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# Stat3 and Stat4 Direct Development of IL-17-Secreting Th Cells[1]

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**IL-17-secreting CD4$^+$ T cells are critically involved in inflammatory immune responses. Development of these cells is promoted in vivo and in vitro by IL-23 or TGF$\beta$1 plus IL-6. Despite growing interest in this inflammatory Th subset, little is known about the transcription factors that are required for their development. We demonstrate that Stat3 is required for programming the TGF$\beta$1 plus IL-6 and IL-23-stimulated IL-17-secreting phenotype, as well as for ROR$\gamma$t expression in TGF$\beta$1 plus IL-6-primed cells. Moreover, retroviral transduction of a constitutively active Stat3 into differentiating T cell cultures enhances IL-17 production from these cells. We further show that Stat4 is partially required for the development of IL-23-, but not TGF$\beta$1 plus IL-6-primed IL-17-secreting cells, and is absolutely required for IL-17 production in response to IL-23 plus IL-18. The requirements for Stat3 and Stat4 in the development of these IL-17-secreting subsets reveal additional mechanisms in Th cell fate decisions during the generation of proinflammatory cell types.** *The Journal of Immunology,* **2007, 178: 4901–4907.**

Interleukin-17-secreting T cells play critical roles in autoimmune disease, immunity to infection, and the homeostatic regulation of inflammatory cells (1–4). The development of these cells, which have been variously called Th17, $Th_{IL-17}$, Tn, and Th1$\beta$, is only beginning to be understood (5–8). A proinflammatory cytokine environment containing TGF$\beta$1 plus IL-6 has been implicated in the generation of IL-17-secreting T cells from naive CD4 T cells (9–11). IL-23, which shares cytokine and receptor subunits with IL-12 (12, 13), also promotes the development of proinflammatory IL-17-secreting cells in vivo and in vitro from Th cells with a memory phenotype (3, 5, 6, 14). Consequently, IL-6- and IL-23p19-deficient mice are protected from inflammatory autoimmune disease (6, 10, 15). The development of the IL-17-secreting Th subset is negatively regulated by IL-4, IL-27, and IFNs (3, 5, 16, 17).

Despite the activation of Stat proteins by IL-6 and IL-23 (12, 13, 18), the development of IL-17-secreting cells was reported to be independent of Stat4 and Stat6, factors required for the normal development of Th1 and Th2 cells, respectively (3, 5). In contrast, T-bet, which promotes the development of Th1 cells, is a critical negative regulator of IL-17-secreting cells. We have shown that the IL-23-primed IL-17-secreting phenotype is not stable after continued in vitro culture and that following Ag receptor-induced T-bet expression, IL-23-primed cells convert to an IFN-$\gamma$-secreting phenotype (7). Recently, the transcription factor ROR$\gamma$t was shown to be important in the development of IL-17-secreting T cells (19). However, cytokine-stimulated transcription factors that promote the IL-17-secreting genetic phenotype have not been identified. In this report, we define requirements for Stat3 and Stat4 in the development of IL-17-secreting Th cells.

## Materials and Methods

### *Mice*

All animal studies in this report were approved by the Indiana University Institutional Animal Care and Use Committee. The generation of *Stat4*$^{-/-}$ mice and *Stat3*$^{fl/fl}$ mice with a CD4-Cre (*Stat3*$^{CD4-/-}$) transgene were previously described (20–23). Wild-type (WT;[4] Harlan Sprague Dawley) and *Stat4*$^{-/-}$ mice were used on a C57BL/6 background. *Stat3*$^{fl/fl}$ mice are on a mixed 129-C57BL/6 genetic background and the presence of the CD4-Cre transgene deletes exons 16–21 in T cells (22). WT mice in experiments using *Stat3*$^{CD4-/-}$ mice were Cre-negative littermates.

### *Analysis of Th cell differentiation*

Naive, memory, or total CD4$^+$ T cells were isolated from WT and *Stat4*$^{-/-}$ C57BL/6 or *Stat3*$^{CD4-/-}$ and WT littermate spleen and lymph nodes (MACS isolation system; Miltenyi Biotec) as indicated. T cells were activated with plate-bound anti-CD3 (2 $\mu$g/ml 145-2C11) and soluble anti-CD28 (0.5 $\mu$g/ml; BD Pharmingen). Th1, IL-23-primed, and TGF$\beta$1 plus IL-6-primed cultures were generated with anti-IL-4 (10 $\mu$g/ml 11B11) and IL-12 (2 ng/ml; PeproTech), IL-23 (4 ng/ml; R&D Systems), or TGF$\beta$1 (1 ng/ml; PeproTech) and IL-6 (100 ng/ml; PeproTech) (9), respectively. After 5 days in culture, $1 \times 10^6$ live cells/ml were restimulated with medium alone, plate-bound anti-CD3 (2 $\mu$g/ml), or cytokines indicated for 18–24 h as described previously (7). Cell-free supernatants were analyzed for IL-17 and IFN-$\gamma$ using ELISA (reagents from BD Pharmingen or R&D Systems). RNA was isolated from Th cultures and message levels of the genes indicated were analyzed by real-time PCR as described elsewhere (7). RNA

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[4] Abbreviation used in this paper: WT, wild type.

was isolated from cells stimulated for 0, 4, or 18 h with anti-CD3 as specified in the figure legends. All reagents were obtained from Applied Biosystems, and real-time PCR was performed according to standard manufacturers' protocols. Results are relative to the condition as indicated in the figure legend. Chromatin immunoprecipitation analysis was performed as described (24) using PCR primers that span the IL-17A promoter.

*Flow cytometry*

Five-day differentiated Th1 and IL-23-primed cells from *Stat3*$^{CD4-/-}$ and WT littermate controls were analyzed for CXCR3-PE (R&D Systems) by flow cytometry. P- and E-selectin ligand expression was assessed from developing Th1, IL-23-primed, or unskewed (activated CD4$^+$ T cells cultured with anti-IL-4 alone) cultures as described previously (25, 26).

*Retroviral transduction*

Mouse stem cell virus long-terminal repeat and Stat3C retroviruses were prepared by transient transfection of Phoenix-GP packaging cells using a calcium phosphate precipitation method (7). Two million CD4$^+$ T cells were differentiated under conditions described previously in the presence of human IL-2 (50 U/ml). Two days after differentiation, cells were transduced with Mouse stem cell virus long-terminal repeat or Stat3C retrovirus containing supernatants by centrifugation for 1 h at 2000 rpm at room temperature. The supernatants were removed after the spin infection and replaced with medium containing cytokines and Abs for continued differentiation. One day later, cells were expanded with fresh medium in the presence of human IL-2 (25 U/ml). After 6 days of differentiation, cells expressing the truncated mouse MHC class I molecule H-2K$^k$ were positively selected using MACSelect K$^k$ MicroBeads (Miltenyi Biotec). Selected cells at 1 million/ml were restimulated with anti-CD3 (2 $\mu$g/ml) for 1 day. The supernatants were collected and stored at $-20$°C until use for cytokine ELISA.

## Results

*Stat3 is required for the differentiation of IL-17-secreting but not IFN-γ-secreting Th subsets*

To define the requirements for transcription factors in the development of IL-17-secreting T cell subsets, we tested the role of Stat3 in TGF$\beta$1 plus IL-6-mediated T cell differentiation using mice that have a floxed Stat3 allele and express a CD4-Cre transgene (23). Splenic T cell populations (CD4$^+$, CD8$^+$, regulatory T cells (CD4$^+$CD25$^+$), NKT, and $\gamma\delta$) appeared normal in mice with T cells lacking Stat3 (data not shown), agreeing with a previous report of Stat3 conditional mutants with a Lck-Cre transgene (27).

Naive CD4$^+$ T cells were isolated from *Stat3*$^{fl/fl}$ CD4-Cre (*Stat3*$^{CD4-/-}$) mice and *Stat3*$^{fl/fl}$ CD4-Cre-negative (WT) littermate controls. Cells were differentiated under Th1 (IL-12 plus anti-IL-4) or TGF$\beta$1 plus IL-6-primed (TGF$\beta$1 plus IL-6 plus anti-IL-4) conditions for 5 days. Immunoblot analysis of Th cultures confirmed a lack of Stat3 expression in *Stat3*$^{CD4-/-}$ cells (data not shown). After differentiation, cells were washed and left unstimulated or stimulated with anti-CD3 for 24 h. IFN-$\gamma$ and IL-17 levels were measured using ELISA. Although IFN-$\gamma$ secretion by Th1 cultures was independent of Stat3, the development of the IL-17-secreting phenotype was greatly decreased in the absence of Stat3 (Fig. 1*A*). Similarly, analysis of IL-17A/F mRNA expression by real-time PCR demonstrated greatly decreased levels in the absence of Stat3, compared with WT cells (Fig. 1*B*). We further examined the expression of other transcription factors that program the IFN-$\gamma$- or IL-17-secreting phenotype. ROR$\gamma$t is preferentially expressed in TGF$\beta$1 plus IL-6-primed cultures, and expression is dependent upon Stat3 (Fig. 1*B*). In contrast to previous reports, we did not observe differences in the expression of T-bet between IL-12- and TGF$\beta$1 plus IL-6-primed cultures (Fig. 1*B*, compared with Ref. 9). Two additional transcription factors, ERM and Hlx, were expressed at greatly reduced levels in TGF$\beta$1 plus IL-6- vs IL-12-primed cultures (Fig. 1*B*).

We next wanted to test the requirement for Stat3 in cultures that were differentiated using IL-23 to promote the IL-17-secreting phenotype. It has previously been established that IL-23 primarily

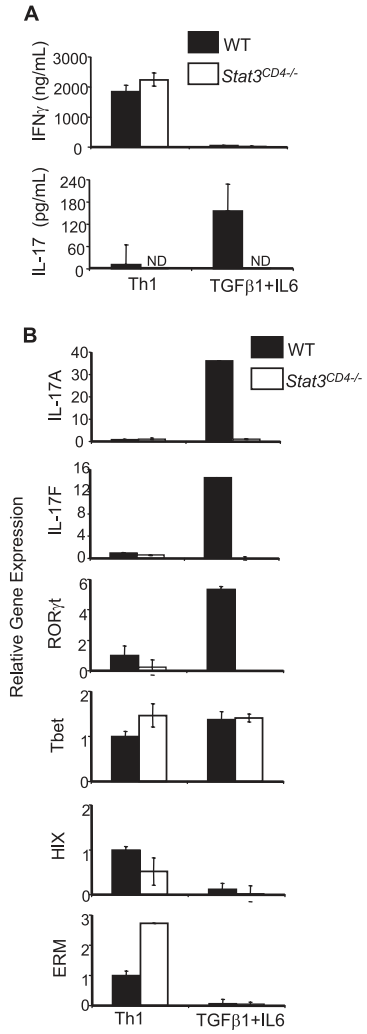


**FIGURE 1.** Stat3 is required for IL-17 production in TGF$\beta$1 plus IL-6-primed cells. *A*, Naive WT and *Stat3*$^{CD4-/-}$ CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 and differentiated under Th1 (IL-12 plus anti-IL-4) or TGF$\beta$1 plus IL-6-primed (TGF$\beta$1 plus IL-6 plus anti-IL-4) conditions for 5 days, washed, and left unstimulated or restimulated with anti-CD3 for 18 h. Cell-free supernatants were analyzed for IFN-$\gamma$ and IL-17 production using ELISA. Unstimulated cells had no detectable cytokine levels. *B*, RNA was extracted from cells cultured as in *A*, activated with anti-CD3 for 4 h, and analyzed for the expression of the indicated genes by real-time PCR. Cycle number is normalized to $\beta_2$-microglobulin expression and results are represented as fold induction relative to the WT-stimulated Th1 conditions. ELISA and real-time PCR data are presented as the mean $\pm$ SD and are representative of two to three experiments. ND, Not detected.

affects memory phenotype T cells (12, 14). To confirm this in our experiments, we differentiated purified memory (CD4$^+$CD62L$^-$) and naive (CD4$^+$CD62L$^+$) T cells under IL-23-primed (IL-23 plus $\alpha$IL-4) conditions for 5 days and stimulated cells with anti-CD3 for 24 h before IFN-$\gamma$ and IL-17 levels were measured using ELISA. IL-17 production was observed primarily in the memory phenotype cultures (Fig. 2*A*). Since results are similar using either purified memory or total CD4$^+$ cells when examining IL-23 function, we used total CD4$^+$ T cells for subsequent experiments. Purified CD4$^+$ T cells from *Stat3*$^{CD4-/-}$ mice and littermate controls were differentiated under Th1 (IL-12 plus anti-IL-4) or IL-23-primed (IL-23 plus anti-IL-4) conditions for 5 days and stimulated with anti-CD3 for 24 h. IFN-$\gamma$ and IL-17 levels were measured using ELISA. Although IFN-$\gamma$ secretion by Th1 or IL-23-primed

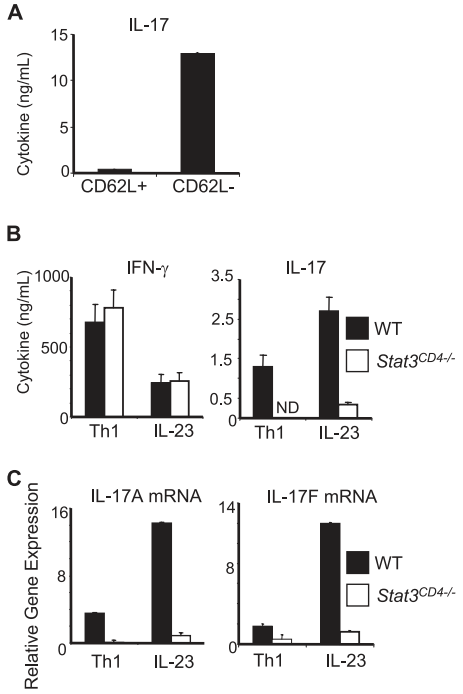


**FIGURE 2.** Stat3 is required for IL-17 production in IL-23-primed cells. *A*, Purified naive ($CD62L^+$) or memory ($CD62L^-$) CD4 T cells were activated with anti-CD3 and anti-CD28 and differentiated under IL-23-primed (IL-23 plus anti-IL-4) conditions for 5 days, washed, and left restimulated with anti-CD3 for 18 h. Cell-free supernatants were analyzed for IL-17 production using ELISA. *B*, WT and $Stat3^{CD4-/-}$ $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 and differentiated under Th1 (IL-12 plus anti-IL-4) or IL-23-primed (IL-23 plus anti-IL-4) conditions for 5 days, washed, and left unstimulated or restimulated with anti-CD3 for 18 h. Cell-free supernatants were analyzed for IFN-$\gamma$ and IL-17 production using ELISA. Unstimulated cells had no detectable cytokine levels. *C*, RNA was extracted from cells activated as in *A* and analyzed for IL-17A/F expression by real-time PCR. Cycle number is normalized to $\beta_2$-microglobulin expression and results are represented as fold induction relative to the WT-unstimulated Th1 conditions. ELISA and real-time PCR data are presented as the mean ± SD and are representative of three experiments. ND, Not detected.

cultures was independent of Stat3, similar to results in Fig. 1, the development of IL-17-secreting Th cells was significantly decreased in the absence of Stat3 ($p < 0.05$) (Fig. 2*B*). IL-17A/F mRNA expression assessed by real-time PCR demonstrated markedly diminished levels in $Stat3^{CD4-/-}$ cultures, compared with control cultures (Fig. 2*C*). Thus, Stat3 is required for the differentiation of IL-17-secreting but not IFN-$\gamma$-secreting Th subsets.

### *Activated Stat3 promotes the IL-17-secreting phenotype*

Although data in Figs. 1 and 2 demonstrate that Stat3 is required for the development of IL-17-secreting T cells, we next wanted to assess the ability of Stat3 to promote the development of IL-17-secreting cells in the absence of other differentiating signals. To test this, we transduced WT T cells differentiated for 6 days under the conditions indicated in Fig. 3, with a bicistronic retrovirus containing either no insert or expressing a constitutively active Stat3 (Stat3C) (28) and H-$2K^k$ (Fig. 3*A*). At the end of the differentiation period, H-$2K^k$-positive cells were purified by magnetic selection and stimulated with anti-CD3. Expression of activated Stat3 in these cultures was confirmed by immunoblot (data not shown). Supernatants from 24-h-stimulated cells were assessed for IL-17 levels by ELISA. Expression of Stat3C in cultures without exogenous cytokines increased IL-17 secretion almost 4-fold (Fig. 3*B*), suggesting that Stat3, in the absence of other differentiating signals could promote the development of IL-17-secreting cells. Similar increased levels of IL-17 secretion by retroviral expression of Stat3C were observed in IL-23-primed and TGF$\beta$1 plus IL-6-primed cultures. Thus, activated Stat3 can promote the development of IL-17-secreting Th cells.

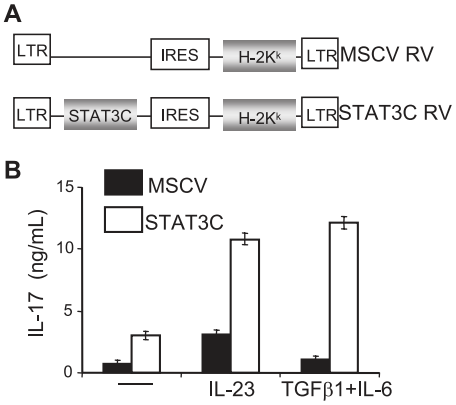


**FIGURE 3.** Active Stat3 promotes the development of IL-17-secreting cells. *A*, Schematic diagram of retroviral vectors used for transduction of primary T cells. *B*, $CD4^+$ T cells were cultured under unskewed, IL-23-primed, or TGF$\beta$1 plus IL-6-primed conditions. After 2 days in culture, cells were transduced with control or Stat3C-expressing retroviruses. On day 6 of differentiation, H-$2K^k$-positive cells were purified by magnetic selection and stimulated with anti-CD3. Supernatants following 24 h of stimulation were tested for IL-17 levels using ELISA. ELISA data are presented as the mean ± SD and are representative of three experiments.

### *Role of Stat3 in the development of IL-12- and IL-23-primed Th cell phenotypes*

Although some genes have been shown to be differentially expressed between IL-12- and IL-23-primed cells (6), the similarity in signaling pathways between IL-12 and IL-23 provided a strong basis to determine whether genes associated with the Th1 phenotype are also expressed in IL-23-primed cultures. To determine whether IL-12 and IL-23 induce similar P- and E-selectin ligand formation, a requirement for inflammation in specific tissues (26), we tested selectin ligand expression on Th1 and IL-23-primed cells. Although these ligands are strongly induced under Th1 conditions in a Stat3-independent manner, IL-23 stimulated minimal selectin ligand expression beyond what was observed in T cells cultured in the absence of either IL-12 or IL-23 (Fig. 4*A* and data not shown). Expression of Th1-specific CXCR3 and the Ets family transcription factor ERM (29) were similar in Th1 and IL-23-primed cells and were independent of Stat3 (Fig. 4, *B* and *C*). IL-18R$\alpha$, T-bet, and Hlx are expressed at lower levels in IL-23-primed cultures than in Th1 cells, and Stat3 is partially required for Hlx in Th1 cells but not T-bet or IL-18R$\alpha$ expression in either culture condition (Fig. 4*C* and data not shown). Thus, Th1 and IL-23-primed cells express overlapping gene sets, although the level of expression may differ. Furthermore, although Stat3 plays an obligate role in the acquisition of the IL-17-secreting phenotype, it may not be required for all aspects of this IL-23-induced differentiated state.

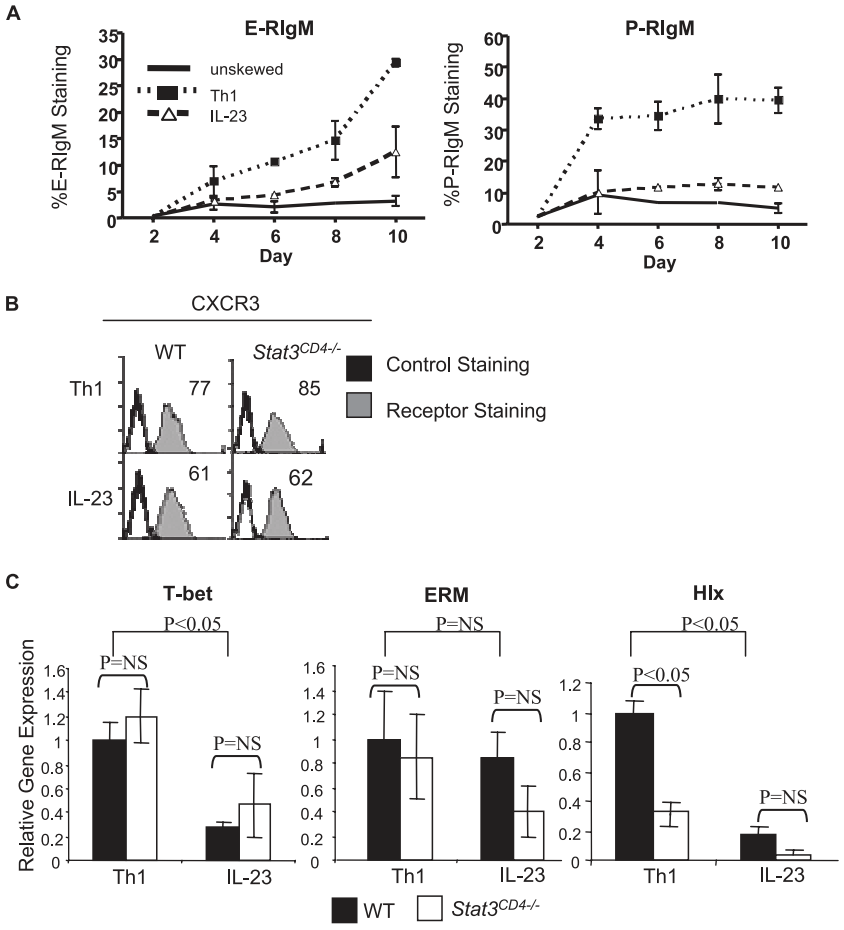


**FIGURE 4.** The role of Stat3 in gene expression from Th1 and IL-23-primed cultures. *A*, Expression of P- and E-selectin ligands on developing Th1, IL-23-primed, or unskewed cultures was determined by flow cytometry using rat IgM (RIgM) chimera proteins. Results are represented as percentage of cells (±SD from two experiments) staining with E- or P-chimeras for each culture condition at the indicated time point. *B*, *Stat3*$^{CD4-/-}$ and WT littermate CD4$^+$ T cells were differentiated for 5 days under Th1 or IL-23-primed conditions and stained for CXCR3-PE before analysis for receptor expression by flow cytometry. Numbers represent mean fluorescence intensities. Black, Control staining; gray, receptor staining. Data are representative of three experiments. *C*, RNA from cells differentiated as in Fig. 2 was analyzed for expression of the indicated genes by real-time PCR. Cycle number is normalized to $\beta_2$-microglobulin expression and results are represented as fold induction relative to the WT Th1 conditions. Results are the mean ± SD of two experiments. Statistics were performed using a two-tailed *t* test and *p* values for comparisons are shown between WT cells of both culture conditions and WT vs *Stat3*$^{CD4-/-}$ cells for both culture conditions.

*Stat4 is required for maximal IL-17 production from IL-23-primed T cells*

Previous work has suggested that Stat4 is not required for the generation of IL-17-secreting Th cells (3, 5). However, IL-23 activates Stat4, decreased levels of IL-17 were observed in *Bacteroides fragilis*-infected Stat4-deficient mice, and Stat4- and IL-23p19-deficient mice share similar resistance to autoimmune diseases (13, 15, 21, 30), supporting a possible role for Stat4 in IL-23-mediated IL-17 production. To define Stat4 dependency in IL-23-stimulated T cell differentiation, WT and *Stat4*$^{-/-}$ CD4$^+$ T cells were differentiated under Th1 or IL-23-priming conditions. After 5 days, cells were left unstimulated or stimulated with anti-CD3 for 24 h. IFN-γ and IL-17 levels were measured using ELISA, and IL-17A/F mRNA expression was assessed by real-time PCR. Stat4$^{-/-}$ IL-23-primed cultures had a significant decrease in IL-17 production compared with WT cultures ($p < 0.05$), indicating that similar to its role in IFN-γ secretion from Th1 cells, optimal IL-17 expression in IL-23-primed cells requires Stat4 (Fig. 5*A*).

Although the precise role of IL-23 in the generation of IL-17-secreting cells is still unclear, evidence suggests that it may play a role in survival or maintenance of Th17 cells. If the role of Stat4 in the generation of IL-23-primed IL-17-secreting cells is strictly in maintaining the phenotype or viability of IL-17-secreting cells, the expression of other Th17-associated genes should also be affected by Stat4 deficiency. To test this, we performed real-time PCR for RORγt and IL-22, two genes expressed in IL-17-secreting populations (19, 31). Basal RORγt expression is similar between WT and *Stat4*$^{-/-}$ IL-23-primed cultures, and while there is a modest decrease in anti-CD3-induced expression in *Stat4*$^{-/-}$ IL-23-primed cultures, the difference from WT cultures is not statistically significant ($p > 0.1$) (Fig. 5*B*). Levels of IL-22 mRNA were indistinguishable between WT and *Stat4*$^{-/-}$ IL-23-primed cultures (Fig. 5*B*). Thus, the role of Stat4 appears to be restricted to programming of the *Il17* gene and does not appear to be required for the expression of other Th17-associated genes.

Our finding of a role for Stat4 in the development of IL-23-primed IL-17 secretion is clearly different from previous reports that suggested no role for Stat4 in the development of IL-17-secreting cells (3, 5). Harrington et al. (5) used an in vitro culture system and restimulated cells with PMA and ionomycin for intracellular cytokine staining to assess IL-17 expression. However, restimulation with PMA and ionomycin has previously been shown to obscure differences between WT and Stat4-deficient Th1 cultures (32). Indeed, when we stimulate our cultures with PMA and ionomycin, we observe similar levels of IL-17 in WT and Stat4-deficient cultures (Fig. 5*C*). Thus, the conditions in previous experiments may not have been optimal for observing Stat4-dependent responses.

We also tested the role of Stat4 in the development of IL-17-secreting cells in a TGFβ1 plus IL-6-primed culture. WT and *Stat4*$^{-/-}$ CD4$^+$ T cells were differentiated under conditions described in Fig. 1. After 5 days, cells were left unstimulated or stimulated with anti-CD3 for 24 h. IFN-γ and IL-17 levels were measured by ELISA. As might be predicted by the lack of Stat4 activation by either TGFβ1 or IL-6, there was no requirement for Stat4 in the generation of IL-17-secreting cells in these cultures (Fig. 5*D*). Thus, although Stat3 is required for the development of IL-17-secreting cells following culture with either TGFβ1 plus

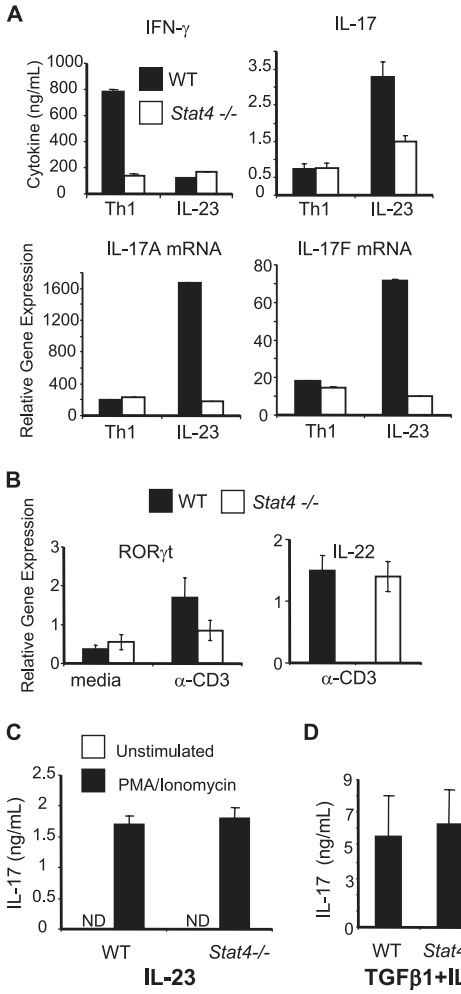


**FIGURE 5.** Stat4 is required for IL-17 production in IL-23-differentiated cells. *A*, WT and *Stat4*$^{-/-}$ CD4$^+$ T cells were differentiated as in Fig. 2 and restimulated with anti-CD3. RNA was extracted from 18-h activated T cells and IL-17A/F expression was quantified using real-time PCR. Cycle number is normalized to $\beta_2$-microglobulin expression and results are represented as fold induction relative to the WT unstimulated Th1 conditions. ELISA and real-time PCR results are presented as the mean ± SD and are representative of two to four experiments. *B*, RNA generated from IL-23-stimulated cultures as in *A* was tested for levels of RORγt and IL-22 mRNA by real-time PCR. Cycle number is normalized to $\beta_2$-microglobulin expression and results are represented as fold induction relative to the IL-23-primed WT unstimulated (*left*) or stimulated (*right*) conditions. Data are averages ± SD of three experiments. *C*, WT and *Stat4*$^{-/-}$ IL-23-primed cultures were differentiated for 5 days and restimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml) or medium alone for 18 h, and cell-free supernatants were analyzed for IL-17 production using ELISA. *D*, CD4$^+$ T cells from WT and *Stat4*$^{-/-}$ mice were differentiated with TGFβ1 plus IL-6 plus anti-IL-4 for 5 days. Cells were restimulated with anti-CD3 and cell-free supernatants were analyzed for IL-17 using ELISA. ND, Not detected. ELISA results for *C* and *D* are presented as the mean ± SD and are representative of two experiments.

IL-6 or IL-23, the requirement for Stat4 is restricted to the action of IL-23.

Restimulation of Th1 cells with IL-12 plus IL-18 synergizes in the Stat4-dependent induction of IFN-γ, whereas IL-23 and IL-18 synergize in the induction of IL-17 from IL-23-primed cells (7). To assess the role of Stat4 in cytokine-induced IL-17 production, Th1 and IL-23-primed cells were restimulated with medium alone, IL-12 plus IL-18, or IL-23 plus IL-18 (2° culture) and assayed for the production of IFN-γ and IL-17 using ELISA. Although individual cytokines had little effect (data not shown), IL-12 plus IL-18 induces Stat4-dependent production of IFN-γ from Th1 cells and to a lesser degree from IL-23-primed cultures (Fig. 6). IL-23 plus IL-18 stimulate IL-17 production only in IL-23-primed cells while promoting minimal IFN-γ production (Fig. 6). IL-23 plus IL-18-induced IL-17 secretion was eliminated in *Stat4*$^{-/-}$ IL-23-primed cultures (Fig. 6), reinforcing the requirement for Stat4 in IL-23-mediated T cell function.

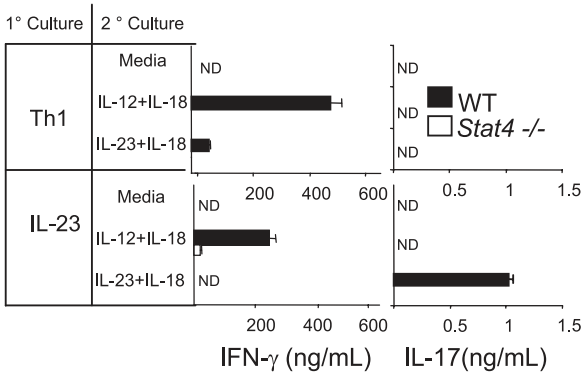


**FIGURE 6.** Stat4 is required for cytokine-induced IL-17 production. WT and *Stat4*$^{-/-}$ Th1 or IL-23-primed cultures were differentiated for 5 days as described in Fig. 5 (1° culture) followed by incubation for 24 h in the absence or presence of the indicated cytokine combinations. Cell-free supernatants were analyzed for IL-17 and IFN-γ using ELISA. ELISA data are presented as the mean ± SD and are representative of four experiments. ND, Not detected.

## Discussion

IL-17-secreting CD4$^+$ T cells are important in the development of autoimmune disease and resistance to some infectious agents. Although it is now recognized that these cells can be differentiated in cytokine environments containing either IL-23 or TGFβ1 plus IL-6, the transcription factors involved in programming Th cells for IL-17 expression have not been completely identified. In this report, we demonstrate that Stat3 is required for the development of IL-17-secreting Th cells in either cytokine environment, while Stat4 is important only for the development of IL-17-secreting Th cells in response to IL-23. Stat4 is also required during cytokine-stimulated production of IL-17. Thus, these results provide the first definitive evidence for cytokine-induced transcription factors required for the development of IL-17-secreting Th cells.

Although Stat3 has not been previously implicated in Th differentiation, our results clearly demonstrate that Stat3 is required for the expression of IL-17A, IL-17F, and RORγt in Th17 cultures. This is further supported by recent data demonstrating that enhanced Stat3 activation in suppressor of cytokine signaling 3 (Socs3) conditional mutant T cells resulted in increased generation of IL-17-secreting cells and a requirement for Stat3 in the acute response to IL-23 (33, 34). Our results suggest that, like the respective roles for Stat4 and Stat6 in Th1 and Th2 development, cytokine-stimulated Stat protein activation is required for the IL-17-secreting Th differentiation pathway. Although we have shown that expression of the major biomarkers of IL-17-secreting cells, IL-17A, IL-17F, and RORγt, require Stat3 signaling for expression, it is certainly possible that other features of the Th17 phenotype may be independent of Stat3. Furthermore, Stat3 plays only a minor role in the development of Th1 cells, partially affecting ERM and Hlx expression (Figs. 1*B* and 4*C*). The modest differences in results between analyses of Th1 cells between Figs. 1*B* and 4*C* could be due to the use of naive or total CD4$^+$ cells in

those respective experiments. Overall, our results establish a critical role for Stat3 in the acquisition of IL-17-secreting but not IFN-γ-secreting Th phenotypes.

The mechanism for Stat3 activation of the IL-17-secreting Th genetic program is still unclear. Stat3 can bind directly to the IL-17A promoter in response to IL-23 stimulation (33), and we have observed that Stat3 is bound to the IL-17A promoter during differentiation of TGFβ plus IL-6-primed cells (data not shown). This suggests that Stat3 binding to the IL-17 locus may directly promote transcription and local chromatin remodeling. Stat3 may also cooperate with other factors activated by IL-23, TGFβ1, or IL-6 in the establishment of this phenotype. Additionally, we have shown that Stat3 is required for the expression of RORγt in Th17 culture conditions (Fig. 1*B*), a factor recently shown to be critical for the development of Th17 cells. Future studies will define the relative roles of each of these factors in Th17 development.

We have observed some differences in the ability of CD4 T cells from different strains of mice to differentiate into IL-17-secreting cells. Although both C57BL/6 and BALB/c T cell cultures can produce higher levels of IL-17 secretion, cells from 129/Sv or mixed 129 × C57BL/6 mice secrete only low levels of IL-17 (Fig. 1*A* and our unpublished observations). This might correlate with differences in the expression of transcription factors controlling the phenotype. Indeed, T-bet expression was not as repressed in the control *Stat3*$^{fl/fl}$ TGFβ plus IL-6 cultures (Fig. 1*B*) as in studies previously reported by Veldhoen et al. (9) using C57BL/6 cells. The basis for these differences is still unclear although they may be reflected in the susceptibility of these strains to certain diseases and infectious agents.

The ability of the constitutively active Stat3 to promote IL-17 secretion suggests that it is sufficient to activate the Th17 development program. However, we have not ruled out the involvement of other Stat proteins in this process. Constitutively active Stat5 and Stat6 were shown to promote Th2 development (35, 36) and given the ability of IL-4 to inhibit Th17 generation (3, 5), it is likely that these would have a negative effect on the development of IL-17-secreting cells. A mutation of Stat1 analogous to the Stat3C mutation does not confer constitutive activity, but rather increased IFN-γ-inducible activity (37). The negative effects of IFN-γ on the development of IL-17-secreting cells (3, 5), as well as the ability of Stat1 to induce T-bet (38), which is itself a negative regulator of IL-17 secretion (3, 5, 7), suggest that active Stat1 would interfere with Th17 differentiation. A constitutively active Stat4 has not been described. Thus, although we have not excluded the involvement of other Stat proteins and our data certainly indicate a role for Stat4, we demonstrate that Stat3 is necessary and sufficient to promote the development of IL-17-secreting Th cells.

As mentioned in *Results*, our data demonstrate a role for Stat4 in the development of IL-23-primed Th cells, which differs from previously published data. One report used PMA plus ionomycin, which has been shown to minimize the effects of Stat4 deficiency in Th1 cultures, to stimulate Stat4-deficient cultures (5, 32). We demonstrate a partial role for Stat4 in the secretion of IL-17 from IL-23-primed cultures, when the cells are restimulated with anti-CD3. Moreover, these cultures are completely dependent on Stat4 for cytokine-induced IL-17 production. Thus, the more physiological the stimulus, the greater the dependence on Stat4 observed. Indeed, using the ChIP assay, we can find Stat4 associated with the IL-17A gene following IL-23 plus IL-18 stimulation (data not shown). A second report by Park et al. (3) used in vivo immunization of WT and Stat4-deficient mice to assess IL-17 production from Ag-stimulated cells. However, this protocol does not distinguish between in vivo generation of IL-17-secreting cells primed by IL-23 or TGFβ1 plus IL-6. Because we have shown that Stat4 dependence is restricted to IL-23-stimulated cells, the lack of a role for Stat4 in an in vivo immunization model may reflect the relative role of these two pathways in the generation of IL-17-secreting cells. Importantly, our results provide a mechanism for decreased IL-17 following *B. fragilis* infection of Stat4-deficient mice and the similarity in phenotype between Stat4- and IL-23p19-deficient mice in autoimmune disease models (15, 21, 30). Thus, Stat4 deficiency, like IL-12p40 deficiency, results in a composite phenotype from defects in IL-12 and IL-23 signaling.

There are many aspects of the development of IL-17-secreting T cells that are still unclear. It has been proposed that TGFβ1 plus IL-6 and IL-23 act sequentially in the development of IL-17-secreting cells, though it is not certain whether IL-23 only acts upon cells primed with TGFβ1 plus IL-6. Indeed, IL-23 has been shown to preferentially affect memory phenotype cells (Fig. 2*A*) and, unlike TGFβ1 plus IL-6, did not promote an IL-17-secreting phenotype from naive cells (9–12, 14). However, IL-23 does not promote an IL-17-secreting phenotype from differentiated Th1 or Th2 cells (Ref. 5 and data not shown), suggesting that some targets of IL-23 are T cells that have been activated but are not committed to a Th lineage. Although it is likely that IL-23 acts on TGFβ1 plus IL-6-primed cells (9), it is also possible that IL-23 promotes an IL-17-secreting phenotype from other T cell populations.

In this report, we have defined cytokine-induced transcription factor requirements for the establishment of an IL-17-secreting Th cell. Stat3 is required for multiple features of Th17 cells including the expression of IL-17 genes and RORγt, analogous to the requirement for Stat4 and Stat6 in Th1 and Th2 differentiation, respectively. Moreover, Stat4 is required for the maximal development of IL-17-secreting cells, but not the expression of other Th17-associated genes, in IL-23-primed cultures. How Stat3 and Stat4 signals integrate to direct the development of IFN-γ- or IL-17-secreting T cells is still unclear. Targeting the balance of Stat activation within developing Th cell subsets could be an important mechanism of treating inflammatory disease.

## Disclosures

The authors have no financial conflict of interest.

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