

The Transcription Factor STAT3 Is Required for T Helper 2 Cell Development

Gretta L. Stritesky,^{1,2} Rajarajeswari Muthukrishnan,¹ Sarita Sehra,¹ Ritobrata Goswami,^{1,2} Duy Pham,^{1,2} Jared Travers,¹ Evelyn T. Nguyen,¹ David E. Levy,³ and Mark H. Kaplan^{1,2,*}

¹Department of Pediatrics, Herman B Wells Center for Pediatric Research

²Department of Microbiology and Immunology

Indiana University School of Medicine, Indianapolis, IN 46202, USA

³Department of Pathology and Microbiology, 550 First Avenue MSB548, New York University School of Medicine, New York, NY 10016, USA

*Correspondence: mkaplan2@iupui.edu

DOI 10.1016/j.immuni.2010.12.013

SUMMARY

Signal transducer and activator of transcription (STAT) family members direct the differentiation of T helper cells, with specific STAT proteins promoting distinct effector subsets. STAT6 is required for the development of T helper 2 (Th2) cells, whereas STAT3 promotes differentiation of Th17 and follicular helper T cell subsets. We demonstrated that STAT3 was also activated during Th2 cell development and was required for the expression of Th2 cell-associated cytokines and transcription factors. STAT3 bound directly to Th2 cell-associated gene loci and was required for the ability of STAT6 to bind target genes. In vivo, STAT3 deficiency in T cells eliminated the allergic inflammation in mice sensitized and challenged with ovalbumin or transgenic for constitutively active STAT6. Thus, STAT3 cooperates with STAT6 in promoting Th2 cell development. These results demonstrate that differentiating T helper cells integrate multiple STAT protein signals during Th2 cell development.

INTRODUCTION

CD4⁺ T helper cells differentiate into several distinct subsets to provide host protection against a variety of pathogens. Each T helper cell lineage expresses specific transcription factors and cytokines that confer specific effector functions. T helper 2 (Th2) cells secrete interleukin (IL)-4, IL-5, and IL-13, which are important for immunity against extracellular parasites and for providing B cell help leading to antibody production. However, nonprotective Th2 cell responses contribute to asthma, allergy, and other allergic diseases (Zhu and Paul, 2008).

The differentiation of Th2 cells occurs when a naive T cell is activated by antigen in the presence of IL-4. The IL-4 receptor is composed of the common cytokine receptor gamma subunit and the IL-4R α chain. IL-4R is expressed at low levels on the surface of naive CD4⁺ T cells, but upon antigen stimulation IL-4R surface expression is increased. IL-4 binding to the IL-4R results in recruitment of signal transducer and activator

of transcription 6 (STAT6). STAT6 then becomes phosphorylated, dimerizes, and translocates to the nucleus where it can activate transcription of Th2 cell-specific genes (Nelms et al., 1999).

There are several transcription factors associated with establishing the Th2 cell phenotype. STAT6 induces expression of GATA3, which is considered the Th2 cell master regulator (Ouyang et al., 2000). Other transcription factors important for Th2 cell development are induced upon TCR stimulation including NFAT family members, IRF4, Jun family members, and c-MAF (Ansel et al., 2006; Lee et al., 2006; Murphy and Reiner, 2002). More recently, BATF was suggested to play a role in promoting the Th2 cell phenotype (Betz et al., 2010). The expression of each transcription factor is necessary for optimal Th2 cell development.

Though particular cytokines are thought to be promoters of specific Th cell lineages, a differentiating Th cell receives multiple cytokine signals. IL-4 and STAT6 clearly play a dominant role in Th2 cell differentiation. 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). Development of allergic disease is completely dependent on IL-4 as shown by the fact that 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). However, IL-2 and STAT5 are also necessary for optimal Th2 cell differentiation through direct effects on Th2 cell cytokines and *Il4ra* expression (Ben-Sasson et al., 1990; Cote-Sierra et al., 2004; Kagami et al., 2001; Liao et al., 2008; Zhu et al., 2003). Moreover, although STAT3 is required for the differentiation and effector function of both Th17 and Tfh cells, IL-6-activated STAT3 promotes *Maf* expression, a factor required for IL-4 expression in Th2 cells (Mathur et al., 2007; Nurieva et al., 2008; Yang et al., 2005, 2007). STAT3 directly binds the *Maf* and *Batf* loci (Durant et al., 2010). IL-6 also induces SOCS1 and NFATc2 that decrease Th1 cell differentiation and increase IL-4 production, respectively, during Th2 cell development (Diehl et al., 2000, 2002). However, the requirement for STAT3 in Th2 cell development has not been defined.

In this report we demonstrate that STAT3 is required for Th2 cytokine production and transcription factor expression. STAT3 was activated throughout Th2 cell differentiation and when ectopically expressed with STAT6 could augment Th2

cell cytokine production. STAT3 was also required for Th2 cell-mediated allergic inflammation. Thus, in the presence of activated STAT6, STAT3 promotes optimal Th2 cell differentiation and cytokine production.

RESULTS

STAT3 Is Activated during Th2 Cell Differentiation

STAT6 activation is critical in Th2 cell differentiation. Though several cytokines important in Th2 cell differentiation and cytokine production signal through STAT3, the activation of STAT3 during Th2 cell development has not been carefully examined. To define STAT3 activation throughout Th2 cell differentiation, wild-type and STAT3-deficient Th2 cells were assessed for intracellular phospho-STAT3 and phospho-STAT6 each day during Th2 cell differentiation. Wild-type Th2 cells were nearly all phospho-STAT6 positive early on in differentiation and remained phospho-STAT6 positive throughout differentiation (Figures 1A and 1B). Wild-type Th2 cells also demonstrated a high percentage of phospho-STAT3-positive cells throughout Th2 cell differentiation. STAT3 phosphorylation occurred early in differentiation, peaked at 48 hr, and decreased by 72 hr (Figures 1A and 1B). There was a second peak of STAT3 phosphorylation after addition of further cytokines at 72 hr (Figures 1A and 1B). The initial induction of STAT3 phosphorylation was independent of IL-4 signaling because it was identical between wild-type and STAT6-deficient cells during the first 3 days of Th2 cell cultures (Figure S1B available online). However, STAT6-deficient Th2 cells displayed reduced phospho-STAT3 during the last 2 days of differentiation, suggesting that genes downstream of STAT6 were at least partially responsible for maintaining STAT3 phosphorylation (Figure S1B). Similar patterns of pSTAT6 and pSTAT3 are observed in Th2 cell cultures of DO11.10 TCR transgenic T cells stimulated with anti-CD3 or ovalbumin (Figures 1C and 1D), although with antigen stimulation, pSTAT6 rose more slowly at culture initiation, and pSTAT3 decreased more dramatically at the end of culture. Taken together these data show that STAT3 becomes phosphorylated during Th2 cell differentiation.

STAT3-Deficient Cells Are Defective in Th2 Cell Differentiation

To determine which cytokines were activating STAT3 during Th2 cell differentiation, we cultured Th2 cells in the presence of antibodies to cytokines known to activate STAT3. A combination of anti-IL-2 and anti-CD25 decreased Th2 cytokine production coincident with a decrease in pSTAT5 (Figure 2A and data not shown), similar to previous results (Cote-Sierra et al., 2004). Antibodies to IL-6 or IL-21 decreased IL-4 and IL-13 production, although they had no effect on IL-5 production (Figure 2A). Although the individual antibodies did not have a substantial effect on pSTAT3, a combination of antibodies to IL-2, CD25, IL-6, and IL-21 decreased pSTAT3, as well as pSTAT5, without affecting pSTAT6 (Figure 2B and data not shown). Thus, several cytokines play redundant roles in the activation of STAT3 during Th2 cell differentiation.

To specifically define whether the activation of STAT3 during Th2 cell 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, proliferation, and apoptosis of STAT3-deficient Th2 cells were not obviously different from wild-type cultures (Figures S2A–S2C). Importantly, STAT6 phosphorylation was not dependent on STAT3; a similar pattern was observed in STAT3-deficient cultures (Figures 1A and 1B). To examine differentiation, naive CD4⁺ T cells were isolated from spleens of wild-type and *Stat3^{Cd4-/-}* mice and cultured under Th1, Th2, or Th17 cell conditions. STAT3-deficient Th1 cells produced similar amounts of the cytokines IFN- γ and GM-CSF as did wild-type Th1 cells, although STAT3 was required for the generation of cells secreting IL-17A and IL-17F (Figure S2D). Although wild-type Th2 cells secreted high amounts of IL-4, IL-5, and IL-13, STAT3-deficient CD4⁺ T cells cultured under Th2 cell-skewing conditions had diminished IL-4, IL-5, and IL-13 production and gene expression and also diminished expression of the gene encoding the Th2 cell-associated cytokine IL-24 (Figures 2C–2E). STAT3-deficient Th2 cells had only a modest increase in IFN- γ production, suggesting that they were not differentiating into Th1 cells and did not acquire expression of *Foxp3* mRNA (Figure S2E and data not shown).

STAT3 Is Required for Th2 Cell Transcription Factor Expression

Previous studies have demonstrated that STAT3-deficient CD4⁺ cells have reduced CD25 expression (Akaishi et al., 1998) and IL-2 signaling is required for Th2 cell differentiation at multiple levels including the expression of *Il4ra* (Liao et al., 2008). To determine whether CD25 or IL-4R α expression was decreased on STAT3-deficient cells during Th2 cell differentiation, we examined surface expression throughout differentiation. The percent of receptor-positive cells was comparable between wild-type and STAT3-deficient Th2 cells (Figure S3), and although the MFI of CD25 was decreased on the fourth day of differentiation, adding exogenous IL-2 during differentiation did not rescue Th2 cytokine production in STAT3-deficient cultures (data not shown). Normal IL-4R α expression in STAT3-deficient Th2 cell cultures was consistent with normal STAT6 activation in the absence of STAT3 (Figures 1A and 1B). These data suggest that the reduction in Th2 cytokine production in STAT3-deficient Th2 cells is not due to reduced CD25 or IL-4R α expression.

We then examined the expression of transcription factors associated with establishing the Th2 cell phenotype including *Gata3*, *Batf*, *Maf*, *Gfi1*, *Irf4*, and the IL-6 target *Socs1* during the differentiation process. Expression of *Gata3* was decreased throughout differentiation; *Maf* and *Batf* expression were more affected during the last 3 days of differentiation (Figure 3A). GATA3 protein was also decreased 24 hr after the initiation of culture, but endogenous IL-2 or IL-4 production was not affected by STAT3 deficiency at this time point (Figures S4A and S4B and data not shown). *Gfi1* and *Irf4* expression were less affected by STAT3 deficiency, and *Socs1* expression was decreased only during the last 3 days of differentiation (Figure S4C), agreeing with our data that IFN- γ was not induced in STAT3-deficient Th2 cell cultures (Diehl et al., 2000). The expression of *Gata3*,

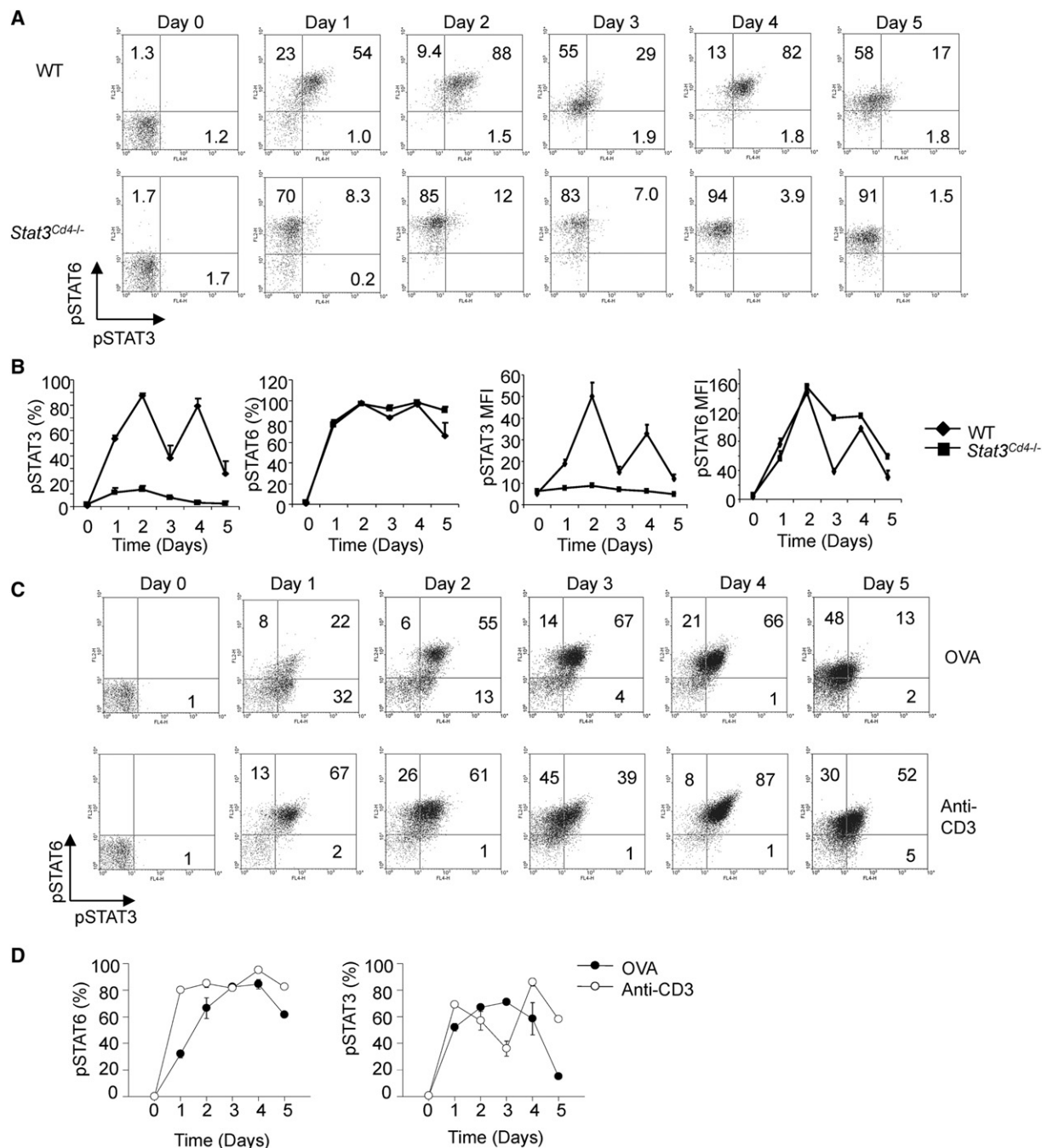


Figure 1. STAT3 Is Activated during Th2 Cell Differentiation

(A) Wild-type (WT) and *Stat3^{Cd4-/-}* naive CD4⁺ T cells were activated with anti-CD3 and anti-CD28 and cultured in IL-4 and anti-IFN- γ (Th2 cell conditions). Each day during differentiation, cells were stained for intracellular phospho-STAT3 and phospho-STAT6. Numbers in flow cytometry dot plots indicate the percentages of cells in each quadrant.

(B) Graphical representation of percent phospho-STAT-positive cells and mean fluorescent intensity (mean $\pm$ SD). Data are an average of two mice and are representative of two to three experiments.

(C) Naive DO11.10 TCR transgenic T cells were stimulated with anti-CD3 or ovalbumin (OVA) in the presence of antigen-presenting cells and cultured under Th2 cell conditions. Each day during differentiation, cells were stained for intracellular phospho-STAT3 and phospho-STAT6. Numbers in flow cytometry dot plots indicate the percentages of cells in each quadrant.

(D) Graphical representation of average percent phospho-STAT-positive cells for DO11.10 TCR transgenic T cells stimulated with OVA or anti-CD3 (mean $\pm$ SD). Data are an average of two mice and are representative of two experiments.

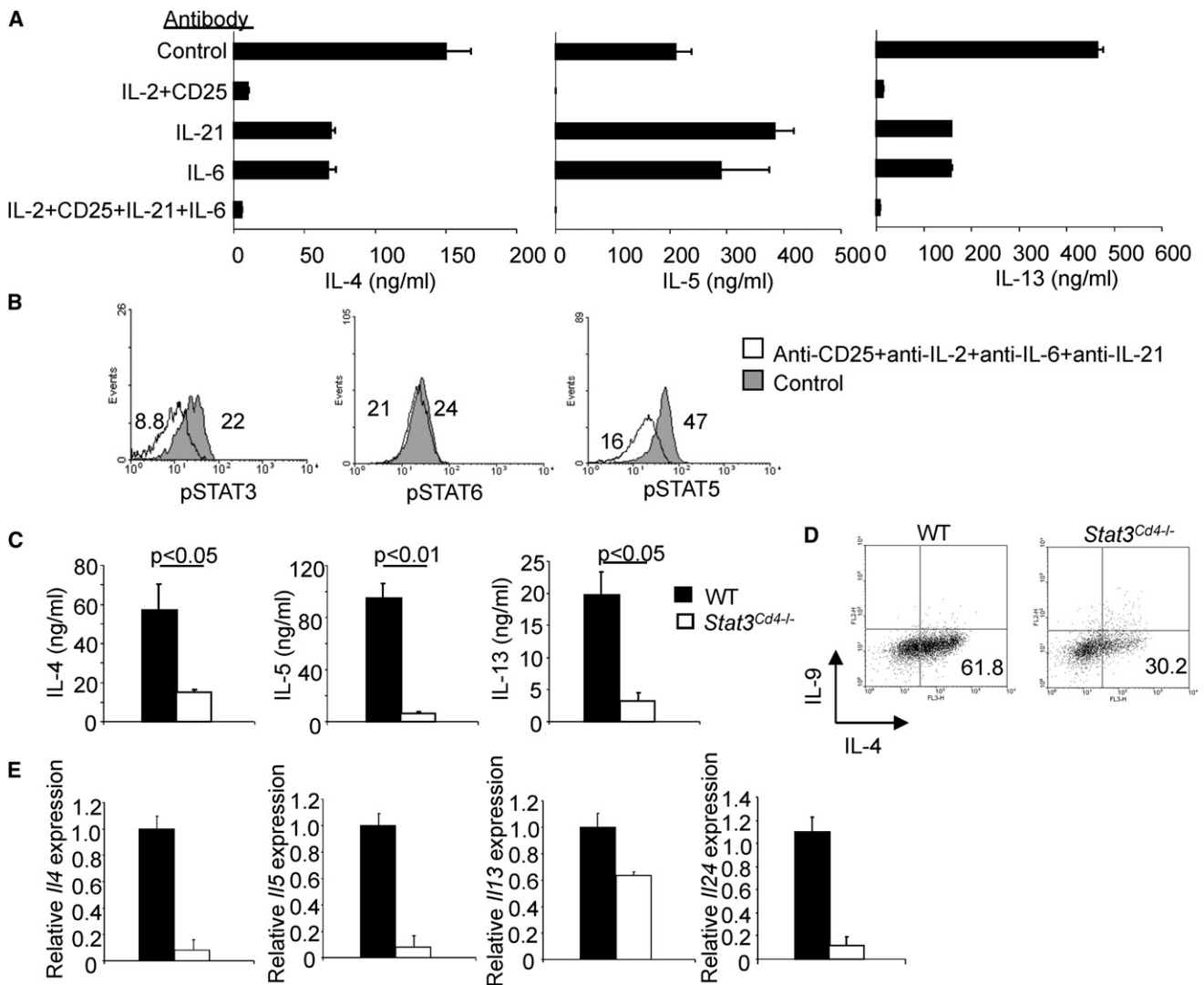


Figure 2. Reduced Th2 Cytokine Production in the Absence of STAT3

(A) WT naive CD4⁺ T cells were activated with anti-CD3 and anti-CD28 and cultured under Th2 cell conditions, in the presence or absence of antibodies to the indicated cytokines and receptors (20 μ g/ml per antibody) for 5 days before stimulation with anti-CD3. 24 hr after stimulation supernatants were tested for cytokine concentration via ELISA (mean $\pm$ SD). Results are representative of experiments with four mice.

(B) Cells cultured as in (A) with control antibody or the combination of antibodies indicated were tested for amount of pSTAT by intracellular staining on day 4 of Th2 cell culture. Numbers represent mean fluorescence intensity. Results are representative of experiments with four mice.

(C) WT and *Stat3^{Cd4-/-}* naive CD4⁺ T cells were activated with anti-CD3 and anti-CD28 and cultured with IL-4 + anti-IFN- γ (Th2 cell conditions). After 5 days of differentiation, cells were collected and counted. Differentiated cells (1×10^6) were then restimulated with anti-CD3 (4 μ g/ml) for 24 hr. Cell-free supernatant was collected and tested for various cytokines via ELISA. Data are the mean $\pm$ SD of results from two mice and representative of more than five experiments. Student's t test was performed to calculate p values.

(D) Cells activated and cultured as in (A) were restimulated with anti-CD3 for 5 hr. After stimulation, cells were stained with anti-IL-4 and anti-IL-9. Numbers in flow cytometry dot plots indicate the percentages of cells in the quadrant.

(E) After restimulation of differentiated Th2 cells with anti-CD3 and recovery of supernatants as described in (A), cell pellets were collected, RNA was isolated, and quantitative PCR was performed for the indicated cytokines (mean $\pm$ SD). Data are representative of two experiments.

Maf, *Batf*, and *Irf4* is also decreased at day 5 of differentiation (Figure 3A; Figure S4D). The loss of *Gata3* and *Maf* expression in STAT6-deficient Th2 cells was greater than in STAT3-deficient Th2 cells. However, STAT3 but not STAT6 was required for optimal *Irf4* expression (Figures S4D and S1C).

To further define the effects of STAT3 deficiency on the loci most affected, we examined the presence of histone modifica-

tions trimethyl-H3K4 and -H3K36 that are associated with active genes, and trimethyl-H3K27, which is associated with repressed genes, at the *Gata3*, *Batf*, and *Maf* loci. In naive cells, there was either an increased or unchanged amount of trimethyl-H3K4 and -H3K36, suggesting that STAT3 was not required for these modifications in unstimulated cells (Figure 3B). In contrast, there was a marked increase in H3K27

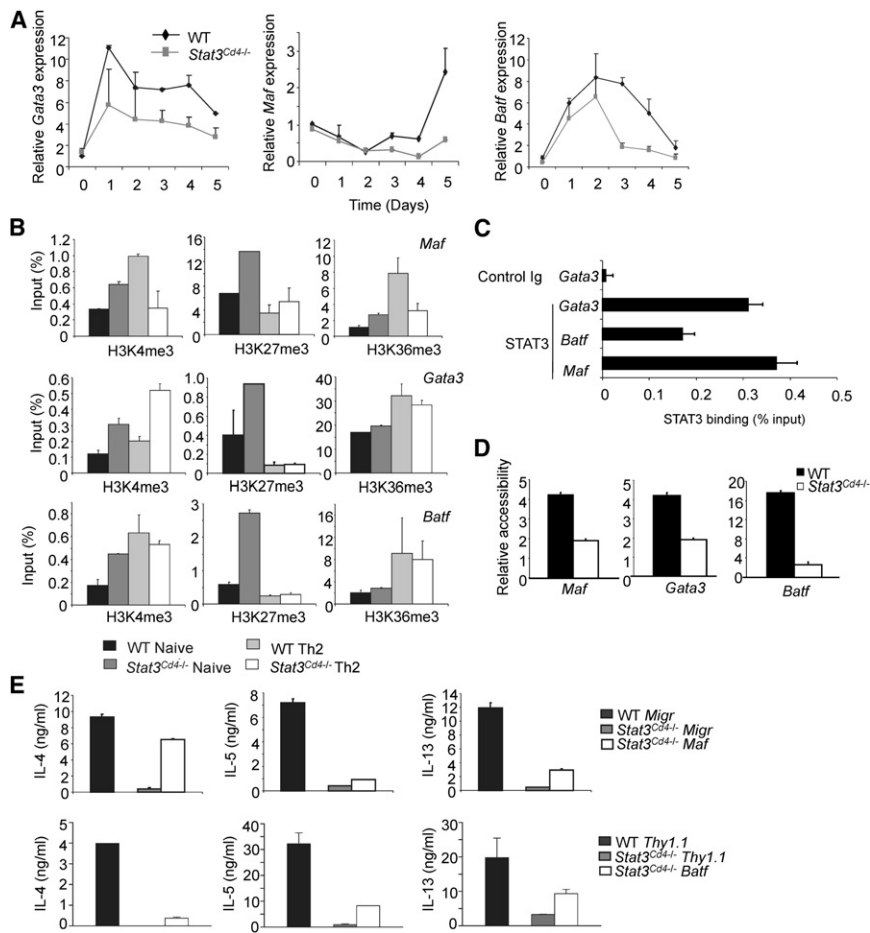


Figure 3. Reduced Th2 Cell-Specific Transcription Factor Expression in the Absence of STAT3

(A) WT and *Stat3*^{Cd4-/-} naive CD4⁺ T cells were differentiated under Th2 cell conditions and cells were isolated each day during differentiation. RNA isolated from cells was analyzed for gene expression with qPCR (mean $\pm$ SD). Data are representative of experiments with four mice.

(B) WT and *Stat3*^{Cd4-/-} naive CD4⁺ T cells were used directly or differentiated as in (A) for 5 days. Chromatin immunoprecipitation was performed for the indicated histone modifications and qPCR was used to determine the amounts of each modification (mean $\pm$ SD).

(C) Chromatin immunoprecipitation from WT naive CD4⁺ T cells with control or STAT3 antibodies followed by qPCR for detection of the indicated loci (mean $\pm$ SD).

(D) WT and *Stat3*^{Cd4-/-} naive CD4⁺ T cells were differentiated as in (A) for 5 days. Nuclei were isolated and left untreated or treated with micrococcal nuclease for 10 min. Relative accessibility is defined as 2 to the power of the difference between the Ct value of qPCR of untreated and treated samples (mean $\pm$ SD).

(E) WT and *Stat3*^{Cd4-/-} naive CD4⁺ T cells were differentiated as in (A). On day 2, cells were transduced with control, *Batf*-, or *Maf*-expressing retrovirus. Sorted transduced cells were restimulated with anti-CD3 for 24 hr. Supernatant was tested for Th2 cytokines with ELISA (mean $\pm$ SD). Data are representative of two independent experiments.

trimethylation in naive STAT3-deficient T cells (Figure 3B), suggesting that STAT3 plays a role in preventing this modification, and correlating with STAT3 bound to these loci in naive cells (Figure 3C). In contrast, differences in H3K27 methylation between wild-type and STAT3-deficient Th2 cells were not observed. The *Gata3* and *Batf* loci did not have decreased H3K4 and H3K36 methylation in STAT3-deficient Th2 cells compared to wild-type Th2 cells. However, activating histone modifications were decreased at the *Maf* locus (Figure 3B). To determine whether STAT3 was also affecting the accessibility of chromatin, we performed micrococcal nuclease assays with nuclei from wild-type and STAT3-deficient Th2 cells. We observed decreased accessibility at all three loci, with the greatest difference at the *Batf* locus (Figure 3D).

We then tested whether transduction of any of these factors into STAT3-deficient Th2 cell cultures would result in a recovery of cytokine production. Although transduction of *Gata3* into STAT3-deficient Th2 cells did not alter cytokine production (data not shown), transduction of either *Maf* or *Batf* resulted in a partial recovery of Th2 cytokine production, with *Maf* having the greatest effects on IL-4 production (Figure 3E). Thus, *Maf* and *Batf* probably represent STAT3 targets during Th2 cell development, and the defects in Th2 cytokine production in the absence of STAT3 are the result of effects on multiple downstream transcription factors.

STAT3 and STAT6 Cooperate in Promoting Th2 Cell Cytokine Production

To determine whether STAT6 and STAT3 are cooperating to promote Th2 cytokine production, we used two 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 (Bromberg et al., 1999; Bruns et al., 2003). We first transduced STAT3C into naive CD4⁺ T cells activated under non-skewing conditions (anti-IFN- γ), which has previously been shown to increase IL-17 production (Mathur et al., 2007). Under non-skewing conditions, transduction of STAT3C induced the production of Th2 cytokines (Figure 4A).

To directly test whether the combination of constitutively active STAT6 and STAT3 could increase Th2 cytokine production compared to constitutively STAT6 alone, naive T cells cultured under Th1 cell conditions were transduced with retroviruses expressing STAT6VT and STAT3C alone or in combination. Under these conditions, STAT3C alone did not increase IL-4 production, had modest effects on IFN- γ production, and did not induce IL-17 production (Figure 4B and data not shown). Transduction of STAT6VT increased IL-4 production and decreased IFN- γ , and cotransduction of STAT3C further increased the amount of IL-4 produced in these cultures (Figure 4B).

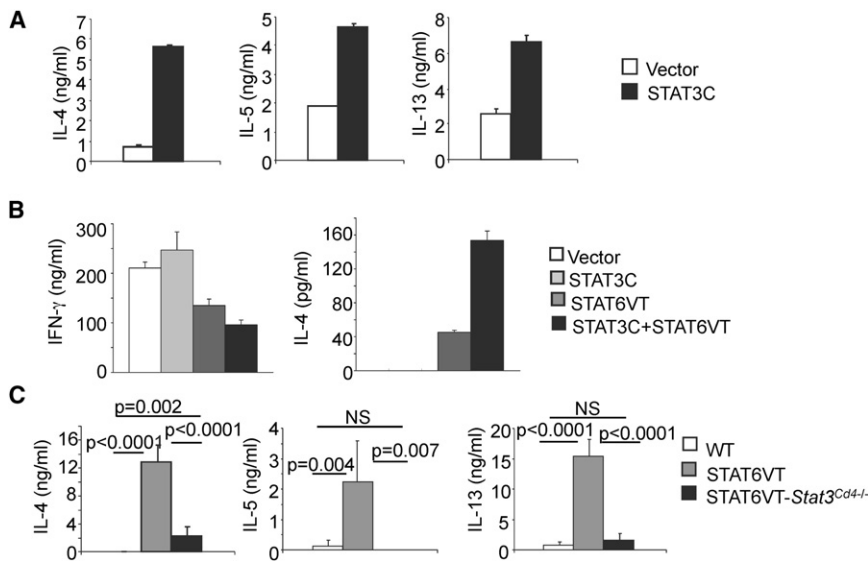


Figure 4. STAT3 and STAT6 Cooperate in Promoting Th2 Cytokine Production

(A) Naive CD4⁺ T cells were activated for 48 hr in the absence of skewing cytokines before being transduced with control or STAT3C-expressing retrovirus. After 5 days in culture, cells were sorted and restimulated with anti-CD3 (4 μg/ml) for 24 hr before cell-free supernatant was tested for cytokines via ELISA (mean ± SD).

(B) Naive CD4⁺ T cells were activated for 48 hr under Th1 cell conditions (5 ng/ml IL-12 + 10 μg/ml anti-IL-4) before being transduced with control, STAT6VT-expressing, STAT3-expressing, or both retroviruses. After 5 days in culture, cells were sorted and restimulated with anti-CD3 (4 μg/ml) for 24 hr before cell-free supernatant was tested for cytokines with ELISA (mean ± SD).

(C) CD4⁺ cells were isolated from WT, STAT6VT, and STAT6VT-Stat3Cd4-/- mice. Cells were then restimulated with anti-CD3 for 24 hr. Cell-free supernatant was collected and tested for various cytokines via ELISA. Data are representative of two experiments (average of 2–6 mice ± SD). Student's t test was performed to calculate p values.

We then wanted to confirm the requirement for STAT3 for optimal Th2 cell differentiation *in vivo* in the presence of a constitutively active STAT6. Peripheral T cells in STAT6VT transgenic mice have an increased propensity toward a Th2 cytokine-secreting phenotype (Bruns et al., 2003; Sehra et al., 2008). To directly test whether 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 hr before cytokine production was assessed with 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 4C). These results demonstrate that STAT3 cooperates with STAT6 to promote Th2 cytokine production.

STAT3 Binds Th2 Cell-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 via chromatin immunoprecipitation. In Th2 cells, similar to binding in naive T cells (Figure 3C), STAT3 directly binds a number of the same loci bound in Th17 cells, including *Maf*, *Batf*, and *Irf4*, which also contribute to Th17 cell development (Bauquet et al., 2009; Betz et al., 2010; Brustle et al., 2007; Schraml et al., 2009). STAT3 was also bound to *Gata3* and *Il21*, a cytokine produced by multiple Th cell subsets, but was more prominent at the *Il17* and *Il17f* loci in Th17 cells than in other Th cell subsets (Figure 5A).

We then tested whether STAT3 has an effect on STAT6 binding to target genes. In Th2 cells, STAT6 binds to the *Gata3*, *Maf*, *Batf*, and *Irf4* genes (Figure 5B). However, in the absence of STAT3, STAT6 binding was either undetectable or greatly diminished (Figure 5B). This was concurrent with decreased locus accessibility in the absence of STAT3 (Fig-

ure 3D) and suggests that STAT3 is required to allow access for STAT6 to bind these loci and increase gene expression.

We then tested whether an active STAT6 was capable of inducing expression of Th2 cell 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 from wild-type mice. 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 5C). Together, these data suggest that STAT3 facilitates the ability of STAT6 to bind target genes and promote the Th2 cell genetic program.

STAT3 in T Cells Is Required for the Development of Allergic Inflammation

To test whether STAT3 is also required for *in vivo* Th2 cell differentiation, wild-type and Stat3^{Cd4-/-} mice were sensitized with alum-adsorbed OVA. Two weeks after the second immunization, and after intranasal challenges, we observed that pulmonary inflammation, assessed by total cell number and by number of eosinophils in the bronchoalveolar lavage (BAL), was decreased in Stat3^{Cd4-/-} mice, compared to wild-type mice (Figure 6A). Amounts of Th17 and Th2 cytokines were decreased in the BAL fluid and in cultures of splenocytes stimulated *in vitro* with OVA analyzed via ELISA (Figures 6B and 6C). Although Th2 cell immunity is clearly decreased *in vivo*, the simultaneous requirement for Th17 cells in this model (Lewkowich et al., 2005; Schnyder-Candrian et al., 2006) complicates the interpretation of the requirement for STAT3-dependent Th2 cell-mediated inflammation *in vivo*.

To investigate the requirement for STAT3 in allergic inflammation further, we used mice expressing STAT6VT in T cells that spontaneously develop multiorgan allergic inflammation, including pulmonary inflammation, blepharitis, and skin inflammation, all of which are completely dependent on IL-4 (Sehra et al., 2008, 2010). The incidence of blepharitis in STAT6VT

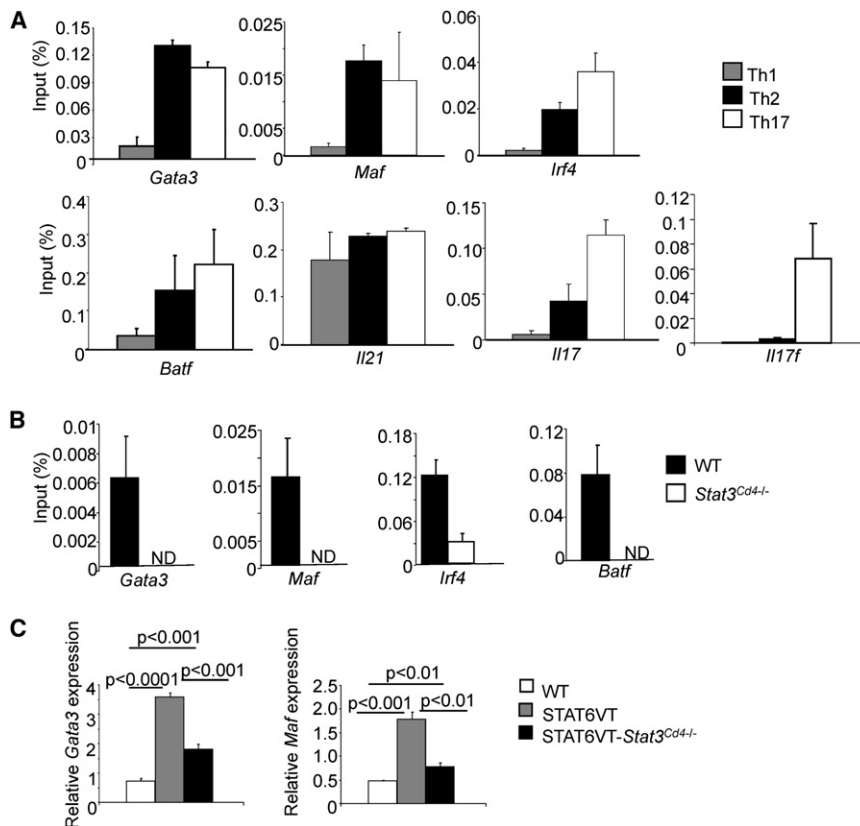


Figure 5. STAT3 Binds Th2 Cell-Associated Gene Loci and Defines the STAT6 Binding Pattern

(A) Naive CD4⁺ T cells activated with anti-CD3 and anti-CD28 and cultured under Th1, Th2, or Th17 cell conditions for 3 days were used for ChIP performed with normal rabbit IgG or anti-STAT3 before qPCR was performed for the indicated genes. Data are expressed as percent input $\pm$ SD and control Ig background values are subtracted from the values indicated. Data are representative of three to four experiments with similar results.

(B) Naive CD4⁺ T cells from WT and Stat3^{Cd4-/-} activated with anti-CD3 and anti-CD28 and cultured under Th2 cell conditions for 3 days were used for ChIP performed with normal rabbit IgG or anti-STAT6 and qPCR was performed for the indicated genes. Data are expressed as percent input $\pm$ SD and control Ig background values are subtracted from the values indicated. Data are representative of three to four experiments with similar results. ND, not detected.

(C) CD4⁺ cells were isolated from WT, STAT6VT, and STAT6VT-Stat3^{Cd4-/-} mice. Cells were then restimulated with anti-CD3 for 6 hr before RNA was isolated and quantitative PCR was performed. Data are representative of two experiments (average of 2–6 mice $\pm$ SD). Student's t test was performed to calculate p values.

mice is nearly 75% and is never observed in wild-type mice. STAT6VT-Stat3^{Cd4-/-} mice were protected from blepharitis and have 0% incidence even in older mice (Figure 6D). Approximately 40% of STAT6VT transgenic mice developed skin inflammation resembling atopic dermatitis, a condition not observed in wild-type mice. As with blepharitis, STAT6VT transgenic mice lacking STAT3 in T cells were protected from skin inflammation, thickening of the skin, and immune infiltration (Figures 6D and 6F). STAT6VT transgenic mice develop lung inflammation characterized by peri-bronchial and peri-arterial accumulation of eosinophils and lymphocytes. However, STAT6VT-Stat3^{Cd4-/-} mice, like wild-type mice, had very few eosinophils infiltrating the lungs (Figures 6E and 6G). These results demonstrate that the loss of STAT3 in T cells protects mice from the development of Th2 cell-mediated inflammatory diseases. Taken together, STAT3 and STAT6 proteins are both necessary for optimal Th2 cell development, and in the context of the STAT6 signal, STAT3 enhances Th2 cell development.

DISCUSSION

The paradigm that STAT family members promoted specific Th effector cell phenotypes was developed when the number of known effector subsets was more limited. We and other researchers initially defined that STAT4 is required for Th1 cell development, and STAT6 is required for Th2 cell development (Kaplan et al., 1996a, 1996b; Shimoda et al., 1996; Takeda et al., 1996; Thierfelder et al., 1996). However, this simple one

STAT-one subset paradigm became more complicated when it was shown that STAT1 also contributed to Th1 cell 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., 2005; Zhu et al., 2003). This was an important finding because STAT5, which is also 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 our understanding of the integration of STAT signals by demonstrating that STAT3, which clearly promotes Th17 cell development in the absence of signals that promote other phenotypes, is required for the function of STAT6 during Th2 cell development.

Based on our data, we propose the following model of Th2 cell development. STAT3 is bound to multiple Th2 cell-associated transcription factor loci even in naive cells, which limits repressive histone modifications. Upon activation in a pro-Th2 cell environment, STAT3 is activated by multiple cytokines promoting chromatin remodeling and allowing STAT6 to bind and activate target genes. STAT3 also has direct effects on histone modifications at the *Maf* locus. Interestingly, we find that H3K4 methylation is STAT3 dependent at the *Maf* but not the *Batf* locus, whereas in Th17 cells the opposite pattern was observed (Durant et al., 2010). The similarity in STAT3 target genes in Th2 and Th17 cells suggests that STAT3 is able to initiate differentiation to both phenotypes, and it is the presence

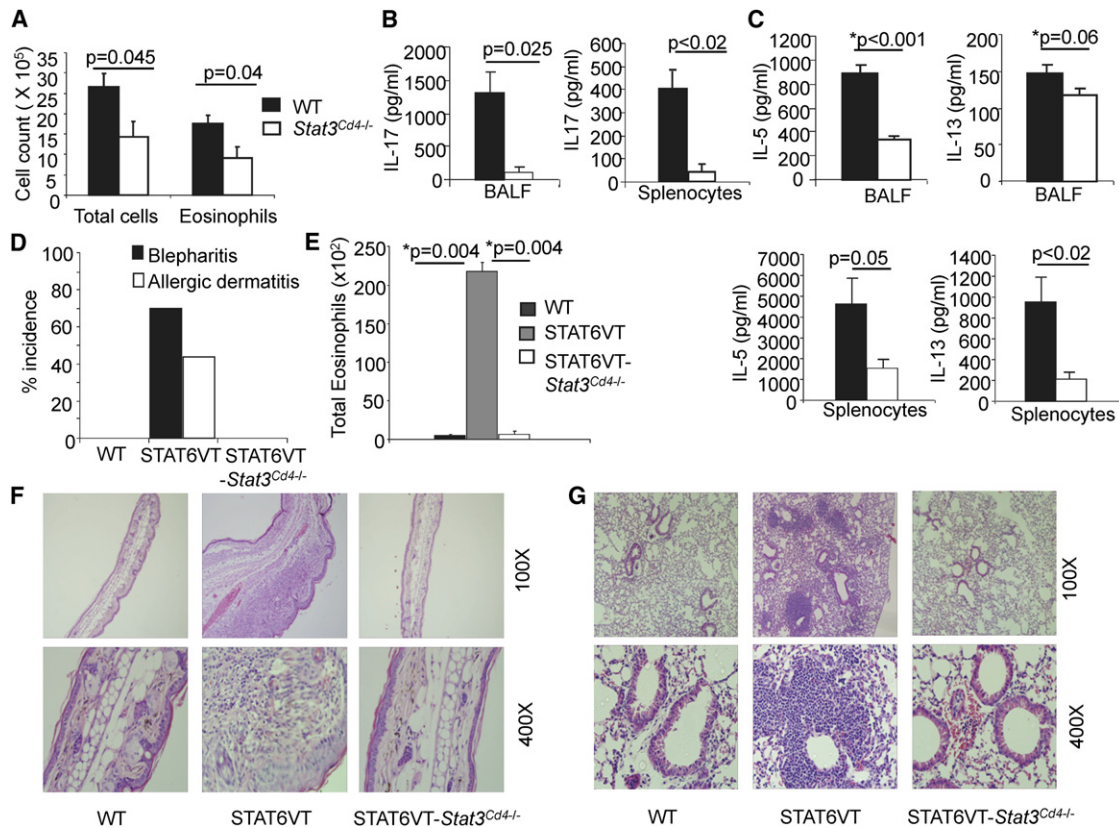


Figure 6. STAT3 Promotes the Development of Allergic Inflammation

(A–C) WT and *Stat3*^{Cd4-/-} mice were immunized with OVA and Alum on days 0 and 7 and challenged as described in Experimental Procedures. After challenges, BAL cell numbers were determined by cell counting and flow cytometry (A), and cytokine levels were measured in BAL fluid and in culture supernatants from splenocytes stimulated with OVA for 72 hr via ELISA. Data are average of 5–6 mice per group $\pm$ SEM.

(D) Incidence of blepharitis and atopic dermatitis of WT, *Stat3*^{Cd4-/-}, and *Stat3*^{Cd4-/-} + IL-4 mice are shown. Incidence was determined by visual examination of mice (n = 25 per group).

(E) Numbers of eosinophils (defined by flow cytometry) recovered in BAL. BAL data are representative of two independent experiments and shown as the average of two mice per group $\pm$ SD. For (A)–(C) and (E), Student's t test was performed to calculate p values.

(F) Ear tissue from WT, *Stat3*^{Cd4-/-}, and *Stat3*^{Cd4-/-} + IL-4 mice were fixed and paraffin-embedded sections were stained with hematoxylin-eosin. Magnification is indicated in the panel and photomicrographs are representative of 10 mice per group.

(G) Lungs from WT, *Stat3*^{Cd4-/-}, and *Stat3*^{Cd4-/-} + IL-4 mice were embedded in paraffin and stained with H&E. Magnification is indicated in the panel and photomicrographs are representative of 10 mice per group.

of the IL-4/STAT6 signal that promotes Th2 cell development at the expense of the Th17 cell program. IL-4 signaling has a similar effect on Treg cell development by decreasing STAT5 binding to the Foxp3 locus (O'Malley et al., 2009) and promoting an alternative T helper cell subset. Thus, STAT6 plays a definitive role in the outcome of Th cell differentiation in the presence of IL-4.

The exact targets of STAT3 required for Th2 cell development are not entirely clear and probably several targets are important. 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 Th2 cytokine production. BATF, a transcription factor recently shown to promote Th2 cell development, also requires STAT3 for normal expression in Th2 cells, and transduction of *Batf* resulted in a partial recovery of Th2 cytokine production. 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 cell cultures is more complex than the absence of one factor and recovery of Th2 cytokine expression may require the coordinated function of several factors.

The requirement for STAT3 in Th2 cell development is in contrast to the hyper-IgE syndrome that develops in patients with dominant-negative STAT3 mutations (Ma et al., 2008; Milner et al., 2008). Although human STAT3 mutations are autosomal dominant, it is reasonable to expect that some STAT3 function is retained in these patients because, at least in mice, STAT3 deficiency is embryonic lethal (Takeda et al., 1997). Moreover, it is yet unclear how STAT3 mutants result in hyper-IgE syndrome. Like mice with STAT3-deficient T cells (Mathur et al., 2007; Yang et al., 2007), patients with hyper-IgE syndrome lack Th17 cells, though effects on Th2 cells in patients have not been clearly defined. However, mice with STAT3-deficient T cells do not have increased serum IgE (data not shown),

suggesting either that human STAT3 mutants are not functionally equivalent to STAT3 deficiency or that mutant STAT3 promotes hyper-IgE in cells other than T cells. Although mutant STAT3 in human B cells is required for IL-21-stimulated IgE production and generation of memory B and plasma cells, STAT3 deficiency in mouse B cells affects IgG responses but does not result in hyper-IgE syndrome (Avery et al., 2008, 2010; Fornek et al., 2006). The pathogenesis of hyper-IgE syndrome is clearly complex and additional mechanistic insight into STAT3-dependent functions probably requires introduction of STAT3 mutations into a mouse model.

Multiple signals contribute to the generation of differentiated T helper cell subsets. However, in this model there is a dominant signal, IL-4 in the case of Th2 cells, which 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., 2005, 2007). However, IL-4 provides a dominant signal that diminishes Th17 cell 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 cell effects of STAT3 are reduced, whereas the pro-Th2 effects of STAT6 are amplified. Mechanistically this occurs through the binding of STAT3 to Th2 cell genes that facilitate the ability of STAT6 to activate genes necessary for Th2 cell 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.

EXPERIMENTAL PROCEDURES

Mice

Stat3^{fl/fl} mice (Chiarle et al., 2005) with a *Cd4-Cre (Stat3^{Cd4-/-})* transgene and wild-type mice (Harlan Laboratories, Indianapolis, IN) were on a C57BL/6 background. WT mice in experiments with *Stat3^{Cd4-/-}* mice were Cre-negative littermates. STAT6VT (STAT6 with V547 and T548 mutated to alanine) transgenic mice, previously described (Bruns et al., 2003), were backcrossed to C57BL/6 mice (Harlan Laboratories). *Stat3^{Cd4-/-}* mice were mated to STAT6VT transgenic mice to generate STAT6VT-*Stat3^{Cd4-/-}* mice. All mice were maintained in specific-pathogen-free conditions and experiments were approved by the Indiana University Institutional Animal Care and Use Committee.

Quantitative RT-PCR

Total RNA was isolated from either unstimulated or anti-CD3 (2 μ g/ml)-restimulated cells with Trizol and reverse transcribed according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Quantitative PCR was performed with Taqman Fast Universal PCR Master Mix and commercially available primers for genes with the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). RNA was normalized to expression levels of β 2-microglobulin and relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method.

Murine T Helper Cell Differentiation

Naive CD4⁺CD62L⁺ T cells were purified from spleens via magnetic isolation (Miltenyi Biotec, Auburn, CA). Naive CD4⁺ T cells (1×10^6 cells/ml of complete RPMI-1640 medium) were cultured with plate-bound anti-CD3 (4 μ g/ml 145-2C11) and soluble anti-CD28 (1 μ g/ml; BD PharMingen) under Th2 cell (IL-4 at 10 ng/ml [PeproTech, Rocky Hill, NJ] and anti-IFN- γ at 10 μ g/ml), Th1 cell with anti-IL-4 (10 μ g/ml 11B11) and IL-12 (5 ng/ml; R&D Systems), or Th17

cell with anti-IFN- γ , anti-IL-4, TGF- β 1 (2 ng/ml; R&D Systems), and IL-6 (100 ng/ml; PeproTech). Cells were expanded on day 3 after stimulation by adding half the dose of the original cytokines in fresh medium. After 5 days of culture, differentiated cells were restimulated with plate-bound anti-CD3 at 4 μ g/ml for 1 or 3 days, and the cell-free supernatant was collected after centrifugation and stored at -20°C until use. The levels of cytokines produced were determined via ELISA (reagents from BD PharMingen or eBioscience). For cytokine-blocking experiments, Th2 cells were cultured as above in the presence of control antibody or blocking antibodies to IL-2, IL-6, IL-21, and/or CD25 (20 μ g/ml, BD PharMingen or R&D Systems) from the initiation of culture and additional antibodies added at day 3 during cell expansion. Naive CD4⁺ T cells from DO11.10 mice were stimulated with plate-bound anti-CD3 and anti-CD28, or 100 μ g/ml OVA protein and anti-CD28, and cultured with anti-IFN- γ and IL-4 and CD4-depleted irradiated APCs (1:5). On day 3 of culture, cells were expanded and an additional dose of IL-4 was added.

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 I.C. Ho) and EGFP, or STAT3C (Mathur et al., 2007) or BATF (Williams et al., 2003) subcloned into the bicistronic retroviral vector containing the marker Thy1.1 and Thy1.1 empty vector (kindly provided by S. Goenka) were used to generate virus as described (Mathur et al., 2007). After 2 days of differentiation, T helper cells were transduced with retroviral supernatant containing polybrene. On day 5, transduced cells (EGFP⁺, Thy1.1⁺, or hCD4⁺ cells) were sorted by flow cytometry before cytokine production and gene expression analyses.

Analysis of Inflammation and Th2 Cell Responses In Vivo

Immunization and challenge of mice was performed as described (Chang et al., 2010; O'Malley et al., 2009). Splenocytes from the mice were stimulated with OVA (100 μ g/ml) for 72 hr and Th2 cytokines in cell-free supernatants were assessed via ELISA. Paraffin-embedded sections were stained with hematoxylin and eosin for evaluation of the infiltration of inflammatory cells by light microscopy. BAL was performed and eosinophils in the BAL were identified as described (Chang et al., 2010; O'Malley et al., 2009).

Intracellular Staining

After differentiation, cells were restimulated with anti-CD3 (4 μ g/ml) for 5 hr. Monensin was added to the cells for the final 2 hr of stimulation. After stimulation, cells were fixed with paraformaldehyde and permeabilized with saponin. Cells were then stained with fluorochrome-conjugated anti-mouse IL-4 (BVD6-24G2; eBioscience). For intracellular phospho-STAT staining, T cells were collected and fixed with 1.5% formaldehyde for 10 min at room temperature. After fixation, cells were permeabilized for 15 min at 4°C with 100% methanol. Cells were then stained for phospho-STAT3 or phospho-STAT6 (BD PharMingen) for 30 min at room temperature. Cells were analyzed by flow cytometry with FACSCalibur (Beckton Dickinson) and results were analyzed with WinMDI.

Chromatin Immunoprecipitation

ChIP assay was performed as previously described (Yu et al., 2007). Immunoprecipitations were performed with rabbit polyclonal antibodies (anti-STAT3 sc-482, anti-Stat6 sc-1698, rabbit IgG [Santa Cruz], H3K4me3, and M3K27me3 [Millipore], H3K36me3 [Abcam]). Quantification of DNA was performed with SYBR Green Fast PCR Master Mix (primer sequences provided in Table S1) via ABI 7500 Fast Real-time PCR System. 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 control was subtracted from the amount of the immunoprecipitated DNA from the specific antibody ChIP followed by normalizing against the amount of the input DNA.

Nuclease Accessibility Assay

Nuclei were isolated from naive CD4⁺ T cells or Th2 cells by using preparation buffer (0.3 M sucrose, 60 mM KCl, 0.1 mM ethylene glycol tetraacetic acid [EGTA], 15 mM Tris-HCl [pH 7.5], 15 mM NaCl, and 5 mM MgCl₂) containing 0.4% NP-40. Nuclei were pelleted by centrifugation at 200 rpm for 5 min

and washed three times in preparation buffer without NP-40-containing protease inhibitors before samples were stored in preparation buffer at -80°C . Aliquots of nuclei (1×10^6) were incubated with 0.2 U of micrococcal nuclease S7 (MNase) (Roche) in MNase digestion buffer (60 mM KCl, 15 mM Tris-HCl [pH 7.5], 15 mM NaCl, and 1 mM MgCl_2 , 0.5 mM Spermidine, 0.1 mM PMSF) containing 5 mM CaCl_2 and protease inhibitor cocktail for 10 min at 37°C , and the reactions were terminated by the addition of stop buffer (20 mM EDTA, 1% SDS, and 100 $\mu\text{g/ml}$ proteinase K). Genomic DNA was then purified with the high pure PCR product purification kit (Roche). Relative accessibility was quantified with SYBR Green Fast PCR Master with ABI 7500 Fast Real-time PCR System. Relative accessibility is defined as 2 to the power of the difference between the Ct value of qPCR of nuclease untreated and treated samples.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at doi:10.1016/j.immuni.2010.12.013.

ACKNOWLEDGMENTS

The authors thank I.C. Ho and E.J. Taparowsky for providing the *c-Maf* and *Batf* plasmids, respectively, B. Zhou for DO11.10 mice, J. Blum, A. Dent, and S. Goenka for review of this paper. This work was supported by U19 AI070448. G.L.S. was supported by T32 AI060519. D.P. was supported by T32 HL07910.

Received: June 30, 2010

Revised: October 18, 2010

Accepted: November 4, 2010

Published online: January 6, 2011

REFERENCES

- Afkarian, M., Sedy, J.R., Yang, J., Jacobson, N.G., Cereb, N., Yang, S.Y., Murphy, T.L., and Murphy, K.M. (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4⁺ T cells. *Nat. Immunol.* 3, 549–557.
- Ahyi, A.N., Chang, H.C., Dent, A.L., Nutt, S.L., and Kaplan, M.H. (2009). IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *J. Immunol.* 183, 1598–1606.
- Akaishi, H., Takeda, K., Kaisho, T., Shineha, R., Satomi, S., Takeda, J., and Akira, S. (1998). Defective IL-2-mediated IL-2 receptor alpha chain expression in Stat3-deficient T lymphocytes. *Int. Immunol.* 10, 1747–1751.
- Akimoto, T., Numata, F., Tamura, M., Takata, Y., Higashida, N., Takashi, T., Takeda, K., and Akira, S. (1998). Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J. Exp. Med.* 187, 1537–1542.
- Ansel, K.M., Djuretic, I., Tanasa, B., and Rao, A. (2006). Regulation of Th2 differentiation and Il4 locus accessibility. *Annu. Rev. Immunol.* 24, 607–656.
- Avery, D.T., Ma, C.S., Bryant, V.L., Santner-Nanan, B., Nanan, R., Wong, M., Fulcher, D.A., Cook, M.C., and Tangye, S.G. (2008). STAT3 is required for IL-21-induced secretion of IgE from human naive B cells. *Blood* 112, 1784–1793.
- Avery, D.T., Deenick, E.K., Ma, C.S., Suryani, S., Simpson, N., Chew, G.Y., Chan, T.D., Palendira, U., Bustamante, J., Boisson-Dupuis, S., et al. (2010). B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *J. Exp. Med.* 207, 155–171.
- Bauquet, A.T., Jin, H., Paterson, A.M., Mitsdoerffer, M., Ho, I.C., Sharpe, A.H., and Kuchroo, V.K. (2009). The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat. Immunol.* 10, 167–175.
- Ben-Sasson, S.Z., Le Gros, G., Conrad, D.H., Finkelman, F.D., and Paul, W.E. (1990). IL-4 production by T cells from naive donors. IL-2 is required for IL-4 production. *J. Immunol.* 145, 1127–1136.
- Betz, B.C., Jordan-Williams, K.L., Wang, C., Kang, S.G., Liao, J., Logan, M.R., Kim, C.H., and Taparowsky, E.J. (2010). Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. *J. Exp. Med.* 207, 933–942.
- Bromberg, J.F., Wrzeszczynska, M.H., Devgan, G., Zhao, Y., Pestell, R.G., Albanese, C., and Darnell, J.E., Jr. (1999). Stat3 as an oncogene. *Cell* 98, 295–303.
- Bruns, H.A., Schindler, U., and Kaplan, M.H. (2003). Expression of a constitutively active Stat6 in vivo alters lymphocyte homeostasis with distinct effects in T and B cells. *J. Immunol.* 170, 3478–3487.
- Brusselle, G., Kips, J., Joos, G., Bluethmann, H., and Pauwels, R. (1995). Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am. J. Respir. Cell Mol. Biol.* 12, 254–259.
- Brustle, A., Heink, S., Huber, M., Rosenplanter, C., Stadelmann, C., Yu, P., Arpaia, E., Mak, T.W., Kamradt, T., and Lohoff, M. (2007). The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* 8, 958–966.
- Burchill, M.A., Yang, J., Vogtenhuber, C., Blazar, B.R., and Farrar, M.A. (2007). IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3⁺ regulatory T cells. *J. Immunol.* 178, 280–290.
- Chang, H.C., Zhang, S., Thieu, V.T., Slee, R.B., Bruns, H.A., Laribee, R.N., Klemsz, M.J., and Kaplan, M.H. (2005). PU.1 expression delineates heterogeneity in primary Th2 cells. *Immunity* 22, 693–703.
- Chang, H.C., Sehra, S., Goswami, R., Yao, W., Yu, Q., Stritesky, G.L., Jabeen, R., McKinley, C., Ahyi, A.N., Han, L., et al. (2010). The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat. Immunol.* 11, 527–534.
- Chiarle, R., Simmons, W.J., Cai, H., Dhall, G., Zamo, A., Raz, R., Karras, J.G., Levy, D.E., and Inghirami, G. (2005). Stat3 is required for ALK-mediated lymphomagenesis and provides a possible therapeutic target. *Nat. Med.* 11, 623–629.
- Cote-Sierra, J., Foucras, G., Guo, L., Chiodetti, L., Young, H.A., Hu-Li, J., Zhu, J., and Paul, W.E. (2004). Interleukin 2 plays a central role in Th2 differentiation. *Proc. Natl. Acad. Sci. USA* 101, 3880–3885.
- Diehl, S., Anguita, J., Hoffmeyer, A., Zapon, T., Ihle, J.N., Fikrig, E., and Rincon, M. (2000). Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity* 13, 805–815.
- Diehl, S., Chow, C.W., Weiss, L., Palmethofer, A., Twardzik, T., Rounds, L., Serfling, E., Davis, R.J., Anguita, J., and Rincon, M. (2002). Induction of NFATc2 expression by interleukin 6 promotes T helper type 2 differentiation. *J. Exp. Med.* 196, 39–49.
- Durant, L., Watford, W.T., Ramos, H.L., Laurence, A., Vahedi, G., Wei, L., Takahashi, H., Sun, H.W., Kanno, Y., Powrie, F., and O'Shea, J.J. (2010). Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* 32, 605–615.
- Fornek, J.L., Tygrett, L.T., Waldschmidt, T.J., Poli, V., Rickert, R.C., and Kansas, G.S. (2006). Critical role for Stat3 in T-dependent terminal differentiation of IgG B cells. *Blood* 107, 1085–1091.
- Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., and Weaver, C.T. (2005). Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6, 1123–1132.
- Kagami, S., Nakajima, H., Suto, A., Hirose, K., Suzuki, K., Morita, S., Kato, I., Saito, Y., Kitamura, T., and Iwamoto, I. (2001). Stat5a regulates T helper cell differentiation by several distinct mechanisms. *Blood* 97, 2358–2365.
- Kaplan, M.H., Schindler, U., Smiley, S.T., and Grusby, M.J. (1996a). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4, 313–319.
- Kaplan, M.H., Sun, Y.L., Hoey, T., and Grusby, M.J. (1996b). Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382, 174–177.
- Kuperman, D., Schofield, B., Wills-Karp, M., and Grusby, M.J. (1998). Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are

- protected from antigen-induced airway hyperresponsiveness and mucus production. *J. Exp. Med.* 187, 939–948.
- Lee, G.R., Kim, S.T., Spilianakis, C.G., Fields, P.E., and Flavell, R.A. (2006). T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity* 24, 369–379.
- Lewkowich, I.P., Herman, N.S., Schleifer, K.W., Dance, M.P., Chen, B.L., Dienger, K.M., Sproles, A.A., Shah, J.S., Kohl, J., Belkaid, Y., and Wills-Karp, M. (2005). CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J. Exp. Med.* 202, 1549–1561.
- Liao, W., Schones, D.E., Oh, J., Cui, Y., Cui, K., Roh, T.Y., Zhao, K., and Leonard, W.J. (2008). Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. *Nat. Immunol.* 9, 1288–1296.
- Lighvani, A.A., Frucht, D.M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B.D., Nguyen, B.V., Gadina, M., Sher, A., Paul, W.E., and O'Shea, J.J. (2001). T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc. Natl. Acad. Sci. USA* 98, 15137–15142.
- Ma, C.S., Chew, G.Y., Simpson, N., Priyadarshi, A., Wong, M., Grimbacher, B., Fulcher, D.A., Tangye, S.G., and Cook, M.C. (2008). Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J. Exp. Med.* 205, 1551–1557.
- Mathur, A.N., Chang, H.C., Zisoulis, D.G., Stritesky, G.L., Yu, Q., O'Malley, J.T., Kapur, R., Levy, D.E., Kansas, G.S., and Kaplan, M.H. (2007). Stat3 and Stat4 direct development of IL-17-secreting Th cells. *J. Immunol.* 178, 4901–4907.
- Milner, J.D., Brenchley, J.M., Laurence, A., Freeman, A.F., Hill, B.J., Elias, K.M., Kanno, Y., Spalding, C., Elloumi, H.Z., Paulson, M.L., et al. (2008). Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 452, 773–776.
- Murphy, K.M., and Reiner, S.L. (2002). The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2, 933–944.
- Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., and Paul, W.E. (1999). The IL-4 receptor: Signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* 17, 701–738.
- Nurieva, R.I., Chung, Y., Hwang, D., Yang, X.O., Kang, H.S., Ma, L., Wang, Y.H., Watowich, S.S., Jetten, A.M., Tian, Q., and Dong, C. (2008). Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29, 138–149.
- O'Malley, J.T., Sehra, S., Thieu, V.T., Yu, Q., Chang, H.C., Stritesky, G.L., Nguyen, E.T., Mathur, A.N., Levy, D.E., and Kaplan, M.H. (2009). Signal transducer and activator of transcription 4 limits the development of adaptive regulatory T cells. *Immunology* 127, 587–595.
- Ouyang, W., Lohning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A., and Murphy, K.M. (2000). Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity* 12, 27–37.
- Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., and Dong, C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6, 1133–1141.
- Rocken, M., Racke, M., and Shevach, E.M. (1996). IL-4-induced immune deviation as antigen-specific therapy for inflammatory autoimmune disease. *Immunol. Today* 17, 225–231.
- Schnyder-Candrian, S., Togbe, D., Couillin, I., Mercier, I., Brombacher, F., Quesniaux, V., Fossiez, F., Rytffel, B., and Schnyder, B. (2006). Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* 203, 2715–2725.
- Schraml, B.U., Hildner, K., Ise, W., Lee, W.L., Smith, W.A., Solomon, B., Sahota, G., Sim, J., Mukasa, R., Cemerski, S., et al. (2009). The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* 460, 405–409.
- Sehra, S., Bruns, H.A., Ahyi, A.N., Nguyen, E.T., Schmidt, N.W., Michels, E.G., von Bulow, G.U., and Kaplan, M.H. (2008). IL-4 is a critical determinant in the generation of allergic inflammation initiated by a constitutively active Stat6. *J. Immunol.* 180, 3551–3559.
- Sehra, S., Yao, Y., Howell, M.D., Nguyen, E.T., Kansas, G.S., Leung, D.Y., Travers, J.B., and Kaplan, M.H. (2010). IL-4 regulates skin homeostasis and the predisposition toward allergic skin inflammation. *J. Immunol.* 184, 3186–3190.
- Shimoda, K., van Deursen, J., Sangster, M.Y., Sarawar, S.R., Carson, R.T., Tripp, R.A., Chu, C., Quelle, F.W., Nosaka, T., Vignali, D.A., et al. (1996). Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 380, 630–633.
- Takatori, H., Nakajima, H., Hirose, K., Kagami, S., Tamachi, T., Suto, A., Suzuki, K., Saito, Y., and Iwamoto, I. (2005). Indispensable role of Stat5a in Stat6-independent Th2 cell differentiation and allergic airway inflammation. *J. Immunol.* 174, 3734–3740.
- Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T., and Akira, S. (1996). Essential role of Stat6 in IL-4 signalling. *Nature* 380, 627–630.
- Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., and Akira, S. (1997). Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc. Natl. Acad. Sci. USA* 94, 3801–3804.
- Tepper, R.I., Levinson, D.A., Stanger, B.Z., Campos-Torres, J., Abbas, A.K., and Leder, P. (1990). IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. *Cell* 62, 457–467.
- Thierfelder, W.E., van Deursen, J.M., Yamamoto, K., Tripp, R.A., Sarawar, S.R., Carson, R.T., Sangster, M.Y., Vignali, D.A., Doherty, P.C., Grosveld, G.C., and Ihle, J.N. (1996). Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382, 171–174.
- Williams, K.L., Zullo, A.J., Kaplan, M.H., Brutkiewicz, R.R., Deppmann, C.D., Vinson, C., and Taparowsky, E.J. (2003). BATF transgenic mice reveal a role for activator protein-1 in NKT cell development. *J. Immunol.* 170, 2417–2426.
- Yang, Y., Ochando, J., Yopp, A., Bromberg, J.S., and Ding, Y. (2005). IL-6 plays a unique role in initiating c-Maf expression during early stage of CD4 T cell activation. *J. Immunol.* 174, 2720–2729.
- Yang, X.O., Panopoulos, A.D., Nurieva, R., Chang, S.H., Wang, D., Watowich, S.S., and Dong, C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J. Biol. Chem.* 282, 9358–9363.
- Yao, Z., Kanno, Y., Kerenyi, M., Stephens, G., Durant, L., Watford, W.T., Laurence, A., Robinson, G.W., Shevach, E.M., Moriggl, R., et al. (2007). Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 109, 4368–4375.
- Yu, Q., Thieu, V.T., and Kaplan, M.H. (2007). Stat4 limits DNA methyltransferase recruitment and DNA methylation of the IL-18Ralpha gene during Th1 differentiation. *EMBO J.* 26, 2052–2060.
- Zhu, J., and Paul, W.E. (2008). CD4 T cells: Fates, functions, and faults. *Blood* 112, 1557–1569.
- Zhu, J., Cote-Sierra, J., Guo, L., and Paul, W.E. (2003). Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 19, 739–748.

Supplemental Information

The Transcription Factor STAT3 Is Required for T Helper 2 Cell Development

Gretta L. Stritesky, Rajarajeswari Muthukrishnan, Sarita Sehra, Ritobrata Goswami, Duy Pham, Jared Travers, Evelyn T. Nguyen, David E. Levy, and Mark H. Kaplan

Inventory of Supplemental Data

Supplemental Figure 1. The Activation of STAT3 in Th2 Cells is Independent of STAT6. Data demonstrate the activation of STAT3 in WT and *Stat6*^{-/-} Th2 cells (related to data in Figure 1) and the expression of Th2-associated transcription factors in WT and *Stat6*^{-/-} Th2 cultures (related to data in Figure 3).

Supplemental Figure 2. Effect of STAT3-deficiency on Th2 cell growth, and Th1 and Th17 cell differentiation. Data demonstrate comparable cell growth, proliferation and apoptosis between wild type and STAT3-deficient Th2 cultures, and that STAT3 is required for Th17 cytokine secretion, but not for Th1 cytokine secretion (control data for Figure 2).

Supplemental Figure 3. STAT3 Deficient Th2 Cells Have CD25 and IL-4R α Expression Similar to Wild type Th2 Cells. Data demonstrate the expression of CD25 and CD124 on differentiating WT and STAT3-deficient Th2 cells. Companion to data in Figure 3.

Supplemental Figure 4. STAT3-dependent expression of transcription factors. Data demonstrate expression of transcription factors and cytokine 24 hours after the initiation of culture (A and B), during differentiation (C), and after five days of differentiation (D). Related to data in Figure 5.

Supplemental Table 1. Sequences of oligonucleotides used for ChIP analysis.

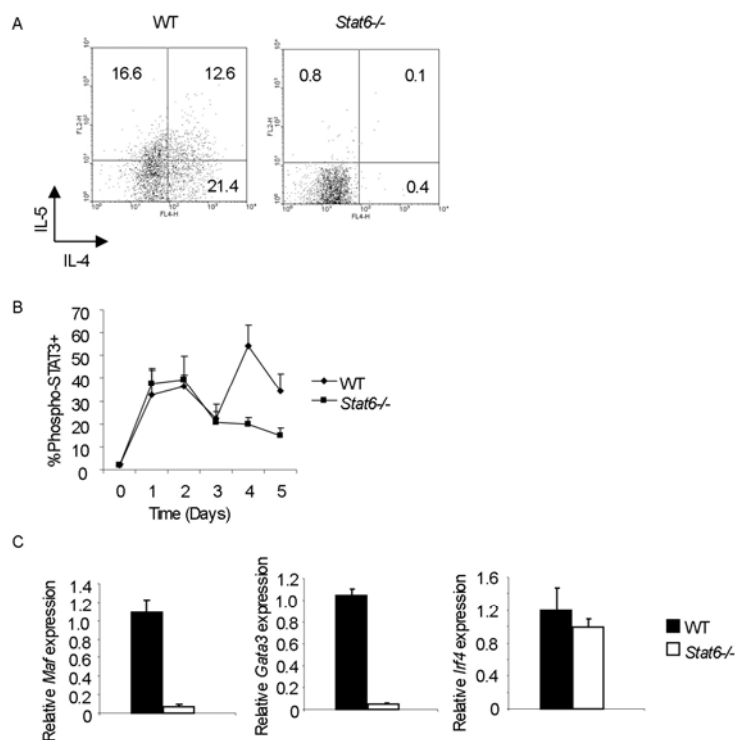


Figure S1 . The Activation of STAT3 in Th2 Cells is Independent of STAT6.

(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 before intracellular staining for IL-4 and IL-5.

(B) Cells were differentiated as in A. Each day during differentiation cells were stained for intracellular phospho-STAT3.

(C) Cells were differentiated as in A. On day 5 cells were re-stimulated with anti-CD3 for 4 hours. Following stimulation RNA was isolated and real-time PCR was performed.

Data are representative of 2-4 experiments (A,B) or average of 6 mice $\pm$ SEM (C).

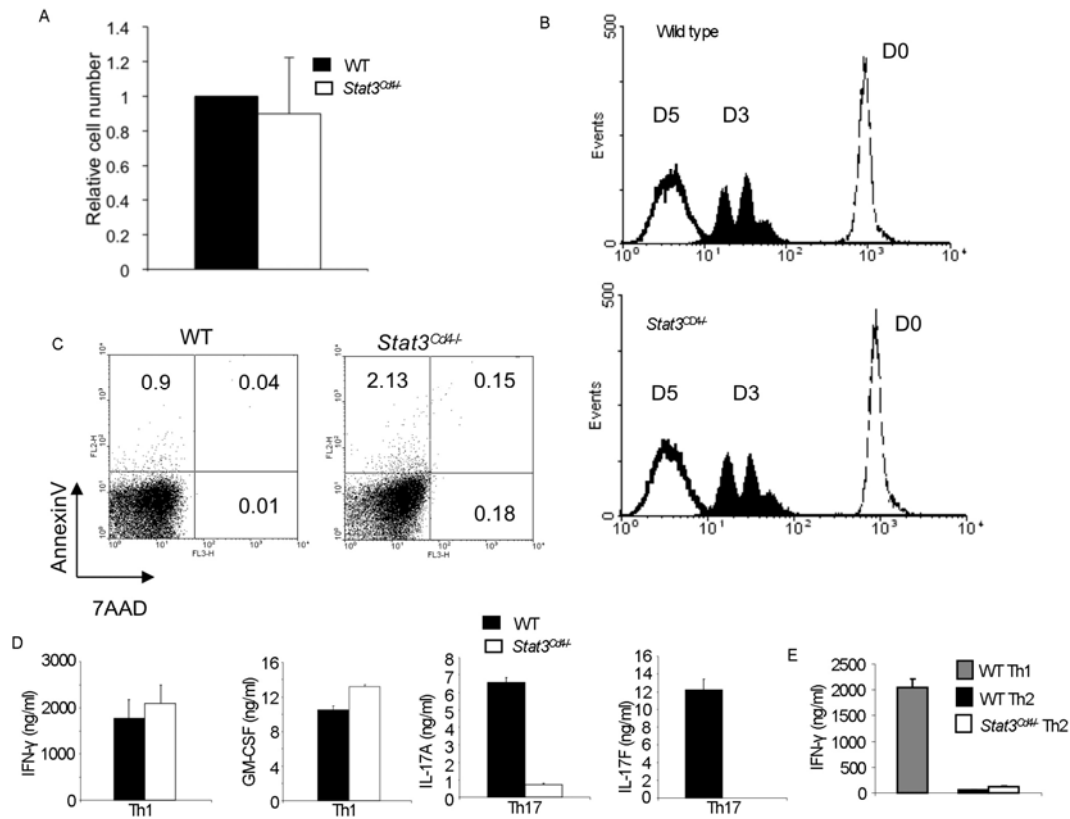


Figure S2. Effect of STAT3-deficiency on Th2 growth, and Th1 and Th17 differentiation

(A-C) Wild type and *Stat3^{CD4}* naïve CD4 T cells were cultured under Th2 conditions for five days.

(A) Viable cells were counted at the end of five days of Th2 culture using trypan blue staining. Results are shown as an average of 10 experiments $\pm$ SEM where wild type cell numbers are normalized to 1.

(B) CD4⁺ T cells were labeled with CFSE and fluorescence was examined in cells at the initiation of culture, on day 3, and at the end of the five day Th2 culture. Results are representative of experiments with four mice.

(C) Th2 cells were cultured for five days and stained for annexin V and 7AAD. Numbers indicate the percent positive cells in the indicated quadrant. Results are representative of experiments with three mice.

(D) Naïve CD4⁺ T cells 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 μ g/ml) for 24 hours. Cell-free supernatant was collected and tested for various cytokines using ELISA.

(E) Wild type Th1 or wild type and *Stat3^{CD4}* Th2 cells differentiated for 5 days were re-stimulated with anti-CD3 (4 μ g/ml) for 24 hours. Cell-free supernatant was collected and tested for IFN- γ .

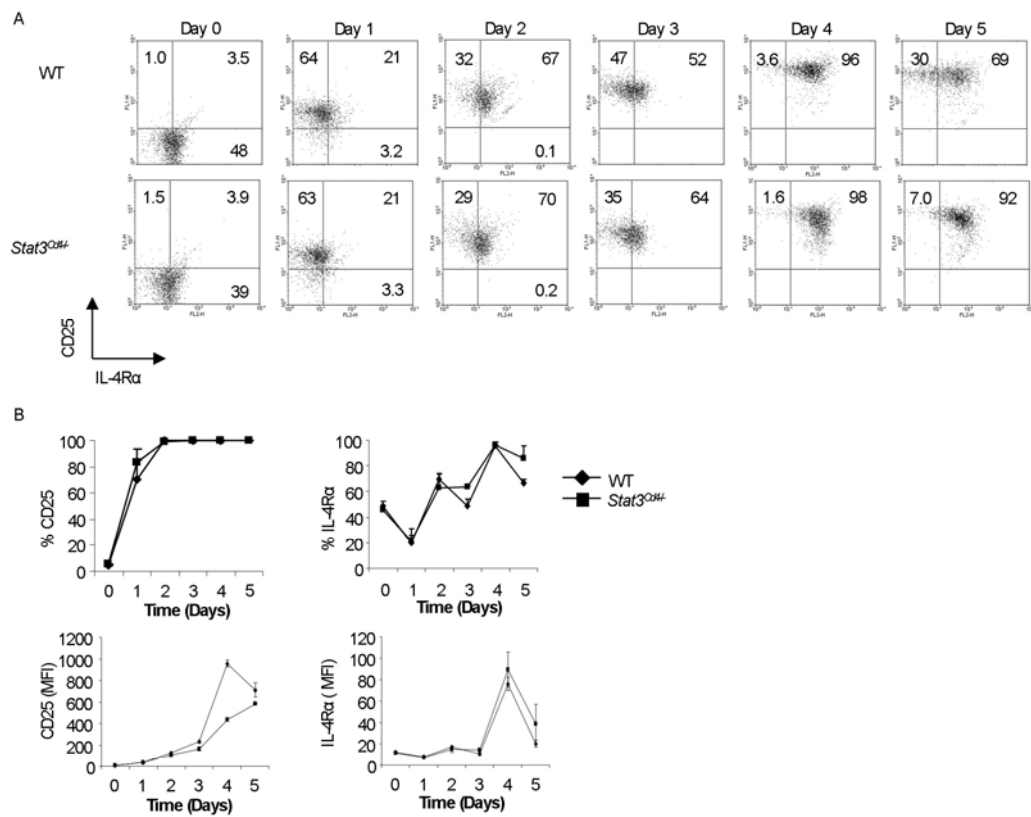


Figure S3. STAT3 Deficient Th2 Cells Have CD25 and IL-4Rα Expression Similar to Wild type Th2 Cells

(A) Wild type (WT) and *Stat3^{Cd4-/-}* naïve CD4⁺ T cells were isolated and cultured as in Figure 1. Each day during differentiation cells were surface stained for CD25 and IL-4Rα. (B) Graphical representation of % positive and mean fluorescence intensity of CD25 and IL-4Rα.

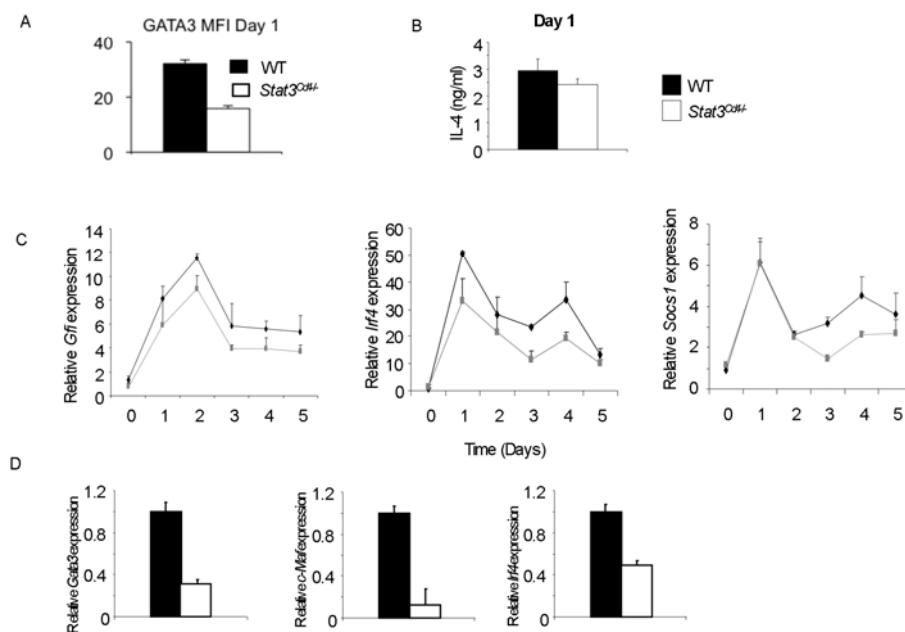


Figure S4. STAT3-dependent expression of transcription factors

(A) Wild type (WT) and *Stat3^{Cd4/-}* naïve CD4+ T cells were cultured under Th2 conditions for 24 hours and GATA3 protein expression was determined by intracellular staining.

(B) Wild type (WT) and *Stat3^{Cd4/-}* naïve CD4+ T cells were activated by anti-CD3 and anti-CD28 for 24 hours before supernatants were tested for IL-4 concentration by ELISA. Results are an average of 6 mice $\pm$ SEM.

(C) Wild type (WT) and *Stat3^{Cd4/-}* naïve CD4+ T cells were cultured under Th2 conditions. RNA was isolated from cells during each day of differentiation and tested for the expression of the indicated genes using qPCR.

(D) Wild type (WT) and *Stat3^{Cd4/-}* naïve CD4+ T cells were cultured under Th2 conditions for 5 days before RNA was isolated from cells and tested for the expression of the indicated genes using qPCR.

Supplemental Table 1

Primers	Sequence
<i>Gata3</i> FOR <i>Gata3</i> REV	5' CGTATATGATGAGTCTTCTCTGGGACTTG 3' 5' AAATCTCAGAACACACACATTTCCAGG 3'
<i>Maf</i> FOR <i>Maf</i> REV	5' AACAGTGTTGGCTTTGTCTACTATGGGAT 3' 5' GTAGGCGGTGCTCTGATATATTGCTGTT 3'
<i>Irf4</i> FOR <i>Irf4</i> REV	5' GACCTCCTTACTGACTGTAAGTTGTGG 3' 5' TTCCATTGTCAGAGCCCTGGTAGTAAAC 3'
<i>Il17f</i> FOR <i>Il17f</i> REV	5' GATCTGGTCCATTTCCGTCATCTCCC 3' 5' GACCTCCTTACTGACTGTAAGTTGTGG 3'
<i>Il24</i> FOR <i>Il24</i> REV	5' TCTTGAAACTGTCACCAGCTGCAAAGT 3' 5' TCAGTCTAGGTAGCCACTCCATTCTATGTCTA 3'
<i>Il21</i> FOR <i>Il21</i> REV	5' TGCCGCTGCTTTACTCATTG 3' 5' GCACCGTCAGCTTTCAGAGA 3'
<i>Batf</i> FOR <i>Batf</i> REV	5' CATTGCTGTGATGATGATGGTGACGAT 5' AAGGCTGGCAAGATGACTCAGAGAATA
<i>Il17</i> FOR <i>Il17</i> REV	5' GTGCTGACCTCATTTGAGGATGGAATC 5' TACCTCTGTGGTCACTTACGTCAAGAG