Sensitivity and Resistance to Regulation by IL-4 during Th17 Maturation

Laura A. Cooney, Keara Towery, Judith Endres and David A. Fox

J Immunol 2011; 187:4440-4450; Prepublished online 26 September 2011;
doi: 10.4049/jimmunol.1002860
http://www.jimmunol.org/content/187/9/4440
Sensitivity and Resistance to Regulation by IL-4 during Th17 Maturation

Laura A. Cooney,¹ Keara Towery,² Judith Endres, and David A. Fox

Th17 cells are highly pathogenic in a variety of immune-mediated diseases, and a thorough understanding of the mechanisms of cytokine-mediated suppression of Th17 cells has great therapeutic potential. In this article, we characterize the regulation of both in vitro- and in vivo-derived Th17 cells by IL-4. We demonstrate that IL-4 suppresses reactivation of committed Th17 cells, even in the presence of TGF-β, IL-6, and IL-23. Downregulation of IL-17 by IL-4 is dependent on STAT6 and mediated by inhibition of STAT3 binding at the Il17a promoter. Although Th1 cytokines were shown to induce IFN-γ expression by Th17 cells, IL-4 does not induce a Th2 phenotype in Th17 cells. Suppression by IL-4 is stable and long-lived when applied to immature Th17 cells, but cells that have undergone multiple rounds of stimulation, either in vivo during a Th17-mediated inflammatory disease, or in vitro, become resistant to suppression by IL-4 and lose the ability to signal through IL-4R. Thus, although IL-4 is a potent suppressor of the Th17 genetic program at early stages after differentiation, prolonged stimulation renders Th17 cells impervious to regulatory cytokines. The Journal of Immunology, 2011, 187: 4440–4450.

T

h17 cells, which express the proinflammatory cytokine IL-17, play an important role in the pathogenesis of a variety of autoimmune and inflammatory diseases. Naive CD4⁺ T cells differentiate into Th17 cells in response to TGF-β, IL-6, and IL-21 and become more pathogenic in the presence of IL-23 (1–7). In addition, Th17 cell development depends on the transcription factors STAT3, IRF4, RORγt, and RORα (8–11). In vitro, induction of Th17 differentiation is inhibited by the Th1 cytokines IFN-γ and IL-12, as well as the Th2 cytokines IL-4 and IL-13 (12–14).

Most of what is known about the cross-regulation of Th17 cells is limited to the earliest stages of differentiation, which occur within the first few hours to days following the initial T cell activation. However, it is also important to address the role of cytokine-mediated regulation of committed Th17 cells. Little is known about the regulation of secondary stimulation, which may be more representative of a Th17 cell that has become activated and differentiated in a lymph node in the presence of TGF-β and IL-6, exited the lymph node, and traveled to a site of inflammation with a distinct cytokine milieu, such as an inflamed joint. It was also shown that cytokine-regulatory networks can change as T cells mature. For example, IL-27 suppresses Th17 development from naive CD4⁺ T cells, but it fails to suppress reactivation of committed Th17 cells (15). Understanding the regulation of pre-existing activated T cells may also have therapeutic applications in the control of chronic T cell-mediated diseases, such as rheumatoid arthritis.

Several groups recently demonstrated a high degree of plasticity in Th17 cells, such that stimulation with IL-12 upregulates T-bet and IFN-γ and induces a Th1-like phenotype, whereas stimulation with TGF-β upregulates Foxp3 and induces a regulatory T cell-like phenotype (16, 17). One report suggested that the Th17 phenotype is unstable, and Th17 cells will spontaneously convert to Th1 cells in lymphopenic hosts (18). However, another group demonstrated that in vitro-generated Th17 cells quickly lose IL-17 expression unless IL-23 is added and opposing cytokines are blocked, whereas in vivo-generated Th17 cells continue to express IL-17, regardless of which cytokines are added (19). Similarly, in vitro-generated Th17 cells could convert to a Th1 or Th2 phenotype when stimulated under Th1- or Th2-skewing conditions, whereas in vivo-generated Th17 cells were refractory to Th1- and Th2-polarizing signals.

Some Th17-to-Th1 conversion may not be surprising, considering the evidence for the close relationship between these two lineages. Th17 cells and Th1 cells were thought to share a common lineage precursor (20), and IL-17/IFN-γ double-positive cells are quite common in humans (21, 22). Similarly, the shared dependence on TGF-β implies some regulatory T cell–Th17 commonality. However, there is much less evidence of any Th2–Th17 commonality. Unlike the frequent occurrence of IL-17/IFN-γ double-positive cells, IL-17/IL-4 double-positive cells have only rarely been encountered (23), suggesting that the relationship between Th1 and Th17 may be very different from the relationship between Th2 and Th17.

Although IL-4 was shown to inhibit Th17 differentiation and IL-17 expression, nothing is known about the molecular mechanisms of this suppression or about the effects of IL-4 on other Th17-family genes, such as RORγt and IL-22. Similarly, the effects of IL-4 on developing versus committed Th17 cells have not been explored. In this study, we demonstrate that IL-4 suppresses...
a subset of Th17 genes distinct from IFN-γ. Suppression of IL-17 depends on STAT6, but not GATA-3, and correlates with a loss of STAT3 binding at the Il17a promoter. IL-4 induces a stable, irreversible loss of IL-17 expression without inducing conversion to a Th2 phenotype. However, repeated stimulation renders both in vivo- and in vitro-derived Th17 cells refractory to suppression by IL-4.

### Materials and Methods

**Mice**

For in vitro Th17 differentiation and in vivo keyhole limpet hemocyanin (KLH) immunization, male 6–8-wk-old BALB/c mice were obtained from Jackson Laboratories. For cII/CFA immunization, male 8–10 wk-old DBA1 mice were obtained from Jackson Laboratories. STAT6-deficient and IL-4R mutant mice on a BALB/c background were obtained from Jackson Laboratories. All animals were housed in specific pathogen-free conditions, and all procedures were approved by the University Committee for the Use and Care of Animals, University of Michigan. Single-cell suspensions from spleens and thymys of CD4+ T cells were then labeled with CD4 FITC, CD25 PE, CD44 PE-Cy7, and CD62L allophycocyanin (BioLegend). The CD4+CD25+CD44+CD62L+ cells were sorted on a FACSVerse, FACSaria, or FACSDiva.

**Generation of bone marrow-derived dendritic cells**

Bone marrow was isolated from femurs and tibias, treated with hyptonic ammonium chloride buffer to lyse erythrocytes, and cultured for 6 d at 1 × 10^6 cells/ml with 10 ng/ml recombinant mouse IL-4 and GM-CSF (PeproTech) in RPMI 1640 (10% FCS, 2% L-glutamine, 1% penicillin/streptomycin, and 55 mM 2-ME). The cells were then collected using a cell scraper, and CD11c+ cells were positively selected by two rounds of MACS (Miltenyi Biotec).

**Purification of naive T cells**

Spleens were collected, and CD4 T cells were magnetically isolated by negative selection using the EasySep kit from Stem Cell Technologies. The purified CD4+ T cells were then labeled with CD4 FITC, CD25 PE, CD44 PE-Cy7, and CD62L allophtocyanin (BioLegend). The CD4+CD25+CD44+CD62L+ cells were sorted on a FACSVerse, FACSaria, or FACSDiva.

**Th17 differentiation**

Bone marrow-derived dendritic cells (BM-DCs) and naive T cells were plated in six-well plates in RPMI 1640 at 0.125 × 10^6 BM-DCs and 0.25 × 10^6 naive T cells/ml with 4 μg/ml anti-CD3 (145-2C11), 10 μg/ml anti-IL-4 (11B11), 10 μg/ml anti–IFN-γ (R4-6A2), 1 ng/ml recombinant human TGF-β1 (PeproTech), 20 ng/ml recombinant mouse IL-6 (PeproTech), and 10 ng/ml recombinant mouse IL-23 (eBioscience). For inhibition of Th17 differentiation, anti–IL-4 was omitted from the culture, and recombinant mouse IL-4 (PeproTech) was added at 10 ng/ml, unless stated otherwise. Alternatively, anti–IFN-γ was omitted from the culture, and recombinant mouse IFN-γ (PeproTech) was added at 10 ng/ml, unless stated otherwise. Cells were stimulated for 6 d and then collected, washed twice with cold 2% newborn calf serum/PBS, and put back into culture in the same volume without stimulation for 2 d. For inhibition of Th17 restimulation, IL-4 was added to the culture during the 2-d rest period or during a 2-d restimulation with anti-CD3 following the rest period.

**Th17 maturation**

Naive T cells underwent 6 d of Th17 differentiation, followed by 2 d of rest, according to the protocol described above. To induce maturation, the cells were then expanded in a 2-fold culture volume with the addition of fresh BM-DCs, restimulated with the same cytokine and neutralizing Ab mixture for 5 d, and then washed and rested for 2 d. This cycle of 5 d of stimulation and 2 d of rest was repeated, for a total of 3 wk of culture. At the end of 3 wk, the Th17 cells were restimulated for 2 d with anti-CD3 and rIL-4.

**ELISA**

IL-17A was measured by ELISA. Plates were coated with purified anti–IL-17A (clone TC11-18H10.1, BioLegend), blocked, and then loaded with tissue culture supernatants or serum. The plates were washed and treated with biotin-conjugated anti–IL-17A (clone TC11-8H4, BioLegend) mixed with streptavidin HRP (BioLegend). The plates were developed with OptEIA TMB substrate (BD) and absorbance at 450 nm was quantitated with a Bio-Rad (Hercules, CA) plate reader using KC4 software (Biotek, Winooski, VT).

**Flow cytometry**

For intracellular cytokine staining (ICS), cells were stimulated for 6 h with 5 ng/ml PMA and 500 ng/ml ionomycin, with 10 μg/ml brefeldin A added for the last 5 h (all chemicals were purchased from Sigma). Cells were then treated with mouse Fc Block anti-CD16/32, stained with FITC- or PE-conjugated anti-CD4 (clone GK1.5), and fixed overnight. The next day, cells were permeabilized with saponin and stained with fluorescently labeled anti–IL-17 (clone TC11-18H10.1), anti–IFN-γ (clone XMG 1.2), and anti–IL-4 (clone 11B11) or the appropriate isotype control (all Abs from BioLegend). Staining was measured with a FACSCalibur, and data were analyzed using Cell Quest software (BD).

**Real-time PCR**

Gene expression at the mRNA level was analyzed by TaqMan-based real-time PCR with specific primers and probes. First, RNA was collected from frozen cell pellets with the RNeasy Mini kit and treated with DNase (Qiagen). cDNA was generated using the High Capacity cDNA archive kit (Applied Biosystems). Relative quantification using the comparative cycle threshold method was carried out using TaqMan Universal PCR Master Mix or Gene Expression Master Mix (Applied Biosystems) and run on an AB7500 machine. The following primer and probe sets were obtained from Applied Biosystems: IL-17A, IL-17F, IL-22, RORγ, IL-23R, IL-4, IL-4R, STAT6, GATA3, β-actin, and GAPDH.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was carried out according to the EZ ChIP protocol (Upstate). Briefly, Th17 cells were fixed with formaldehyde and lysed with SDS. Lysates were sonicated to shear DNA and immuno-precipitated with protein G and Abs to STAT3. Eluted DNA was quantitated by real-time PCR with SYBR Green Master Mix (Applied Biosystems) and the following primers: Il17a promoter forward: 5'-AGATTCATGGACCCCGACA-GAGG-3', Il17a promoter reverse: 5'-CACGACTCCAGACGATCC-TACATG-3', Il17aR promoter reverse: 5'-GACTGACCTACATTGGGC-C-3', Rorc promoter forward: 5'-AGGCCTTCTGACTTATTGATT-G-3', and Rorc promoter reverse: 5'-AGGGGGTGCCTGATTAATC-3'.

**Western blot**

Th17 cells were washed and rested overnight in RPMI 1640 with 2% FCS plus cytokine-neutralizing Abs to minimize background levels of STAT activation. The cells were then incubated with 50 ng/ml IL-4. The reaction was stopped with cold PBS plus 1 mM NaVO₃, and the cells were lysed with Proteasome exclusion reagent (Novagen) supplemented with protease inhibitor mixture (Calbiochem). Lysates were reduced and denatured by boiling with SDS loading buffer with 100 mM DTT. Samples were run on a 10% Precise Tris-Heps-SDS gel (Pierce, Rockford, IL) and transferred to polyvinylidene fluoride membrane (Millipore). Membranes were stained with the following primary Abs at 1:1000, unless noted otherwise: anti-STAT6 (Cell Signaling), anti–phospho-Tyr641 STAT6 (Calbiochem), and anti–GAPDH (1:400; BioLegend). Secondary Abs were goat anti-rabbit IgG HRP (1:1000; Cell Signaling) or rabbit anti-goat IgG HRP (1:10,000; Abcam). Chemiluminescence was developed with Pierce ECL. Western blotting substrate and detected on blue autoradiography film (MidSci). Band intensities were quantified using Kodak 1D 3.6 software.

**Immunizations**

CFA was prepared by mixing heat-inactivated mycobacterial strain H37Ra in IFA at 4 mg/ml. For KLH immunization, Imject mKLH subunits (Pierce) were diluted in PBS to 2 mg/ml and mixed at a 1:1 ratio with CFA. Mice were immunized i.p. with 100 μg KLH. For collagen immunization, lyophilized chicken collagen (Chondrex, Redmond, WA) was dissolved overnight in acetic acid at 4 mg/ml. CFA and collagen were mixed at a 1:1 ratio to form an emulsion, and 100 μg collagen was injected intradermally (i.d.) at the base of the tail.

**Statistical analysis**

The p values were calculated by the unpaired two-way Student t test, one-sample t test, one-way ANOVA, or two-way ANOVA with the Dunnett posttest, using Prism, as described in the figure legends.
Results

Th1 and Th2 cytokines suppress reactivation of in vivo-derived Th17 cells

Previous work showed that IL-4 can inhibit in vitro Th17 differentiation from naive CD4+ T cells (12), but much less is known about the effects of IL-4 on pre-existing or memory Th17 cells, such as those that are likely to persist during many inflammatory diseases. To examine the cytokine-mediated regulation of Th17 cells generated in vivo in the context of an IL-17–dependent autoimmune disease, we immunized DBA1/LacJ mice i.d. with chick type II collagen emulsified in CFA, following the standard protocol for induction of collagen-induced arthritis (CIA). After 2 wk, spleen cells were restimulated in vitro with collagen, in the presence or absence of rTh1 and rTh2 cytokines or neutralizing Abs. After 5 d of culture, IL-17 production was measured by ELISA. The results in Fig. 1A demonstrate that in vivo-derived, committed Th17 cells are susceptible to counter-regulation by a number of other Th1 and Th2 cytokines. In particular, IL-4 and IL-12 were potent suppressors, and IFN-γ was a weak suppressor, of collagen-specific IL-17 production. IL-13, another important Th2 cytokine, also inhibited IL-17 production, confirming recent data from Newcomb et al. (14) and furthering the idea that Th2 cells can suppress Th17 activity through multiple mechanisms. In addition, neutralizing Abs to IL-4, IFN-γ, or IL-12 upregulated IL-17 production, implying that the collagen-specific Th17 response is constrained by endogenous cytokines, possibly from collagen-specific T cells of opposing lineages (Fig. 1B). To confirm that these observations are not restricted to DBA mice, the collagen-specific response, or an autoimmune disease, we measured Th17 responses in BALB/c mice 2 wk after i.p. immunization with KLH. Despite the well-known Th2 bias in BALB/c mice, immunization induced significant KLH-specific IL-17 production in the spleen, which was negatively regulated by both endogenous and exogenous Th1 and Th2 cytokines during Ag-specific rechallenge, recapitulating our previous findings in the collagen system (Fig. 1C). These data implied that, even after differentiation, Th17 cells are still susceptible to cytokine-mediated counter-regulation, which raises many interesting questions about the stability of lineage commitment, the mechanisms of suppression, and the role of these regulatory pathways in chronic inflammation.

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** Ag-specific IL-17 production is regulated by endogenous and exogenous Th1 and Th2 cytokines. DBA mice were immunized i.d. with cII/CFA, and spleens were collected 2 wk later. Single-cell suspensions were restimulated in vitro with 50 μg/ml heat-denatured collagen in the presence of recombinant cytokines (A) or 10 μg/ml purified cytokine-neutralizing Abs (B) for 5 d. Supernatants were collected, and IL-17 was measured by ELISA. Error bars represent SEM of triplicate culture samples. *p < 0.05, **p < 0.01, ***p < 0.001, versus 0 ng/ml (A) or isotype control (B) (one-way ANOVA). C, BALB/c mice were immunized i.p. with KLH in CFA, and spleens were collected 2 wk later. Single-cell suspensions were restimulated in vitro with 50 μg/ml heat-denatured KLH in the presence of 10 ng/ml recombinant cytokines or 10 μg/ml purified cytokine-neutralizing Abs for 5 d. Supernatants were collected, and IL-17 was measured by ELISA. Error bars represent SEM of triplicate culture samples. **p < 0.01, ***p < 0.001, versus KLH or isotype (one-way ANOVA).
Suppression by IL-4 and IFN-γ dominates over strong stimulation of Th17 cells

Although several groups showed that the combination of TGF-β and IL-6 acts on naive T cells to induce Th17 differentiation (2, 3, 24), it is not clear what effect TGF-β and IL-6 have on memory Th17 cells. Therefore, we decided to restimulate collagen-immunized spleen cells in the presence of TGF-β and/or IL-6, with the assumption that 2 wk after immunization most collagen-specific T cells have already differentiated; thus, TGF-β and IL-6 are more likely to be acting on activated or memory Th17 cells rather than inducing new Th17 differentiation in naive T cells. The results showed that IL-6 alone, and, to a much greater extent, TGF-β alone, was able to upregulate IL-17, a result consistent with the presence of some endogenous IL-6. However, the combination of TGF-β and IL-6 was significantly better at inducing IL-17 than was either cytokine alone, and addition of IL-23 to the mixture enhanced IL-17 production further (Fig. 2A). Moreover, TGF-β, IL-6, and IL-23, alone or in combination, induced considerable IL-17 production, even in the absence of exogenous collagen, suggesting that the right cytokine milieu can stimulate pre-existing Th17 cells without the need for concomitant TCR stimulation. This inference assumes that Ag from the in vivo immunization does not persist in these cultures. The potential for Th17 cells to become Ag independent could have important implications for the development of chronic inflammation.

Given that TGF-β, IL-6, and IL-23 can greatly enhance the activation of pre-existing Th17 cells, we wondered how these positive signals might interact with negative signals coming from Th1 and Th2 cytokines. Thus, we asked whether IL-4, IFN-γ, and IL-12 would continue to suppress IL-17 production in the presence of TGF-β, IL-6, and IL-23. ELISAs of cytokines produced in spleen cell cultures showed that the suppressive signals from IL-4 overpowered any combination of activating signals, and even very elevated production of IL-17 was still potently downregulated (Fig. 2B). Interestingly, IFN-γ continued to suppress IL-17 in the presence of TGF-β, IL-6, and IL-23, whereas IL-12 did not (Fig. 2C). These results point to different mechanisms of suppression downstream of the IFN-γ and IL-12 receptors and may reflect competition for shared signaling pathways by IL-12 and IL-23.

IL-4 and IFN-γ selectively inhibit unique subsets of Th17 genes

To more carefully investigate the regulatory mechanisms at work during different stages of Th17 development, we next analyzed in vitro-derived Th17 cells. Purified naive CD4+ T cells were differentiated into Th17 cells for 5 or 6 d, as described in Materials and Methods. The cells were then rested for 2 d, followed by
challenge with rTh1 and rTh2 cytokines in the presence or absence of anti-CD3 for 2 d. This protocol of 5 d of priming/2 d of rest/2 d of restimulation allows us to specifically focus on the effects of opposing cytokines on activation of pre-existing Th17 cells, rather than the effects on differentiation of naive T cells into Th17. Th17 activity was measured by ICS, ELISA, and real-time PCR. As predicted, the results clearly demonstrated that adding IL-4 or IFN-γ during restimulation inhibited the expression of IL-17 protein, sometimes by as much as 95%, and with a more potent inhibition by IL-4 (Fig. 3A). We also found that IL-4 and IFN-γ inhibited expression of IFN-γ, IL-17F, and RORγt mRNA (Fig. 3C). Interestingly, however, these cytokines selectively inhibited the expression of a unique subset of Th17 genes: IL-4 suppressed IL-23R but not IL-22, whereas IFN-γ suppressed IL-22 but not IL-23R. The differential regulation of IL-22 and IL-23R implied that the Th17 gene-expression program is not completely reversed in the presence of IFN-γ and IL-4, leaving open the possibility that these conflicting cytokine milieus yield either a mixed population of cells or cells of a mixed phenotype.

**Suppression by IL-4 depends on STAT6 but not GATA3**

Because IL-4R signaling is mediated primarily by STAT6, we hypothesized that STAT6 was required for suppression of IL-17 by IL-4; to address this question, we repeated our previous in vitro experiments using STAT6-deficient Th17 cells. In the restimulation of in vitro-derived Th17 cells, IL-4 had no effect on IL-17 expression in the absence of STAT6 (Fig. 3B). Similarly, by real-time RT-PCR, IL-4 failed to robustly suppress IL-17A, IL-17F, RORγt, and IL-23R expression in STAT6-deficient Th17 cells (Fig. 3C). Spleen cells from STAT6-deficient mice stimulated with anti-CD3 in the absence of exogenous IL-4 also had increased IL-17 expression by ELISA compared with wild-type T cells, indicating that endogenous cytokines also inhibit IL-17 expression via STAT6 (Fig. 3D). STAT5 can also be activated by IL-4 and was reported to mediate suppression of IL-17 downstream of IL-2 (25, 26). However, using STAT3-deficient spleen cells stimulated with anti-CD3, we found that STAT5 was dispensable for suppression of IL-17 by IL-4 (Supplemental Fig. 3). Similarly, IL-4R signaling was shown to activate insulin receptor substrate (IRS)-2; however, when we used mice bearing a point mutation in the IL-4R at the IRS-2 binding site, thus preventing recruitment and activation of IRS-2 by IL-4 without impacting STAT6 activation, rIL-4 continued to inhibit IL-17 expression, demonstrating that IRS-2 activation is not required for suppression of IL-17 (Supplemental Fig. 1).

Previous data from other groups suggested that suppression of Th1 development by IL-4 is mediated by GATA-3, the transcription factor induced by IL-4 that acts as the master regulator of Th2 differentiation (27–30). In addition, we found that GATA-3 mRNA is upregulated in Th17 cells as early as 2 h after the addition of IL-4, prior to downregulation of IL-17 mRNA (data not shown). Thus, we hypothesized that GATA-3 may play a role in suppression of IL-17 by IL-4. Mice bearing a floxed Gata3 gene with inducible Cre recombinase under control of the IFN-inducible Mx1 promoter (Gata3fl/fl:TgMx1cre mice) were treated with polynosinic-polycytidylic acid to induce excision of the Gata3 gene, which resulted in a 70% decrease in total splenic levels of GATA3 mRNA (Supplemental Fig. 2). Although the in vivo conditional deletion was less than ideal, published reports using these mice demonstrated a near-complete suppression of early T lineage progenitor development, suggesting that the incomplete deletion is sufficient to significantly alter T cell function (31). Spleen cells from these mice and control mice that are unable to delete GATA3 were cultured with anti-CD3 in the presence or absence of Th17-

![Figure 3](http://www.jimmunol.org/Download)
skewing cytokines, and the effect of IL-4 on IL-17 expression was measured by ICS and real-time PCR. However, contrary to our prediction, the results in Fig. 4 show that deletion of Gata3 had no effect on suppression of IL-17 by IL-4.

**Suppression by IL-4 results in loss of STAT3 binding at the Il17a promoter**

Th17 development in mice depends on the transcription factor STAT3, which is activated by IL-6 and IL-23. STAT3 has multiple roles in Th17 development: in activated Th17 cells stimulated with IL-23, it binds directly to the Il17a promoter and induces IL-17 expression, and in naive T cells stimulated with TGF-β and IL-6, it is required for induction of RORγt expression (10, 32–34). Thus, we hypothesized that IL-4 may mediate suppression of Th17 activity by inhibiting STAT3 binding at Th17 gene loci. To test this hypothesis, we restimulated Th17 cells with anti-CD3 and Th17-skewing cytokines in the presence or absence of IL-4 for 6 or 24 h and measured STAT3 binding at the Il17a promoter, Il17a/f intergenic region, and Rorc promoter by ChIP. The results in Fig. 5 demonstrate very strong STAT3 binding at all three regions. Interestingly, IL-4 inhibited STAT3 binding at the Il17a promoter but had no effect at the Il17a/f intergenic region or the Rorc promoter.

**Suppression by IL-4 is stable but does not induce Th2 conversion**

Several groups recently demonstrated a high degree of plasticity in Th17 cells (17, 35); thus, we decided to ask whether prolonged culture with IL-4, the chief Th2-skewing cytokine, would induce Th17-to-Th2 conversion. Th17 cells were generated in vitro, with 5 d of differentiation followed by 2 d of rest. Following the rest period, the Th17 cells were restimulated in Th0, Th2, or Th17 conditions for 2, 4, or 6 d. Th2 conversion was assessed by real-time PCR for IL-4 and GATA-3, as well as IL-17 and RORγt. The results in Fig. 6A show that low levels of mRNA for IL-4 and GATA-3 were expressed in Th2-stimulated Th17 cultures, but similar levels of IL-4 and GATA-3 message were also expressed in Th17 cells stimulated with anti-CD3 alone. Although IL-4 mRNA expression persisted longer in cultures restimulated with Th2 conditions versus anti-CD3, these results may indicate a small number of contaminating Th0 or Th2 cells in the culture, rather than induction of the Th17-to-Th2 conversion. In addition, the levels of IL-4 mRNA expressed by Th17 cells restimulated in Th2 conditions were significantly lower than the levels of IL-4 mRNA expressed by naive T cells differentiated under Th2 conditions (Supplemental Fig. 3). We also examined IL-4 expression in Th17 cells by ICS and found that <2% of the cells expressed IL-4 after 2 d of restimulation in Th0 or Th2 conditions. Furthermore, the IL-4 expression on day 2 was extinguished by day 4 or 6 of Th2 culture, and no cells coexpressed IL-4 and IL-17, implying that there was no Th17-to-Th2 conversion (Fig. 6B, data not shown). Thus, although low levels of IL-4 and GATA-3 mRNA persist, IL-4 protein expression is quickly extinguished, suggesting a role for posttranscriptional regulation.

Although we demonstrated that Th17 cells stimulated in the presence of IL-4 do not convert to Th2 cells, it was not clear whether IL-17 expression was only temporarily downregulated, requiring constant IL-4R signaling to maintain suppression, or persistently extinguished, even after removal of IL-4. Thus, we again generated Th17 cells in vitro (primary culture), which were restimulated in secondary culture with Th2 conditions (anti-CD3, 10 ng/ml IL-4, anti–IFN-γ), Th0 conditions (anti-CD3, anti–IL-4, anti–IFN-γ), or “Th23 conditions” (anti-CD3, 20 ng/ml IL-23, anti–IL-4, anti–IFN-γ) to maintain the existing Th17 cell population without inducing new differentiation. After 2 d of secondary culture, the cells were washed and rested in tertiary culture for 1, 2, or 3 d to allow the Th17 cells to regain IL-17 expression. After each phase of culture, cells were restimulated with PMA, ionomycin, and brefeldin A for ICS. The results in Fig. 7 show that secondary stimulation in Th0 or Th23 conditions imprinted Th17 cells for increasing levels of IL-17 expression after stimulation was removed, whereas secondary stimulation in Th2 conditions resulted in a continual decline in IL-17 expression, even after IL-4 was removed. These results demonstrated that IL-4-mediated suppression of IL-17 is stable and does not require continual exposure to IL-4, suggesting that IL-4 may induce heritable changes in chromatin structure at the Il17a locus. Cell numbers and viability were consistent within groups and over time during tertiary culture, implying that there was no selective proliferation or cell death. Thus, IL-17 expression is easily suppressed by low doses of IL-4, despite the presence of activating cytokines, and the suppression by IL-4 is also persistent and not passively reversed.
Repeated stimulation renders Th17 cells resistant to suppression by IL-4

The results shown thus far suggested that Th17 cells are very sensitive to suppression by IL-4, which raises the question of how Th17 cells manage to persist in vivo during an inflammatory disease where they may encounter many suppressive cytokines. One explanation may be the process of maturation or stabilization. Previous reports from other groups demonstrated that developing Th1 cells progress through several stages of maturation, gradually stabilizing their phenotype in response to cytokine stimulation.

**FIGURE 6.** Th17 cells restimulated in Th2 conditions do not convert to Th2 cells. A, Th17 cells were restimulated for 2, 4, or 6 d in Th0, Th2, or Th17 conditions, and expression of IL-4 and GATA-3 was measured by real time PCR. Results were normalized to ß-actin expression. Error bars represent the SEM of triplicate PCRs. B, IL-17A and IL-4 expression was measured by ICS.

**FIGURE 7.** Suppression of IL-17 expression persists after removal of IL-4. Th17 cells were generated in vitro and treated with IL-4 to suppress IL-17 expression, as described, and then washed and put back into culture for 1, 2, or 3 d to allow cells to regain IL-17 expression. Primary culture = 5 d of Th17 differentiation and 2 d of rest, as described. Secondary culture = 2 d restimulation in Th0 conditions (anti-CD3, anti-IL-4, anti-IFN-γ), Th23 conditions (anti-CD3, anti-IL-4, anti-IFN-γ, 20 ng/ml IL-23), or Th2 conditions (anti-CD3, 10 ng/ml IL-4, anti-IFN-γ). Tertiary culture = 1, 2, or 3 d resting culture. After each phase of culture, cells were restimulated with PMA, ionomycin, and brefeldin A, and IL-17 expression was assessed by ICS.
Early in the culture, IL-4 suppresses Th1 differentiation and IFN-\(\gamma\) expression, but after prolonged stimulation, Th1 cells lose the ability to respond to IL-4 and become resistant to suppression (29, 36, 37). Previous data from our laboratory on the effect of IL-4–transduced dendritic cells on IL-17 production during CIA suggested that Th17 cells are less susceptible to regulation by IL-4 after the onset of arthritis (38). Thus, we decided to address the maturation of Th17 cells in vitro and in vivo, as measured by insensitivity to suppression by IL-4. Th17 cells were generated in vitro with 5 d of differentiation and 2 d of rest, as in previous experiments. To induce maturation, we repeated this process two additional times, for a total of 3 wk of culture, and then assayed the effect of IL-4 on IL-17 expression during a 2-d restimulation with anti-CD3, followed by ICS. Fig. 8A shows representative dot plots for IL-17 expression in Th17 cells treated with IL-4 after 1 or 3 wk of culture. The results showed that after three rounds of stimulation, Th17 cells are largely resistant to suppression by IL-4. Interestingly, the results in Fig. 8B demonstrate that Th17 cells cultured for 3 wk become resistant to suppression by IL-4 and IFN-\(\gamma\) but remain sensitive to suppression by IL-12.

We already showed that IL-4 suppresses IL-17 expression by collagen-immunized splenocytes, thus demonstrating that Ag-specific Th17 cells in the spleen 2 wk after immunization have not fully matured. However, we decided to look for ex vivo maturation of splenocytes from immunized mice by stimulating with Ag for 1 or 3 d to induce maturation and then washing and challenging with Ag in the presence or absence of IL-4 for 2 d, followed by IL-17 ELISA of the supernatants. As the results in Fig. 8C show, we found that 3 d of ex vivo restimulation with Ag was sufficient to induce Th17 maturation and resistance to suppression by IL-4 in whole-spleen cultures from 2-wk immunized mice. Similar results were observed for KLH-immunized BALB/c mice (data not shown).

The ability of Th17 cells to become resistant to suppression could have important implications for the development of autoimmune disease, and IL-4–transduced dendritic cells were shown to suppress collagen-specific IL-17 production from mice before the onset of arthritis but not after (38). Thus, we asked whether Th17 maturation correlated with disease progression or severity in CIA. To address this question, we collected spleens from mice at different time points after immunization with cII/CFA and assessed the Th17 sensitivity to suppression by IL-4 on day 0, 1, or 3 of culture. We found that, 6 wk after immunization, splenic Th17 cells were moderately sensitive to suppression by IL-4 directly ex vivo. However, Th17 responses of cells from mice that had undergone prolonged immunizations matured more quickly ex vivo than did Th17 responses of cells from very short immunizations (Fig. 8D, Table I).

**Mature Th17 cells express IL-4R but lose the ability to phosphorylate STAT6**

Our observation of desensitization of Th17 cells to challenge with IL-4 begged the question of whether signaling through IL-4R...
remained intact following maturation. To measure IL-4R signaling, Th17 cells were rested in low-serum media with cytokine-neutralizing Abs overnight to minimize the background level of activation; stimulated with IL-4 for 15, 30, or 120 min; and lysed in the presence of protein phosphatase inhibitors. STAT6 activation was measured by Western blot with phospho-STAT6 specific Abs. The results in Fig. 9A show that IL-4 induced significantly less STAT6 activation in mature Th17 cells cultured for 3 wk versus immature Th17 cells cultured for 1 wk, regardless of whether phospho-STAT6 levels were normalized to total STAT6 or to the loading control GAPDH. In addition, when total STAT6 expression was normalized to GAPDH, we found that STAT6 was actually upregulated in mature Th17 cells (data not shown). Thus, the loss of STAT6 activation was not simply due to downregulation of STAT6 expression.

One plausible explanation for the loss of STAT6 activation is downregulation of IL-4Rα. Therefore, we decided to measure IL-4Rα expression at different stages of Th17 maturation by flow cytometry. The results in Fig. 9B show that IL-4Rα expression was not significantly downregulated in 3-wk Th17 cultures versus 1- or 2-wk cultures.

Discussion

The data presented demonstrate a remarkable degree of complexity in the cytokine-mediated regulation of Th17 cells. Initial reports suggested that Th cell cross-regulation is rather black and white: TGF-β, IL-6, and IL-23 promote Th17 cells, whereas IL-4, IFN-γ, and IL-12 inhibit Th17 cells. However, upon closer inspection, we see many shades of gray. For instance, Th17 cells developing in the presence of IL-4 may continue to express IL-22, whereas Th17 cells developing in the presence of IFN-γ may continue to express IL-23R, suggesting that there may be an array of Th cell subset phenotypes that include intermediate states that are not fully polarized to one of the well-defined Th subsets. It is also particularly interesting that IL-4 and IFN-γ continue to suppress IL-17 production in the presence of TGF-β, IL-6, and IL-23, whereas IL-12 does not. Another important difference appears in the process of Th17 maturation, which results in resistance to suppression by IL-4 and IFN-γ but not to IL-12.

Th1 cytokines can suppress IL-17 expression but also induce a Th1-like phenotype in Th17 cells. In contrast, IL-4 induces potent and stable suppression without inducing any Th2 conversion, even in the face of strong Th17 stimuli. This is consistent with previous work from our laboratory demonstrating that a single injection of IL-4-transduced dendritic cells induced long-lasting protection from CIA, which is likely mediated by suppression of collagen-specific Th17 responses (38, 39). These results suggest a fundamental difference in the mechanisms underlying regulation of Th17 cells by Th1 and Th2 cytokines. How Th17 cells integrate a complex array of positive and negative signals is an interesting area for future research and may depend on the ability of key transcription factors, such as T-bet and GATA-3, to bind to Th17 gene loci and interact with RORγt.

**Table I.** In vivo experience correlates with ex vivo development of resistance to IL-4

<table>
<thead>
<tr>
<th>Time Since Immunization (wk)</th>
<th>Culture until IL-4 Resistance (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;3</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0–1</td>
</tr>
</tbody>
</table>

DBA mice were immunized with cII/CFA and spleens were collected after 1, 2, 4, or 6 wk. Spleen cells were restimulated with collagen and IL-4 was added on d 0, 1, or 3 of culture. IL-17 was measured by ELISA on d 5. A designation of resistance required that IL-17 production in the presence of IL-4 was ≥70% of IL-17 production in the absence of IL-4. Spleens from 1-wk–immunized mice continued to respond to IL-4 when it was added after 3 d of culture, whereas spleens from 6-wk–immunized mice failed to respond when IL-4 was added on day 0 or 1.

**FIGURE 9.** Impaired IL-4R signaling in mature Th17 cells. **A,** Th17 cells were generated in vitro with 1 or 3 wk of stimulation and rested overnight in low-serum media with cytokine-neutralizing Abs to reduce the background level of STAT activation. Cells were then washed and stimulated with 50 ng/ml IL-4 for 15, 30, or 120 min and lysed with PhosphoSafe Lysis Buffer (Novagen). Lysates were reduced and run on 10% SDS-PAGE gels and stained with Abs for phospho-STAT6, total STAT6, or GAPDH. Band intensities were quantitated with Kodak software, and phospho-STAT6 intensity was normalized either to total STAT6 intensity or GAPDH intensity; p < 0.05 for the average band intensity between mature and immature Th17 cells (two-way t test). **B,** IL-4R expression in immature and mature Th17 cells was measured by flow cytometry. Bold lines represent IL-4R; dotted lines represent isotype control.
The molecular mechanisms mediating Th17 suppression and/or lineage conversion downstream of Th1 and Th2 cytokines remain largely unknown. We showed that IL-4-mediated suppression is dependent on STAT6 and independent of STAT5, IRS-2, and GATA-3. One potential mechanism of suppression downstream of IL-4R could be direct binding of STAT6 to Th17 gene loci and inhibition of transcription. However, we performed multiple STAT6 ChIPs, scanning wide regions of the Il17a and Rorc promoters, and found no evidence for direct binding (Supplemental Fig. 4). Given that IL-17 expression is dependent on STAT3, we hypothesized that IL-4R signaling may suppress IL-17 expression by inhibiting STAT3 DNA-binding activity, which we confirmed by ChIP for STAT3 at the Il17a promoter. Interestingly, STAT3 binding was specifically downregulated at the Il17a promoter, whereas there was no loss of STAT3 binding at the Rorc promoter or Il17ad intergenic region, suggesting that STAT3 may be displaced from the Il17a promoter by a STAT6-induced transcriptional repressor that specifically binds this region. We screened several candidates but were unable to identify the direct target of STAT6 that ultimately mediates IL-17 gene silencing.

Although much attention has been given to the cytokines that regulate Th cell differentiation from naive T cells, there is a paucity of data on how cells are regulated beyond this early window. For instance, current dogma states that TGF-β and IL-6 act on naive T cells to induce Th17 differentiation, whereas IL-23 acts on existing Th17 cells; however, our observations suggested that TGF-β and IL-6 may be equally as important as IL-23 for augmenting cytokine production by effector or memory Th17 cells. In addition, recent observations demonstrated a remarkable degree of fluidity within Th cell lineages in vivo, with cells converting from one lineage to another or stably expressing multiple cytokines characteristic of different lineages. Thus, much more work is needed to address the role of cytokine-mediated regulation throughout the full lifespan of each of the Th cell subsets, as well as the ways in which our notions of divergent Th lineages break down and the lines between distinct subsets become blurred.

In our experiments, Th17 cells cultured under Th2 conditions did not take on a Th2 phenotype and were unable to re-express IL-17 after the suppressive signals were removed. However, we also found that three rounds of in vitro stimulation rendered Th17 cells resistant to suppression by IL-4 as a result of desensitization of IL-4R, suggesting that there may be a specific window of opportunity for Th17 suppression. Importantly, we observed a similar maturation process in Th17 cells generated in vivo, and maturation state correlated with disease progression. The in vitro maturation kinetics closely mirrored what was demonstrated for Th1 cells, suggesting that this process is not Th17 specific, but rather may be a universal property of chronically activated Th cells (to our knowledge, there is no evidence regarding Th2 maturation). Although we found that Th17 cells generated both in vitro and in vivo gradually become more stable and resistant to suppression, data from other groups suggested that in vitro-derived Th17 cells quickly lose their IL-17 expression and can be converted to other lineages, even after three rounds of stimulation. However, in vivo-derived Th17 cells maintained their phenotype and were resistant to suppression (17–19). The conflicting observations of lineage stability following in vitro differentiation may be due to differences in culture conditions or APC populations. For example, other investigators used peptide plus irradiated spleens as controls, whereas we used anti-CD3 and BM-DCs. Several groups showed that in vitro-derived Th17 cells differ greatly from in vivo-derived Th17 cells, and one of these reports suggested that in vivo-derived memory Th17 cells are more resistant to suppression and lineage conversion than in vitro-derived Th17 cells (19, 40, 41). In this regard, our in vitro culture conditions may more closely approximate the natural setting, with respect to Th17 cell maturation, compared with more short-term Th17 cultures.

Similar to published data on mature Th1 cells (36, 37, 42), we were able to demonstrate a loss of STAT6 activation in response to IL-4 in mature Th17 cells, despite normal levels of all of the IL-4R-signaling components. One potential mechanism for loss of STAT6 activation in mature Th17 cells is upregulation of a member of the suppressor of cytokine signaling (SOCS) family of proteins. Most SOCS family proteins preferentially interact with one or more specific cytokine receptors, thereby inhibiting activation of unique STAT molecules. For example, SOCS3, was shown to suppress Th17 differentiation by binding to IL-6R and inhibiting STAT3 activation, whereas SOCS5 was shown to bind to IL-4R and inhibit STAT6 activation in Th1 cells (42). However, there can be some redundancy, because previous work also demonstrated that inhibition of IL-4R signaling in Th1 cells may be mediated by SOCS1 (43, 44). In fact, there is disagreement on the underlying mechanisms of IL-4R desensitization in mature Th1 cells: Seki et al. (42) demonstrated selective upregulation of SOCS5 in committed Th1 cells and SOCS5-dependent inhibition of IL-4R signaling, whereas Huang et al. (37) found no increase in expression of SOCS1, SOCS3, or SOCS5 and suggested that recruitment of STAT6 to IL-4R was impaired through an unknown mechanism. In our in vitro-matured Th17 cells, there was upregulation of both SOCS1 and SOCS5; however, Th17 cells from SOCS5-deficient mice (a kind gift from Dr. Sandra Nicholson, Walter and Eliza Hall Institute) showed no loss of IL-4R desensitization upon maturation (data not shown). Future experiments are needed to address the role of SOCS1 in Th17 maturation. This line of inquiry is made difficult by the lethal phenotype of SOCS1−/− mice, which has been attributed to overexpression of IFN-γ (45).

The simple observation that inflamed joints from arthritic mice coexpress large quantities of IL-17, IFN-γ, and IL-4 (46) suggests that Th17 cells at the site of inflammation are resistant to suppression and that more work is needed to determine the role of Th17 maturation in disease. Similarly, much work is needed to assess the sensitivity and resistance to suppression by IL-4 in human Th17 cells from patients with various immune-mediated diseases. The concept of distinct stages of human Th17 maturation raises many exciting new questions and ideas about the etiology of Th17-mediated disease. Better understanding of the molecular mechanisms mediating stabilization of committed cytokine production may lead to new approaches for targeted therapies.

Acknowledgments

We thank the following people for kind donation of mutant mouse tissues and reagents: Arian Laurence, John O’Shea, Chia-Jui Ku, Tomonori Hoseya, Douglas Engel, and Sandra Nicholson. We also thank the staff at the University of Michigan hybridoma core facility and the flow cytometry core facility for technical assistance. We are grateful to Donna Cash for assistance with preparation of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


Downloaded from http://www.jimmunol.org by guest on April 15, 2017


