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STAT3 impairs STAT5 activation in the development of IL-9-secreting T cells

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Abstract

T helper (Th) cell subsets develop in response to multiple activating signals, including the cytokine environment. IL-9-secreting T cells develop in response to the combination of IL-4 and TGF-β, though they clearly require other cytokine signals, leading to the activation of transcription factors including STAT5. In Th17 cells, there is a molecular antagonism of STAT5 with STAT3 signaling, though whether this paradigm exists in other Th subsets is not clear. In this report, we demonstrate that STAT3 attenuates the ability of STAT5 to promote the development of IL-9-secreting T cells. We demonstrate that production of IL-9 is increased in the absence of STAT3, and cytokines that result in a sustained activation of STAT3, including IL-6, have the greatest potency in repressing IL-9 production in a STAT3-dependent manner. Increased IL-9 production in the absence of STAT3 correlates with increased endogenous IL-2 production and STAT5 activation, and blocking IL-2 responses eliminates the difference in IL-9 production between wild type and STAT3-deficient T cells. Moreover, transduction of developing Th9 cells with a constitutively active STAT5 eliminates the ability of IL-6 to reduce IL-9 production. Thus, STAT3 functions as a negative regulator of IL-9 production through attenuation of STAT5 activation and function.

INTRODUCTION

Differentiation of CD4 T cells into T helper (Th) subsets is induced upon ligation of the T cell receptor and is significantly influenced by the cytokines present in the environment during activation and expansion. Initial studies demonstrated that IL-4 and IL-12 were sufficient to drive the differentiation of CD4 T cells into IL-4-producing Th2 cells or IFN-γ-producing Th1 cells, respectively resulting in the simple paradigm where one cytokine activated one STAT protein that subsequently induced the expression of a single differentiation program (1). This paradigm was insufficient to explain Th subsets described later, such as Th17 cells, which required multiple cytokine signals for their development.

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Our current understanding suggests that T cell differentiation is likely the result of the integration of multiple cytokine signals that leads to induction of a unique profile of transcription factor expression that drives distinct cell fates.

In the framework of a paradigm where a single STAT protein yields multiple outcomes depending on additional cytokines in the environment, cytokine signaling through STAT3 is a key regulator in maintaining the balance of transcription factors in T helper cell differentiation. STAT3 is required for differentiation of IL-17-producing Th17 cells as well as T follicular helper (TFH) cells (2–4). STAT3 plays an important role in directly transactivating key Th17- and TFH-associated genes, including *Il17a*, *Il17f*, *Rorc*, *Bcl6* and *Il21* (3–5). However, STAT3 also plays an equally important role in these cells as an antagonist to IL-2-induced STAT5 signaling that is detrimental to both Th17 and TFH differentiation. STAT3 can directly compete with STAT5 for DNA binding, which deters activation of *Il17a/f* and *Il2ra* (6, 7). Additionally, induction of STAT3 in T cells also alters capacity of the cell to produce IL-2 and express the high affinity IL-2R (i.e. CD25) (8, 9), thereby reducing the risk of autocrine responsiveness to IL-2 and prolonging lineage commitment.

Despite the role of STAT3 as a STAT5 antagonist in Th17 and TFH cell differentiation, our laboratory demonstrated that STAT3 is an important positive regulator of Th2 fate determination in the presence of the differentiating STAT6 signal (10). In Th2 cells, STAT3 augmented STAT6 binding to key Th2-associated gene promoters, including *Gata3* and *Maf*. Further, selective STAT3-deficiency in T cells resulted in reduced Th2 differentiation in vitro and a marked reduction in allergen-induced airway hypersensitivity in vivo (10). These results suggest that in the presence of a dominant cytokine pathway, such as the IL-4-STAT6 pathway, the function of other STAT proteins might be altered.

Like Th2 cells, IL-9-producing CD4 T cells, termed Th9 cells, also differentiate in response to IL-4/STAT6-mediated signals. However, this IL-4 mediated signal is altered by additional TGF-β signaling and results in a unique pattern of gene expression including elevated expression of the transcription factor IRF4, suggesting that these cells are a distinct lineage from Th2 cells (11, 12). Th9 cells play a critical role in allergic airway inflammation, parasite immunity and also promote anti-tumor responses (12). However, relatively little is known about the underlying molecular mechanisms that govern the differentiation of Th9 cells. Because Th9 cells share a requirement for STAT6 signaling with Th2 cells, and our previous work suggested that STAT3 was required for Th2 differentiation, we initially asked if STAT3-activating cytokines enhanced Th9 differentiation. Surprisingly, induction of STAT3 by particular cytokines inhibited Th9 differentiation and deletion of STAT3 in Th9 cultured cells augmented Th9 differentiation by altering IL-2 and STAT5 activation.

METHODS

Mice

Stat3 $^{fl/fl}$ mice expressing the CD4-Cre transgene (*Stat3* $^{fl/fl}$ Cre+) mice were originally provided by Dr. David Levy (13) and maintained on the C57Bl/6 background under specific pathogen free conditions. Bcl6$^{fl/fl}$ CD4-Cre mice (14) were provided by Dr. Alexander Dent.

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All mice were used with the approval of the Indiana University Institutional Animal Care and Use Committee.

T cell culturing conditions

Naïve CD4 T cells were isolated from the spleens of mice using magnetic enrichment following direction of the supplier (Miltenyi Biotec, Auburn, CA, USA). These cells were cultured in complete RPMI 1640 media at 1×10^6 cells per ml on tissue culture plates coated with 2μg/ml of anti-CD3 (145-2C11, BioXCell, Labanon, NH, USA) and soluble anti-CD28 (5μg/ml, 37.51, BioXCell) for Th0/unpolarized conditions. In addition to CD3/CD28 stimulation, Th2 cells were cultured with murine IL-4 (20ng/ml, Peprotech, Rocky Hill, NJ, USA) and anti-IFN-γ (XMG1.2, 10μg/ml, BioXCell); Th9 cells received IL-4 and anti-IFN-γ as per Th2 cells, but also were treated with 2ng/ml of human TGF-β (Miltenyi Biotec); Th17 cells were cultured with murine IL-6 (50ng/ml, Peprotech), human TGF-β (2ng/ml), anti-IFN-γ (10μg/ml) and anti-IL-4 (11B11, 10μg/ml, BioXCell); iTregs were cultured in the presence of human TGF-β (2ng/ml) and human IL-2 (50U/ml). After three days of initial polarization, cells were expanded in 3 volumes fresh media containing half concentrations of the cytokines listed above.

In some experiments, cells were also treated with various STAT3 agonist cytokines including IL-6, IL-10, IL-21. IL-23, IL-27 and GM-CSF (all from Peprotech), or with IL-2 blocking reagents (anti-IL-2: 10ug/ml, JES6-5H4, Biolegend, anti-CD25: 10ug/ml, PC61, Biolegend).

Intracellular cytokine staining, transcription factor staining, and flow cytometry

At various time points during the culture period, we harvested cells to assess their capacity to produce a number of cytokines by intracellular cytokine staining (ICS). 1-2×10^5 cells were cultured in media containing Phorbol 12-myristate 13-acetate (PMA, 5ng/ml, Sigma), ionomycin (500ng/ml, Sigma) and monensin (2μM, Sigma) for 5–6 hours at 37ºC. After incubation, cells were washed in PBS and stained with a fixable viability dye (eBioscience) followed by washing and fixation with 4% formaldehyde for 10 min at room temperature. Cells were then permeabilized with 0.1% saponin and stained for intracellular cytokines (IL-4; 11B11, BioLegend, IL-9; RM9A4, Biolegend, IL-2; JES6-5H4, Biolegend, IL-17A; eBio17B7, eBioscience, IFN-γ; XMG1.2, eBioscience).

Real-time PCR

Total RNA was isolated from cells cultured for 5 days in various conditions using Trizol (Life Technologies). RNA was reverse transcribed according to manufacturers directions (Invitrogen). Quantitative real-time PCR was performed with commercially available primers (Life Technologies,) with a 7500 Fast-PCR machine (Life Technologies).

Retroviral Transduction

Bicistronic retroviral vectors containing constitutively active murine STAT5 (caSTAT5) and human nerve growth factor receptor (hNGFR, (15)) were a gift from Dr. J. Sun and were used to generate retrovirus as previously described (10). In order to transduce Th9 cells with these retroviruses, naïve cells were cultured under Th9 conditions for 2 days and infected with either the control hNGFR empty vector control or caSTAT5-hNGFR virus in the

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presence of polybrene (8μg/ml, Sigma) by centrifugation at 2,000 rpm for 1 hr at room temperature. After centrifugation, the retrovirus was removed and conditioned media was added back to the cells. On the next day, cells were expanded as per above and assessed for function and phenotype on day 5 of culture.

Data Analysis and Statistics

Flow cytometry data was collected using the Attune Flow Cytometer (Life Sciences), and data was analyzed on FlowJo v 8.87 (Tree Star). All data was graphed and analyzed statistically using Prism software v6 (Graph Pad). Unless noted, all statistical comparisons were done using a Student's t-test and considered significantly different if $p<0.05$.

RESULTS

STAT3 is a negative regulator of IL-4-mediated IL-9 production

Our previous work demonstrated that STAT3 was required for IL-4/STAT6-mediated Th2 differentiation (10). Because differentiation of Th9 cells are also derived in the presence of IL-4 and requires STAT6 (16), we wanted to determine if STAT3 was also required in this process. To this end, we generated T helper subsets in vitro that differentiate in culture in the absence of IL-4 (i.e. Th0, Th17 and iTreg) or require IL-4 for their differentiation (i.e. Th2, Th9) and defined their capacity for IL-9 production after short restimulation with anti-CD3. We observed that both IL-4-derived Th2 and Th9 cells had a significantly increased ($p<0.05$) capacity to produce IL-9 after re-stimulation, with an ~7- and ~3.5-fold increase in the proportion of cells producing IL-9 under Th2 and Th9 cells, respectively (Figure 1A, B). Interestingly, deficiency of STAT3 in IL-4-independent T helper subsets (Th0, Th17, iTregs) exhibited no increased capacity to produce IL-9 ($p>0.05$, Figure 1A, B). Further, STAT3-deficient Th2 and Th9 cells expressed a marked increase ($p<0.05$) in *Il9* mRNA levels as compared to controls (Figure 1 C). Together, these data indicate that STAT3 is a negative regulator of IL-9 production in cells differentiated with IL-4.

STAT3-agonist cytokines dampen Th9 differentiation

To further address the role of STAT3 in the regulation of IL-9 production, we asked if adding known STAT3 activating cytokines to Th9 cultures would negatively affect their differentiation. As a first step, we assayed whether a panel of known STAT3 agonist cytokines could induce phosphorylation of STAT3 in naïve cells cultured under Th9 conditions for 0.5 hrs (e.g. initial activation) or 24 hrs (e.g. sustained activation). IL-6, -10, -21, and -27 all significantly ($p<0.05$) induced acute phosphorylation of STAT3, whereas IL-23 and GM-CSF did not (Figure 2A, B). However, only IL-6 and IL-27 resulted in increased phosphorylation of STAT3 at 24 hrs after stimulation. We next asked if the cytokines that were able to induce STAT3 phosphorylation (IL-6, 10, 21, 27) were able to inhibit Th9 differentiation when added to these cultures. Interestingly, IL-6 exhibited the greatest capacity to inhibit Th9 differentiation, followed by IL-21, whereas addition of IL-10 and IL-27 had no effect on IL-9 production (Figure 2C). The ability of these cytokines to inhibit Th9 differentiation generally correlates with the induction of sustained STAT3 activation (Figure 2A,B). Furthermore, IL-6 dramatically reduced Th9 differentiation even at low concentrations (1 ng/ml), whereas IL-21 had no effect at this dose (Figure 2C).

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These results raised the possibility that the dose of these cytokines may be important in their ability to inhibit Th9 differentiation. To further explore this, we increased the dose of IL-21 to determine if higher doses of IL-21 could match the effects seen of low-dose IL-6 treatment. Initially, we compared the capacity of these cytokines to promote sustained phosphorylation of STAT3 at 24 hrs after stimulation. While IL-6 treatment resulted in maximal activation of STAT3 at relatively small doses (1 ng/ml), this level of STAT3 phosphorylation was not seen at even 50 times the dose of IL-21 (50 ng/ml) (Figure 2D). We also assessed IL-9 production after 5 days of culture in Th9 cells cells cultured with increasing doses of IL-6 and IL-21. IL-6, at very low doses (1 ng/ml), resulted in maximal decreases of IL-9 production whereas IL-21 generally required 50 times the dose to reduce IL-9 secretion to similar levels (Figure 2E). Taken together, these data indicate that STAT3 agonist cytokines impair Th9 differentiation, correlating with their capacity to activate sustained STAT3 phosphorylation during differentiation.

IL-6/IL-21-mediated disruption of Th9 differentiation requires STAT3, but not BCL6

IL-6 and IL-21 were previously reported to induce phosphorylation of both STAT1 and STAT3 (17), and in some instances, cytokines that activate STAT1 have been also shown to impair Th9 differentiation (18, 19). We therefore wanted to determine if IL-6 and IL-21-mediated suppression of Th9 differentiation required STAT3. In these experiments, naïve CD4 T cells were isolated from *Stat3*$^{fl/fl}$-Cre- or *Stat3*$^{fl/fl}$-Cre+ mice and cultured under Th9 conditions in the presence or absence of IL-6 or IL-21 for five days, followed by the assessment of these cells for IL-9 production after restimulation. As before, both IL-6 and Il-21 differentially decreased the frequency of IL-9-producing in STAT3-sufficient cells; however, this effect was completely lost in STAT3-deficient cells (Figure 3A, B). Further, IL-6 was capable of repressing the Th9-associated factor IRF4 protein expression in WT Th9 cells, but not in STAT3-deficient cells whereas IL-21 had no effect on IRF4 expression (Figure 3C). Importantly, addition of IL-6 or IL-21 to Th9 culture conditions in either WT or STAT3-deficient cells did not result in an increase in the capacity of these cells to produce IL-17, suggesting that activated STAT3 selectively acts to modulate IL-9 and not divert these cells towards a Th17 phenotype (Figure 3A). As a whole, these data indicate that STAT3 is a critical mediator downstream of IL-6/21 signaling.

Recent work demonstrated that IL-21 mediates suppression of Th9 differentiation via induction of BCL6 (20). IL-6 has also been reported to induce early BCL6 expression in T follicular helper cells (17). We therefore wanted to ask in our system if IL-6/21 suppresses Th9 differentiation via induction of BCL6. In our studies, IL-6, but not IL-21 enhanced Bcl6 mRNA expression in Th9 cells, whereas neither cytokine induced detectable BCL6 protein expression (data not shown). Further, treatment of conditional BCL6-deficient Th9 cells with IL-6 or IL-21 resulted in a decreased frequency of IL-9-producing cells and IRF4 protein expression similar to that observed in control cells (p>0.05, Figure 3A, D, E). These data suggest that although STAT3 is required for suppression of Th9 differentiation by both IL-6 and IL-21, this inhibition is independent of BCL6.

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STAT3 regulates STAT5 activation during Th9 differentiation

Previous studies have shown that STAT5 is required for Th9 differentiation but inhibits differentiation of Th17 and T follicular helper cells (20–24). During Th17 differentiation, STAT3 acts to directly induce transcription of Th17-associated genes but also aids differentiation by antagonizing binding of STAT5 to key Th17-associated genes (6). To determine the mechanism of STAT3 function in Th9 cells, we examined phosphorylated STAT5 protein levels over the course of Th9 differentiation in control or STAT3-deficient CD4 T cells, or in control cells in the presence or absence of IL-6 or IL-21. At the onset of the experiment, naïve control and STAT3-deficient cells had virtually no detectable pSTAT5 expression and indicates that naïve cells isolated from these mice are similar directly after isolation. At day 3 of culture, all groups had elevated but similar levels of pSTAT5. However, when cells were expanded in fresh media at day 3 of culture, IL-6-treated, and to a lesser extent IL-21-treated cells rapidly lost pSTAT5 expression, compared to IL-21-untreated cells ($p<0.05$, Figure 4A). Interestingly, STAT3-deficient Th9 cells exhibited significantly higher levels of pSTAT5 expression at day 5 of culture ($p<0.05$, Figure 4A). Importantly, STAT6 activation is not impaired in the absence of STAT3 (16).

We also examined pSTAT5 at day 5 of culture in control or STAT3-deficient Th9 cells treated with or without IL-6 or IL-21 to determine if the decrease in pSTAT5 induced by these cytokines was STAT3-dependent. Indeed, while control cells treated with IL-6 or IL-21 exhibited measurable decreases in pSTAT5, STAT3-deficient Th9 cells treated with these cytokines had no decrease as compared to untreated controls (Figure 4B, C).

To determine if the decreased pSTAT5 expression observed in IL-6-treated cells correlated with a decrease in the expression of STAT5 target genes, we examined protein levels of CD25, IRF4 and FOXP3 at day 5 of culture in both control and STAT3-deficient cells left untreated or treated with IL-6 or IL-21. Figures 4D–F show that IL-6 markedly decreased expression of CD25, IRF4 and FOXP3 ($p<0.05$) as compared to untreated controls. Interestingly, IL-21 had virtually no effect on STAT5-target gene expression despite a reduced STAT5 activation ($p>0.05$). Further, IL-6-mediated suppression of these genes is completely dependent on STAT3, as STAT3-deficient cells exhibited no decrease in CD25, IRF4 or FOXP3 protein expression ($p>0.05$, Figure 4D–F). These data suggest that STAT3 regulates the activation of STAT5 that is likely to directly impact IL-9 production.

Augmented STAT5 activation and IL-9 production in STAT3-deficient Th9 cells requires IL-2 production

As IL-2 is the major STAT5-activating cytokine produced by T cells and is critically important for Th9 differentiation (20, 25–27), we initially determined if STAT3-deficiency or activation of STAT3 by agonist cytokines altered IL-2 production during Th9 differentiation. In these experiments, STAT3-deficient or control cells were cultured under Th9 conditions in the presence or absence of IL-6. At days 0, 3, 4 and 5 of culture we re-stimulated cells to determine their capacity to produce IL-2 by intracellular cytokine staining. Figure 5A shows that IL-6 treatment decreased the early (days 3 and 4 of culture) capacity of Th9 cells to produce IL-2 as compared to untreated controls ($p<0.05$). In

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contrast, STAT3-deficient Th9 cells have an increased capacity to produce IL-2 as compared to controls (p<0.05, Figure 5A).

To determine if the reduced capacity of IL-6 and IL-21-treated Th9 cells to produce IL-2 was dependent on STAT3, we examined the capacity of STAT3-sufficient or -deficient cells to produce IL-2 at day 4 of culture after re-stimulation. Indeed, STAT3-sufficient cells exhibited a loss of IL-2 production when treated with IL-6 or IL-21 (p<0.05), but this loss in function was abrogated in STAT3-deficient cells (p>0.05, Figure 5B). Therefore, IL-6 and IL-21, through STAT3 signaling, impair the production of IL-2 in developing Th9 cells.

Based on the above data, we hypothesized that augmented IL-2 production in the absence of STAT3 may lead to enhanced Th9 differentiation. To test this hypothesis, we differentiated Th9 cells from control or STAT3-deficient mice, and on day 3 expanded these cells cell in fresh media with or without blocking antibodies to IL-2 and CD25. After 2 additional days of culture, we assessed pSTAT5 levels in resting cells and IL-9 production after stimulation with PMA and ionomycin. Figure 5C–D show that IL-2/CD25 blockade significantly reduced both pSTAT5 and IL-9 production of STAT3-deficient Th9 cells to levels observed in control cells that underwent the same treatment.

The deficiency in IL-2 production in IL-6-treated cultures suggests that supplementation of the cultures with exogenous IL-2 might rescue IL-9 production. To test this, we added increasing doses of IL-2 to IL-6-treated Th9 cultures, but did not observe a recovery in IL-9 production (Fig. 5E). This lack of responsiveness is consistent with decreased CD25 expression in IL-6-treated Th9 cultures (Fig. 4D). Taken together, these data suggest that STAT3 controls IL-2 production that then acts to activate STAT5 and promote Th9 differentiation.

Expression of constitutively active STAT5 protects Th9 cells from deleterious effects of IL-6

Above we showed that STAT3 controls STAT5 activation and Th9 differentiation by regulating the production of IL-2. We therefore hypothesized that ectopic expression of a constitutively active STAT5 (caSTAT5) would neutralize the deleterious effects of IL-6 on Th9 differentiation. To test this hypothesis, we cultured STAT3-sufficient Th9 cells in the presence or absence of IL-6 for two days, and then transduced these cells with a retrovirus expressing human nerve growth factor (hNGFR) alone or hNGFR-IRES-caSTAT5. At day 5 of culture we assayed IL-9 production after restimulation. In control-transduced cells, IL-6 treatment reduced the proportion of IL-9 producing cells as compared to untreated controls (p<0.05, Figure 6A, B). Transduction of cells with caSTAT5 resulted in an increased proportion of IL-9-producing cells as compared to cells transduced with a control retrovirus (p<0.05), therefore confirming the positive role of STAT5 in Th9 differentiation seen in other studies (20, 21, 27). However, when caSTAT5-expressing cells were treated with IL-6, there was no appreciable decrease in the frequency of IL-9 producing cells as compared to control-transduced cells (p>0.05, Figure 6A, B, C). Additionally, caSTAT5 expression in these cells attenuates the IL-6-mediated loss of FOXP3, IRF4, GATA3 and CD25 expression (p<0.05, Figure 6C). Together, these data indicate that IL-6/STAT3 suppression of Th9 differentiation is mediated by suppression of IL-2 production and STAT5 signaling.

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DISCUSSION

These studies define a role for IL-6 as a potent negative regulator of Th9 development and demonstrate that STAT3 was required for the ability of IL-6 to inhibit IL-9 production. Other STAT3-activating cytokines were not as effective as IL-6, and this correlated with either a lack of long-term activation of STAT3 or increased activation of STAT1. IL-6 diminished endogenous IL-2 production, resulting in decreased CD25 expression and diminished STAT5 activation, a molecule that promotes Th9 development. Moreover, responsiveness to exogenous IL-2 was diminished, possibly due to decreased CD25 expression. Importantly, the ability of IL-6 to impair Th9 development is attenuated in the presence of active STAT5. Thus, IL-6-induced STAT3 interferes with the activation of STAT5 and impairs the differentiation of IL-9-producing T cells.

The concepts of STAT protein cooperation and antagonism have been identified during the development of multiple T helper cell lineages. Both STAT3 and STAT5 cooperate with STAT6 for optimal Th2 differentiation (10, 15) through mechanisms that include cooperative binding to DNA and is similar to that observed in cooperative STAT1 and STAT3 DNA binding downstream of the IL-21 receptor (28). In contrast, antagonism between STAT3 and STAT5 are critical for appropriate Th17 and TFH differentiation where STAT3 and STAT5 compete for binding to key Th17- and TFH-associated genes and balance the expression of Blimp-1 and BCL6 (5, 24, 29–31). Antagonism also occurs during STAT activation. In TFH cell differentiation, STAT3-deficient CD4 T cells exhibit a profound increase in expression of activated STAT1 and STAT1 target gene expression that interferes with normal TFH differentiation (7), suggesting compensatory activation of alternative STAT molecules. Thus, there is considerable evidence for STAT proteins being key integrators of multiple extracellular signals.

Our data supports a mechanism wherein the IL-6/STAT3 pathway represses both IL-2 production and IL-2 responsiveness in Th9 cultures, resulting in decreased IL-9 production. This is further substantiated by the ability of active STAT5 to bypass the requirement for IL-2 signaling even in the presence of IL-6. The mechanism through which IL-6 regulates IL-2 is not entirely clear. STAT3 can induce FoxO1 and FoxO3 to repress IL-2 in some T cell culture conditions (9), but we did not observe increased expression of either factor with added IL-6 in Th9 cultures (data not shown). We did observe IL-6 induced expression of *Ikzf3*, the Ikaros family member Aiolos, in Th9 cultures that was shown to represses IL-2 in Th17 cultures (32). However, knockdown of *Ikzf3* in Th9 cultures by siRNA did not rescue IL-9 production (data not shown). We further examined a potential role for SOCS3 to regulate IL-9 using conditional mutant T cells. Although IL-9 production was increased in the absence of SOCS3, IL-6 was still capable of repressing IL-9 in SOCS3-deficient T cells (data not shown). This does not exclude the potential role of other SOCS proteins, or of IL-6-induced phosphatases that might negatively regulate IL-2 signaling. Thus, although the effect of IL-6 in this system is clear, the mechanism is still not completely defined.

We showed that various STAT3-activating cytokines had different capacities to repress Th9 differentiation that correlated with their capability for sustained STAT3 activation. IL-6, the most potent repressor of IL-9, induced phosphorylation of STAT3 that was sustained from

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0.5 to 24 hours. In contrast, IL-21 stimulation induced similar short-term activation of STAT3, but did not result in STAT3 activation after 24 hours of stimulation, correlating with a lesser ability to inhibit IL-9 production. Interestingly, IL-27, which was previously shown to inhibit IL-9 production by Th9 cells at higher doses (18), also induced long-term STAT3 activation in a greater percentage of cells than IL-21 but exhibited no inhibitory effect at the doses used in our study. Stimulation with IL-27 also resulted in the long-term activation of STAT1, whereas IL-6 and IL-21 induced acute STAT1 activation that was diminished after 24 hours (data not shown). As STAT3 and STAT1 have been shown to antagonize reciprocal function (7, 28, 33), it is possible that sustained STAT1 activation interferes with the ability of STAT3 to repress IL-2 expression, resulting in a minimal effect of IL-27 on IL-9 production.

The role of BCL6 in Th9 development is not entirely clear. Our results, using mice that are conditionally deficient for BCL6 in T cells, observed no increased IL-9 in the absence of BCL6 and demonstrated that STAT3-activating cytokines did not require BCL6 for decreasing IL-9 production. In contrast, two previous reports identified BCL6 as a repressor of IL-9 production (20, 27). These studies used BCL6-specific shRNA in T cell cultures or T cells isolated from mice with a germ line mutation in BCL6 to demonstrate a role for BCL6 in limiting IL-9 production (20, 27). These differences in methodology raise two potential reasons for the distinct findings. First, shRNA treatment would diminish BCL6 expression later in culture, or for a shorter time period, than the T cells used in our studies that are developmentally deficient in BCL6 (14). Second, mice with a germ line deficiency in BCL6 have dramatic inflammatory disease, and recent reports have supported that at least some observations in these mice are a result of the inflammatory cytokine environment, rather than an intrinsic role of BCL6 within T cells (34, 35). Notably, *Bcl6* germline deficient mice over-produce IL-6 and this can skew T cell differentiation to other Th lineages (36). Another explanation for the difference in results may be due to the timing of cytokine addition. In our studies IL-6 and IL-21 were added at the onset of culture, whereas in Liao et al IL-21 was added 4 hours prior to analysis (20). Based on these differences, it is tempting to speculate that STAT3-activating cytokines may have different requirements for BCL6 depending on whether acute activation of IL-9 or longer-term differentiation is being examined.

Our previous work demonstrated that although STAT3 is required for Th2 development, it is not required for Th9 differentiation (10, 16). In this report, we further that observation by demonstrating that IL-6-induced STAT3 is a potent inhibitor of IL-9 production in both Th2 and Th9 cells. In our unpublished data, we also demonstrated that the expression of the IL-9-inducing transcription factor *Sfpi1* was increased in Th2 cells in the absence of STAT3 and is reminiscent of STAT3 acting as a switch factor in other Th cell lineages. In the differentiation of Th17 cells, STAT3 represses Treg-associated Foxp3 and induces *Rorc* and *Il17* expression by directly binding to each of these genes (2, 3, 5). The mechanism of STAT3 action in Th2 and Th9 cells is not entirely clear but logically include the ability of STAT3 to promote STAT6 binding to Th2 target genes including GATA3 that might promote differentiation towards the Th2 phenotype and away from a Th9 phenotype (10, 16). Moreover, expression of STAT6 target genes in STAT3-deficient Th2 cells resembles the effects of TGF-β treatment on Th2 cells resulting in reduced GATA3 expression and reduced IL-4 production (37, 38). Based on these data, it is also possible that STAT3 might also de-

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sensitize Th2 cells to TGF-β signaling, however, the expression of other known TGF-β-induced genes (i.e. *Itgae*) is not significantly changed in STAT3-deficiencent Th2 cells as compared to controls (data not shown). Therefore, this scenario is less likely. Ultimately, STAT3 might limit IL-9 production through several mechanisms.

Although T helper cell differentiation is often examined in a reductionist system, examining the effects of single cytokines, T cell differentiation in vivo occurs in a complex cytokine environment. The T cell must intrinsically assimilate multiple signals to generate responses that are effective at combating pathogens. The mechanisms of signal assimilation include regulation of cytokine receptors to alter cellular responses, but extend to co-operation and competition among transcription factors activated by extracellular signals. Many cytokines have been shown to positively or negative impact Th9 differentiation although the precise mechanisms are not well defined (12). In this report, we show that the IL-6/STAT3 pathway impairs IL-9 production. STAT3 functions by limiting IL-2 production and impairing STAT5 activation, a factor necessary for the expression of *Il9*. This finding parallels the paradigm in Th17 cells that STAT5 impairs STAT3-dependent differentiation and activation of *Il17-Il17f* (6, 23). Our findings suggest that STAT3-STAT5 antagonism impact T helper cell differentiation pathways distinct from Th17 development. The relative importance of this pathway in vivo will be the focus of future studies.

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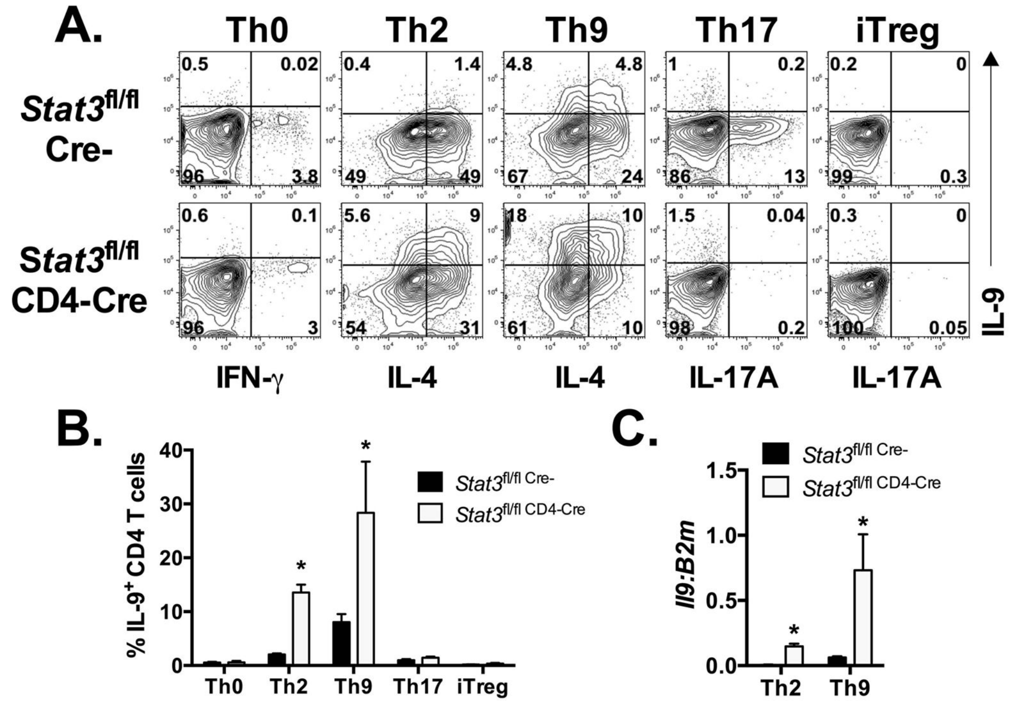

Figure 1. Stat3 is a negative regulator of IL-9 in Th2 and Th9 cells

STAT3 is a negative regulator of IL-9 production in Th2 and Th9 cells. Naïve CD4 T cells were isolated and cultured under Th0, Th2, Th9, Th17 and iTreg conditions for 5 days followed by stimulation with PMA and ionomycin in the presence of monensin for 5.5 hours. A) Representative contour plots and (B) quantitation of intracellular cytokine staining. C) *Il9* mRNA expression in resting Th2 and Th9 cells at day 5 of culture. *, $p<0.05$ comparing $Stat3^{fl/fl}$ Cre- vs $Stat3^{fl/fl}$ Cre+. Error bars represent standard deviation of the mean and data is representative of one of 3 individual experiments with 3–4 mice per experiment.

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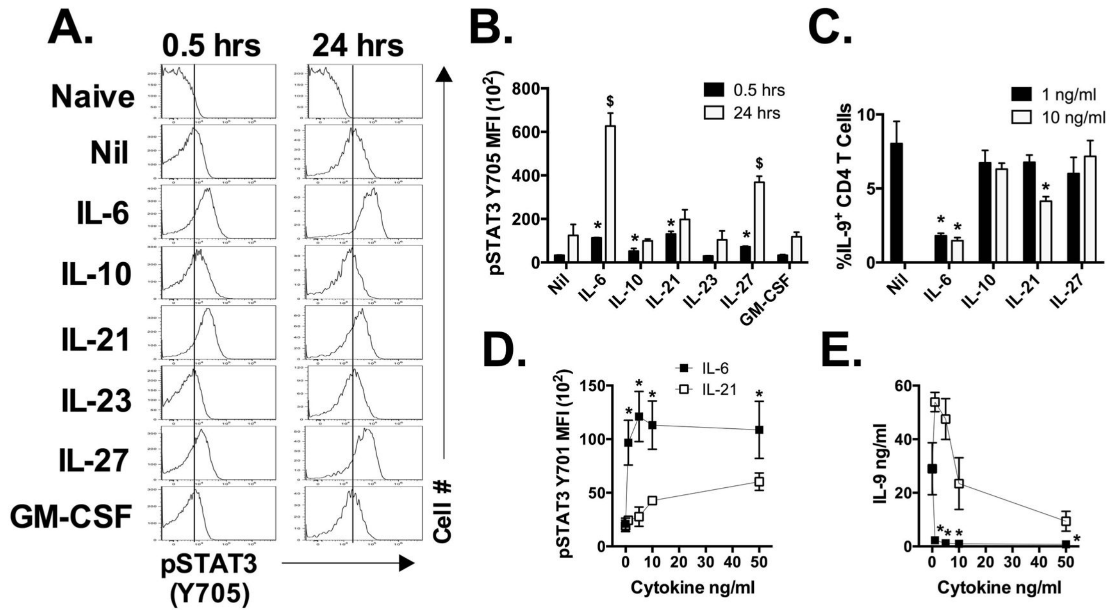

Figure 2. IL-6 is a negative regulator of IL-9 in Th2 and Th9 cells

STAT3 agonist cytokines activate STAT3 in under Th9 conditions and limit Th9 differentiation. Naïve CD4 T cells were cultured under Th9 conditions alone and with addition of 10ng/ml of STAT3 agonist cytokines. At 0.5 and 24 hrs of culture, we examined phospo-STAT3 (pSTAT3 Y705) via flow cytometry. A) Representative histograms and (B) quantitation of pSTAT3 (Y705) staining. *, $p<0.05$ significantly different at 0.5hrs of stimulation as compared to Nil-treated controls. $, $p<0.05$ significantly different at 0.5hrs of stimulation as compared to Nil-treated controls. C) Quantitation of the frequency of IL-9^{+} CD4 T cells treated with various STAT3-activating cytokines at 1ng/ml or 10ng/ml. *$p<0.05$ significantly different as compared to untreated (Nil) controls. D) pSTAT3 (Y705) MFI at 24 hours of culture with increasing doses of IL-6 and IL-21. E) IL-9 secretion measured after 24 restimulation of day 5 cultured Th9 cells in the presence of increasing doses of IL-6 and IL-21 at day 0. *$p<0.05$ significantly different comparing IL-6 treatment vs IL-21 treatment. Error bars represent standard deviation of the mean and data is representative of one of 2 individual experiments with 3 mice per experiment.

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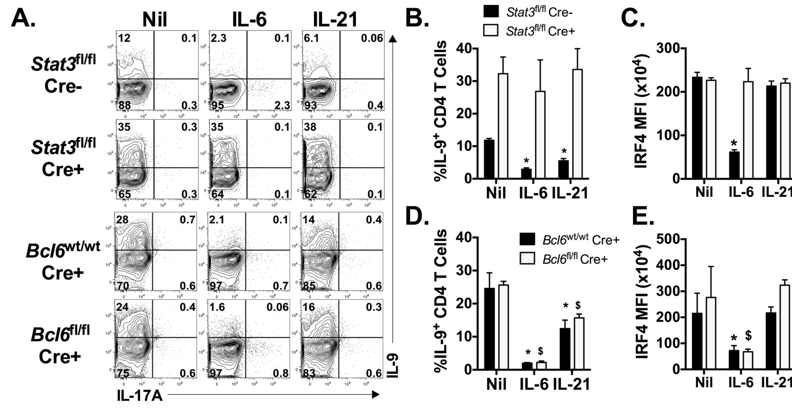

Figure 3. IL-6-mediated IL-9 silencing is dependent on STAT3 but no BCL6

IL-6-mediated disruption of Th9 differentiation requires STAT3 but not BCL6. Naïve CD4 T cells were isolated from Stat3$^{fl/fl}$ Cre-, Stat3$^{fl/fl}$ Cre+, BCL6$^{wt/wt}$ Cre+ and BCL6$^{fl/fl}$ Cre+ mice and differentiated under Th9 conditions in the presence or absence of 10ng/ml of IL-6 or IL-21. A) Representative contour plots of IL-9 and IL-17A production after restimulation of day 5 cells with PMA and ionomycin. Quantitation of the (B) proportion of IL-9^{+} CD4 T cells and (C) the MFI of IRF4 of STAT3-sufficient and deficient cells and the % of IL-9^{+} (D) and IRF4 MFI (E) of BCL6-sufficient and deficient cells. *, p<0.05 significantly different as compared WT untreated controls. $, p<0.05 significantly different as compared to STAT3- or BCL6-deficient untreated controls. Error bars represent standard deviation of the mean and data is representative of one of 2–3 individual experiments with 3–4 mice used in each experiment,.

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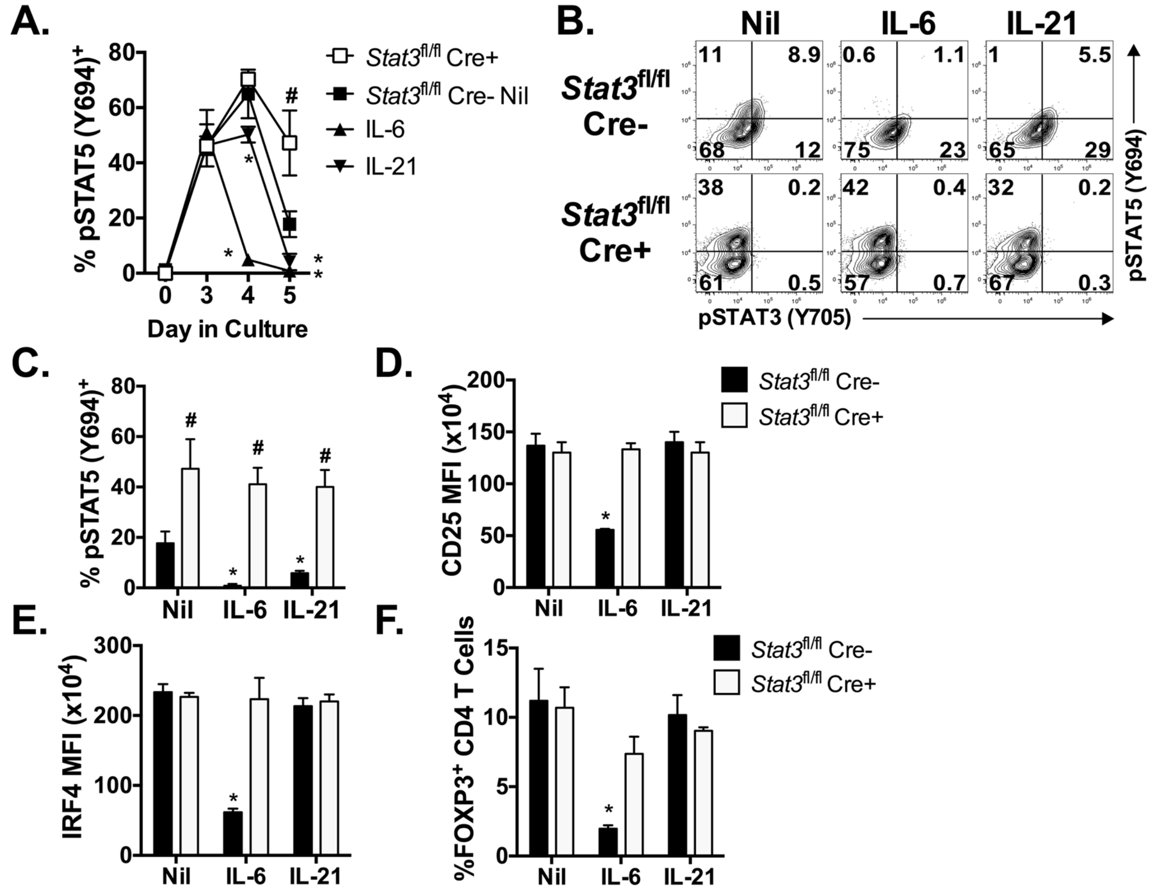

Figure 4. STAT3 regulates STAT5 activation during Th9 differentation

STAT3 regulates STAT5 activation during Th9 differentiation. Th9 cells derived from Stat3$^{fl/fl}$ Cre- and Stat3$^{fl/fl}$ Cre+ mice were cultured in the presence or absence of IL-6 or IL-21 as above and monitored pSTAT5 (Y694) expression over the time course of differentiation (A). B) Representative contour plots and quantitation (C) of pSTAT5 (Y694) and pSTAT3 (Y705) at day 5 of differentiation in STAT3$^{fl/fl}$ Cre- and Cre+ Th9 cells treated with or without IL-6 or IL-21 as above. Expression of CD25 (MFI, D), IRF4 (MFI, E), and FOXP3 (%, F) as cells treated as per panel B. *, $p<0.05$ significantly different as compared to STAT3$^{fl/fl}$ Cre- untreated controls. #, $p<0.05$ significantly different as compared to STAT3$^{fl/fl}$ Cre- cells. Error bars represent standard deviation of the mean and data is representative of one of 3 individual experiments with cells isolated from 3 mice per each experiment.

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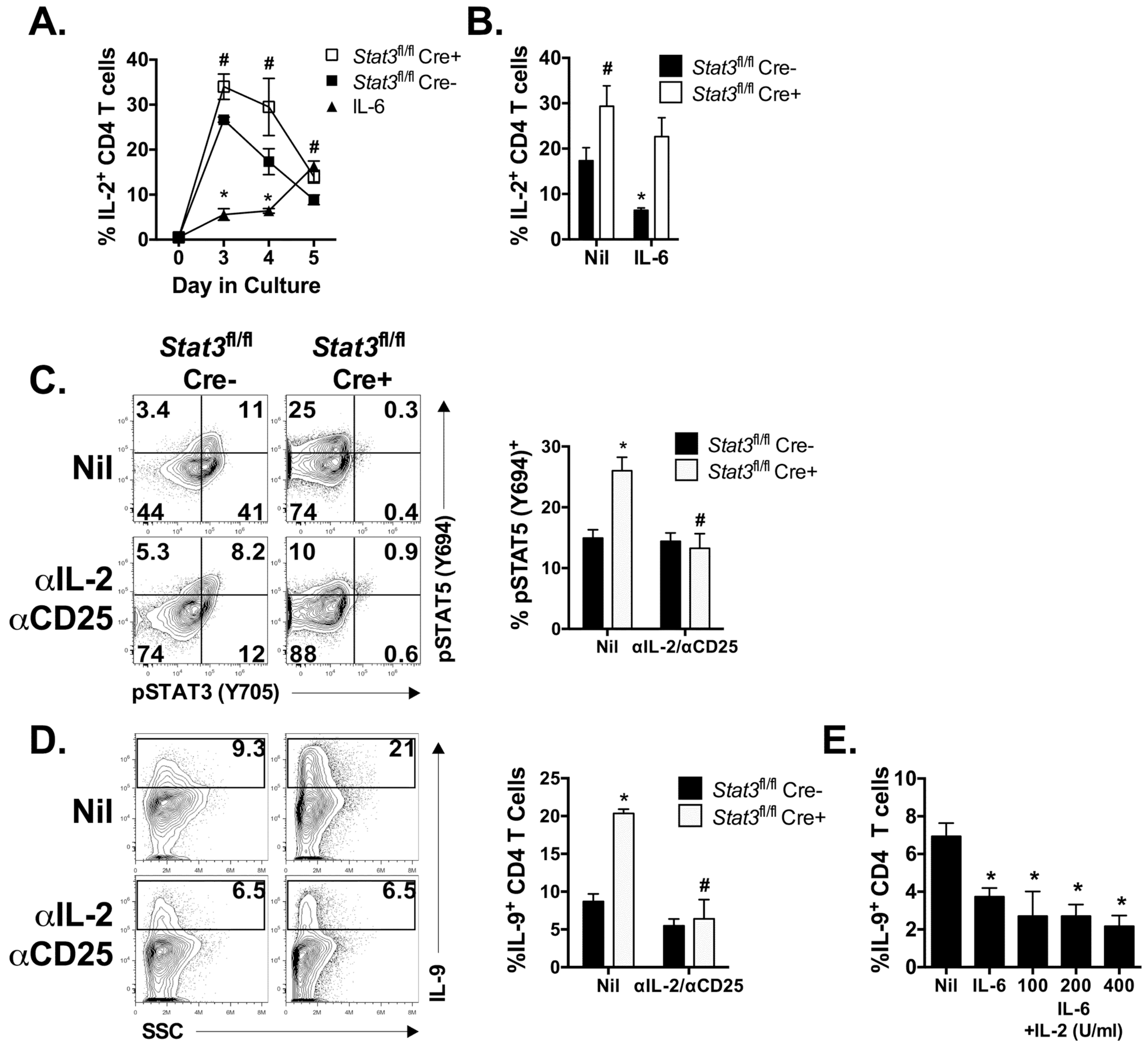

Figure 5. STAT3 is a negative regulator of IL-2 production in Th9 cells

IL-2 secretion is required for enhanced STAT5 activation and IL-9 production by STAT3-deficient Th9 cells. Th9 cells were cultured as per Figure 5 and at each time point cells were re-stimulated with PMA and ionomycin and assessed for IL-2 production by ICS (A). B) The proportion of IL-2+ cells at day 5 of culture in STAT3-sufficient and -deficient cells cultured with or without IL-6. *, $p<0.05$ significantly different as compared to Stat3$^{fl/fl}$ Cre- untreated controls. #, $p<0.05$ significantly different as compared to Stat3$^{fl/fl}$ Cre- cells. C) Stat3$^{fl/fl}$ Cre- and Cre+ cells were cultured under Th9 conditions, at day 3 of culture cells were expanded in fresh media with or without blocking antibodies to IL-2 and CD25. Two days after antibody blockade we examined pSTAT5 (Y694) expression in resting cells (C) or the proportion of IL-9^{+} cells after PMA and ionomycin (D). (E), Th9 cell were cultured in the presence or absence of IL-6 for 3 days as per above. On day 3 of culture, cells were

removed for the αCD3-coated plates and given fresh media with ½ concentration of cytokines. In addition, some IL-6-treated Th9 cells were also given increasing doses of exogenous recombinant human IL-2 and cultured for an additional 2 days. The frequency of IL-9^+ CD4 T cells is shown after stimulation with PMA and ionomycin. *, $p<0.05$ significantly different as compared to STAT3$^{fl/fl}$ Cre- untreated controls. #, $p<0.05$ significantly different as compared to STAT3$^{fl/fl}$ Cre+ cells. Error bars represent standard deviation of the mean and data is representative of one of 2–3 individual experiments with cells isolated from 3 mice per experiment.

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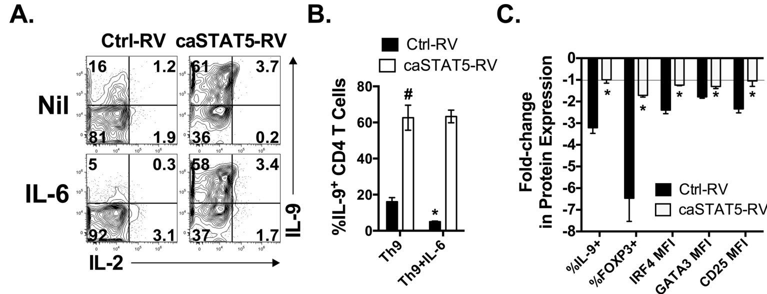

Figure 6. Constitutive active STAT5 protects Th9 cells against deleterious effects of IL-6/STAT3

Transduction of Th9 cells with constitutively active (caSTAT5) protects against deleterious effects of IL-6 on Th9 differentiation. Th9 cells cultured with or without IL-6 were transduced with a hNGFR-expressing empty vector control virus or hNGFR-caSTAT5 virus on day two of culture. After 3 days, cells were stimulated with PMA and ionomycin and assessed for IL-9 production by ICS. A) Representative contour plots (A) and quantitation of IL-9 production (B) by control and caSTAT5-transduced Th9 cells. C) IL-6-induced changes in Th9-associated factor protein expression assayed by flow cytometry. Fold changes in protein expression in panel C were determined by dividing the % positive or MFI from Nil (either control or STAT5-transduced) treated cells by that of IL-6-treated cells (either control or STAT5-transduced) and assigning it as a negative value, representing a decrease in expression. A value of -1 indicates no change. Error bars represent standard deviation of the mean and data is representative of one of 2 individual experiments with cells isolated from 3 mice per experiment.