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Th cell effector subsets develop in response to specific cytokine environments. The development of a particular cytokine-secreting pattern requires an integration of signals that may promote the development of opposing pathways. A recent example of this paradigm is the IL-9-secreting Th9 cell that develops in response to TGF-β and IL-4, cytokines that, in isolation, promote the development of inducible regulatory T cells and Th2 cells, respectively. To determine how the balance of these factors results in priming for IL-9 secretion, we examined the effects of each pathway on transcription factors that regulate Th cell differentiation. We demonstrated that TGF-β induces the PU.1-encoding Spool locus and that this is independent of IL-4-induced STAT6 activation. IL-4-activated STAT6 is required for repressing the expression of T-bet and Foxp3 in Th9 cells, transcription factors that inhibit IL-9 production, and STAT6 is required for the induction of IRF4, which promotes Th9 development. These data established a transcription factor network that regulates IL-9 and demonstrated how combinations of cytokine signals generate cytokine-secreting potential by altering the expression of a panel of transcription factors. The Journal of Immunology, 2012, 188: 000–000.

Materials and Methods

Mice

Wild-type (WT) female mice (C57BL/6 and BALB/c) were purchased from Harlan Biosciences (Indianapolis, IN). Mice with conditional Stat3 deletion, which were provided by Dr. David Levy (New York University, New York, NY) and described previously (12), were mated to Cd4-cre mice (13). Ranit2−/− mice were mated to dLck-Cre mice (14). Stat6−/− and Purp14-deficient mice were described previously (15, 16) and are on a BALB/c and C57BL/6 background, respectively. Il4−/− mice were purchased from Jackson Laboratories and were bred in the Indiana University animal facility. Mice were maintained in pathogen-free conditions, and studies were approved by the Indiana University Institutional Animal Care and Use Committee.

Differenetiation of murine T cells

Naive CD4+CD62L+ T cells were purified from spleens and lymph nodes by magnetic selection (Miltenyi Biotec). Naive CD4+ T cells (1 × 10^6 cells/ml complete RPMI 1640 medium) were activated with plate-bound anti-CD3 (2 μg/ml; 145-2C11; BioXcell) and soluble anti-CD28 (1 μg/ml; 37.51; BD Biosciences) and cultured under Th9 conditions (IL-4 [20 ng/ml; PeproTech], TGF-β [2 ng/ml; R&D Systems], and anti–IFN-γ [10 μg/ml; XMG; BioXcell]); Th2 conditions (IL-4 and anti–IFN-γ); Th1 conditions (IL-12 [5 ng/ml; R&D Systems], IL-2 [50 U/ml; PeproTech], and anti–IL-4 [10 μg/ml]; 11B11; BioXcell]; Th17 conditions (TGF-β, IL-6 [100 ng/ml; R&D Systems], IL-1β [10 ng/ml; eBioscience], IL-23 [10 ng/ml; R&D Systems], anti–IFN-γ, and anti–IL-4); and Treg conditions (TGF-β and anti–IL-4). After 3 d, cultures were expanded with fresh complete RPMI 1640 medium with IL-4 and TGF-β added to the Th9...
cells; half the dose of IL-1β, IL-23, and IL-6 added to the Th17 cells; and IL-2 added to Tregs. After 5 d of differentiation, cells were stimulated with plate-bound anti-CD3 (4 μg/ml) for 1 d. Cell-free supernatant was collected, and the amount of IL-9 was assessed by ELISA using anti-IL-9 capture Ab (D8402E8; BD Biosciences) and biotin-labeled anti-IL-9 (D9302C12; BioLegend) as the secondary Ab.

**Intracellular cytokine staining**

After 5 d of culture, cells were stimulated with PMA (phorbol 12-myristate 13-acetate) and ionomycin for 5 h. Monensin was added to the cells for the last 3 h of stimulation. Cells were fixed with paraformaldehyde after stimulation and permeabilized with saponin. Cells were then stained with fluorochrome-conjugated anti-mouse IL-9 (RM9A4; BioLegend), anti-mouse IL-4 (11B11; BioLegend), and anti–IL-17A (eBio17B7; eBioscience). For intracellular phospho-STAT staining, Th2 and Th9 cells were harvested each day during the 5-d differentiation, fixed with 1.5% paraformaldehyde for 10 min at room temperature, and permeabilized for 10 min with 100% methanol at 4°C. Cells were then stained for phospho-STAT3 and phospho-STAT6 (BD Pharmingen) after 30 min at room temperature. Cells were analyzed by flow cytometry with FACSCalibur (Beckton Dickinson).

**Retroviral transduction**

Bicistronic retroviral vectors encoding mouse GATA3 and human CD4, T-bet, Foxp3, JunB, or c-Maf (kindly provided by I.C. Ho, Brigham and Women’s Hospital, Boston, MA) and enhanced GFP or Runx3 and Thy1.1 were used to generate virus, as described previously (17, 18). Naive CD4+ T cells from WT and/or IL-4-deficient and Stat6-deficient mice were cultured under Th9 cell conditions; after 2 d of differentiation, Th cells were transduced with retroviral supernatant containing polybrene. On day 5, transduced cells (eGFP+, Thy1.1+, or hCD4+) were analyzed by flow cytometry for IL-9, IL-4, and IL-17A secretion.

**Quantitative RT-PCR**

Total RNA was isolated from unstimulated or stimulated (plate-bound anti-CD3; 2 μg/ml) cells using TRIzol and reverse transcribed, according to the manufacturer’s instructions (Invitrogen Life Technologies). For quantitative RT-PCR (qPCR), TaqMan Fast Universal Master Mix and commercially available primers were used for Gata-3, Maf, T-bet, Runx3, Foxp3, and IL9, and RNA expression was normalized to the expression of β2-microglobulin. The relative expression was calculated by the change-in-change (ΔΔCt) method.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChiP) assay was performed, as previously described (19), with minor modifications. Cells were kept unstimulated before cross-linking of protein–chromatin complexes. Immunocomplexes were precipitated with protein A-agarose beads at 4°C overnight. The supernatant was incubated in the presence of rabbit polyclonal Abs (anti-PU.1 [Santa Cruz Biotechnology] and rabbit IgG [Millipore]). DNA cross-links were reversed by incubating precipitates overnight at 65°C, and DNA was purified by phenol-chloroform extraction and ethanol precipitation.

DNA quantification was performed with SYBR Green Fast PCR Master Mix via an ABI 7500 Fast Real-time PCR system. A standard curve was generated from serial dilutions of input DNA to quantify immunoprecipitated DNA. The amount of immunoprecipitated DNA from the specific Ab ChiP and normalized against the amount of input DNA to calculate percentage input.

**Immunoblot**

Total cell lysates were prepared from Th9, Th2, or Th1 cells after 5 d of differentiation. The lysates were resuspended in SDS-PAGE loading buffer, boiled at 100°C for 5 min, and run on SDS-PAGE gels (Invitrogen Life Technologies) before immunoblot.

**Results**

**Differential requirement for STAT proteins in Th2 and Th9 cells**

In addition to the requirement for STAT6 in the development of Th2 and Th9 cells (5, 7, 16, 20, 21), we recently showed that STAT3 is also required for the development of Th2 cells (13). To compare the phosphorylation of STAT3 and STAT6 during the development of Th2 and Th9 cells, we cultured naive CD4+ T cells under Th2 or Th9 conditions for 5 d and analyzed STAT phosphorylation each day during culture. The percentages of cells that were phospho-STAT6+, as well as the intensity of phospho-STAT6 staining, were identical between Th2 and Th9 cells (Fig. 1A). STAT3 was also phosphorylated during Th9 differentiation, although with slightly lower percentages of cells and mean fluorescence intensity than in Th2 cells (Fig. 1A). To define the role for each STAT protein in the development of IL-9–secreting T cells, we differentiated naive T cells from mice with conditional deletion of Stat3 in T cells (Stat3(−/−)) and littermate controls or from WT and Stat6(−/−) mice. Consistent with previous observations, IL-9 production was absent in cells lacking expression of STAT6 (Fig. 1B, 1C). In contrast, and despite the activation of STAT3 throughout Th9 differentiation, STAT3 was not required for the development of IL-9–secreting cells (Fig. 1B, 1C).

We next examined the requirement for STAT6 cofactors in the development of Th9 cells. PARP-14, previously identified as...
a collaborator of STAT6 (22, 23), is a poly-ADP ribosyl polymerase. PARP-14 interacts with STAT6 target genes and increases STAT6-dependent gene expression by facilitating the clearance of repressive molecules from gene promoters and increasing binding of STAT6 (24). Because STAT6 was required for Th9 development, we next compared the differentiation of WT and Parp14-/- Th9 cells. Consistent with the role of PARP-14 as a coactivator, we observed diminished production of IL-9 in the absence of PARP-14 (Fig. 1B, 1C). Together, these data suggested that although STAT6 is required for both Th2 and Th9 development,

FIGURE 3. Transcription factor binding to the Il9 locus. A, Schematic diagram of the Il9 gene, with CNS used for ChIP analysis indicated. B, Naive CD4+ T cells from WT mice were activated with anti-CD3 and anti-CD28 and cultured under Th2 and Th9 cell conditions for 5 d. ChIP was performed for STAT6 before qPCR for two Il9 CNS sites, or for the Irf4 or Maf promoters, to determine the amount of Stat6 binding. Data are average ± SD of four mice from two experiments. C, Naive CD4+ T cells from WT and Stat6-/- mice were cultured under Th9 conditions for 5 d. ChIP was performed for PU.1 (top panel) and IRF4 (bottom panel) before qPCR for two Il9 CNS sites. Data are average ± SD of six to eight mice from three or four experiments.
STAT6, although activated in both developing subsets, is only required for Th2 development.

**Th2 transcription factors repress IL-9 in Th9 cultures**

Because STAT6 was required for the development of Th2 and Th9 cells, we examined the expression of transcription factors downstream of STAT6 in Th2 cells for a role in Th9 cells. GATA3 was shown to be necessary for the development of IL-9-secreting T cells, but it also had low expression in Th9 cultures (5, 7). Expression of other Th2 transcription factors has not been carefully examined. To explore the role of these factors further, we tested the expression of Gata3, Maf, and Junb in polarized Th cell cultures by qPCR. Gata3 was expressed in the highest amount in Th2 cells, as has been well documented (25, 26), although it was also detected in Th9 cultures (Fig. 2A). Because the expression of Gata3 in Th9 cultures was previously described to be low, we confirmed our results by analyzing expression of GATA3 protein using intracellular staining for flow cytometry. We observed that Th2 and Th9 cultures had similar levels of GATA3 protein before stimulation with anti-CD3 (Fig. 2B). However, GATA3 protein doubled following stimulation of Th2 cultures but was unaffected by stimulation of Th9 cultures (Fig. 2B). Maf, which is important for the production of IL-4 and IL-21 in Th2 and Th17 cells (27–29), was expressed at the highest levels in Th9 cultures assessed by qPCR (Fig. 2A). Junb, which also contributes to IL-4 production (30, 31), did not show differential expression among Th subsets, although it was enriched in Treg cultures (data not shown). We examined the induction of expression of Gata3, Maf, and If4, the latter a factor previously documented to be expressed in Th9 cells and to regulate IL-9 (9), for differences in kinetics during the differentiation of Th2 and Th9 cells. We observed similar patterns of induction during differentiation, with greater induction of Gata3 and Maf in Th2 and Th9 cells, respectively, toward the end of differentiation (Fig. 2A, 2C). If4 also showed a similar pattern of induction in Th2 and Th9 cells, although expression was higher in Th9 cells throughout differentiation (Fig. 2C). To determine whether these factors might account for the IL-4/STAT6–dependent induction of IL-9, we examined the expression of each of these genes, as well as Sfpi1, the gene encoding PU.1, a transcription factor that we recently defined as promoting Th9 differentiation (8), in WT and Stat6−/− Th9 cultures. We observed that, although expression of Sfpi1 was not dependent upon STAT6, expression of If4, Gata3 and Maf, as well as GATA3 protein expression, was decreased in the absence of STAT6 (Fig. 2D, 2E).

We then determined whether STAT6 could be regulating IL-9 production by binding directly to the I9 gene or whether it affected the binding of other factors to the I9 gene. We performed ChIP experiments, testing the association of STAT6 to two conserved noncoding sequences (CNS) upstream of the I9 locus (32). Although STAT6 binding could be detected to CNS1 (I9 promoter) more than CNS0, the percent input values were very low (Fig. 3A, 3B), and <5% of the STAT6 binding detected to the Maf and If4 promoters in Th2 and Th9 cells (Fig. 3B). In agreement with this finding, STAT6 binding was not detected in the I9 locus in Th2 cells analyzed using published ChIP-sequencing datasets (33). These data suggested that I9 is not a major binding target of STAT6.

Because PU.1 and IRF4 were shown to regulate I9 directly (8, 9), we tested whether STAT6 deficiency affected the binding of either factor to I9 regulatory elements. We demonstrated that binding of PU.1 to the I9 promoter (CNS1) was indistinguishable between WT and Stat6−/− Th9 cultures (Fig. 3C). In contrast, IRF4 binding to the I9 gene was decreased in Stat6−/− Th9 cultures (Fig. 3C). This suggested that IRF4 could be a relevant transcription factor downstream of IL-4/STAT6. However, transduction of IRF4 into Stat6−/− Th9 cultures did not rescue IL-9 production (data not shown), suggesting that it is not sufficient to induce I9 in the absence of STAT6.

To determine whether either GATA3 or c-Maf contributed to IL-9 production, we transduced differentiating Th9 cells with bicistronic retroviruses and examined the effects on IL-9 production using flow cytometry. Transduction of GATA3 resulted in a decrease in IL-9–producing cells compared with Th9 cultures transduced with control retrovirus (Fig. 4A). We observed a similar result when we transduced Th9 cultures with a c-Maf–expressing retrovirus (Fig. 4B). We then determined whether either GATA3 or c-Maf contributed to IL-9 production, we transduced differentiating Th9 cells with bicistronic retroviruses and examined the effects on IL-9 production using flow cytometry. Transduction of GATA3 resulted in a decrease in IL-9–producing cells compared with Th9 cultures transduced with control retrovirus (Fig. 4A). We observed a similar result when we transduced Th9 cultures with a c-Maf–expressing retrovirus (Fig. 4B).
expressing retrovirus (Fig. 4B). Transduction with a JunB-expressing retrovirus did not affect IL-9 production (Fig. 4C). We speculated that because both GATA3 and c-Maf can induce the production of IL-4, these factors might be inducing IL-4–secreting cells in Th9 cultures at the expense of IL-9–secreting T cells. However, neither GATA3 nor c-Maf transduction increased the percentages of IL-4–secreting cells (Fig. 4A, 4B). It was also possible that increases in endogenous IL-4 during the culture period were affecting the differentiation of IL-9–secreting T cells. To test this, we performed transductions into differentiating Il4−/− Th9 cultures. Differentiation of IL-9–secreting T cells, in the absence of endogenous IL-4, was normal (Fig. 4A, 4B). Moreover, transduction of GATA3 or c-Maf into Il4−/− Th9 cultures was as effective at reducing IL-9 production as was transduction into WT cells (Fig. 4A, 4B). Thus, although GATA3 and Maf are expressed in Th9 cells, ectopic expression actually decreased IL-9 production through effects that are independent from the induction of IL-4.

**Repression of IL-9 by Th1-associated transcription factors**

Because IL-4 promotes Th2 differentiation, it interferes with the expression and function of other transcription factors, such as T-bet and Runx3, which promote IFN-γ expression and Th1 differentiation (34, 35). Although T-bet is predominantly expressed in Th1 cells, with expression in Th9 cells similar to other lineages, Runx3 is expressed in most Th lineages, with the highest expression in Th1 cells but detectable expression in Th9 cells (Fig. 5A, 5B). To determine whether T-bet and Runx3 limit Th9 development, we transduced differentiating Th9 cells with retroviruses expressing each factor. Ectopic expression of either T-bet or Runx3 decreased the percentages of IL-9–secreting T cells (Fig. 5C). Consistent with this observation, differentiation of Th9 cells using naive CD4+ T cells deficient in T-bet or Runx3 resulted in increased percentages of IL-9–secreting T cells compared with control cultures (Fig. 5D). To determine whether STAT6 regulates the expression of either of these factors in Th9 cells, we tested mRNA from WT and Stat6−/− Th9 cultures. Runx3 expression was decreased in the absence of STAT6; in contrast, T-bet expression was increased in the absence of STAT6 (Fig. 5E). Together, these data suggested that both T-bet and Runx3 can limit IL-9 production and that the STAT6 signal represses T-bet expression in Th9 cells.

A balance of IL-4 and TGF-β signals is required for the development of IL-9–secreting T cells

Although STAT6 was not required for expression of Sfpi1 in Th9 cells, it is required to modify TGF-β–induced Treg development, because IL-9–secreting T cells develop from a balance between TGF-β– and IL-4–induced signals (5, 7). When the dose of IL-4 was held constant at 0 ng/ml (Treg condition) or 10 ng/ml (Th9 condition), increasing doses of TGF-β resulted in increased production of Foxp3+ cells, attenuated by the presence of IL-4 in Th9 conditions, and increasing amounts of IL-9 when IL-4 was present (Fig. 6A). Although a small percentage of dim IL-9+ cells was detectable in Treg cultures, there was no IL-9 detectable from Treg cultures using ELISA (Fig. 6A). There was a similar increase in the expression of Sfpi1 in response to TGF-β, with slightly higher expression under Treg conditions than under Th9 conditions, consistent with our previous work (Fig. 6B) (8). In contrast, with a constant dose of TGF-β, altering the dose of IL-4 did not affect Sfpi1 expression (Fig. 6B), consistent with STAT6–independent expression (Fig. 2D). These results suggested that IL-9–secreting cells arise when there is a balance between the TGF-β signal that induces Sfpi1 expression and the IL-4 signal that limits Foxp3 expression. This is consistent with the requirement for STAT6 in the ability of IL-4 to limit Foxp3 expression.
(Fig. 6C) (36, 37) and suggested that Foxp3 may be incompatible with IL-9 production. To test this, we transduced developing Th9 cells with Foxp3-expressing or control retroviruses. We observed that ectopic expression of Foxp3 reduced IL-9 production without the induction of the Th17 cytokine IL-17A or IL-4 (Fig. 6D, data not shown). Thus, it is likely that an additional role for STAT6 during the development of Th9 cells is the repression of Foxp3 expression that would otherwise reduce IL-9 production.

Discussion

Transcription factors direct the development of Th subsets with distinct cytokine-secreting potentials. A multitude of factors has been identified that can promote one lineage while decreasing differentiation of another. The developing Th subset integrates multiple, sometimes conflicting, signals as it adopts a particular effector fate. This is particularly true in the development of IL-9–secreting Th9 cells, where the cell integrates the Treg-inducing TGF-β signal and the Th2-inducing IL-4 signal to adopt a phenotype discrete from either individual signal.

In this study, we documented specific targets of both Th9-inducing signals (Fig. 6E). The TGF-β signal, which is well documented to induce Foxp3, also induces expression of Sfipl (Fig. 6). The IL-4 signal targets multiple genes, including the induction of Irf4, Gata3, and Maf, and is required for the repression of T-bet and Foxp3. Each of these factors likely plays combinatorial roles in the regulation of Il9 gene expression. Foxp3 represses IL-9 production and would contribute to the lack of appreciable IL-9 production from Tregs, despite their expression of Sfipl. Thus, the IL-4 signal regulates the expression of both positive and negative regulators of IL-9 production.

T-bet and Runx3 repress IL-9 in Th9 cells and likely contribute to diminished Il9 expression in Th1 cells, as they do at the Il4 locus (35). This could occur through both direct and indirect mechanisms. In support of a direct effect, in previously generated ChIP-sequencing datasets of Runx3/CBF and T-bet binding in Th1 cells (14), both factors bound directly to the Il9 gene: T-bet at a major peak around −22.5 kb and smaller peaks between +7 and +9 kb and Runx3 at a major peak around +5 kb and minor peaks at −25, −17, −7, and +11 kb. They might also have indirect effects by regulating other transcription factors required for IL-9 production. However, it seems unlikely that Runx3 contributes to the lack of IL-9 production in Stat6−/− Th9 cultures, because expression of Runx3 was decreased in the absence of STAT6 (Fig. 5E). Increased expression of Tbx21 in the absence of STAT6 suggested that the IL-4/STAT6 signal is required to limit T-bet-mediated inhibition in Th9 cells.

Similar to data in Th2 cells, STAT6 is required for the expression of Gata3 and Maf in Th9 cells. However, the role of these factors in Th9 cells remains unclear. GATA3 was required for Th9 development, although initial reports noted it was not expressed in Th9 cultures (5, 7). We found Gata3 expressed in Th9 cells, less than in Th2 cells but in greater amounts than observed in other Th lineages (Fig. 2). Expression of Gata3 in Th9 cells was confirmed by intracellular staining. However, ectopic expression of GATA3 reduced IL-9 production in WT cells and did not induce IL-9 production when transduced into Stat6−/− Th9 cultures, suggesting that it was not directly regulating the Il9 gene. It is possible that GATA3 is an intermediate in the IL-4/STAT6-dependent reduction of Foxp3, as was demonstrated for GATA3 in Tregs (38). The expression of Maf was higher in Th9 cultures than in either Th2 or Th17 cells, for which the function of c-Maf has been previously examined (27–29). c-Maf also repressed IL-9 production, suggesting that it was not involved in regulation of the Il9 gene. c-Maf might be involved in the regulation of other
genes in Th9 cells. We showed that Th9 cells can secrete IL-21 (39), and because this cytokine is a c-Maf target in other Th lineages, there might be a role for c-Maf in promoting IL-21 production in Th9 cells. There could also be kinetic effects of the expression of each factor during Th cell differentiation that might be obscured by ectopic expression during in vitro culture. Although we did not observe significant differences in expression of these factors until the final days of Th2 and Th9 differentiation, it is still possible that a balance of these factors, at particular times during the differentiation process, is critical for appropriate IL-9 production.

IRF4 is one of the factors downstream of IL-4 and STAT6 that promotes IL-9 production. It was previously shown to bind the Il9 promoter and is required for the expression of IL-9 (9). We confirmed these observations and demonstrated that, in the absence of STAT6, there is decreased IRF4 expression, as well as decreased binding of IRF4 to the Il9 promoter. However, IRF4 is not the only factor “missing” in Th9 cells. Transduction of IRF4 into Stat6−/− Th9 cultures did not rescue IL-9 production (data not shown). Moreover, IRF4 transduction did not decrease Foxp3 expression and only modestly affected T-bet expression, further suggesting that additional factors downstream of IL-4 and STAT6 are required for the development of IL-9-secreting T cells.

The Th9 subset of Th cells was only recently described, and the factors that promote its development are still being defined. In this article, we defined a transcription factor network downstream of the Th9-inducing cytokines TGF-β and IL-4 (Fig. 6E). TGF-β induction of Sip1 is complemented by the IL-4–dependent repression of Foxp3 and T-bet and the induction of IRF4, as well as other likely factors that are not yet defined. Further work in defining these factors will add needed insight to the regulation of IL-9.

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Disclosures
The authors have no financial conflicts of interest.

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