA from cells stimulated as in A for three rounds of stimulation was examined for relative levels of *Il12rb2* expression. Levels are relative to three-round Th1

cultures. (C) Cells stimulated and cultured as in A for three rounds of stimulation were then stimulated with IL-12 for 1 hour. Phospho-STAT4, total STAT4, and actin were detected by immunoblot. (D) Cells were stimulated and cultured for three rounds as in A. After 3 days of culture in Th1, Th2, or IL-23 plus IL-1β, RNA was isolated from control or switched cultures for quantitative PCR to test for expression of the indicated genes. Expression is relative to the level of expression of each gene in IL-23 plus IL-1β cultured cells before the fourth round of culture. Data are representative of 2 experiments.

T-BET and STAT4 are not required for the repression of IL-17 in Th17 cells cultured with Th1 priming conditions

Th17 cells cultured for multiple rounds of stimulation secrete IFN-γ when cultured with IL-12 (Figures 9 and 10). Both T-bet and STAT4 are important for IFN-γ production from Th1 cells. To determine if T-bet and STAT4 are required for the repression of IL-17 and induction of IFN-γ from Th17 cells cultured with IL-12, we cultured T-bet-deficient and STAT4-deficient naïve T cells under Th17 conditions for 3 rounds of stimulation. For the 4^{th} round of stimulation Th17 cells were cultured with IL-12, IL-4, or IL-23+IL-1β. T-bet- and STAT4-deficient Th17 cultured in IL-23+IL-1β for the 4^{th} round of stimulation secreted high levels of IL-17. However, T-bet- and STAT4-deficient Th17 cells cultured in IL-4 had a significant decrease in IL-17 production and secreted increased levels of IL-4 (Figure 12). Similar to culture with IL-4, IL-12 cultured Th17 cells deficient in T-bet or STAT4 had significantly decreased IL-17 production. However, in the absence of T-bet or STAT4, Th17 cells cultured with IL-12 did not induce IFN-γ production (Figure 12). Therefore, T-bet and STAT4 are not required for IL-12

repression of IL-17, however they are required for IL-12 induced IFN-γ production in Th17 differentiated cells.

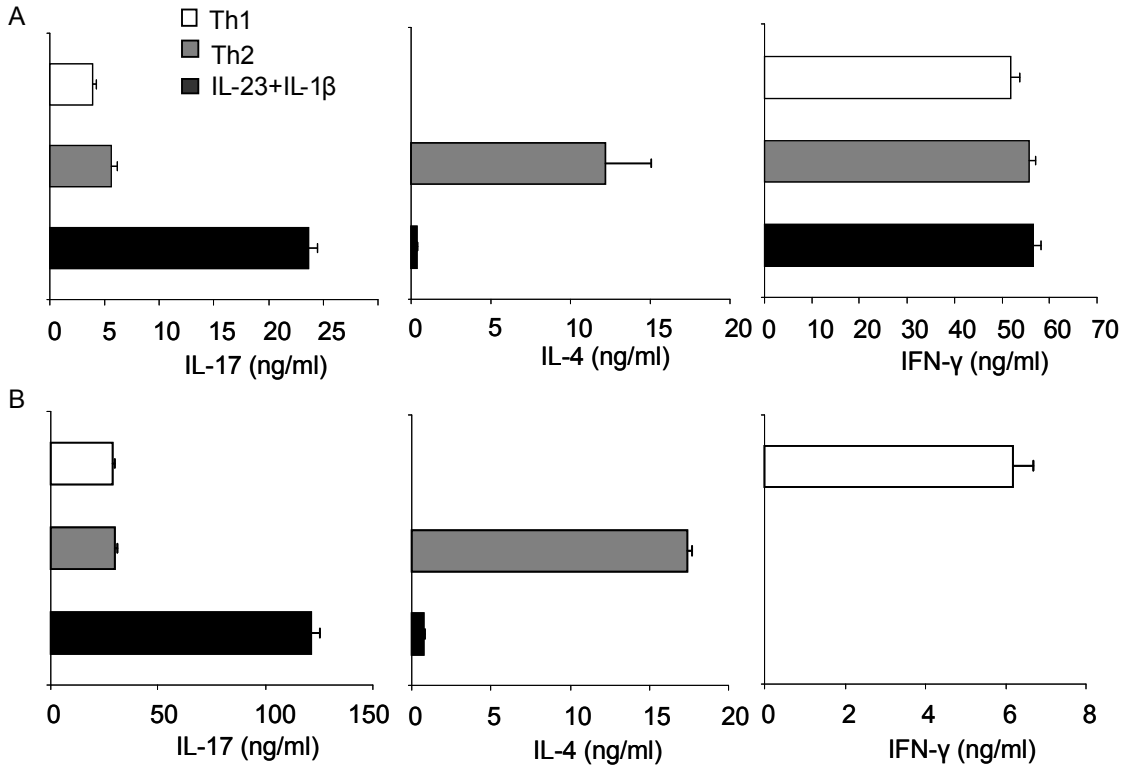

Figure 12. *Tbx21* and *Stat4* are not required for the reduction of IL-17 following culture with IL-12 or IL-4. (A) Naive T cells from WT and *Stat4*-/- (B) WT and *Tbx21-/-* mice were activated, primed with TGF-β plus IL-6 plus IL-1β for the first round followed by two rounds of stimulation in IL-23 plus IL-1β, were cultured for an additional round of stimulation in Th1 or Th2 conditions, or IL-23 plus IL-1β. Supernatants from anti-CD3 stimulated cells were tested for cytokine production using ELISA. Data are representative of 2 mice and 1 experiment.

PART II: Role of STAT4 in IL-17 Producing Memory Cells

Stat4 is required for memory cell IL-17 production

STAT4 is required for the differentiation of Th1 cells (Kaplan et al., 1996b; Thierfelder et al., 1996). Th1 cells deficient in STAT4 have decreased IFN-γ and IL-10 production compared to wild type Th1 cells (Figure 13A and B). However, expression of either STAT4 isoform, α or β, in Th1 cells lead to IFN-γ production similar to wild type Th1 cells (Figure 13A).

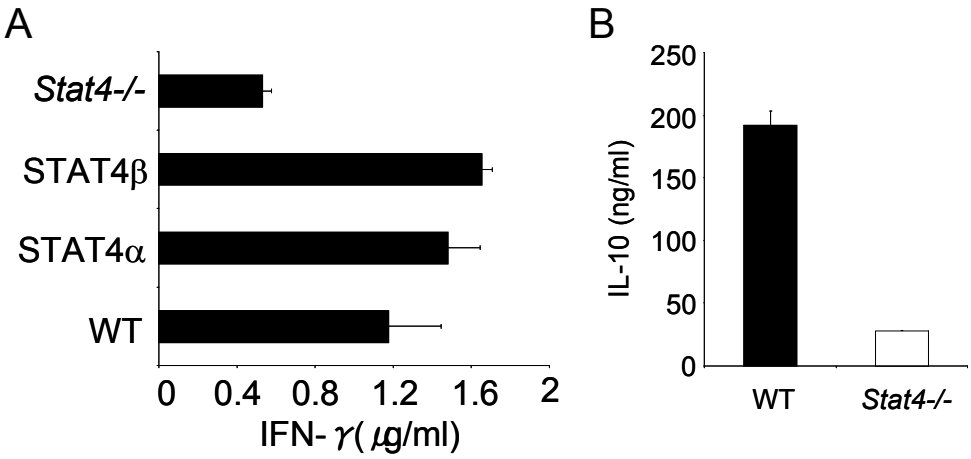

Figure 13. STAT4 is required for Th1 differentiation. (A) Naïve CD4 T cells from wild type, *Stat4-/-*, STAT4α, and STAT4β were cultured under Th1 priming conditions (IL-12, anti-IL-4) and with irradiated APCs for 5 days. On day 5 cells were re-stimulated with anti-CD3 for 24 hours. Cell free supernatant was collected and IFN-γ and production was assayed using ELISA. (B) Naïve CD4 T cells from WT and *Stat4-/-* mice were cultured under Th1 priming conditions for 5 days. On day 5 cells were re-stimulated with anti-CD3 for 24 hours. Cell free supernatant was collected and IL-10 production was assessed using ELISA. Data are representative of 2-4 experiments. Experiments were done in collaboration with Dr. John T. O'Malley.

Although STAT4 is clearly required for Th1 differentiation and IFN-γ production, initial reports showed Th17 development and IL-17 production occurs independent of STAT4 (Harrington et al., 2005; Park et al., 2005). We have shown that IL-23 plays an important role in maintaining IL-17 production from activated or effector Th17 cells (see PART I). IL-23, although to a lesser extent than IL-12, signals through STAT4 (Oppmann et al., 2000; Parham et al., 2002), thus we sought to test the role of STAT4 in IL-17 production from effector/memory cells. Wild type (WT) and STAT4-deficient naïve (CD4+CD44-CD62L+) and activated/memory (CD4+CD44+CD62L-) cells were isolated and sorted from spleens. Naïve CD4 cells were stimulated and cultured with TGF-β+IL-6 and memory/activated T cells were cultured with IL-23. Naïve Th17 cells differentiated independent of STAT4 determined by the production of IL-17 (Figure 14A). Wild type memory cells produced high amounts of IL-17. However, STAT4 deficient memory cells had significantly reduced IL-17 production compared to wild type memory cells (Figure 14A). Following differentiation, sorted cells were re-stimulated with PMA+ionomycin for 6 hours and stained for IL-17 and IFN-γ. Similar to ELISA results, STAT4-deficient Th17 cells had similar percentage of IL-17+ cells compared to wild type. However, memory cells from STAT4-deficient mice had significantly reduced numbers of IL-17+ cells (Figure 14B). Taken together, these data show that STAT4 is required for IL-17 production from IL-23 cultured memory cells.

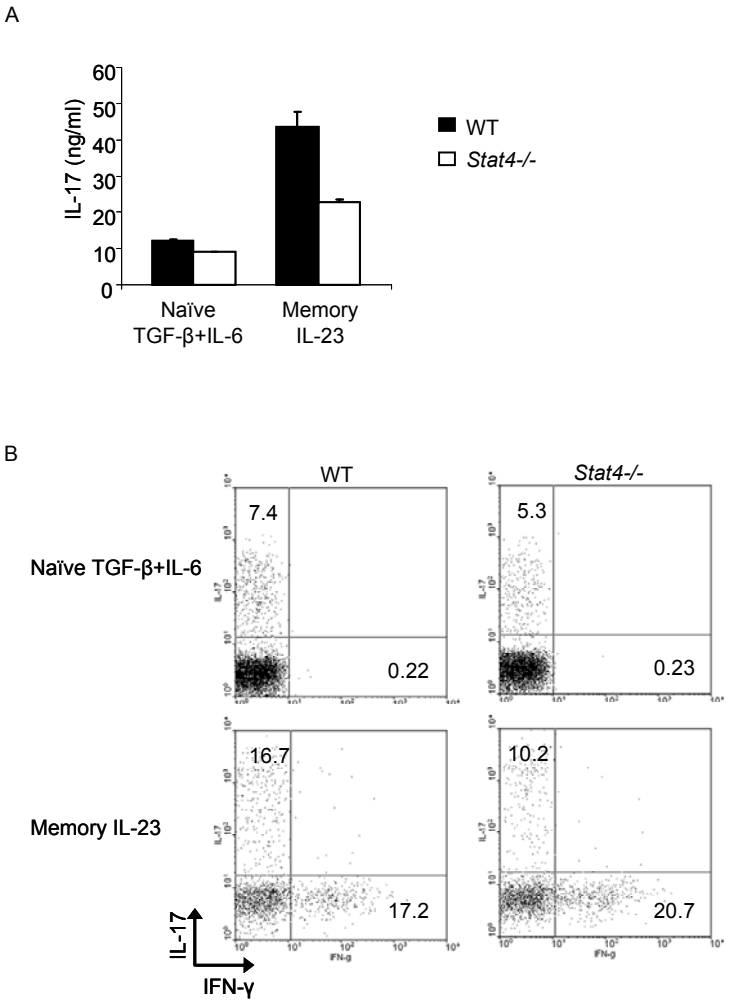

Figure 14. STAT4 is required for IL-17 production from effector/memory cells cultured with IL-23. (A) CD4+ T cells were isolated from WT and *Stat4-/-* spleens. CD4+ cells were stained and sorted for naïve (CD4+CD44-CD62L+) and memory (CD4+CD44+CD62L-). Naïve cells were cultured for 5 days with TGF-β+IL-6 and memory cells with IL-23. On day 5 cells were re-stimulated with anti-CD3 for 24 hours. Cell free supernatant was assessed for IL-17 production using ELISA. (B) Cells sorted and cultured as in A were re-stimulated on day 5 with PMA+ionomycin for 4 hours. Cells were then stained for IL-17 and IFN-γ. Data are representative of 1 (B) or 2 (A) experiments.

We previously published that re-stimulation of Th17 cells with IL-18 and IL-23 results in IL-17 production (Mathur et al., 2007). Naïve Th17 cells from wild type and STAT4-deficient mice cultured with TGF-β+IL-6 had similar amounts of IL-17

production after IL-18+IL-23 re-stimulation, shown by both ELISA and intracellular staining (Figure 15A and B). Conversely, *Stat4* deficient memory cells cultured with IL-23 had reduced IL-17 production after IL-23+IL-18 re-stimulation compared to wild type memory cells (Figure 15A and B). Collectively, these data show that STAT4 is required for IL-17 production from memory cells re-stimulated with IL-23+IL-18.

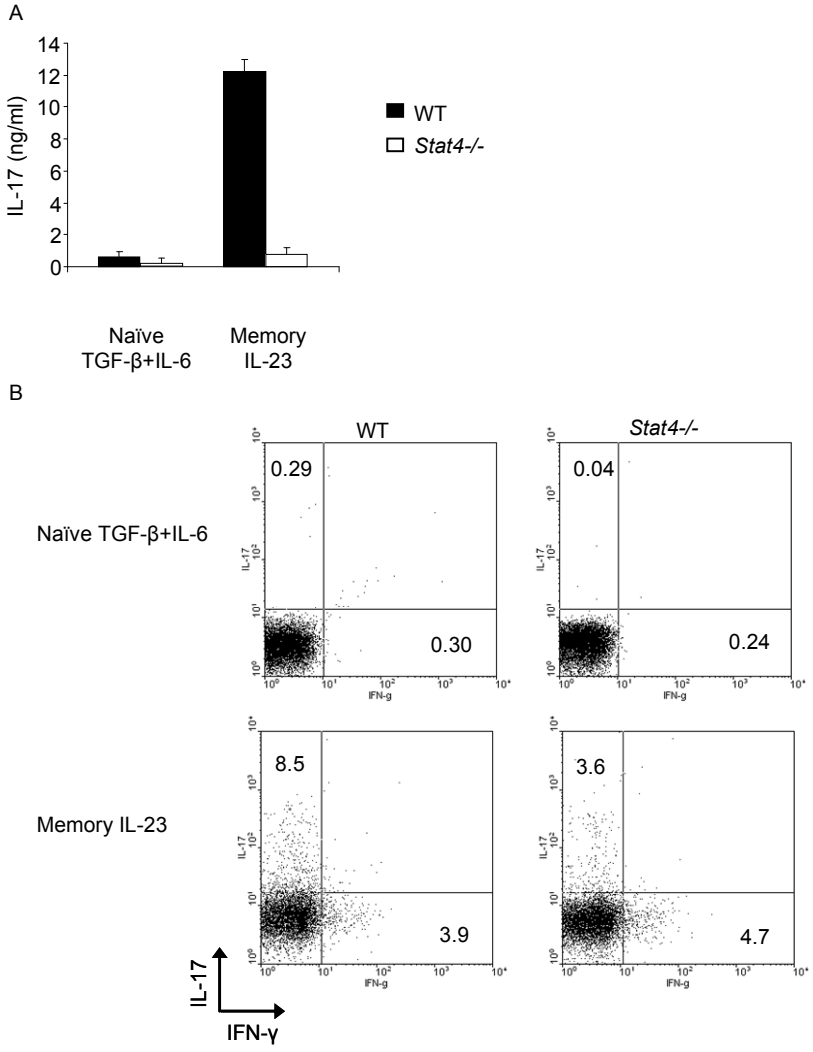

Figure 15. STAT4 is required for IL-17 production after IL-23+IL-18 re-stimulation from effector/memory cells cultured with IL-23. (A) CD4+ T cells were isolated from WT and *Stat4-/-* spleens. CD4+ cells were isolated for naïve (CD4+ CD62L+) and memory (CD4+ CD62L-). Naïve cells were cultured for 5

days with TGF-β+IL-6 and memory cells with IL-23. On day 5 cells were re-stimulated with IL-18+IL-23 for 24 hours. Cell free supernatant was assessed for IL-17 production using ELISA. (B) Cells sorted and cultured as in Figure 14A were re-stimulated on day 5 with IL-18+IL-23 for 4 hours. Cells were then stained for IL-17 and IFN-γ. Data are average ± SD of 2 mice and representative of 3-4 experiments (A) or representative of 1 experiment (B).

STAT4-deficient memory cells have reduced *Rorc* expression

STAT4 deficient memory cells have reduced IL-17 production after 5 days of culture with IL-23. It is possible that STAT4 is required for *Il23r* expression and thus responsiveness to IL-23. To directly test this we isolated memory cells (CD62L-) from wild type and *Stat4-/-* spleens. Immediately following the magnetic sort we isolated RNA from the two populations. *Il23r* expression was assessed using real time PCR. Wild type and *Stat4* deficient memory cells had comparable *Il23r* expression (Figure 16A). These data show that the expression of *Il23r* in memory cells is independent of STAT4. We also wanted to evaluate *Rorc* expression of wild type and STAT4-deficient memory cells. Directly *ex vivo*, *Stat4-/-* memory cells had reduced *Rorc* expression compared to wild type memory cells (Figure 16B). These data suggest that *Stat4* is required for *Rorc* expression in memory cells.

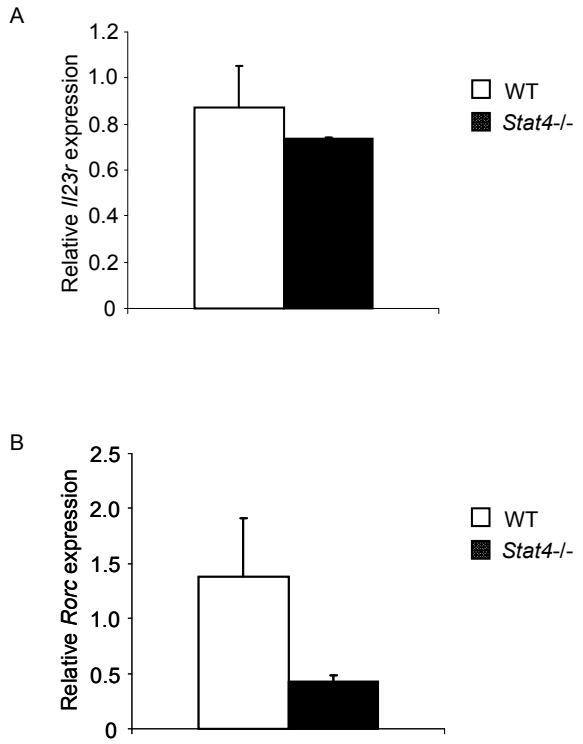

Figure 16. *Stat4-/-* memory cells have reduced *Rorc* expression. (A) Naïve (CD62L+) and memory (CD62L-) were isolated from spleen of wild type and *Stat4*-/- mice. Following magnetic selection RNA was isolated and real time PCR was performed on *Il23r* (B) *Rorc*. Data are average of 2 mice ± SD.

IL-23 stimulation activates STAT4 in IL-23 cultured memory, but not TGF-β+IL-6 cultured naïve CD4 T cells

IL-23 signaling leads to the activation of several STATs including STAT1, STAT3, and STAT4 (Parham et al., 2002). Since STAT4 is differentially required for IL-17 production in naïve versus memory cells we wanted to test if the activation of STAT4 following IL-23 stimulation is different between naïve CD4 T cells cultured with TGF-β+IL-6 and memory CD4 T cells cultured with IL-23. Naïve T cells were cultured with TGF-β+IL-6 and memory T cells (CD62L-) with IL-23 for 5

days. After 5 days of differentiation cells were washed and re-stimulated with IL-23 for 1 hour. Following the 1 hour stimulation cells were intracellularly stained for pSTAT3 and pSTAT4. Both naïve and memory cultured cells had a small percentage of pSTAT3 positive cells following IL-23 stimulation. However, only the memory cells cultured with IL-23 had a shift, albeit modest, in pSTAT4 positive cells (Figure 17). These data suggest that naïve and memory cultured cells have different STAT activation patterns following IL-23 stimulation.

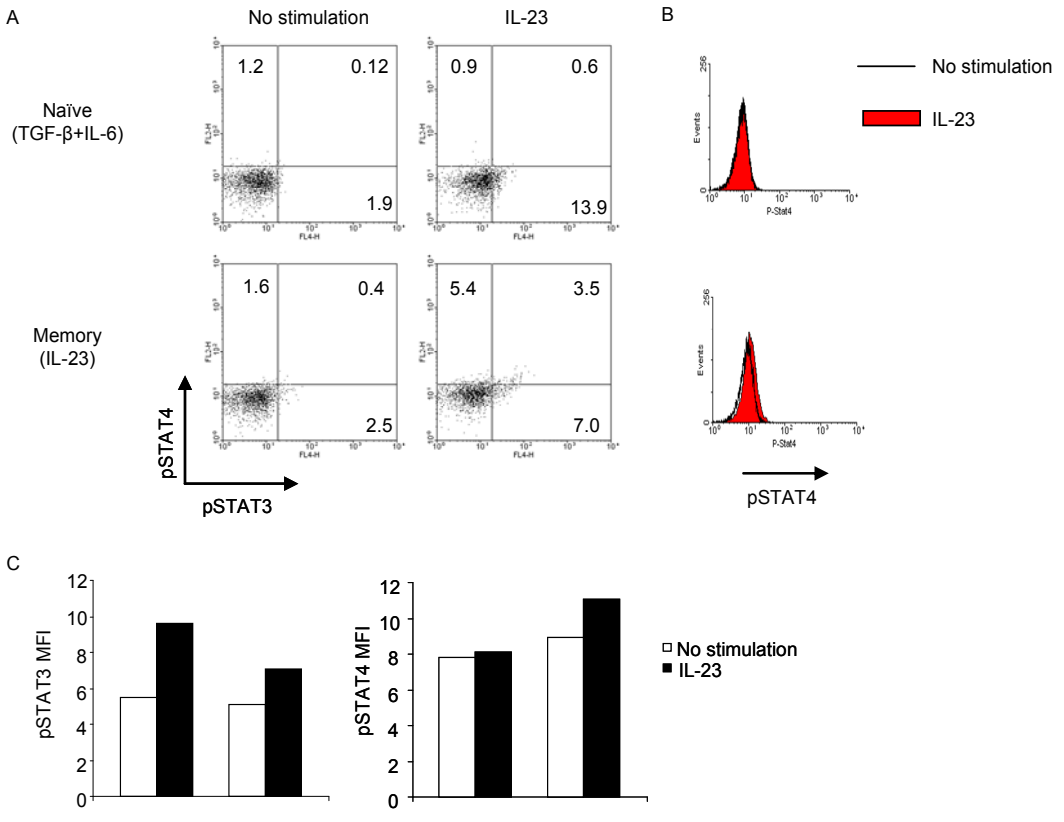

Figure 17. IL-23 stimulation leads to the phosphorylation of STAT4 in memory, but not naïve cells. (A) Naïve CD4+ T cells were activated and cultured in TGF-β+IL-6 and memory (CD62L-) CD4 T cells with IL-23. On day 5 cells were washed and re-stimulated with IL-23 for 1 hour. Following stimulation cells were intracellularly stained for phospho-STAT3 and phospho-STAT4. (B)

Histogram representation of dot plot graphs in A. (C) Graphical representation of mean fluoresce intensity of dot plots in A.

IL-23, not TGF-β+IL-6 leads to increased *Il23r* expression following 5 day culture

Previous publications show that T cell activation, IL-6, TGF-β, IL-21, and IL-23 increase the expression of *Il23r* (Bettelli et al., 2007b; Chen et al., 2007; Ivanov et al., 2006; Parham et al., 2002; Yang et al., 2007; Zhou et al., 2007; Zhou et al., 2008). It is possible the differences observed in cultured naïve and memory Th17 phospho-STAT4 following IL-23 re-stimulation are because of a difference in *Il23r* expression. In order to determine *Il23r* expression in naïve and memory T cells we isolated CD4+CD62L+ naïve T cells and CD4+CD62L- memory cells from wild type spleens and cultured them with TGF-β+IL-6 or IL-23, respectively. Following 5 days of culture RNA was isolated from both populations and real time PCR was performed to assess *Il23r* expression. Naïve T cells cultured with TGF-β+IL-6 had significantly lower *Il23r* expression compared to memory T cells cultured with IL-23 (Figure 18). These data suggest the differences in phospho-STAT4 following IL-23 stimulation may be due to differences in *Il23r* expression.

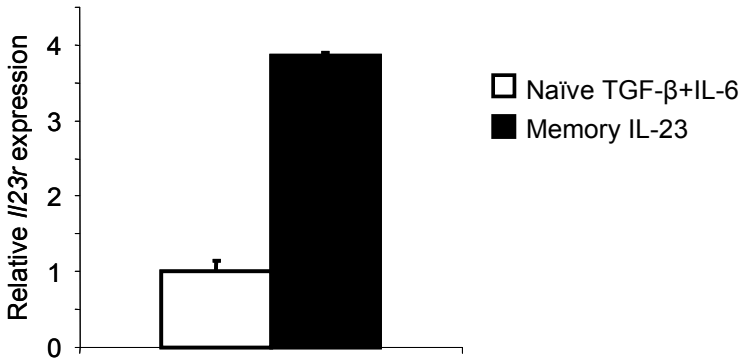

Figure 18. Naïve cells cultured with TGF-β+IL-6 have reduced *Il23r* compared to memory cells cultured with IL-23. Naïve and memory cells were isolated from wild type mice. Naïve cells were cultured with TGF-β+IL-6 and memory cells with IL-23 for 5 days. After 5 days of culture RNA was isolated and real time PCR was performed to assess *Il23r* expression.

Stat4 deficient mice have reduced IL-17 production upon *ex vivo* recall response

Stat4 is required for memory IL-17 production *in vitro* (Figure 14). In order to test the role of *Stat4* in IL-17 production *in vivo* we immunized wild type and *Stat4*-/- BALB/C mice with ovalbumin + Alum. Day 7 after immunization half of the mice were sacrificed. The remaining mice were immunized with another i.p. injection of OVA + Alum on day 7 and were sacrificed on day 14. After day 7 or day 14 inguinal and mesenteric lymph nodes were isolated. Total lymph node cells were re-stimulated with 250 or 500 µg/ml OVA for 72 hours. Cell free supernatant was collected and IL-17 production was assessed. Wild type mice immunized once with OVA + Alum had increased IL-17 production from lymph node cells after *ex vivo* re-stimulation with OVA compared to PBS injected mice. Wild type mice immunized two times with OVA + Alum had increased IL-17 production compared to PBS injected mice, albeit in lower amounts than mice immunized a single time

(Figure 19). *Stat4* deficient mice immunized either once or twice with OVA + Alum had increased IL-17 production compared to wild type PBS injected mice. However, the levels of IL-17 produced by *Stat4* deficient lymph node cells were reduced compared to wild type lymph node cells (Figure 19). The decrease in IL-17 production did not reach statistical significance, however the trend towards significance was consistent. Taken together *Stat4* is required for optimal *ex vivo* IL-17 production.

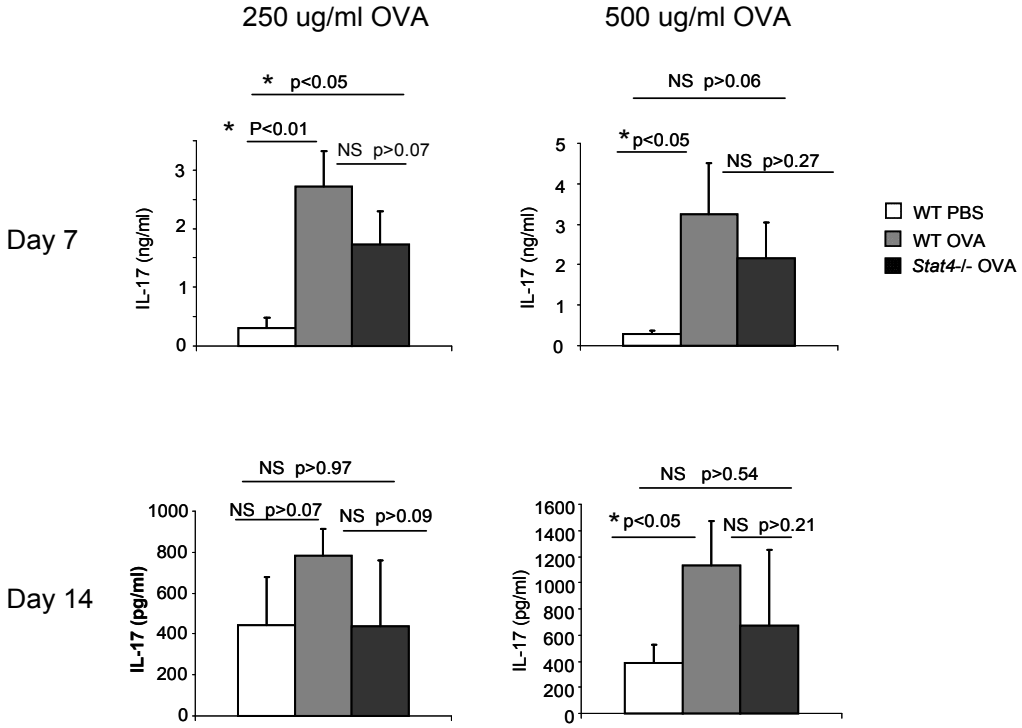

Figure.19. Immunized *Stat4*-/- mice have reduced IL-17 production after OVA re-stimulation. Wild type and *Stat4* deficient mice were immunized i.p. with Ovalbumin (OVA) (20 µg) + Alum (1 mg) or PBS control. At day 7 half of the mice were challenged with OVA (20 µg) + Alum (1 mg) or PBS again and sacrificed at day 14. The other mice were sacrificed on day 7. Inguinal and mesenteric lymph nodes were isolated from all mice. Total lymph node cells were then re-stimulated with 250 or 500 µg/ml OVA. Following 72 hours of stimulation cell-free supernatant was assessed for IL-17 production using ELISA. Data are average of 2-4 mice per group ± SD.

Part III: STAT3 is required for Th2 differentiation

STAT3 is activated during Th2 differentiation

STAT6 activation is critical in Th2 cell differentiation. Though several cytokines important in Th2 differentiation and cytokine production signal through STAT3, the activation of STAT3 during Th2 development has not been carefully examined. To define STAT3 activation throughout Th2 differentiation, wild type and STAT3 deficient Th2 cells were assessed for intracellular phospho-STAT3 and phospho-STAT6 each day during Th2 differentiation. Wild type Th2 cells were nearly all phospho-STAT6 positive early on in differentiation and remained phospho-STAT6 positive throughout differentiation (Figure 20A and B). Wild type Th2 cells also demonstrated a high percentage of phospho-STAT3 positive cells throughout Th2 differentiation. STAT3 phosphorylation occurs early in differentiation, peaks at 48 hours and falls by 72 hours (Figure 20A and B). There is a second peak of STAT3 phosphorylation following expansion of cells in culture and addition of further cytokines at 72 hours, as assessed by percent phospho-STAT6 positive and mean fluorescence intensity (Figure 20). Overall these data show that STAT3 becomes phosphorylated during Th2 differentiation.

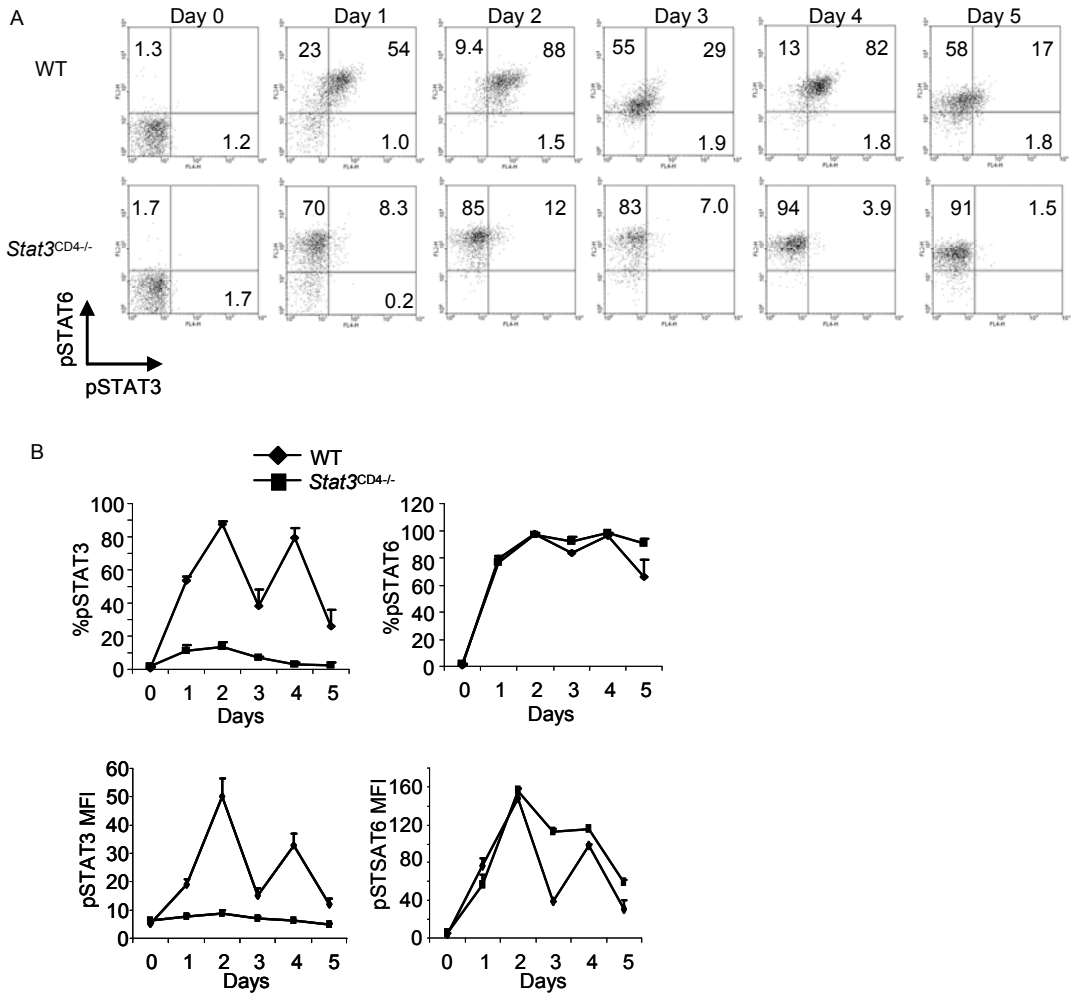

Figure 20. STAT3 is activated during normal Th2 differentiation. (A) Wild type (WT) and *Stat3*$^{CD4-/-}$ naïve CD4+ T cells were activated with anti-CD3 and anti-CD28 and cultured in IL-4 + anti-IFN-γ. Each day during differentiation cells were stained for intracellular phospho-STAT3 and phospho-STAT6. (B) Graphical representation of % pSTAT positive cells and mean fluorescence intensity from A. Data are average of 2 mice per group. Data are representative of 2-3 independent experiments.

The phosphorylation of STAT3 occurs early on in differentiation and the highest peaks of phospho-STAT3 take place after the addition of IL-4. These data suggest that IL-4 can directly activate STAT3. In order to test if IL-4 can directly activate STAT3, wild type cells were cultured under Th2 skewing conditions. On

day 5 cells were washed and stimulated for 30 minutes or 1 hour with recombinant IL-4. Cells were then intracellularly stained for both phospho-STAT6 and phospho-STAT3. Cell stimulated with IL-4 were nearly all phospho-STAT6 positive. However, stimulation with exogenous IL-4 did not lead to the phosphorylation of STAT3 (Figure 21).

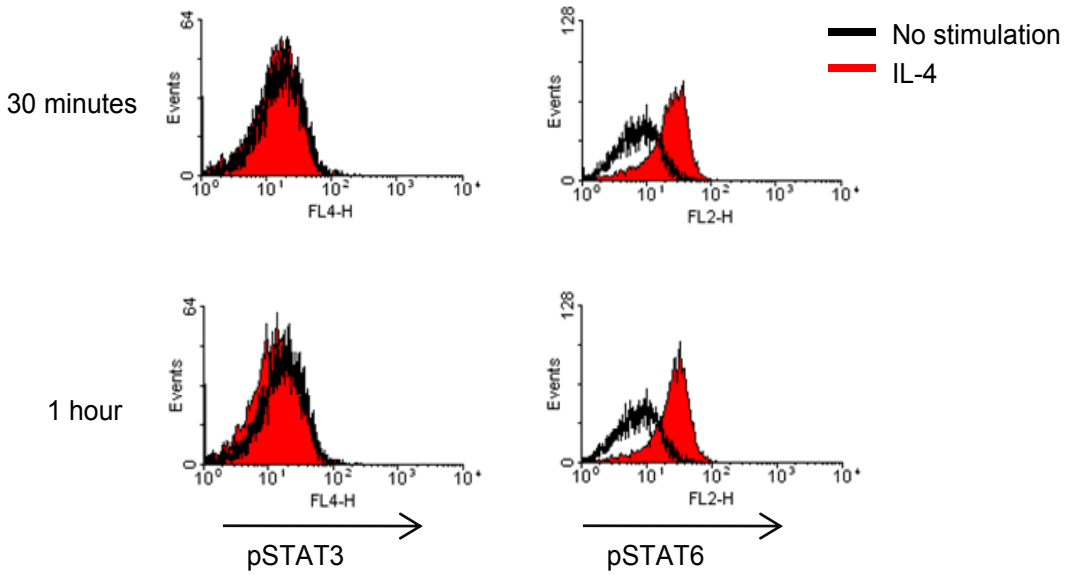

Figure 21. STAT6, but not STAT3 is phosphorylated following IL-4 stimulation. Wild type naïve CD4+ cells were differentiated under Th2 conditions for 5 days. On day 5 cells were stimulated with IL-4 for 30 minutes or 1 hour as indicated. Following stimulation cells were assessed for phospho-STAT3 and phospho-STAT6 by intracellular staining. Data are representative of 2-3 experiments.

Since IL-4 does not directly activate STAT3, we sought to determine what cytokines are responsible for the phosphorylation of STAT3 throughout Th2 differentiation. To test if cytokines known to be secreted by Th2 cells were

responsible for the phosphorylation of STAT3, IL-21 and IL-6 were neutralized throughout Th2 differentiation using neutralizing antibodies. Th2 cells cultured with anti-IL-6 and anti-IL-21 had significantly decreased percent of phopho-STAT3 positive cells compared to Th2 cells. The effect of IL-21 and IL-6 blocking antibodies is specific to STAT3 because phospho-STAT6 was not effected (Figure 22A). Th2 cytokine production was also significantly decreased when IL-6 and IL-21 were neutralized, recapitulating the STAT3-deficient phenotype (Figure 22B). However, the neutralization of either IL-6 or IL-21 alone had minimal effect on Th2 cytokine production. Taken together, IL-6 and IL-21 are required for maximum STAT3 activation throughout Th2 differentiation and for the production of Th2 cytokines.

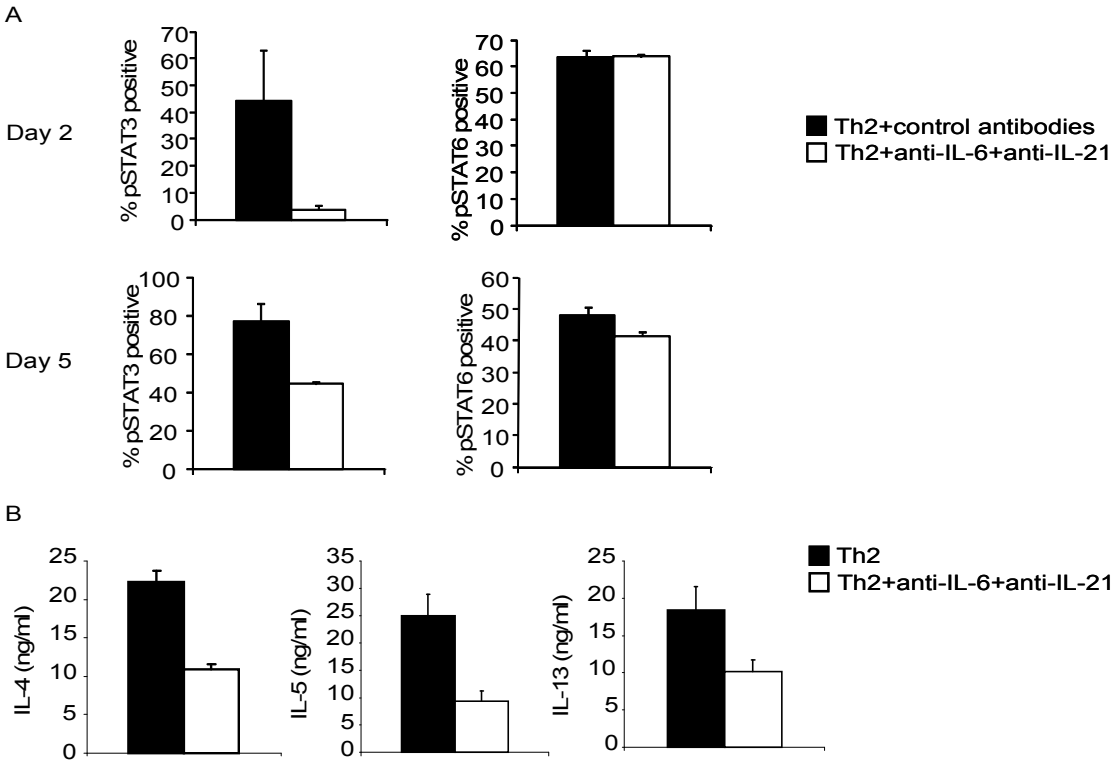

Figure 22. IL-21 and IL-6 are required for the phosphorylation of STAT3 and Th2 cytokine production. (A) Naïve CD4+ cells were isolated from WT spleens. Naïve cells were activated with anti-CD3 and anti-CD28 and cultured in IL-4 + anti-IFN-γ or IL-4, anti-IFN-γ, anti-IL-6, and anti-IL-21. On day 2 and day 5 cells were stained for intracellular pSTAT3 and pSTAT6. (B) Cells were stimulated and cultured same as A. After 5 days of differentiation cells were collected and counted. $1x10^6$ cells were then re-stimulated with anti-CD3 (4 μg/ml) for 24 hours. Cell-free supernatant was collected and tested for various cytokines using ELISA.

STAT3-deficient cells are defective in Th2 differentiation

To define if the activation of STAT3 during Th2 development reflected a requirement for STAT3 in this process, we used mice that have a floxed *Stat3* allele, mated to mice expressing Cre from a CD4 transgene (referred to as $Stat3^{CD4-/-}$ mice). As previously described, T cell development in mice with STAT3-deficient T cells is undistinguishable from wild type mice (Mathur et al., 2007). Moreover, growth of STAT3-deficient cells is not different from wild type cultures. Importantly, STAT6 phosphorylation was not dependent on STAT3 as a similar pattern was observed in STAT3-deficient cultures (Figure 20A and B). To examine differentiation, naïve CD4 T cells were isolated from spleens of wild type and $Stat3^{CD4-/-}$ mice and cultured under Th1, Th2, or Th17 conditions. STAT3-deficient Th1 cells had IFN-γ and GM-CSF production similar to amounts produced by wild type Th1 cells (Figure 23A). In agreement with previously

published reports, STAT3 is required for the generation of cells secreting both IL-17A and IL-17F (Figure 23A). Although wild type Th2 cells secreted high levels of IL-4, IL-5 and IL-13, STAT3-deficient CD4 T cells cultured under Th2 skewing conditions had significantly reduced IL-4, IL-5, and IL-13 production and gene expression (Figure 23B and C).

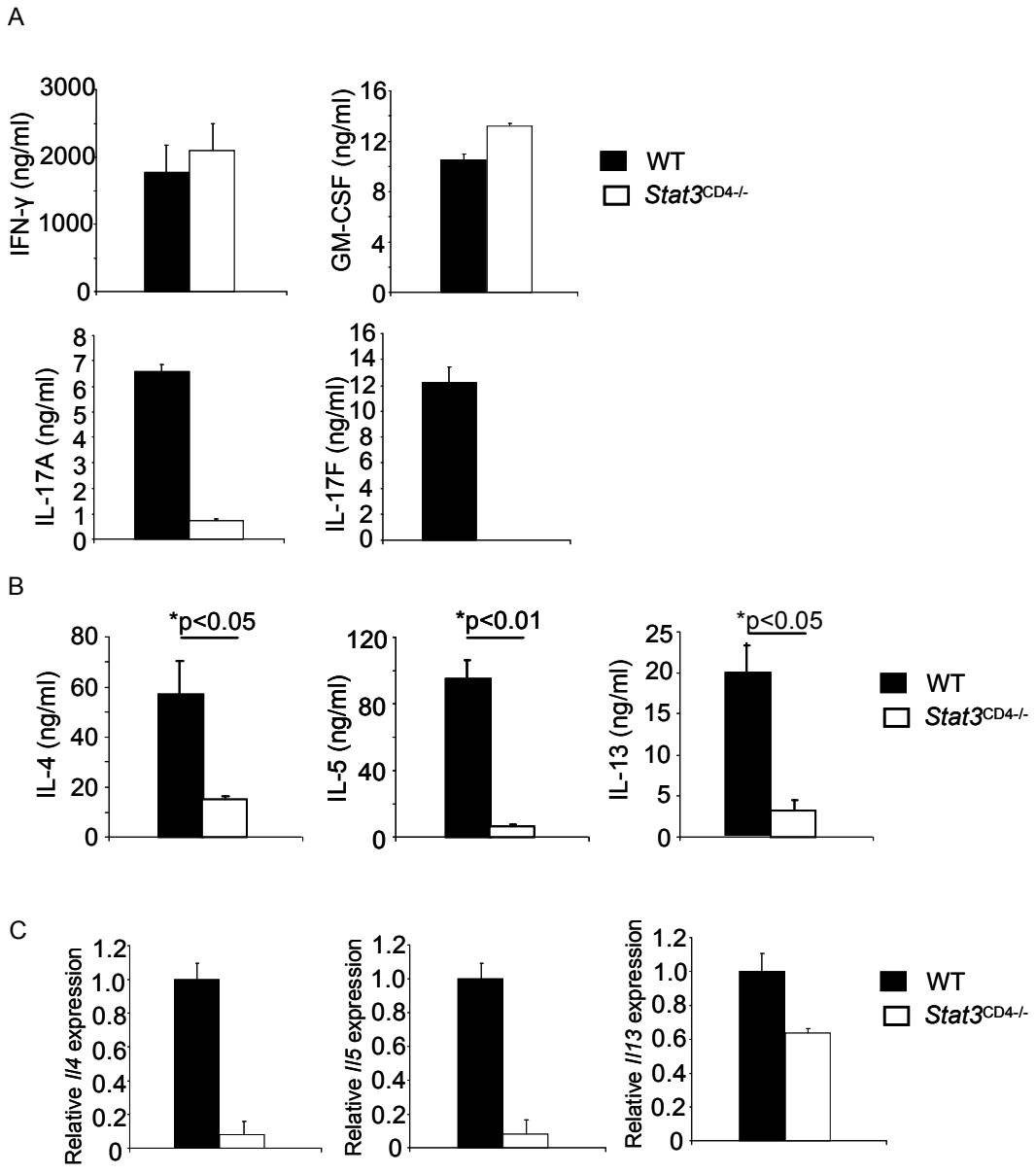

Figure 23. STAT3-deficient Th2 cultures have reduced Th2 cytokine production. (A) Naïve CD4+ T cells from WT and *Stat3*$^{CD4-/-}$ mice were activated with anti-CD3 and anti-CD28 and cultured in IL-12 + anti-IL-4 (Th1) or TGF-β+IL-6 (Th17). After 5 days of differentiation cells were collected and counted. Cells were then re-stimulated with anti-CD3 (4 μg/ml) for 24 hours. Cell-free supernatant was collected and tested for various cytokines using ELISA. (B) WT and *Stat3*$^{CD4-/-}$ naïve CD4+ T cells were activated with anti-CD3 and anti-CD28 and cultured with IL-4 + anti-IFN-γ (Th2 conditions). After 5 days of differentiation cells were collected and counted. Differentiated cells ($1x10^6$) were then re-stimulated with anti-CD3 (4 μg/ml) for 24 hours. Cell-free supernatant was collected and tested for various cytokines using ELISA. (C) After re-stimulation of differentiated Th2 cells with anti-CD3 and recovery of supernatants as described in B, cell pellets were collected, RNA was isolated, and quantitative PCR was performed for the indicated cytokines. *p< 0.05 (two-tailed Student's *t*-test). Data are representative of 2 independent experiments (A), representative of more than 5 independent experiments (B average and SD of two mice), representative of 2 independent experiments (C).

We then examined the kinetics of both IL-4 and IL-5 gene expression and secretion on day 5 following anti-CD3 stimulation. The gene expression of *Il4* and *Il5* is similar between the wild type and STAT3-deficient Th2 cells early after stimulation. However, the expression of both *Il4* and *Il5* is reduced in STAT3-deficient Th2 cells compared to wild type Th2 cells 6, 8, 12, or 24 hours after stimulation (Figure XA). Wild type and STAT3-deficient Th2 cells cytokine production kinetics were similar to those observed in gene expression. STAT3-deficient Th2 cells had similar levels of IL-4 and IL-5 production as wild type Th2 cells during early time points following stimulation. Conversely, following 12 or 24 hours of anti-CD3 stimulation, STAT3-deficient Th2 cells have significantly reduced IL-4 and IL-5 production compared to wild type Th2 cells (Figure XB).

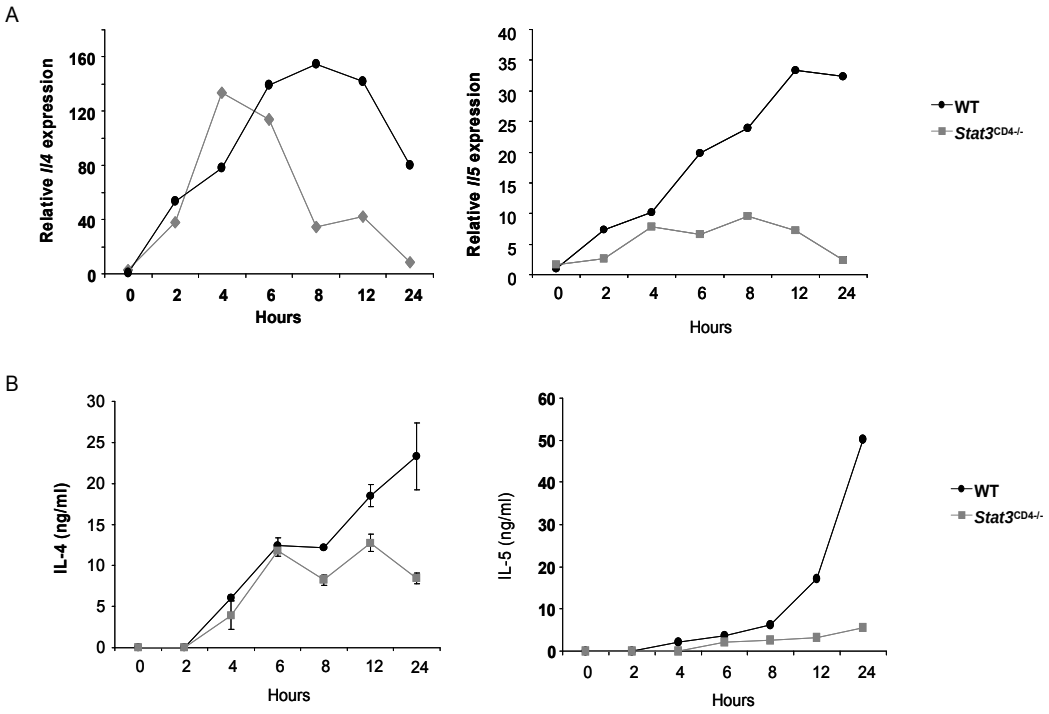

Figure 24. STAT3-Deficient Th2 cells have similar expression and production of IL-4 and IL-5 initially after stimulation compared to wild type. Wild type and STAT3-deficient CD4 T cells were activated and cultured as in figure 23B. On day 5 cells were re-stimulated for 0, 2, 4, 6, 8, 12, or 24 hours with anti-CD3. RNA and supernatants were collected and tested for IL-4 and IL-5 gene expression or cytokine production, respectively. Data are representative of 1 (A) or 2 (B) experiments.

To test the requirement for STAT3 in the expression of other Th2 associated genes quantitative PCR was performed to assess the expression of Growth factor independence 1 (*Gfi1*), C-C chemokine receptor type 4 (*Ccr4*), and IL-24 (*Il24*) in wild type and STAT3-deficient cultures. The expression of both *Gfi1* and *Ccr4* were not statistically different between wild type and STAT3-deficient cells, $p>0.09$ and $p>0.24$, respectively (Figure 25). The expression of *Il24* was significantly reduced in Th2 cells lacking STAT3 (Figure 25). Thus, STAT3 is

required for the production of Th2 cytokines, but not for the expression of all Th2 associated genes.

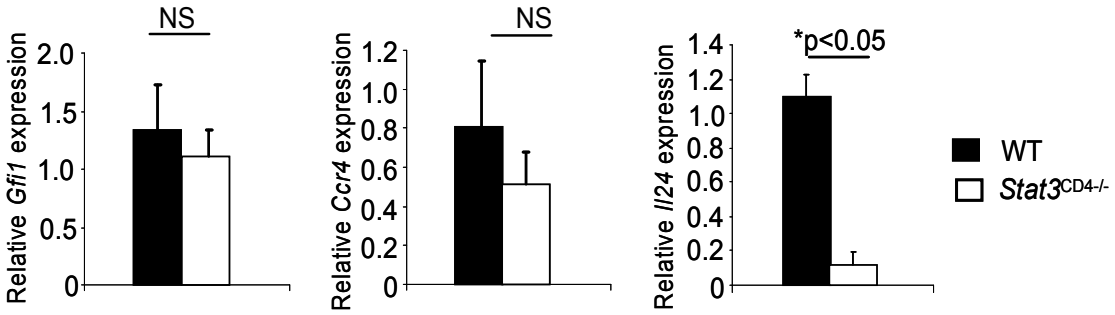

Figure 25. STAT3 is not required for the expression of all Th2 associated genes. RNA was isolated from Th2 cells differentiated for five days before re-stimulation (*Gfi1* and *Ccr4*) or after 6 hours stimulation with anti-CD3 (2 µg/ml) (*Il24*) and quantitative PCR was performed. **p*< 0.05 (two-tailed Student's *t*-test) Data are the average of ± SD of 2-5 experiments.

It is possible that *Stat3* deficient Th2 cells are converting to another T helper lineage. In order to test if Th2 cells lacking *Stat3* are becoming Th1 cells, wild type and STAT3-deficient naïve CD4 T cells were differentiated under Th2 skewing conditions. Following 5 days of differentiation IFN-γ production was assessed using ELISA. S*tat3* deficient Th2 cells had a two-fold increase in IFN-γ production. However, the amount of IFN-γ produced by the STAT3-deficient Th2 cells is significantly lower than wild type Th1 cells (Figure 26A). Therefore, STAT3-deficient Th2 cells are not converting to Th1 cells. In order to test if STAT3-deficient Th2 cells are converting to T regulatory cells, *Foxp3* expression was assessed from wild type and *Stat3* deficient Th2 cells using real-time PCR.

Foxp3 expression was not significantly different between wild type and STAT3-deficient Th2 cells (Figure 26B). These data show that *Stat3* deficient Th2 cells are not simply converting to Th1 or T regulatory cells.

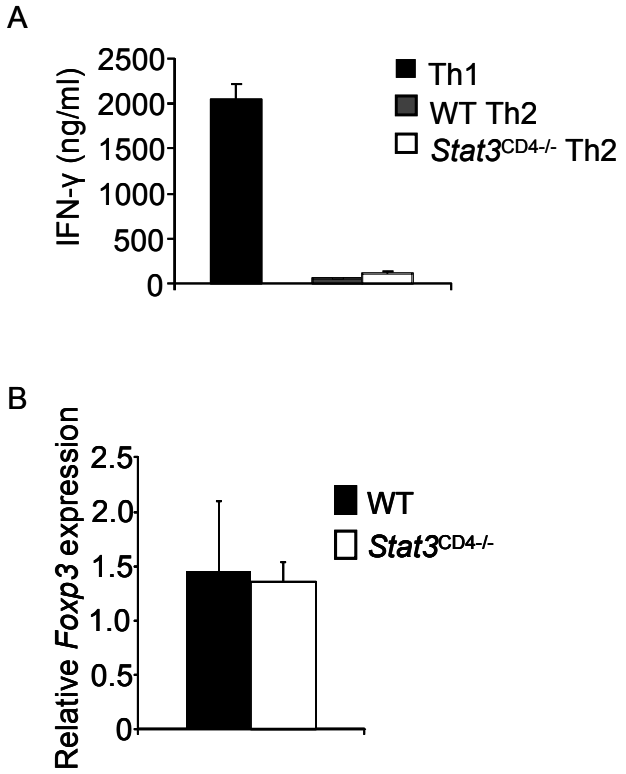

Figure 26. *S*TAT3-deficient Th2 cells do not convert to Th1 or T regulatory cells. (A) Wild type and STAT3-deficient naïve CD4 T cells were differentiated with IL-4 and anti-IFN-γ or IL-12 and anti-IL-4 for 5 days. On day 5 cells were re-stimulated with anti-CD3 for 24 hours. Cell-free supernatants were tested for IFN-γ production using ELISA. (B) Th2 cells cultured as in A were collected and RNA was isolated. Real time PCR was performed to assess *Foxp3* expression. Data are representative of 2-4 experiments.

To test if STAT3 is also required for *in vivo* Th2 differentiation, wild type and *Stat3*$^{CD4-/-}$ mice were immunized twice with alum-adsorbed OVA. Two weeks

after the second immunization, splenocytes from these mice were stimulated *in vitro* with OVA and cytokine production was analyzed using ELISA. The production of Th2 cytokines was significantly decreased in mice that lacked expression of STAT3 in T cells (Figure 27). These data suggest that STAT3 is required for Th2 development *in vivo*.

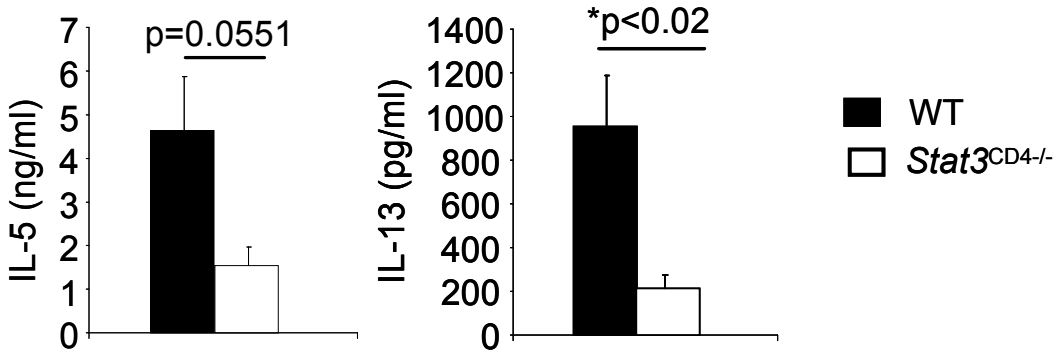

Figure 27. STAT3 is required for *in vivo* Th2 differentiation. WT and *Stat3*$^{CD4-/-}$ mice were immunized with OVA+Alum on days 0 and 7 and challenged as described in methods. After challenges, splenocytes were re-stimulated with OVA for 72 hrs. Cell free supernatant was assessed for cytokines using ELISA.

STAT3 is required for Th2 transcription factor expression

To define the mechanism for how STAT3 promotes Th2 development, we first tested the expression of receptors required for Th2 development. Previous studies have demonstrated STAT3-deficient CD4 T cells have reduced CD25 expression (Akaishi et al., 1998). Moreover, IL-2 signaling is required for Th2 differentiation at multiple levels including the expression of *Il4ra* (Liao et al., 2008). To determine if CD25 expression was decreased on STAT3-deficient cells during Th2 differentiation, we examined CD25 surface expression

throughout differentiation. The percent of CD25 positive cells was comparable between wild type and STAT3-deficient Th2 cells, however the MFI of CD25 was decreased on one day during differentiation (Figure 28A and B). Previous studies have also shown that CD25 expression can be recovered in STAT3-deficient T cells with the addition of exogenous IL-2 (Akaishi et al., 1998). In order to test if the slight decrease in the level of CD25 expression on STAT3-deficient Th2 cells was responsible for the decreased Th2 cytokine production we supplemented the STAT3-deficient Th2 cells with high dose of exogenous IL-2. WT or STAT3-deficient T cells were differentiated in Th2 skewing conditions in the presence or absence of exogenous IL-2. The addition of exogenous IL-2 did not rescue Th2 cytokine production, suggesting that the decrease in CD25 expression was not effecting Th2 cytokine production in the STAT3-deficient Th2 cells (Figure 28C). We also examined IL-4Rα expression in STAT3-deficient Th2 cultures. IL-4Rα expression in STAT3-deficient Th2 cells was similar to that observed in wild type cultures (Figure 28A and B), and this is consistent with normal STAT6 activation in the absence of STAT3 (Figure 20A and B). These data suggest that the reduction in Th2 cytokine production in STAT3-deficient Th2 cells is not due to reduced CD25 or IL-4Rα expression.

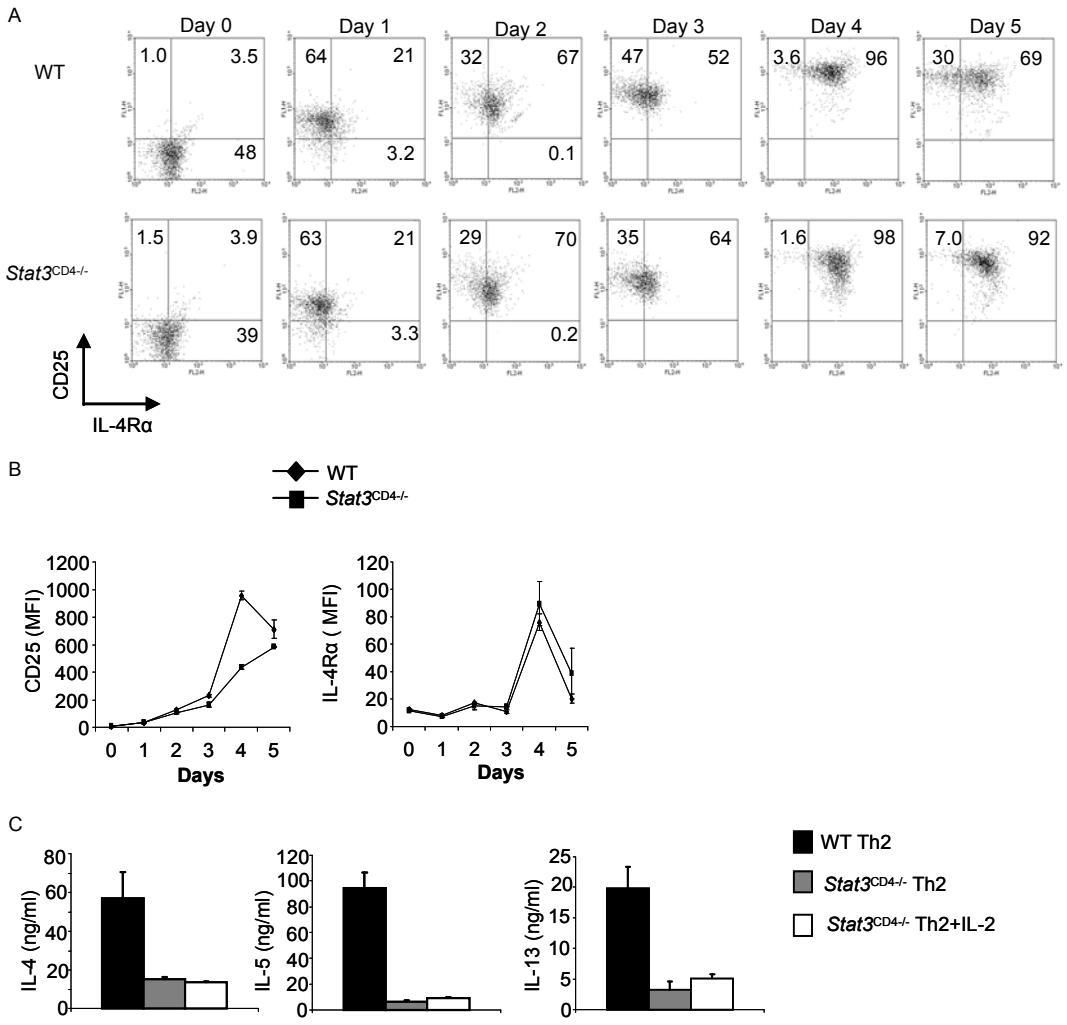

Figure 28. STAT3-deficient Th2 cells have CD25 and IL-4Rα expression similar to wild type Th2 cells. (A) Wild type (WT) and *Stat3*$^{CD4-/-}$ naïve CD4+ T cells were isolated and cultured as in Figure 20. Each day during differentiation cells were surfaced stained for CD25 and IL-4Rα. (B) Graphical representation of mean fluorescents intensity of CD25 and IL-4Rα. (C) Wild type (WT) and *Stat3*$^{CD4-/-}$ naïve CD4+ T cells were isolated and cultured with IL-4 ± IL-2 (100 ng/ml) for 5 days. On day 5 cells were re-stimulated with anti-CD3 for 24 hours. Supernatant was collected and cytokine production was assessed using ELISA. Data are representative of 2 mice (A) or average or two mice ±SD (B,C).

We then examined the expression of transcription factors associated with establishing the Th2 phenotype, including *Gata3*, *Irf4*, *Maf* and *Batf*. In order to

determine the importance of STAT3 in Th2 transcription factor expression, RNA was isolated from wild type and STAT3-deficient Th2 cultures. Following Th2 differentiation, STAT3-deficient Th2 cells had similar levels of *Gata3*, *Irf4*, and *Batf*, but reduced *Maf* expression (Figure 29A). Conversely, the expression of Th2 transcription factors *Gata3*, *Irf4*, *Maf,* and *Batf* were all significantly decreased in the STAT3-deficient Th2 cultures following anti-CD3 re-stimulation compared to wild type stimulated Th2 cells (Figure 29B).

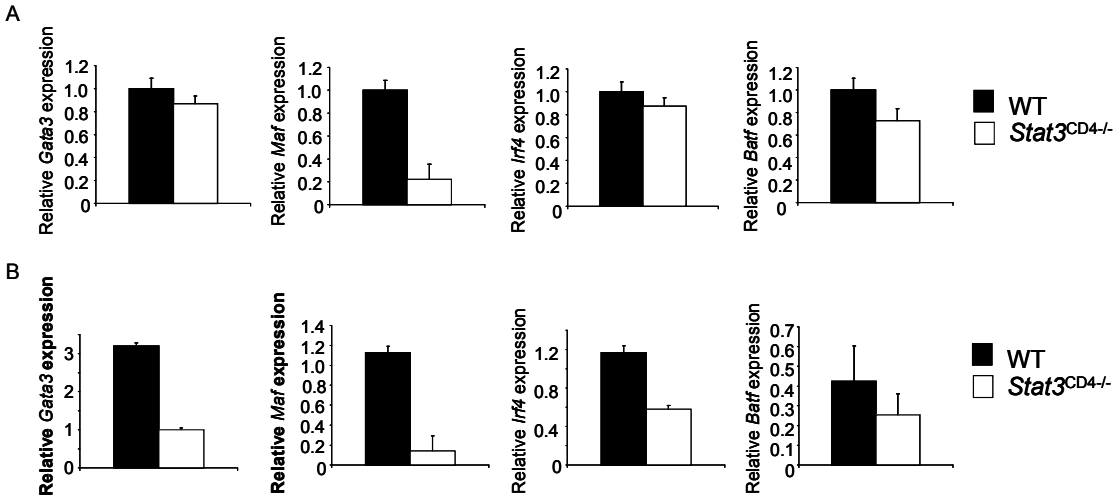

Figure 29. STAT3-deficient Th2 cells have reduced Th2 specific transcription factor expression. WT and STAT3-deficient naïve CD4 T cells were differentiated as in Figure 23B. RNA was isolated for quantitative PCR (A) before or (B) after 4 hour anti-CD3 stimulation. Data are representative of 2-3 independent experiments.

Since STAT3 is known to bind the *Maf* gene, we directly tested if reduced *c-Maf* expression in STAT3-deficient cells was responsible for reduced Th2 cytokine production by retro-virally expressing *Maf* in STAT3-deficient Th2 cells. Intracellular staining showed that *c-Maf* expression was reduced in STAT3-

deficient Th2 cells, compared to wild type Th2 cells, but that expression was recovered when *c-Maf* was transduced (Figure 30A). Similar to what was observed with non-transduced cells (Figure 29), STAT3-deficient Th2 cells transduced with vector control had reduced Th2 cytokine production compared to wild type Th2 cells transduced with vector control. STAT3-deficient cells transduced with *c-Maf* had increased IL-4 production, consistent with the published reports that c-maf can directly activate IL-4 expression. However, the IL-4 production was still lower than control-transduced wild type cells. Transduction of *c-Maf* in STAT3-deficient Th2 cells had modest effects on IL-5 and IL-13 production (Figure 30B). To test if ectopic expression of other Th2 transcription factors could rescue Th2 development in STAT3-deficient T cells, *Irf4* or *Gata3* were retro-virally transduced into STAT3-deficient Th2 cells. STAT3-deficient cells transduced with vector control had reduced *Irf4* and *Gata3* expression compared to wild type cells transduced with vector control. The transduction of *Irf4* or *Gata3* rescued their expression in STAT3-deficient cells (Figure 30C and E). However, the transduction of *Irf4* or *Gata3* did not rescue Th2 cytokine production by the STAT3-deficient Th2 cells (Figure 30D and F). Overall we conclude that decreased expression of a single Th2 transcription factor cannot completely account for the STAT3-deficient Th2 phenotype.

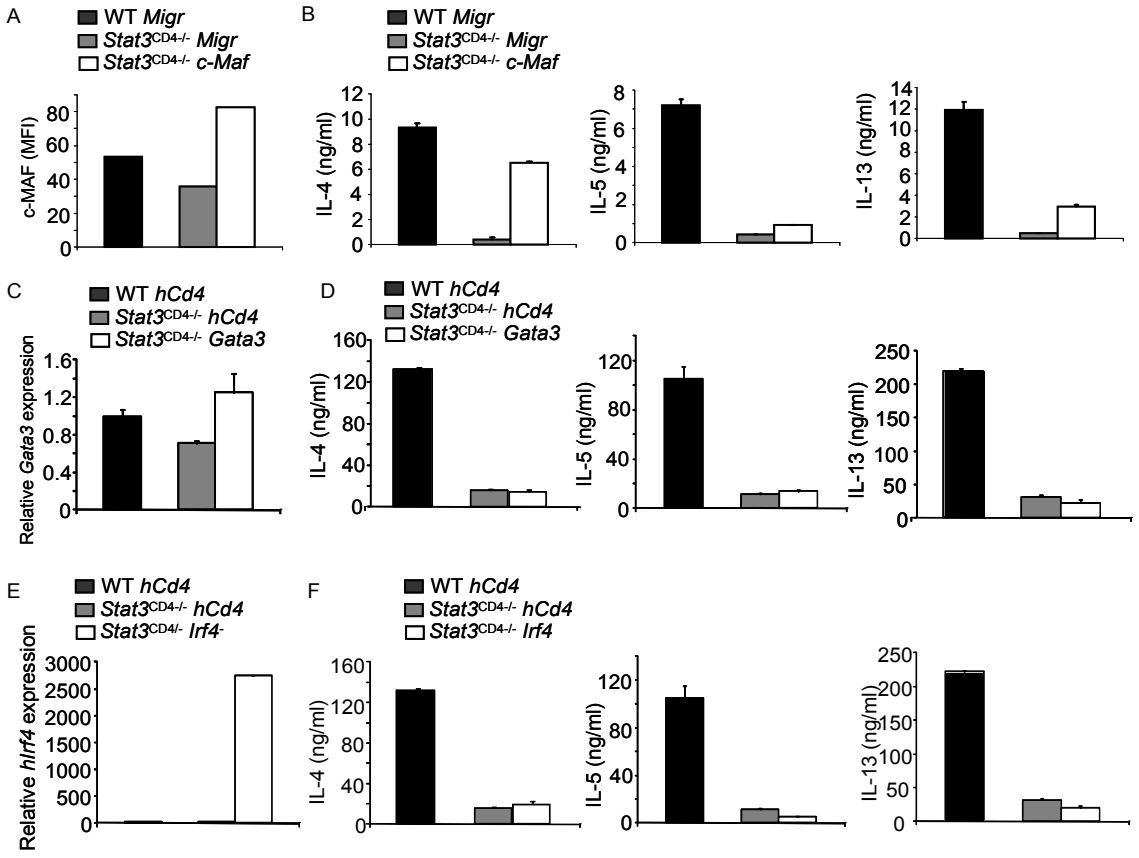

Figure 30. Over-expression of *c-Maf, Gata3,* or *Irf4* in STAT3-deficient Th2 cells does not rescue Th2 cytokine production. (A-F) WT and STAT3-deficient T cells were differentiated as in Figure 23B. On Day 2 cells were transduced with control or *c-Maf*-expressing, *Gata3*-expressing, or *Irf4*-expressing retrovirus. (A) Transduced cells were sorted and stained for intracellular c-maf. (B) Sorted transduced cells were re-stimulated with anti-CD3 for 24 hours. Supernatant was tested for Th2 cytokines using ELISA. (C,E) Transduced cells were sorted and RT PCR was performed. (D,F) Sorted transduced cells were re-stimulated with anti-CD3 for 24 hours. Supernatant was tested for Th2 cytokines using ELISA. Data are representative of 2 independent experiments.

Phosphorylation of STAT3 is independent of STAT6

STAT6 is required for Th2 differentiation (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1997). Similar to the previous reports STAT6-deficient Th2 cultured cells have reduced percentage of IL-4 and IL-5 secreting cells (Figure

31A). In agreement with Th2 cytokine production, STAT6-deficient Th2 cells have significantly reduced expression of Th2 transcription factors *Gata3* and *c-Maf*. However, the expression of *Irf4* in Th2 cells is not effected in the absence of STAT6 (Figure 31B). The reduced expression of *Gata3* and *c-Maf* is more severe than STAT3-deficient Th2 cells, however *Stat3* and not *Stat6* is required for optimal *Irf4* expression (Figures 29 and 31B). To assess phospho-STAT3 in the absence of STAT6, wild type and STAT6-deficient naïve CD4 T cells were differentiated into Th2 cells. On each day during differentiation, cells were stained for phospho-STAT3. STAT6-deficient Th2 cells activated STAT3 with similar kinetics and amounts as wild type Th2 cells during the first 3 days of differentiation. However, STAT6-deficient Th2 cells displayed reduced phospho-STAT3 following expansion of cells during the last 2 days of differentiation (Figure 31C). These data taken together suggest that STAT3 and STAT6 have distinct but overlapping effects on the expression of Th2 cytokines and transcription factors. Furthermore, the activation of STAT3 in Th2 cells is at least initially independent of STAT6.

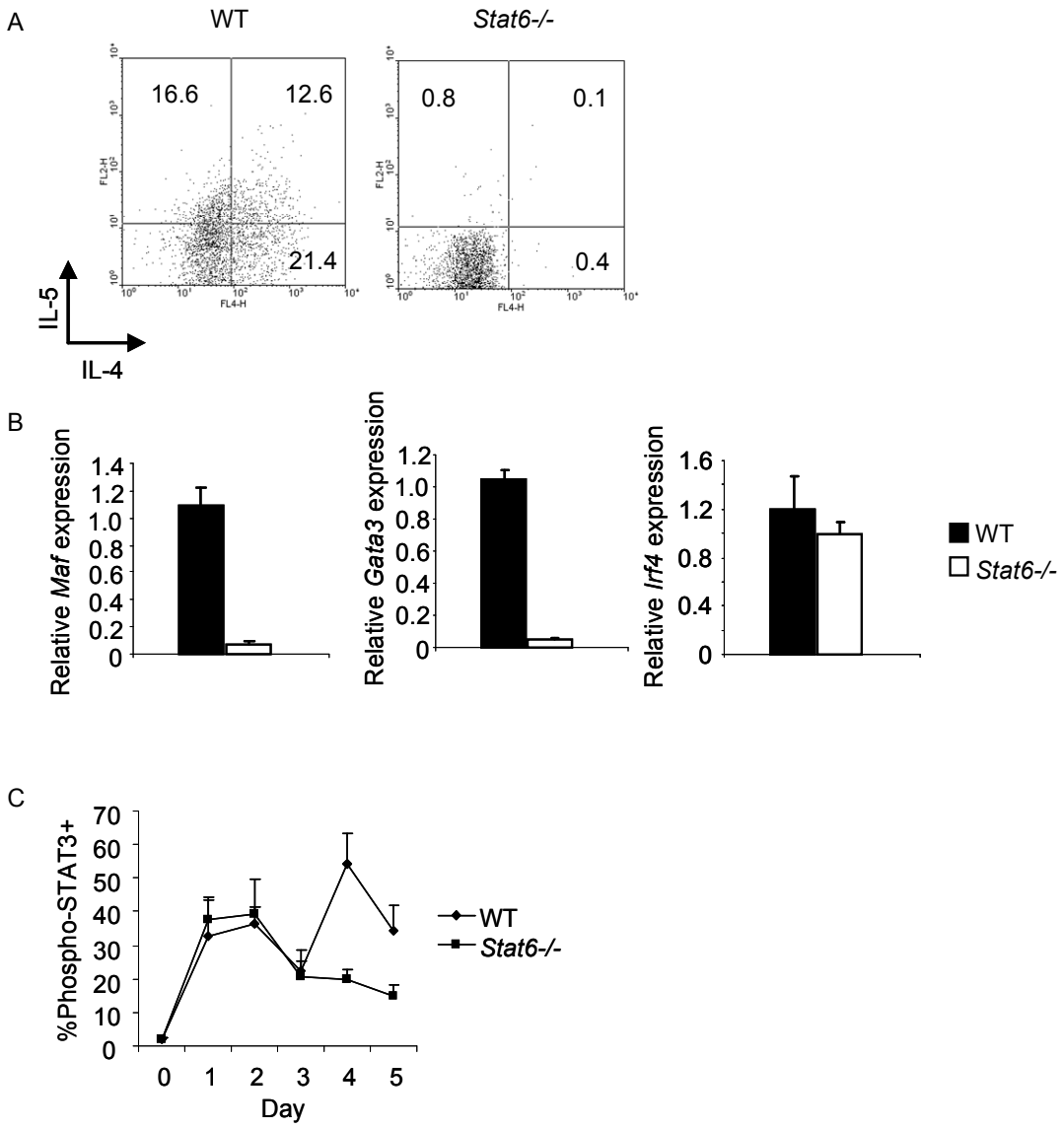

Figure 31. The activation of STAT3 in Th2 cells is independent of STAT6. (A) WT and STAT6-deficient T cells were activated and cultured with IL-4 and anti-IFN-γ. On day 5, cells were re-stimulated with anti-CD3 for 4 hours and intra-cellularly stained for IL-4 and IL-5. (B) Cells were differentiated as in A and on day 5 were re-stimulated with anti-CD3 for 4 hours. Following stimulation RNA was isolated and real-time PCR was performed. (C) Cells were differentiated as in A. Each day during differentiation cells were intracellularly stained for phospho-STAT3. Data are representative of 2-4 experiments (A,B) or average of 6 mice ± SEM (C).

STAT3 and STAT6 cooperate in promoting Th2 cytokine production

Our results suggest that STAT3 and STAT6 are required for optimal Th2 differentiation. To determine if STAT6 and STAT3 are cooperating to promote Th2 cytokine production we used 2 previously described constitutively active STAT mutants. Both the STAT6VT and STAT3C have two amino acid mutations within the SH2 domain, which renders them constitutively active in the absence of a stimulus. Previous reports have already shown that constitutively active STAT6 can promote Th2 cytokine production (Bruns et al., 2003; Sehra et al., 2008; Zhu et al., 2001). Our previous data show STAT3 is required for Th2 differentiation (Figure 23B and C). In order to test if constitutively active STAT3 can induce Th2 cytokine production, naïve CD4 T cells were activated under non-skewing conditions (anti-IFN-γ). On day 2 of differentiation cells were transduced with vector control or STAT3C. Sorted cells were then re-stimulated with anti-CD3 to assess cytokine production (Figure 32).

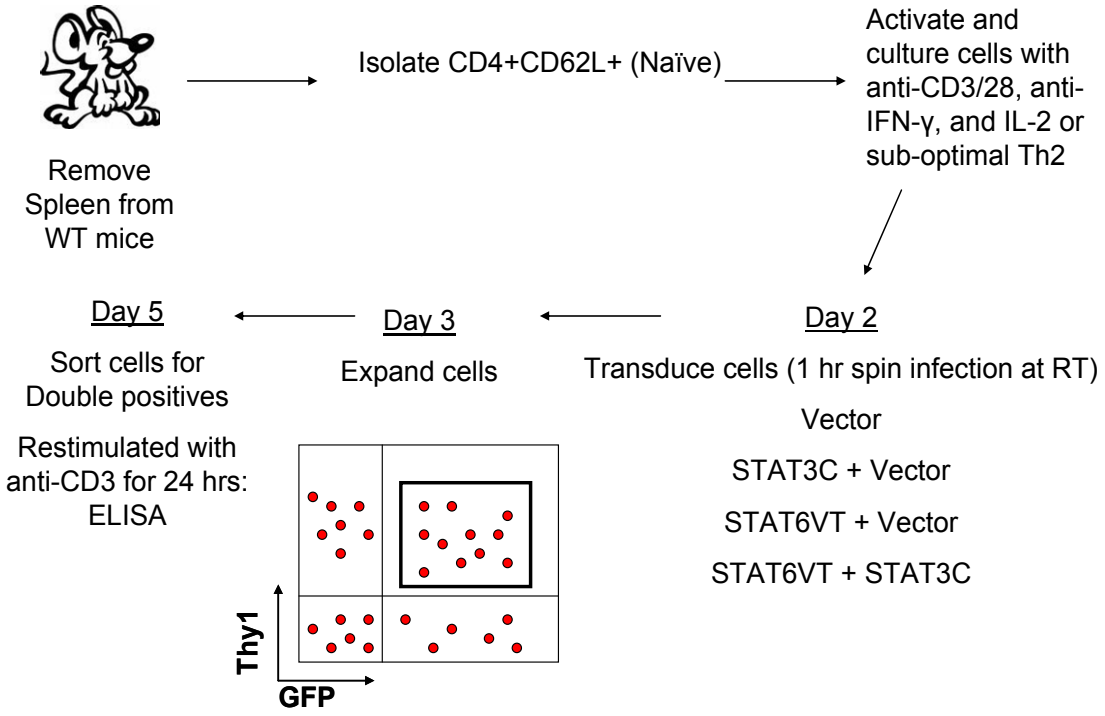

Figure 32. Procedure for retro-viral expression of STAT6VT and STAT3C. Wild type naïve CD4 T cells were activated under non-skewing conditions (anti-IFN-γ + IL-2) or sub-optimal Th2 (anti-IFN-γ+ IL-4 (1 ng/ml)). On day 2 cells were transduced with STAT3C, STAT6VT, or both. Cells were collected and sorted on day 5. Sorted cells were re-stimulated with anti-CD3 for 24 hours and cell free supernatant was tested for cytokines using ELISA.

Under non-skewing conditions STAT3C induced Th2 cytokines IL-4, IL-5, and IL-13 (Figure 33A). To directly test if the combination of constitutively active STAT6 and STAT3 could increase Th2 cytokine production compared to constitutively STAT6 alone, naïve T cells cultured under sub-optimal Th2 conditions were transduced with retroviruses expressing STAT6VT alone or STAT6VT and STAT3C. Sorted transduced cells were re-stimulated with anti-CD3. Th2 cytokine production was increased in cells transduced with STAT6VT only, and cells transduced with both STAT6VT and STAT3C had enhanced Th2 cytokine production compared to those transduced with STAT6VT alone (Figure 33B).

Previous reports have shown that in non-skewed or Th17 cultured cells, transduction with STAT3C enhanced IL-17 production (Mathur et al., 2007). Under sub-optimal Th2 conditions STAT3C expression still has the ability to induce IL-17 production, however in the presence of STAT6VT, STAT3C did not induce IL-17 production (Figure 33C). These data suggest that STAT6 and STAT3 cooperate to enhance Th2 cytokine production.

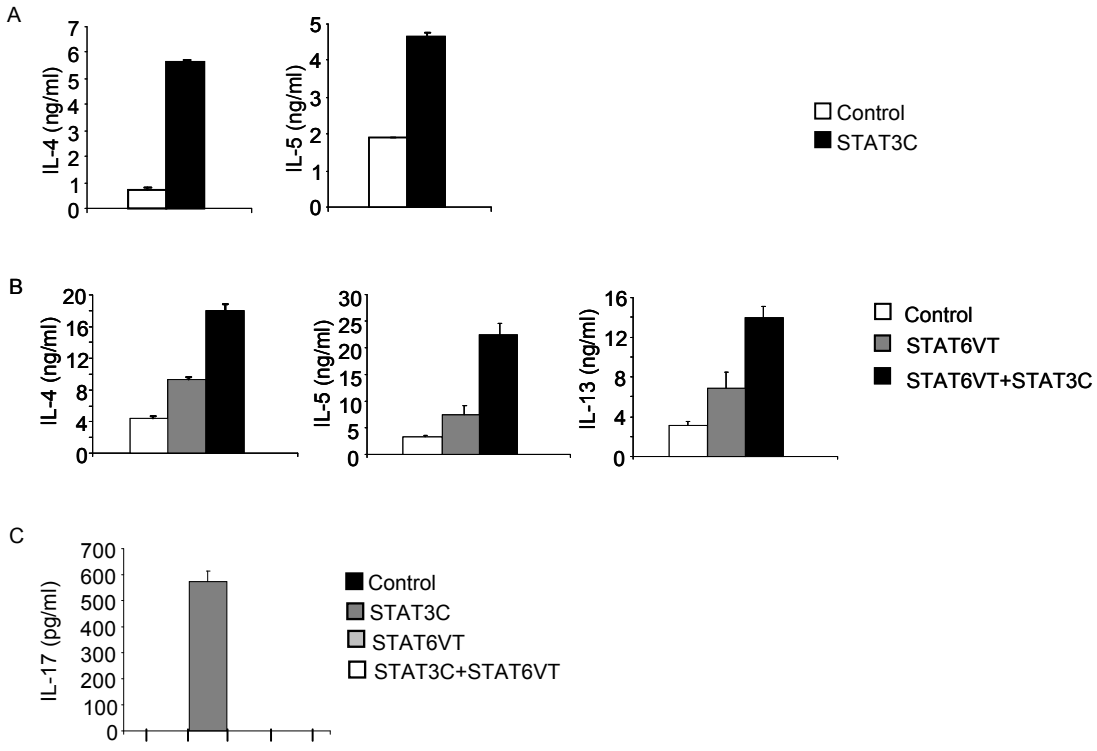

Figure 33. STAT3 and STAT6 cooperate in promoting Th2 cytokine production. (A) Naïve CD4+ T cells were activated for 48 hours with anti-IFN-γ before being transduced with control vector or constitutively active *Stat3* (STAT3C). After 5 days in culture, cells were sorted and re-stimulated with anti-CD3 (4 μg/ml) for 24 hours before cell-free supernatant was tested for cytokines using ELISA. (B) Naïve CD4+ T cells were activated for 48 hours with a low dose of IL-4 (1 ng/ml) before being transduced with control vectors, constitutively active *Stat6* (STAT6VT), or both constitutively active *Stat6* and constitutively active *Stat3* (STAT3C). After 5 days in culture, cells were sorted and re-stimulated with anti-CD3 (4 μg/ml) for 24 hours before cell-free supernatant was tested for cytokines using ELISA. (C) Naïve cells activated as in B were

transduced with control vectors, STAT3C, STAT6VT, or both STAT3C and STAT6VT. After 5 days in culture, cells were sorted and re-stimulated with anti-CD3 (4 μg/ml) for 24 hours before cell-free supernatant was tested for cytokines using ELISA. Data are representative of 2 independent experiments.

Peripheral T cells in STAT6VT transgenic mice have an increased propensity towards a Th2 cytokine-secreting phenotype (Bruns et al., 2003; Sehra et al., 2008). To directly test if STAT3 is necessary for Th2 cytokine production in this *in vivo* system, we isolated CD4 T cells from the spleens of wild type, STAT6VT transgenic, and STAT6VT transgenic crossed to *Stat3*$^{CD4-/-}$ (STAT6VT-*Stat3*$^{CD4-/-}$) mice, and stimulated cells with anti-CD3 for 24 hours before cytokine production was assessed using ELISA. As shown previously, STAT6VT mice have increased production of IL-4, IL-5 and IL-13, whereas STAT6VT T cells lacking STAT3 produced Th2 cytokines in amounts similar to wild type cells (Figure 34A). The observation of STAT3-dependent increased Th2 cytokine production in the STAT6VT cells was confirmed using intracellular staining. Wild type, STAT6VT, and STAT6VT-*Stat3*$^{CD4-/-}$ CD4+ cells were re-stimulated *ex vivo* with PMA+ionomycin for 6 hours were stained for intracellular IL-4, IL-5, and IL-13. Cells from the STAT6VT mice had a 3-4 fold increase in percent of IL-4, IL-5, and IL-13 producing cells. However, STAT6VT cells deficient in *Stat3* had very few IL-4, IL-5, and IL-13 producing cells (Figure 34B). These results demonstrate that STAT3 cooperates with STAT6 to promote Th2 cytokine production.

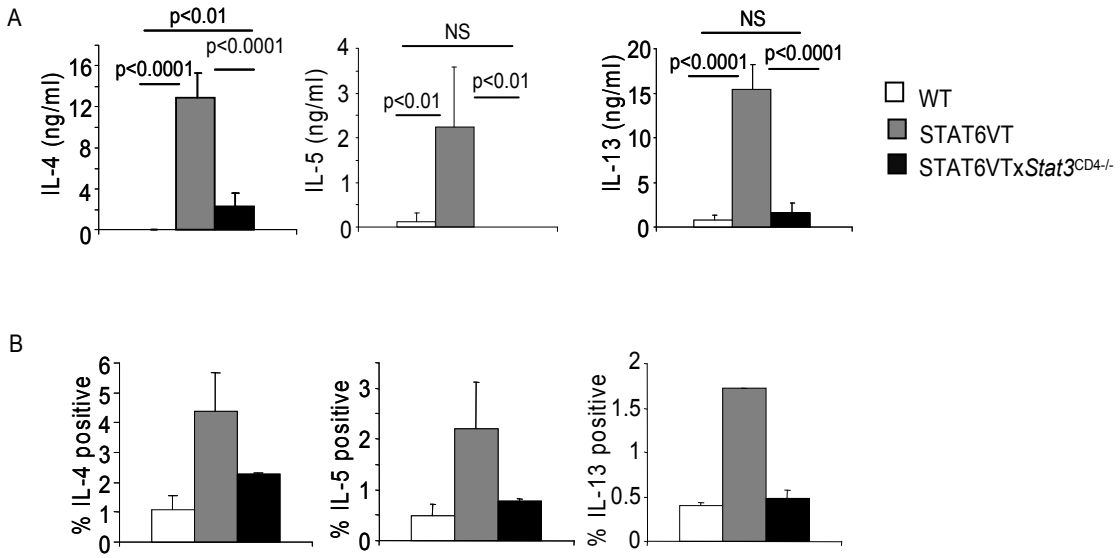

Figure 34. STAT3 is required for promoting Th2 predisposition of STAT6VT CD4 T cells. (A) CD4+ cells were isolated from WT, STAT6VT and STAT6VT-*Stat3*$^{CD4-/-}$ mice. Cells were then re-stimulated with anti-CD3 for 24 hours. Cell-free supernatant was collected and tested for various cytokines using ELISA. (B) Cells from (A) were re-stimulated for 6 hours with PMA+ionomycin. Cells were then intracellularly stained with IL-4, IL-5 and IL-13. Graphs represent % cytokine stain positive. **p*< 0.05 (two-tailed Student's *t*-test). Data are representative of 2 experiments (average of 2-6 mice ± SD) (A) or average of 2 mice ± SD (B).

We then wanted to examine Th2 cytokine production from Th2 cultured cells. CD4 positive T cells from WT, STAT6VT, or STAT6VT-*Stat3*$^{CD4-/-}$ mice were isolated and cultured under Th2 skewing conditions. After 5 days of culture Th2 cytokine production was assessed. In general, Th2 cytokines were increased in CD4 T cells from STAT6VT mice. The increase in Th2 cytokines in STAT6VT cells was diminished in STAT6VT-*Stat3*$^{CD4-/-}$ Th2 cells (Figure 35A and B). However, the STAT3-dependent increase of Th2 cytokines in STAT6VT Th2 cells was variable between cytokines and experiments (Figure 35A and B). These data suggest that Th2 cytokine production following Th2 differentiation is

increased in STAT6VT cells and in the absence of STAT3 the increases are diminished, although the data is not completely consistent.

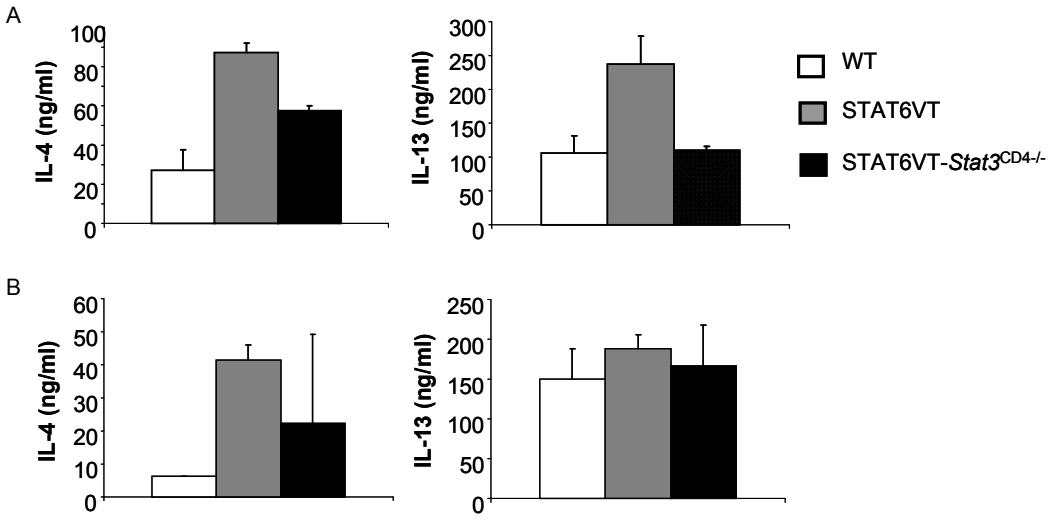

Figure 35. Th2 cytokine production from 5 day differentiated STAT6VT CD4 T cells is reduced in the absence of STAT3. (A,B) CD4 positive T cells were isolated from WT, STAT6VT, or STAT6VT-*Stat3*$^{CD4-/-}$ mice. Cells were activated and cultured under Th2 conditions for 5 days. On day 5 cells were re-stimulated with anti-CD3 24 hours. Cytokine production was assessed using ELISA. Data are average ± SD of 2-3 mice per group.

STAT3 binds Th2-associated gene loci and defines the STAT6 binding pattern

To further examine the cooperation of STAT6 and STAT3 in enhancing Th2 cytokine production, binding of these proteins to gene targets was determined using chromatin immunoprecipitation. In Th2 cells, STAT3 can directly bind the *Gata3* gene, and similar to published reports, the *Maf* promoter region (Figure 36A). We also observed binding to the *Irf4* and *Il24* promoters (Figure 36A). In Th17 cells, STAT3 binding to *Gata3* and *Il24* was lower than in Th2 cells, while

STAT3 binding to *Maf* and *Irf4*, which also contribute to Th17 development (Bauquet et al., 2009; Brustle et al., 2007; Xu et al., 2009), was higher in Th17 than in Th2 cells, or equivalent in both cell types (Figure 36A). However, STAT3 binding to the Th17 genes *Il17f* and *Il21* was only observed in Th17 cultures (Figure 36A). Thus, STAT3 can bind to target genes that are relevant for Th2 development, and the pattern of binding to those genes is different when cells are cultured under different polarizing conditions.

In Th2 cells, STAT6 binds to the *Gata3, Maf* and *Irf4* genes (Figure 36B). However, in the absence of STAT3, STAT6 binding is altered. STAT3-deficient Th2 cells had decreased STAT6 binding to *Gata3* and *Irf4*, but increased binding to *Maf* (Figure 36B). Data were similar for cells examined at three or four days of culture.

We then tested whether an active STAT6 was capable of inducing expression of Th2 transcription factors in the absence of STAT3. Expression of *Gata3* and *Maf* were significantly increased in STAT6VT CD4 T cells examined directly *ex vivo*, compared to cells wild type cells. However, STAT3-deficient STAT6VT CD4 T cells had reduced expression of both *Gata3* and *Maf*, compared to T cells from STAT6VT transgenic mice on a wild type background (Figure 36C). Together, these data suggests that STAT3 and STAT6 do not cooperate in binding the same gene targets. Furthermore, STAT3 dictates STAT6 binding efficiency to

the regulatory regions of Th2 transcription factors and in the absence of STAT3, STAT6 has altered binding and is unable to activate target genes.

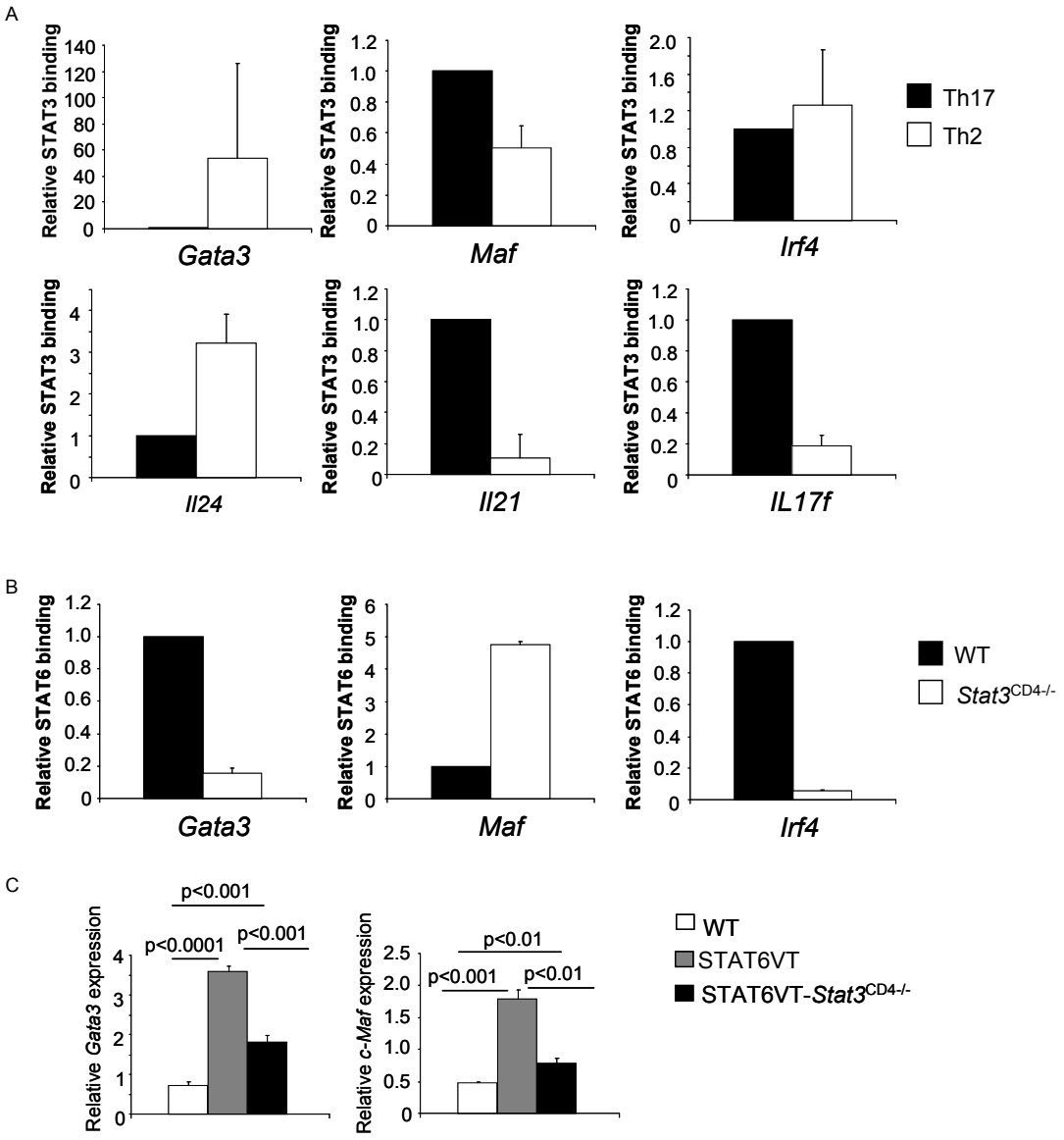

Figure 36. STAT3 binds Th2-associated gene loci and defines the STAT6 binding pattern. (A) Naïve CD4+ T cells were activated with anti-CD3 and anti-CD28 and cultured with under Th2 or Th17 conditions for 3 days. Chromatin immunoprecipitation was performed with control Ig or anti-STAT3 and quantitative PCR was performed for the indicated genes. Data are expressed relative to STAT3 binding in Th17 (1). (B) Naïve CD4+ T from WT and *Stat3*$^{CD4-/-}$ were activated with anti-CD3 and anti-CD28 and cultured under Th2 conditions for 3 days. Chromatin immunoprecipitation was performed with control Ig or anti-

STAT6 and quantitative PCR was performed for the indicated genes. Data are expressed relative to WT STAT6 binding (1). (C) CD4+ cells were isolated from WT, STAT6VT and STAT6VT-*Stat3*$^{CD4-/-}$ mice as in Figure 32A. Cells were then re-stimulated with anti-CD3 (2 μg/ml) for 6 hrs. After stimulation RNA was isolated and quantitative PCR was performed. *p< 0.05 (two-tailed Student's t-test). Data are average ± SD of 2-3 experiments. (A,B) or representative of 2 experiments (average of 2-6 mice ± SD) (C). ChIP experiments were done in collaboration with Dr. Rajarajeswari Muthukrishnan.

STAT3 in T cells is required for the development of allergic inflammation

Since our data suggest that STAT3 is required for Th2 differentiation, we next tested the requirement for STAT3 in Th2-mediated allergic inflammation. The expression of constitutively active STAT6 in lymphocytes of the STAT6VT mice leads to splenomegaly or increased spleen size. This is due to increased B cell number. The spleen size of the STAT6VT compared to the wild type mice are much increased, however STAT6VT mice crossed to mice lacking STAT3 in their T cells have decreased spleen size similar to the size of wild type mice (Figure 37A). The size of the spleen can also be assessed by total splenocyte counts. STAT6VT mice have significantly increased cell number in the spleen compared to wild type mice. In the absence of STAT3, STAT6VT mice splenocyte numbers are reduced to wild type numbers (Figure 37B).

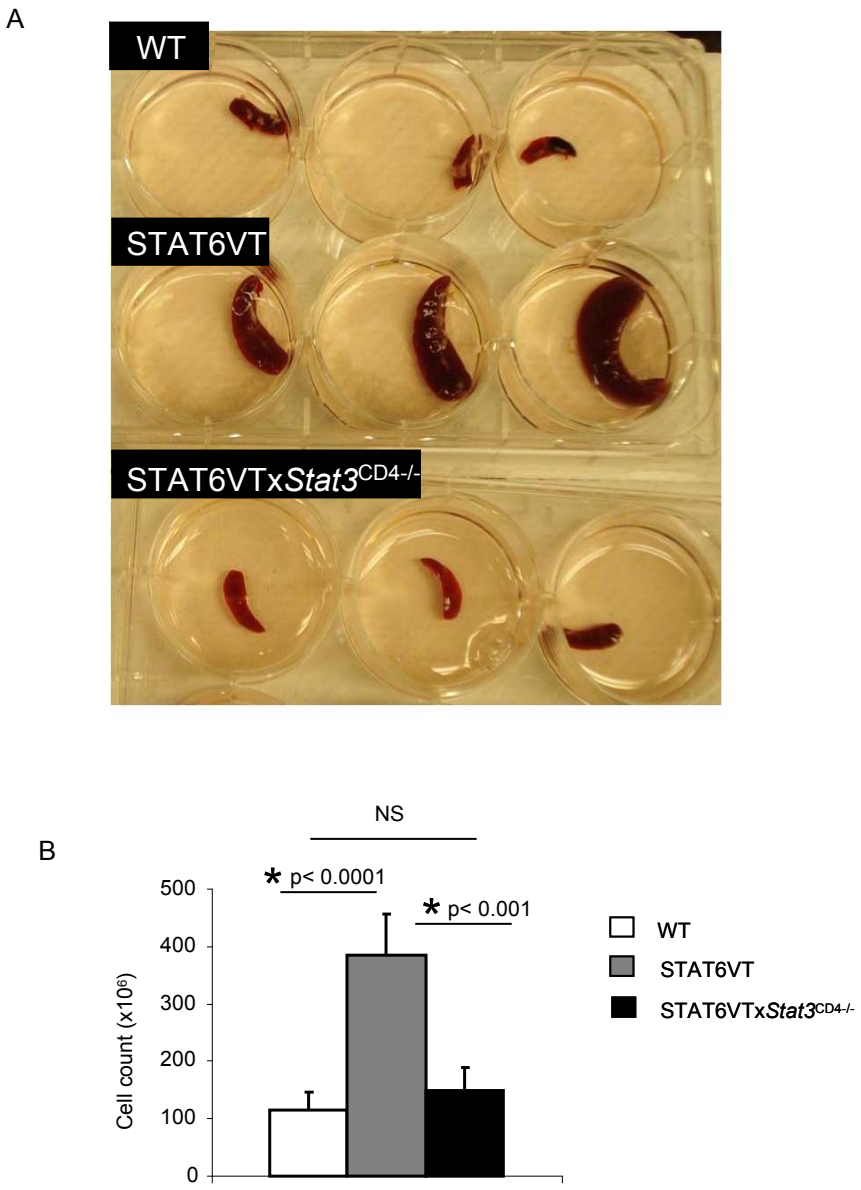

Figure 37. STAT3 deficiency protects STAT6VT mice from splenomegaly. (A) Pictures of wild type, STAT6VT, and STAT6VTx*Stat3*$^{CD4-/-}$ spleens. (B) Total cell counts from spleens of wild type, STAT6VT, and STAT6VTx*Stat3*$^{CD4-/-}$ spleens.

Additionally, mice that express STAT6VT in T cells spontaneously develop multi-organ allergic inflammation, including pulmonary inflammation, blepharitis, and skin inflammation, all of which are completely dependent on IL-4 (Sehra et al., 2008; Sehra et al., 2010). The incidence of blepharitis in STAT6VT mice is

nearly 75%, and is essentially never observed in wild type mice. STAT6VT-*Stat3*$^{CD4-/-}$ mice are protected from blepharitis and have 0% incidence even in older mice (Figure 38A). Approximately 40% of STAT6VT mice develop skin inflammation resembling atopic dermatitis, a condition not observed in wild type mice. As with blepharitis, STAT6VT transgenic mice lacking STAT3 in their T cells are protected from skin inflammation (Figure 38A and B). Histology from sections of the ear demonstrated increased thickness in the STAT6VT transgenic mice and at higher magnification increased cell infiltrate of immune cells can be observed. STAT6VT-*Stat3*$^{CD4-/-}$ mice have ear thickness similar to wild type with minimal cell infiltrate (Figure 38B). STAT6VT mice develop lung inflammation characterized by peri-bronchial and peri-arterial accumulation of eosinophils and lymphocytes. However the STAT6VT-*Stat3*$^{CD4-/-}$ mice, like wild type mice, had very few cells infiltrating the lungs (Figure 38C). Inflammation of the lung was also assessed by the total number of eosinophils in the bronchoalveolar lavage. STAT6VT transgenic mice have increased eosinophils in the BAL, compared to the wild type mice. The loss of STAT3 in the STAT6VT transgenic T cells resulted in reduced cell numbers in the BAL to numbers that were similar to wild type mice (Figure 38C). These results demonstrate that the loss of STAT3 in T cells protects STAT6VT mice from Th2-mediated inflammatory diseases. Taken together, STAT3 and STAT6 proteins are both necessary for optimal Th2 development and in the context of the STAT6 signal, STAT3 enhances Th2 cell development.

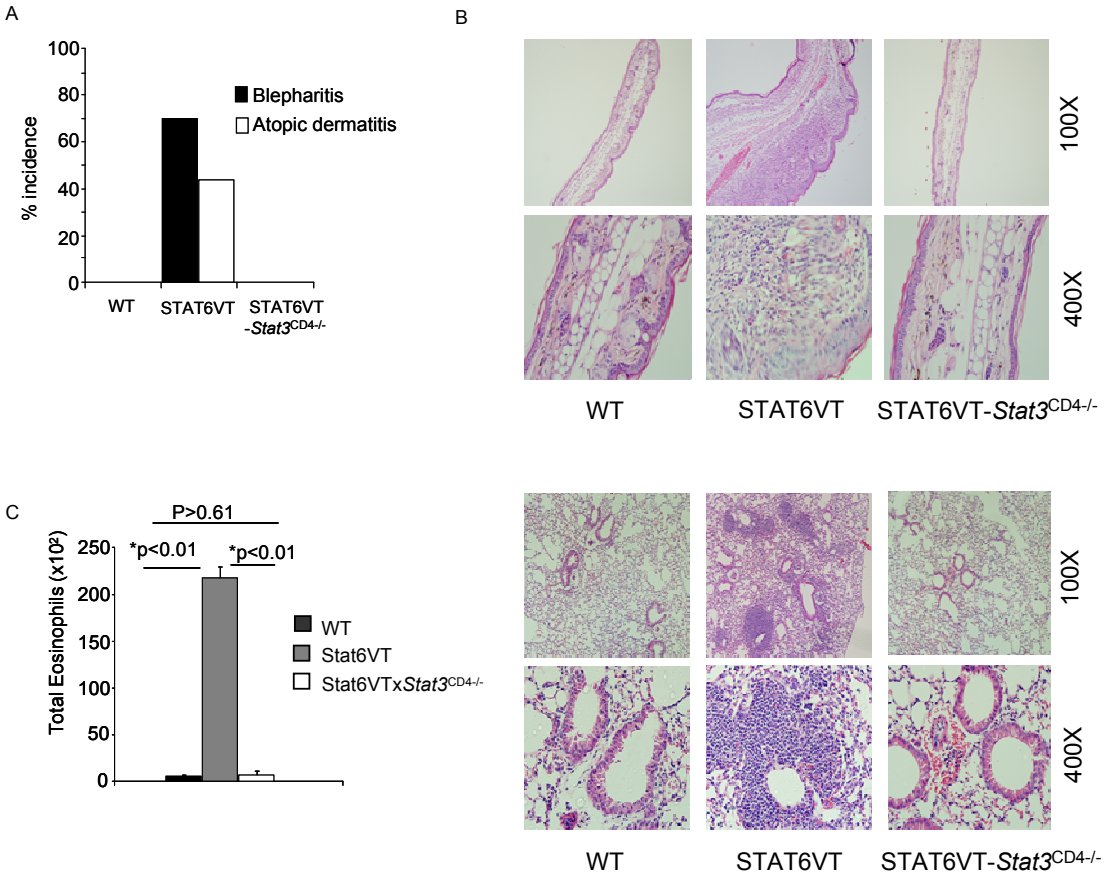

Figure 38. STAT3-deficiency in CD4 T cells of constitutively active STAT6 transgenic mice protects the mice from the development of allergic pulmonary inflammation. (A) Incidence of blepharitis and atopic dermatitis of WT, STAT6VT and STAT6VT-*Stat3*$^{CD4-/-}$ mice are shown. Incidence was determined by visual examination of mice. (B) Ear tissue from WT, STAT6VT and STAT6VT-*Stat3*$^{CD4-/-}$ mice were fixed and paraffin-embedded sections were stained with hematoxylin-eosin. Magnification is indicated in the panel. (C) Lungs from WT, STAT6VT and STAT6VT-*Stat3*$^{CD4-/-}$ mice were embedded in paraffin and stained with H & E. Magnification is indicated in the panel. Numbers of total cells and eosinophils (defined by flow cytometry) recovered in bronchoalveolar lavage. *p< 0.05 (two-tailed Student's t-test). Percentages are from 25 mice per group (A). Histology sections are representative of 10 mice per group (B,C). BAL data are representative of 2 independent experiments and shown as the average ± SEM of 2 mice per group (C). Analysis of allergic inflammation in the STAT6VT transgenic mice was performed in collaboration with Dr. Sarita Sehra.

DISCUSSION

In this thesis we demonstrate that Th17 cells are not committed to the IL-17 secreting phenotype. Cells primed under Th17 conditions for multiple rounds of stimulation convert to a Th1 or Th2 phenotype following stimulation with IL-12 or IL-4, respectively. Additionally, we have defined a role for IL-23 in the Th17 phenotype. Using cytokine capture assays, we have shown that IL-23 maintained the Th17 phenotype without affecting proliferation or survival. The ability of IL-23 to promote IL-17 production, specifically in memory cells, required STAT4. Simulation of memory cells with IL-23 results in the activation of both STAT3 and STAT4. Therefore, in addition to STAT3, STAT4 is required for IL-17 production from Th17 cells.

In agreement with the idea that multiple STAT family members contribute to T helper cell development, we observed a previously unrecognized role for STAT3 in Th2 differentiation. STAT3 is required for Th2 cytokine production and transcription factor expression. STAT3 is activated throughout Th2 differentiation and when ectopically expressed with STAT6 can augment Th2 cytokine production. We also observed that STAT3 is required for Th2 mediated allergic inflammation. Thus, in the presence of activated STAT6, STAT3 promotes optimal Th2 differentiation and cytokine production.

PART I: Role of IL-23 in Th17 Stability

Th17 cells are not committed to the IL-17 secreting phenotype

IL-23 is a critical cytokine in the development of inflammation. However, the direct affects of IL-23 have not been well defined. We have demonstrated that IL-23 maintains a Th17 phenotype in the absence of promoting proliferation or survival, but that IL-23-cultured cells are not committed and can assume other cytokine secreting phenotypes. There are several reasons that it may be advantageous to have a transient Th17 phenotype. In the absence of multiple levels of control, the potent pro-inflammatory activity of these cells, if left unrestrained, could result in excessive tissue damage *in vivo*. In that respect, requiring several cytokines to establish and maintain the phenotype, while the presence of IL-4 or IL-12 effectively diminishes the phenotype, potentially limits the effect of Th17 cells and allows Th1 or Th2 cells to mediate progressive inflammation. The sensitivity of Th17 cells to the cytokine environment is consistent with the earliest reports of Th17 cells that demonstrated the importance of neutralizing IFNs and IL-4 in the culture of IL-17-secreting T cells (Harrington et al., 2005; Park et al., 2005). Our work takes this further by showing that Th1- and Th2-promoting cytokines repress an established Th17 gene program. This sensitivity to other cytokines may also be reflected *in vivo*. In experimental autoimmune encephalomyelitis models, where Th17 cells are clearly important for the initiation of disease, analysis of lymphocytes from the target organ show a heterogeneous population of IL-17- and IFN-γ-secreting cells (Korn et al., 2007). Similarly, while IL-17 is required at early time points in the

establishment of allergic inflammation, Th2 cells establish the cytokine environment characteristic of the allergic response. IL-17 administered to mice with established allergic disease inhibits inflammation (Schnyder-Candrian et al., 2006). Together, these data suggest that, as we have shown for *in vitro* cultured cells, established Th17 cells *in vivo* may be inhibited by the appearance of other Th subsets where a changing cytokine environment results in repression of the Th17 phenotype.

Th17 cells have limited plasticity

The plasticity of Th subsets is of considerable interest and is important in understanding how T cells regulate inflammation over time. In the long-term cultures we have described, the percentages of IL-17+ cells, and total IL-17 secretion, decrease over rounds of stimulation. Importantly, in the absence of cytokines that promote skewing to other phenotypes, cells that were secreting IL-17 do not appear to take on other phenotypes. We did not observe increases in IFN-γ-secreting or Foxp3+ cells and the population seems to acquire cells that do not secrete any of the cytokines examined. Whether this is conversion of Th17 cells to non-secretor phenotypes or expansion of uncommitted cells is unclear. Following exposure to skewing conditions, Th17 cells appear to have differing abilities to acquire other phenotypes. For example, while IL-23-cultured IL-17+ Th17 cells may become Th1 or Th2 phenotype cells, we observe that culture of long-term Th17 cells with TGF-β plus IL-2 did not convert them into Foxp3 expressing cells, but rather increased IL-17 production (Figure 9E), suggesting

that IL-23-cultured cells are not capable of subsequently becoming adaptive regulatory T cells (Tregs). In contrast, natural Tregs have been shown to adapt a Th17 phenotype when cultured with IL-6 (Xu et al., 2007; Yang et al., 2008a), though there is still not a consensus on the ability of adaptive Tregs to acquire an IL-17-secreting phenotype (Yang et al., 2008a; Zheng et al., 2008). Because of the phenotypic overlap, IL-23 may also be important for polarizing Th cells to distinguish the Th17 and Tfh phenotypes (Nurieva et al., 2008; Suto et al., 2008; Vogelzang et al., 2008). Even with our culture conditions optimized for maintaining IL-17 secretion, IL-21 levels still decrease over time, and IL-22 levels are lower than in some previous reports (Figure 7B) (Liang et al., 2006; Zheng et al., 2007a). Although some of these effects might arise from the specific culture conditions, including the concentrations of cytokines added to the culture, it is possible that IL-23, as it maintains IL-17 production, limits IL-21 production, as it does for IL-10 production (McGeachy et al., 2007). Thus, even though IL-23 does not limit conversion to Th1 or Th2 lineages, it might restrict cells from adopting Treg or Tfh phenotypes.

IL-23 promotes IL-17 production and synergizes with IL-1β and IL-18

IL-23 production may promote IL-17 production in at least two ways, by maintaining the IL-17-secreting potential of Th17 cells following Ag receptor stimulation and by directly activating *Il17* in conjunction with IL-18 or IL-1 (Guo et al., 2009; Mathur et al., 2007). The mechanism of IL-23 function at the level of the gene remains unclear. IL-23 requires Stat3 for activity and several reports

have noted that IL-23-induced Stat3 can bind to the *Il17* promoter and this might play a role in chromatin remodeling and histone acetylation at the locus (Chen et al., 2006b; Mathur et al., 2007). Conversely, enhanced Stat3 activity potentiates IL-23 activity (Chen et al., 2006b; Mathur et al., 2007). However, as other Stat3 activating cytokines have distinct effects on Th cell cytokine production, IL-23 must provide a qualitatively unique signal. As the synergy between IL-12 and IL-18 depends on the engagement of MAPK and NF-κB signals to cooperate with Stat4 signaling (Murphy and Reiner, 2002), it is likely that IL-23-activated Stat3 collaborates with other pathways activated by IL-18 or IL-1β. It is also possible that IL-23 inhibits the expression or function of other factors that inhibit the Th17 phenotype. For example, T-bet is a potent inhibitor of IL-17 production and anti-CD3 induced T-bet expression is responsible for changing cells from a Th17 to a Th1 cytokine secreting pattern in IL-23-stimulated cultures (Mathur et al., 2006). In that culture system Ag receptor stimulation was responsible for decreasing IL-17 production and cells cultured in IL-23 without Ag receptor stimulation maintained IL-17-secreting potential. Thus, in the absence of commitment, the balance of activating and inhibitory signals a Th17 cell receives may determine the level of cytokine it will subsequently produce. It is still possible that IL-23 has effects on Th17 cell expansion or survival *in vivo*, though our results suggest that those effects must be indirect.

Epigenetic status of Th17 cells

The epigenetic status of several gene loci encoding cytokines and transcription factors have been examined using high-throughput sequencing or ChIP on chip analysis. Specific modifications are associated with active and inactive genes. Wei et al. focused primarily on trimethylation of histone H3 lysine 2 (H3K4me3) which is found at promoter and enhancer regions of active genes and trimethylation of histone H3 lysine 27 (H3K27me3) which is found in various regions of inactive genes (Wei et al., 2009). Consistent with our findings, *in vitro* differentiated Th17 cells have a bivalent epigenetic status at the *Tbx21* and *Gata3* loci, the Th1 and Th2 master regulators, respectively. The presence of both H3K27me3 and H3K4me3 marks at these loci suggests that Th17 cells have the ability to be reprogrammed to become Th1 or Th2 cells. Conversely, Th1 and Th2 cells have repressive marks at the *Il17a* and *Rorc* loci suggesting the plasticity is uni-directional (Wei et al., 2009). Additionally, Th17 cells stimulated with IL-12 have rapid remodeling of the *Ifng* locus leading to decreased IL-17 production and increased IFN-γ production. IL-12 also induced repressive histone modifications at the *Il17a* and *Il17f* loci of Th17 cells (Mukasa et al., 2010). These data suggest that the plasticity of Th17 cells is at the level of epigenetic modifications of lineage specific genes.

Function of IL-23: Summary

The ability of IL-23 to promote inflammation *in vivo* likely involves several of the functions we have described, including the acute induction of IL-17, functioning in

concert with IL-18 or IL-1β, and the ability of IL-23 to preserve the IL-17-secreting potential of T cells. However, in the absence of IL-23 and the presence of other cytokine environments, IL-17 production can be diminished in favor of establishing new patterns of cytokine secretion. The transient Th17 phenotype may be key to understanding how Th cell control may evolve during inflammation.

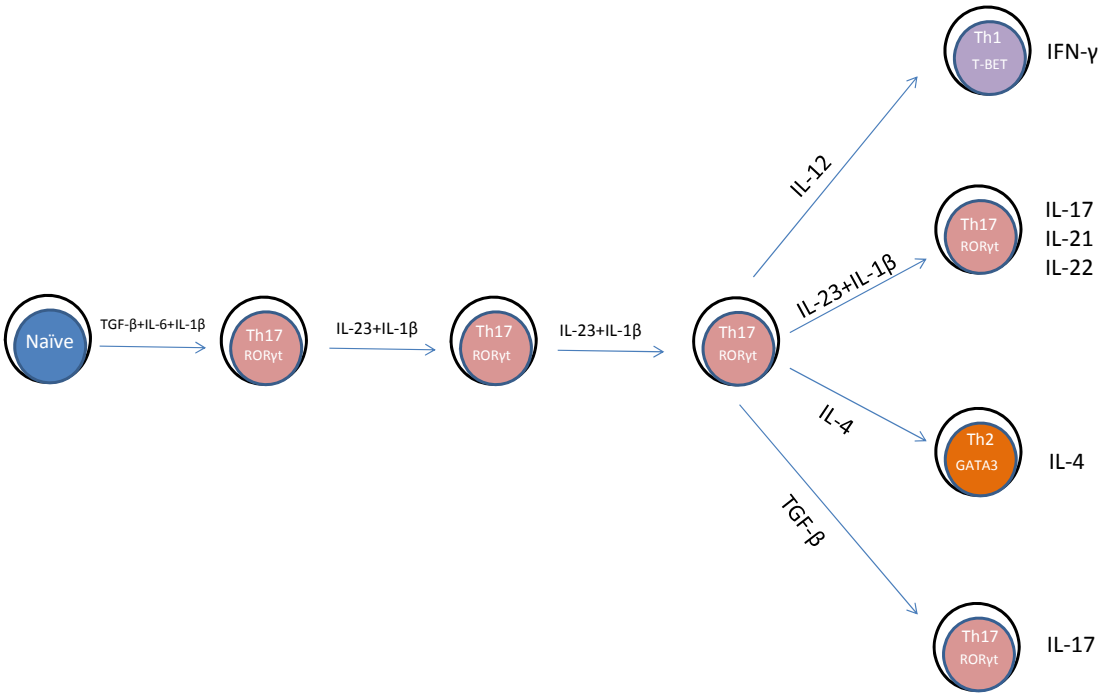

Figure 39. The maintenance and commitment of Th17 cells. The Th17 phenotype is maintained by cells cultured with IL-23+IL-1β for multiple rounds of stimulation. However, Th17 cells cultured with IL-12 or IL-4 convert to the Th1 or Th2 phenotype, respectively. Whereas Th17 cells cultured with IL-23+IL-1β for multiple rounds of stimulation and then cultured with the Treg promoting cytokine TGF-β do not convert to the Treg lineage and have increased IL-17 production.

PART II: Role of STAT4 in IL-17 producing memory cells

STAT4 is required for IL-23 induced IL-17 production in memory cells

IL-23 is clearly required for *in vivo* Th17 cell development and the maintenance of Th17 cells *in vitro*. Initial reports showed that IL-23 signals predominantly through STAT3 and to a lesser extent through STAT4 (Oppmann et al., 2000; Parham et al., 2002). Our data show that STAT4 is required for IL-17 production from memory cells cultured with IL-23, and is dispensable for IL-17 production from naïve cells cultured with TGF-β+IL-6 (Mathur et al., 2007). One of the first papers to characterize Th17 cells as a unique lineage, separate from both Th1 and Th2 subsets, showed Th17 cells develop normally in the absence of STAT4 (Harrington et al., 2005). However, in that report IL-17 production from *Stat4-/-* CD4+ T cells cultured with IL-23, was assessed following PMA+Ionomycin stimulation. The discrepancies between our studies and Harrington et al. may be due to several differences. First, STAT4 dependence can be overcome by stimulation with strong stimuli including PMA+Ionomycin (Mathur et al., 2007). Moreover, our studies focused on CD4+CD62L- cells (effector/memory population) whereas Harrington et al. isolated total CD4 T cells (naïve and effector/memory populations) from TCR transgenic mice. The variations between experimental designs may explain the differences observed when attempting to define the role of STAT4 in IL-23 induced IL-17 production.

Our results show that STAT4 is also required for IL-17 production from IL-23 cultured memory cells following IL-23+IL-18 stimulation. However, when STAT4-

deficient naïve cells cultured with TGF-β+IL-6 were re-stimulated with IL-23+IL-18 they secreted IL-17 amounts similar to wild type cells. Similar to IL-17 production following anti-CD3 stimulation, naïve cells cultured with TGF-β+IL-6 and memory cells cultured with IL-23 have different requirements for STAT4 following IL-18+IL-23 stimulation. Therefore, the requirement for STAT4 in IL-17 production could be restricted to the memory cell population and may be important for re-activation of the antigen experienced cell.

STAT4 is required for *in vivo* Th17 development

To determine if STAT4 is required for Th17 development *in vivo* we immunized wild type and *Stat4-/-* mice with OVA and the adjuvant Alum. Following *ex vivo* stimulation, STAT4-deficient lymph node cells had reduced IL-17 production compared to wild type cells. These data conflict with a previous study using the KLH+CFA immunization model. Splenocytes and lymph node cells from wild type and STAT4-deficient mice immunized with KLH+CFA were re-stimulated *ex vivo* with KLH. STAT4-deficient splenocytes and lymph nodes had similar amounts of IL-17 production compared to wild type cells (Park et al., 2005). These conflicting results could be due to the immunization model. The adjuvant CFA is traditionally associated with a Th1 response whereas Alum promotes a Th2 response. Since STAT4 is required for the Th1 response and promotes IFN-γ production, the STAT4 requirement for IL-23 induced IL-17 production could be masked by the differences in IFN-γ within the system. Whereas in the Alum model, where a Th2 response is induced, STAT4 might be predominantly

activated by IL-23, not IL-12, since there is no Th1 response, and thus the difference in IL-17 production can be observed. Our data clearly show a role for STAT4 in IL-23 induced IL-17 production in the absence of a Th1 response. Therefore, it is important to examine the model, specifically the adjuvant, before clearly defining the role of STAT4 in promoting IL-17 production *in vivo*.

Stat4-/- mice are resistant to the development of EAE. The studies showing that STAT4-deficient mice are protected from the Th17 mediated disease, EAE, were published before the discovery of Th17 cells. The potential explanations given by the authors included: STAT4-deficient mice fail to be primed, STAT4-deficient mice have reduced expression of Th1 cytokines important for EAE and have increased production of Th2 cytokines, and/or STAT4-deficient mice lack chemokine expression important for entering the central nervous system (Chitnis et al., 2001). It is unlikely that STAT4-deficient mice fail to be primed since in response to MOG stimulation STAT4-deficient splenocytes proliferate similar to wild type cells (Mo et al., 2008). However, it is clear that STAT4-deficient mice have less inflammation in the CNS and have reduced Th1 cytokine production, both of which can contribute to their resistance (Chitnis et al., 2001; Mo et al., 2008).

Rorc expression in STAT4-deficent memory cells is reduced

Th17 cells express both the IL-23R and the transcription factor Rorγt, encoded by the gene *Rorc*. STAT4-deficient memory cells express similar levels of IL-23R compared to wild type memory cells, suggesting that both wild type and *Stat4-/-* memory cells can respond to IL-23 stimulation. However, IL-23R expression was assessed using real-time PCR, and therefore it is hard to draw conclusions without assessing surface expression of the IL-23R. IL-23R surface expression is difficult to measure since there is no IL-23R antibody commercially available. The surface expression either by percent positive or expression amount per cell (MFI) might be different between wild type and STAT4-deficient memory cells, which cannot be distinguished by real-time PCR. Therefore, further experiments are necessary to rule out potential differences in IL-23R surface expression. Conversely, STAT4-deficient memory cells have decreased Rorγt expression directly *ex vivo*. The reduction in Rorγt expression could be a result of diminished Rorγt expression in the STAT4-deficient Th17 memory cells or a reduction in total number of Th17 memory cells. STAT3-deficient mice have significantly reduced IL-17 producing memory cells and it is possible that STAT4-deficient mice have a similar phenotype (Yang et al., 2007). Alternatively, STAT4 could be required for Rorγt expression in memory cells. It is clear that STAT3 can bind and activate *Rorc* (Durant et al., 2010), therefore it is possible STAT4 could also bind to the regulatory regions of *Rorc* and might be important for Rorγt expression. Further studies will have to be performed to elucidate the cause for reduced Rorγt expression in STAT4-deficient memory cells.

Memory and naïve cultured Th17 cells have different STAT activation patterns

Memory cells cultured in IL-23 require STAT4 for IL-17 production, however naïve cells cultured in TGF-β+IL-6 produce IL-17 independently of STAT4. After 5 days of culture the pattern of pSTAT3 and pSTAT4 following IL-23 stimulation is distinct between the 2 cell populations. IL-23 stimulation of IL-23 cultured memory cells led to the phosphorylation of STAT4 in a small population of cells. Conversely, IL-23 stimulation of naïve TGF-β+IL-6 cultured cells led to the phosphorylation of STAT3, not STAT4, in a small population of cells. Both cell populations had a small number of pSTAT positive cells and it is possible that the slight shift in pSTAT3 or pSTAT4 positive cells could be a result of limiting IL-23R expression. For future studies it will be important to assess the IL-23R expression and gate or sort IL-23R positive cells to see a more significant shift in pSTAT staining. Nevertheless, the presence of pSTAT4 and pSTAT3 in memory and naïve cultured cells, respectively, suggests that IL-23-induced activation of STAT4 and STAT3 is different between cell populations. The activation of STAT4 specifically in memory cells cultured with IL-23 coincides with the requirement of STAT4 for IL-17 production. It will be important to look at downstream gene targets of both STAT3 and STAT4 to see if memory and naïve cultured cells have different expression of STAT3 and STAT4 dependent genes.

Role of STAT4 in IL-23 signaling: Summary

STAT4 is important for memory cell production of IL-17 following IL-23 priming. Furthermore, IL-17 production following IL-18+IL-23 stimulation from the IL-23 cultured memory cells also required STAT4. Whereas, naïve cells cultured with TGF-β+IL-6, STAT4 is dispensable for IL-17 production following either anti-CD3 or IL-18+IL-23 stimulation. These data correlate with the data showing IL-23 stimulation preferentially activates STAT4 in memory, not naïve cultured cells. Taken together, these data suggest a different requirement for STAT4 in memory versus naïve cultured cells. Although it is not completely clear why, STAT4-deficient memory cells have reduced Rorγt expression compared to wild type memory cells. Furthermore, in an OVA+Alum immunization model, STAT4 is required for IL-17 production from *ex vivo* stimulated lymph node cells. Overall, these data suggest a previously unrecognized role for STAT4 in IL-17 production by memory cells.

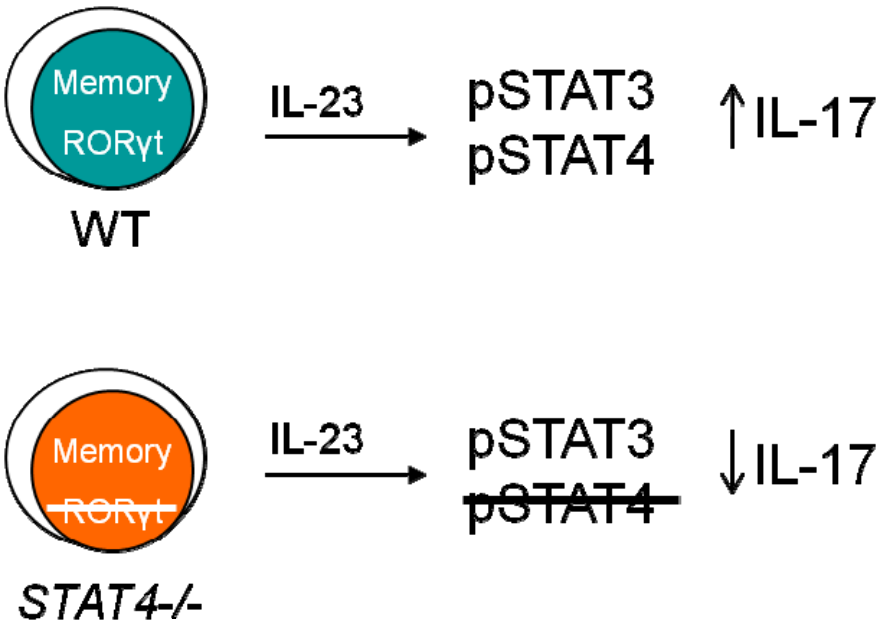

Figure 40. STAT4 is required for IL-17 production following culture with IL-23. STAT4-deficient memory cells have reduced RORγt expression compared to wild type memory cells. Memory cells cultured with IL-23 require STAT4 for optimal IL-17 production. Furthermore, IL-23 stimulation of memory cells cultured for 5 days with IL-23 leads to the phosphorylation of both STAT3 and STAT4.

PART III: STAT3 is required for Th2 differentiation

Multiple STATs are required for T helper cell development

The paradigm that STAT family members promoted specific Th effector phenotypes was developed when the number of known effector subsets was more limited. Researchers initially defined that STAT4 is required for Th1 development and STAT6 is required for Th2 development (Kaplan et al., 1996a; Kaplan et al., 1996b; Shimoda et al., 1996; Takeda et al., 1996; Thierfelder et al., 1996). However, this simple one STAT-one phenotype paradigm became more complicated when it was shown that STAT1 also contributed to Th1 differentiation (Afkarian et al., 2002; Lighvani et al., 2001), and STAT5 could function with STAT6 in the development of Th2 cells (Cote-Sierra et al., 2004; Takatori et al., 2005a; Zhu et al., 2003). This was an important finding as STAT5, which is critical for the development of T regulatory cells (Burchill et al., 2007; Yao et al., 2007), has different functions when activated in the presence of STAT6. Thus, the differentiating T helper cell is able to assimilate multiple signals and acquire the appropriate effector phenotype. In this report, we further expand our understanding of the integration of STAT signals by demonstrating that STAT3, which clearly promotes Th17 development in the absence of other signals, is required for the function of STAT6 during Th2 development.

Gene targets of STAT3 and STAT6

The integration of multiple STAT signals likely includes the ability of each STAT protein to bind both overlapping and distinct sets of genes. STAT6 is required for

the expression of *Gata3*, *Gfi1* and Th2 cytokine genes and has been shown to bind directly to a subset of these loci (Lee and Rao, 2004; Sherman et al., 2002; Zhu et al., 2002). STAT5 is required for *Il4* chromatin accessibility, *Il4ra* expression, and induction of *Socs3* to extinguish Th1 differentiation (Cote-Sierra et al., 2004; Kagami et al., 2001; Takatori et al., 2005b). In Treg cells, STAT5 binds *Foxp3*, although this binding is reduced in the presence of an IL-4 signal (Burchill et al., 2007; O'Malley et al., 2009; Yao et al., 2007). Similarly, STAT3 binds to Th17 genes, though the amount of STAT3 binding to those genes is diminished in Th2 cultures. Conversely, STAT3 binding to Th2 genes is greater in Th2 than in Th17 cells. However, the differences are not as apparent in genes that have roles in both cell types such as *Maf* and *Irf4* (Bauquet et al., 2009; Brustle et al., 2007; Xu et al., 2009). Whether STAT3 directly activates Th2 genes, or whether by binding genes it aids in orchestrating STAT6 binding is not entirely clear. The fact that increased STAT6 binding to *Maf* is observed in STAT3-deficient Th2 cultures, which is insufficient for normal *Maf* expression, coupled with previous data showing that STAT3 activates *Maf* expression (Yang et al., 2005) suggests that STAT3 plays an important role in activating genes during Th2 differentiation. However, the displacement of STAT6 binding from the *Gata3* gene to the *Maf* promoter in the absence of STAT3 suggests that it occupies a transcriptional niche required for appropriate binding of other factors. For example, STAT3 can functionally interact with AP-1 family members (Yoo et al., 2001; Zhang et al., 1999b), though it is not known if STAT6 has a similar capability. It is likely that STAT-interacting transcription factors at each of the

promoters define the ability of STAT3 or STAT6 to function at specific gene promoters.

STAT3 Th2 phenotype cannot be rescued by ectopic expression of Th2 transcription factors

Although the *Maf* gene is a characterized target of STAT3 (Yang et al., 2005), and expression of *Maf* is deficient in the absence of STAT3, ectopic expression of *Maf* resulted in only a partial recovery of IL-4 production, and was insufficient to recover the entire Th2 phenotype. Similarly, we did not see recovery of Th2 cytokine production when *Gata3* or *Irf4* were ectopically expressed. This is distinct from STAT6-deficient cells where expression of GATA3 induces Th2 cytokine production (Chang et al., 2005; Ouyang et al., 2000). Together these data suggest that the defect in STAT3-deficient Th2 cultures is more complex than the absence of one factor, and recovery of Th2 cytokine expression may require the coordinated function c-maf, GATA3 and other factors.

STAT3 is activated throughout Th2 differentiation

It is still not entirely clear which cytokines are responsible for the activation of STAT3 during Th2 differentiation. We have neutralized IL-6 and IL-21 during differentiation of wild type Th2 cultures and saw a 2-fold decrease in Th2 cytokine production (Figure 22B). The neutralization of IL-6 and IL-21 also resulted in decreased phospho-STAT3 but not phospho-STAT6 (Figure 22A). The partial decrease in Th2 cytokines and pSTAT3 suggests that STAT3

activation may result from several cytokines, some in addition to those we have neutralized. Furthermore, it has also been proposed that IL-4 might activate STAT3 in some cell context (Orchansky et al., 1999; Umeshita-Suyama et al., 2000; Wery-Zennaro et al., 1999), although we did not observe IL-4-induced phospho-STAT3 in Th2 cultures (Figure 21). Phospho-STAT3 was observed in STAT6-deficient Th2 cultures (Figure 31C), suggesting that the induction of endogenous STAT3-activating cytokine(s) is not downstream of IL-4 signaling. IL-6 has also been shown to have important roles in proliferation and cell survival (Kishimoto, 2006). STAT3 is required for the proliferation and survival of T cells in response to IL-6, and in the absence of STAT3, IL-6 induced proliferation and survival are reduced (Takeda et al., 1998). It is possible that STAT3 dependent IL-6 functions are important for Th2 proliferation and/or survival. Further examination of STAT3-deficient Th2 cells should be performed in order to determine the contribution of both proliferation and survival in Th2 cytokine production.

The *in vivo* differentiation of Th2 cells requires STAT3

Important in the demonstration that STAT3 promotes Th2 development are the observations that STAT3 is also required for *in vivo* Th2-mediated inflammation. Following immunization with alum-adsorbed ovalbumin we observed that antigen induced Th2 cytokine production was diminished in cultures from *Stat3*$^{CD4-/-}$ mice, compared to those from wild type mice (Figure 27). In studies performed by Sehra et al. it was also observed that allergic pulmonary inflammation was

diminished in mice that were sensitized and challenged with ovalbumin. However, in addition to decreases in Th2 cells, *Stat3*$^{CD4-/-}$ mice had decreased Th17 cells and increased Foxp3 expression in the lung (unpublished data), both of which affect the development of allergic inflammation (Lewkowich et al., 2005; Schnyder-Candrian et al., 2006), thus limiting the ability to draw conclusions from those studies. This led us to use a model we previously developed wherein mice express a constitutively active STAT6 in T cells and develop spontaneous allergic inflammation that is dependent upon IL-4, without any observed decreases in IL-17 expression (Sehra et al., 2010). In this model, STAT3-deficiency in T cells of STAT6VT transgenic mice resulted in decreased *ex vivo* Th2 cytokine production and nearly eliminated allergic inflammation *in vivo*. These data further support a model wherein STAT6 is not sufficient in the absence of STAT3 to promote Th2 development.

STAT3 is required for Th2 development: Summary

Multiple signals contribute to the generation of differentiated T helper subsets. However, in this model there is a dominant signal, IL-4 in the case of Th2 cells that defines the outcome of the differentiation process. It is clear that STAT3 is required for the development of Th17 cells, and that constitutively active STAT3 promotes the development of IL-17-secreting cells (Ma et al., 2008; Mathur et al., 2007; Milner et al., 2008; Nurieva et al., 2008; Yang et al., 2007; Yang et al., 2005). However, IL-4 provides a dominant signal that diminishes Th17 development (Harrington et al., 2005; Park et al., 2005) and decreases

symptoms of autoimmunity in multiple models (Rocken et al., 1996). Thus, when both STAT3 and STAT6 signals are present in a cell, the pro-Th17 effects of STAT3 are reduced, while the pro-Th2 effects of STAT6 are amplified. Mechanistically this occurs through the binding of STAT3 to specific Th2 genes that facilitate the ability of STAT6 to activate genes necessary for Th2 development. Thus, multiple STAT proteins, activated by cytokines present in the milieu of a developing immune response, cooperate in defining the ultimate phenotype of the differentiating effector T cell.

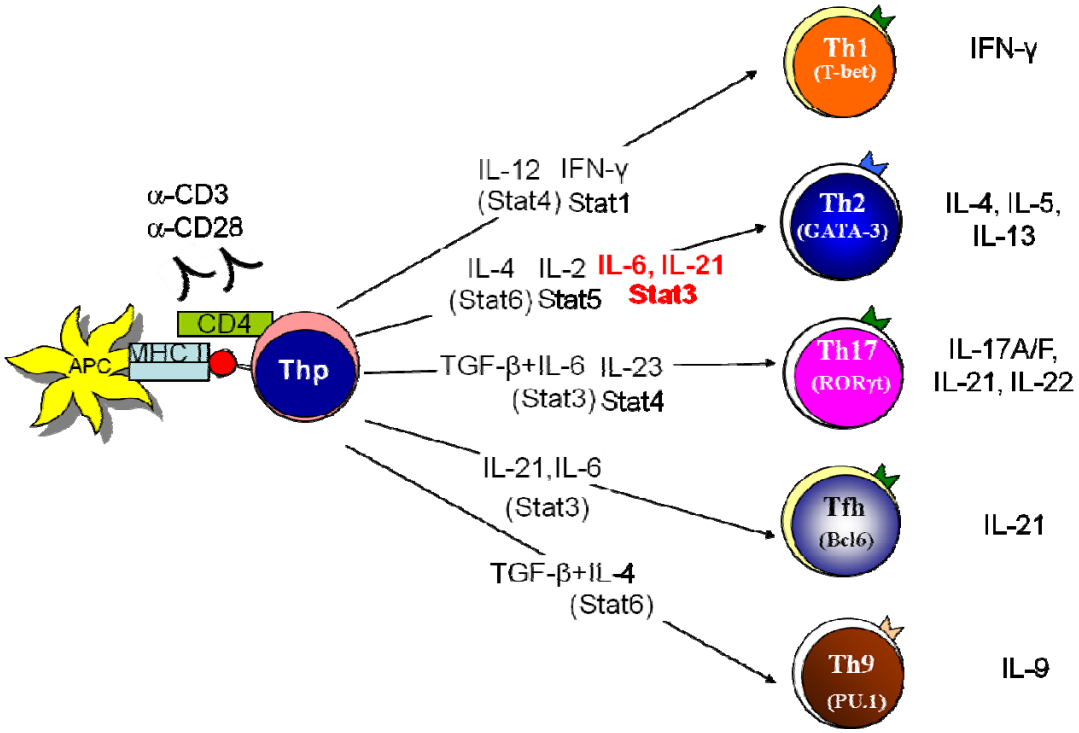

Figure 41. Multiple STAT family members are important in T helper cell development. The cytokines and STAT family members associated with the development of each T helper subset.

Overall conclusions

Our studies have focused on the role of cytokines and STATs in T helper cell differentiation and commitment. The development of both Th2 and Th17 requires numerous signals transduced by multiple STAT family members. Elucidating the cytokines and transcription factors important in the development and regulation of Th17 and Th2 cells has become increasingly important due to the therapeutic potential of targeting these cells in both autoimmunity and allergic disease, respectively. Our studies suggest that Th17 cells are not committed to the IL-17 secreting phenotype. Potential therapies could exploit this characteristic of Th17 cells in order to reduce damage caused by their potent inflammatory response. Additionally, targeting STAT family members has also been proposed as a potential treatment in several diseases (Catlett-Falcone et al., 1999; Morel and Berenbaum, 2004; Nakamura and Hoshino, 2005; O'Shea et al., 2000). However, caution should be used when proposing STAT family members as targets due to the overlap of some STAT family members in several T helper subsets. For example, targeting STAT3 in Th17 mediated autoimmune diseases such as multiple sclerosis or colitis could also affect the protective effects of not only Th17 cells, but, according to our data, also Th2 cells. Overall, our studies have helped elucidate the specific factors involved in T helper cell differentiation and effector function. In the future, our results as well as others in the field will help identify potential therapeutic targets for specific diseases.

FUTURE DIRECTIONS

PART I: Role of IL-23 in Th17 stability

Determining if *in vivo* generated Th17 cells are committed to the Th17 phenotype

Our *in vitro* studies have shown that Th17 cells are not committed to the IL-17 secreting phenotype and when cultured with opposing cytokines can switch to a Th1 or Th2 phenotype. Subsequently it was shown that *in vitro* differentiated Th17 cells transferred into several different mouse models are not stable. Transferred Th17 cells become IFN-γ secreting cells and no longer secrete IL-17. The previous *in vivo* studies of Th17 stability rely on the transfer of *in vitro* differentiated Th17 cells. Many of the studies transferred "Th17 cells" composed of impure populations, and therefore allowing the possibility of outgrowth of IL-17 negative cells. Even in studies that transferred pure populations of IL-17 secreting cells, it is possible that throughout *in vitro* culture they are not receiving the signals necessary for Th17 commitment. In order to further investigate Th17 stability we have designed an IL-17 knock-in mouse. The IL-17 knock-in mouse has the Cre recombinase gene inserted into the IL-17F gene. When crossed to ROSA-EYFP mice, cells that have or are expressing IL-17F will express enhanced yellow fluorescent protein. One advantage of using the IL-17 knock-in mouse is that it allows us to isolate cells that at one time expressed IL-17F. As a result, we would be able to transfer *in vivo* generated Th17 cells in order to assess stability. Furthermore, we could take advantage of the marker protein and be able to assess the current cytokine secreting profile of cells that at one

time secreted IL-17F. These mice could also be used in several disease models such as EAE, CIA, and colitis in order to determining the plasticity of IL-17-secreting cells within *in vivo* disease models.

Determining the stability of Th17 cells in Th1 and Th2 cytokine environments

We have shown that Th17 cells lose their IL-17 secreting phenotype when exposed to IL-12 or IL-4 *in vitro*. To determine if this is also true *in vivo* we could transfer Th17 differentiated cells into several different Th1 and Th2 mouse models. Specifically, STAT6VT and IL-4 transgenic mice or IFN-γ and T-bet transgenic mice provide an environment that promotes Th2 or Th1 development, respectively. Our data suggests that Th17 cells transferred into an environment rich in Th1 or Th2 promoting cytokines would repress the Th17 phenotype and switch to the Th1 or Th2 phenotype. These studies would further define the ability of Th1 and Th2 cytokines to repress established Th17 cells *in vivo*.

Determining the epigenetic status of Th1, Th2 and Th17 associated genes following Th17 cells cultured in opposing cytokines

The epigenetic status of lineage specific genes has been recently characterized. Overall, it has been shown that Th17 cells lack repressive marks in Th1 and Th2 associated genes, however repressive marks were abundant throughout Th17 genes in Th1 and Th2 cells (Wei et al., 2009). Furthermore, Th17 cells stimulated with IL-12 undergo rapid epigenetic remodeling. IL-12 stimulation led

to increased H3K27me3 modifications at the *Il17a-Il17f* locus and increased H3K4me modifications at the *Ifng* locus. These epigenetic modifications coincided with the repression of IL-17A and IL-17F production and increased IFN-γ production (Mukasa et al., 2010). Our data show that Th17 cells cultured in IL-12 or IL-4 have decreased Th17 gene expression and increased Th1 and Th2 gene expression, respectively. The report by Mukasa et al. has clearly demonstrated the epigenetic changes of Th17 and Th1 associated genes that Th17 cells undergo following IL-12 stimulation(Mukasa et al., 2010). However, we would like to expand upon their study and look at epigenetic modifications of Th17 cells following culture with IL-4. Specifically, we would like to look at H3K27me3 and H3K4me modifications to Th2 cytokine and transcription factor genes. Furthermore, the ability of Th17 cells switched to Th1 or Th2 cells to return back to the Th17 phenotype has not been examined. It would be interesting to do the *in vitro* culture experiments to address this question. Moreover, we could assess the epigenetic status of lineage specific genes of Th17 cells switched to Th1 and Th2 and then again after culture with TGF-β+IL-6. Specifically, it would be interesting to look at repressive marks at the *Il17a/f* and *Rorc* loci as well as Th1 and Th2 associated genes of the Th17 cells switched to Th1 and Th2 cells and then back to Th17 cells. These data would determine if the switch of Th17 cells to Th1 or Th2 cells is irreversible.

Defining the ability of Th17 cells to switch to additional lineages

Our data show that Th17 cells can convert to both Th1 and Th2 cells following culture with IL-12 and IL-4, respectively. However, Th17 cells do not convert to Treg cells following culture with TGF-β+IL-2. Recently, additional Th subsets have been defined based on the expression of unique cytokines and transcription factors. To further define the plasticity of Th17 cells, it would be important to determine the ability of Th17 cells to switch to both Tfh and Th9 cells. We could directly test this by culturing Th17 cells for multiple rounds of stimulation and then culture them under Tfh or Th9 conditions. Elucidating the ability of Th17 cells to convert to Tfh and Th9 lineages would be interesting since the differentiation of both cell types require cytokines important in Th17 differentiation.

PART II: Role of STAT4 in IL-17 producing memory cells

The requirement of STAT4 for *in vivo* IL-17 production

According to our results, STAT4 is required for antigen specific IL-17 production following immunization with OVA. However, the amount of IL-17 production following OVA stimulation is low. It is possible that one immunization, with or without an additional boost, does not lead to sufficient priming of antigen specific T cells. In order to increase the number of antigen specific T cells we could increase the number of challenges. Many groups, including our own, do multiple challenges intranasally before analysis (Figure 42). The additional challenges might increase IL-17 production from splenocytes and emphasize the STAT4 dependence. Additionally, we could stimulate the cells *ex vivo* with both antigen

(OVA) and IL-23±IL-18. IL-23 should further increase IL-17 production from activated cells. According to our data, the increase in IL-17 production following the addition of IL-23 will be STAT4 dependent.

Since there are conflicting reports on the dependence on STAT4 for *in vivo* IL-17 production we could directly test the importance of the cytokine environment. Using the OVA immunization model, we could immunize WT and STAT4-deficient mice with OVA plus the adjuvant Alum or CFA. Following *ex vivo* stimulation we could directly compare IL-17 production and the dependence on STAT4 from OVA+Alum or OVA+CFA immunized mice.

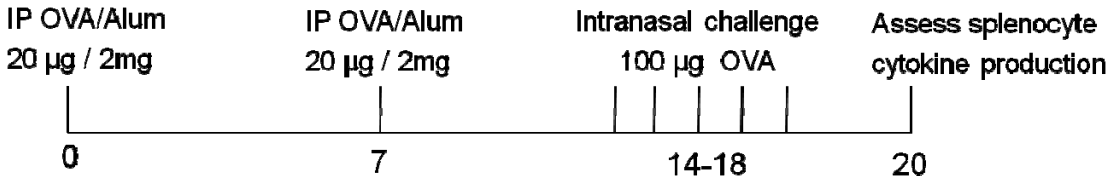

Figure 42. OVA immunization protocol. On day 0 and day 7 WT and STAT4-deficient mice would be injected with OVA and Alum i.p. Mice would then be challenged i.n. with OVA on days 14-18. 48 hours following the last i.n. challenge mice would be sacrificed and splenocytes would be re-stimulated and cytokine production would be assessed.

Characterization of *Stat4-/-* memory cells

STAT4-deficient memory cells have expression of *Il23r* similar to wild type memory cells. However, memory cells from STAT4-deficient mice have a 2-fold decrease in *Rorc* expression compared to wild type memory cells. Further

characterization of STAT4-deficient memory cells is necessary to elucidate the mechanism responsible for reduced IL-17 production. We have shown that IL-17 production from memory cells cultured with IL-23 requires STAT4. However, in order to determine if IL-17 production following direct *ex vivo* stimulation requires STAT4, we will isolate wild type and STAT4-deficient memory cells and stimulate them with anti-CD3 or IL-23+IL-18. IL-17 production can be assessed by both intracellular staining and ELISA. Additionally, it is important to assess pSTAT3 and pSTAT4 of wild type and *Stat4-/-* memory cells following *ex vivo* stimulation with IL-23.

Since *Rorγt* expression is reduced in STAT4-deficient memory cells, the expression of other Th17 transcription factors including *Ahr*, *Rorα*, and *Batf* should be compared between wild type and *Stat4-/-* memory cells. We will also test the requirement for STAT4 in the production of other Th17 cytokines including IL-21, IL-22, and IL-17F by memory cells.

STAT4 dependence of IL-17 production in other cell types

Our results show that STAT4 is required for IL-17 production from CD4 memory T cells. However the requirement of STAT4 for IL-17 production from other cell types has not been examined. To directly test the STAT4 dependence in NK, NKT, and γδ T cell IL-17 production we could sort different populations based on surface markers and then stimulate them with IL-23±IL-18 and assess IL-17 production. Similar to CD4 memory T cells, we could also do longer *in vitro*

cultures with IL-23 and re-stimulate to see IL-17 production following a re-call response from wild type and *Stat4-/-* mice.

PART III: STAT3 is required for Th2 differentiation

Determine if constitutively active STAT3 can rescue Th2 cytokines in STAT6-deficient Th2 cells

We have illustrated that STAT6 and STAT3 are both required for optimal Th2 differentiation. Our data show that constitutively active STAT6 cannot rescue Th2 cytokines production in CD4 T cells lacking STAT3. Furthermore, the expression of constitutively active STAT3 can induce Th2 cytokine production. To directly test if over expression of active STAT3 can recover Th2 cytokine production in STAT6-deficient Th2 cells we could retro-virally transduce STAT6-deficient Th2 cells with constitutively active STAT3. Following transduction with constitutively active STAT3, cells could be re-stimulated and Th2 cytokine production could be compared to wild type Th2 cells.

Affinity of STAT6 and STAT3 to STAT binding sites in Th2 associated genes

STAT6 and STAT3 both directly bind Th2 associated genes. Furthermore, our data suggests that STAT3 is required for directing STAT6 binding. In wild type cells STAT6 binds to *Gata3* and to a lesser extent *c-Maf.* Conversely, STAT3 binds predominantly to *c-Maf.* These data suggest that STAT6 and STAT3 could preferentially bind to the STAT binding site in *Gata3* and *c-Maf*, respectively. To

directly test the affinity of one binding site versus the other we could use DNA affinity purification analysis (DAPA). Biotinylated oligos containing the STAT binding sequences within the *Gata3* and *c-Maf* genes could be incubated with nuclear extracts of Th2 cultured cells. Following incubation and immunoprecipitation, western blots could be performed for both STAT3 and STAT6. Therefore, the binding preference of STAT3 and STAT6 could be elucidated. Additionally, STAT6 binding could be assessed in STAT3-deficient cells in order to determine if STAT6 binding is altered in the absence of STAT3.

Elucidating the requirement of STAT3 in helminth infections

Th2 cells are required for the clearance of extracellular parasites including helminth infections. Since STAT3 is required for Th2 development, STAT3 may also be required for clearance of helminth infections. To directly test if STAT3 is required for immunity to helminth infections we could infect wild type and STAT3-deficient mice (specifically in T cells) with helminthes. Following infection, cytokine response and worm burden could be assessed to establish the role of STAT3 in clearance. Additionally, STAT6VT transgenic mice have increased Th2 development and we hypothesize that infection with helminthes will be cleared as or more efficient as wild type mice. We could also use this model to determine the requirement of STAT3 by using STAT6VTx*Stat3*$^{CD4-/-}$.

Testing the ability of STAT3 inhibitors to suppress allergic disease

Data from our lab has shown that STAT3-deficient mice have reduced allergic inflammation compared to wild type controls (Sehra, unpublished data). Currently there are at least 2 patented STAT3 inhibitors developed for the treatment of different inflammatory diseases. It would be important to test the ability of STAT3 inhibitors to suppress the development of asthma or treat established allergic inflammation. The inhibitors can be given before the induction of asthma or during intranasal challenges of OVA sensitized mice. It is important to consider that STAT3 is expressed in other cell types in addition to T cells. Moreover, it has been shown that STAT3 is required in airway epithelial cells for the induction of allergic inflammation (Simeone-Penney et al., 2007). Conversely, in some models of allergic inflammation the expression of STAT3 is reduced in sensitized mice compared to saline controls. The reduced STAT3 expression correlated with increased SOCS3, a known negative regulator of STAT3 (Paul et al., 2009). It will be important to determine the activation of STAT3 in different cell types throughout the induction of allergic inflammation. Additionally, the requirement of STAT3 can be determined using the STAT3 inhibitors. Elucidating the role of STAT3 in allergic inflammation is important before the use of STAT3 inhibitors to treat allergic inflammation can be considered.

Determining if ectopic expression of multiple transcription factors can rescue the STAT3-deficient Th2 phenotype

STAT3-deficient Th2 cells have reduced expression of Th2 transcription factors *Gata3*, *c-Maf*, and *Irf4*. Ectopic expression of *Gata3*, *c-Maf*, or *Irf4* in STAT3-deficent Th2 cells does not rescue Th2 cytokine production. However, it is possible that several of the Th2 transcription factors are required for optimal Th2 development. To test this directly, STAT3-deficient Th2 cells could be transduced with different combinations of the Th2 transcription factors or all three. If Th2 cytokine production is not rescued in STAT3-deficient Th2 cells transduced with all 3 transcription factors it is possible that other STAT3 dependent factors are required for Th2 development.

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CURRICULUM VITAE

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Education

2005	**B.S.** Biological Sciences, Concordia College	Moorhead, MN
2010	**Ph.D.** Department of Microbiology and Immunology, Indiana University	Indianapolis, IN

Honors, Awards, and Fellowships

2004	Tri Beta Biology National Honor Society Member
2006	Awarded fellowship on Immunology and Infectious Disease Training Grant (T32 AI 060519)
2008	University Travel Fellowship, IUPUI
2009	Keystone Symposia Scholarship
2009	Harold Raidt Graduate Student Teaching Award
2010	Awarded 3rd place in Sigma Xi Graduate Research Competition, IUPUI

Abstracts Presented and Conferences Attended

2006	GL Stritesky, AN Mathur and MH Kaplan. IL-23 induces IL-17 Production from specific T cell subsets. Autumn Immunology Conference.
2007	GL Stritesky, AN Mathur and MH Kaplan. IL-23 enhances the maintenance of IL-17 secretion fromTGFβ+IL-6 primed cells. International Congress of Immunology. Selected for an oral presentation.
2007	GL Stritesky, AN Mathur and MH Kaplan. Defining the role of IL-23 in the Th17 phenotype. Autumn Immunology Conference.
2008	GL Stritesky, N Yeh and MH Kaplan. Determining Th17 lineage commitment. American Association of Immunologists.
2008	GL Stritesky and MH Kaplan. Defining the role of STAT3 in Th2 cell differentiation and function. Autumn Immunology Conference.
2009	GL Stritesky, N Yeh and MH Kaplan. IL-23 promotes maintenance but not commitment to the Th17 lineage. Keystone Symposia: TH17 Cells in Health and Disease.
2010	GL Stritesky, S Sehra and MH Kaplan. STAT3 in T cells is required for the differentiation of Th2 cells and the development of allergic inflammation. Federation of European Biochemical Societies Special Meeting - Jak-Stat Signaling: from Basics to Disease.

Peer Reviewed Publications

2007	Mathur AN, Chang HC, Zisoulis DG, Stritesky GL, Yu Q, O'Malley JT, Kapur R, Levy DE, Kansas GS & Kaplan MH. Stat3 and Stat4

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2010 Chang HC, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, Jabeen R, McKinley C, Ahyi AN, Han L, Nguyen ET, Robertson MJ, Perumal NB, Tepper RS, Nutt SL, Kaplan MH. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. Nat Immunol. 11(6):527-34.

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