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Cutting Edge: The Pathogenicity of IFN-γ–Producing Th17 Cells Is Independent of T-bet

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During the development of experimental autoimmune encephalomyelitis (EAE), the proportion of pathogenic and myelin-specific cells within CNS-infiltrating cytokine-producing Th cells is unknown. Using an IL-17A/IFN-γ double reporter mouse and I-Aβ/myelin oligodendrocyte glycoprotein 38–49 tetramer, we show in this study that IL-17*IFN-γ+ Th cells, which are expanded in the CNS during EAE, are highly enriched in myelin oligodendrocyte glycoprotein–specific T cells. We further demonstrate that IL-23 is essential for the generation and expansion of IFN-γ–producing Th17 cells independently of the Th1-associated transcription factors T-bet, STAT1, and STAT4. Furthermore, Th17 and IL-17*IFN-γ+ Th cells can induce CNS autoimmunity independently of T-bet. Whereas T-bet is crucial for Th1–mediated EAE, it is dispensable for Th17 cell–mediated autoimmunity. Our results suggest the existence of different epigenetic programs that regulate IFN-γ expression in Th1 and Th17 cells. The Journal of Immunology, 2013, 190: 000–000.

Several subsets of cytokine-producing Th cells infiltrate the CNS during the course of experimental autoimmune encephalomyelitis (EAE). However, the breakdown of the blood–brain barrier during EAE facilitates T cell entry into the CNS independent of antigenic specificity. However, it is unclear which proportion of cytokine-producing T cells is directed against myelin Ags and contributes to tissue damage. Th17 cells have been characterized as one of the major pathogenic Th cell populations underlying the development of many autoimmune diseases (1). IL-23 enhances and stabilizes Th17 cells (2–4) and is critical for the development of autoimmune diseases such as EAE (4).

However, several observations have recently challenged the pathogenic role of Th17 cells. First, IL-17A– and IL-17F–deficient mice are only partially resistant to the development of EAE (5, 6). Second, Th17 cells are plastic and have been described to lose IL-17 and acquire IFN-γ expression in a T-bet– and STAT4-dependent manner, questioning whether EAE pathogenicity could be attributed to Th17 or Th1 cells (7–9). Indeed, in several autoimmune diseases, including multiple sclerosis and rheumatoid arthritis, CD4+ effector T cells produce both IL-17 and IFN-γ (10–12), but little is known about their generation. Third, recent studies showed that T-bet is required for the pathogenicity of Th17 cells (7, 13).

Prior to the identification of Th17 cells, several studies investigated the requirement of T-bet for the development of EAE. We and others have demonstrated an essential role for T-bet in EAE disease development (14–16). However, T-bet is also expressed by dendritic cells and B cells, which may directly and/or indirectly affect disease development (17, 18).

In this study we show that during EAE, Th17 cells expressing GM-CSF and/or IFN-γ are highly enriched in myelin oligodendrocyte glycoprotein (MOG)–specific T cells in the CNS and can induce disease independently of Th1 cells. IL-23 signaling is crucial for the development of IL-17*IFN-γ+ T cells, and expression of the IL-23R promotes their expansion and maintains their pathogenic profile. We further demonstrate that although this subset can express IFN-γ and T-bet, its development and pathogenicity is independent of T-bet. Taken together, our data suggest that although the modulation of T-bet might be important to control Th1–mediated autoimmunity, it is ineffective at controlling Th17–mediated autoimmune manifestations.

Materials and Methods

Mice

C57BL/6J (B6), IL-12p40−/−, Tbx21−/−, Stat1−/−, and Stat4−/− mice were purchased from The Jackson Laboratory and Taconic. IL-17A GFP mice are from Biocytogen. IFN-γ knockin Thy1.1, T-betfl/flCD4Cre, and Tbx21−/− eomesodermin (Eomes)−/−CD4Cre− mice were provided by Drs. Casey T. Weaver, Steven Reiner, and Binfeng Lu, respectively. IL-23R GFP reporter mice were previously described (2). All strains are on the C57BL/6J background. All animals were bred and maintained under specific pathogen-free conditions.

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Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; Eomes, eomesodermin; KO, knockout; MOG, myelin oligodendrocyte glycoprotein; ROR, retinoic acid–related orphan receptor; WT, wild-type.

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conditions at the Benaroya Research Institute (Seattle, WA), and all experiments were performed in accordance with the guidelines of the Benaroya Research Institute Animal Care and Use Committee.

**CD4⁺ T cell preparation and T cell differentiation**

For T cell differentiations, naive CD4⁺CD62L⁺CD25⁻ T cells were isolated by FACS sorting (FACSAria; BD Biosciences) and cultured with irradiated spleen cells from IL-12p40⁻/⁻ mice for 7 d in complete RPMI 1640 medium, 2.5 μg/ml anti-CD3 in the presence of 5 ng/ml recombinant human TGF-β (R&D Systems), 30 ng/ml recombinant murine IL-6 (PeproTech), 10 μg/ml anti–IFN-γ, and 10 μg/ml anti–IL-4 (National Institutes of Health/National Cancer Institute Biological Resources Branch Preclinical Repository). For restimulation, T cells were recovered and activated with fresh splenocytes and anti-CD3 with or without IL-23 (20 ng/ml; R&D Systems).

**Abs and flow cytometry**

Intracellular cytokine and intranuclear stainings from CNS-infiltrating cells or in vitro–differentiated cells were performed according to the manufacturer’s instructions (BioLegend/eBioscience). A viability dye (eBioscience) was used to exclude dead cells. For surface cytokine staining, cells were stimulated with PMA/ionomycin and stained with anti–IL-17 and anti–IFN-γ Abs. I-A<sup>+</sup>/MOG<sub>38-49</sub> tetramer was obtained through the National Institutes of Health Tetrramer Facility and used according to their guidelines. All samples were analyzed on an LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

**Statistical analysis**

Statistical analysis was conducted with GraphPad Prism software. The <i>p</i> values were calculated with a Student paired <i>t</i> test. A <i>p</i> value < 0.05 was considered significant. Error bars denote ± SEM as indicated.

**Results and Discussion**

**CNS-infiltrating IL-17⁺IFN-γ⁺ T cells are highly enriched in MOG-specific cells during EAE**

We used an IL-17A/IFN-γ double reporter mouse in which cells expressing IFN-γ and IL-17 can be detected and isolated based on Thy1.1 and GFP expression, respectively, in conjunction with MOG<sub>38-49</sub>/I-Ab tetramer to identify the proportion of Ag-specific and cytokine-producing T cells during EAE. After EAE induction, IFN-γ⁺IL-17⁺ and IL-17⁺IFN-γ⁺ T cells represent most of the CD4⁺ T cells present in the lymph node, with limited expression of GM-CSF and few IL-17⁺IFN-γ⁺ T cells (Fig. 1A, 1B). CNS-infiltrating T cells are further characterized by higher T-bet, retinoic acid–related orphan receptor (Ror)</sub><sup>t</sup>, IL-23R, and GM-CSF expression and lower TGF-β3 expression in comparison with peripheral T cells (Supplemental Fig. 1B). The phenotype of CNS-infiltrating T cells is in sharp contrast to newly in vitro–differentiated Th17 cells (Supplemental Fig. 1C), which do not express T-bet or GM-CSF (Supplemental Fig. 1D).

Numerous studies have investigated the capacity of distinct T cell subsets to induce EAE upon adoptive transfer and after their differentiation or expansion in the presence of appropriate cytokines for a short period in vitro (19, 20). However, these conditions may not fully recapitulate the phenotype of effector T cells infiltrating the CNS in vivo during EAE.

**To determine which T cell subset might play the most important role in EAE pathogenicity, we examined the antigenic specificity of CNS-infiltrating cytokine-producing T cells using MOG<sub>38-49</sub>/I-Ab<sup>+</sup> tetramer (Fig. 1A, 1B).** Double-negative T cells, few cells (6.2 ± 2.1%) were MOG specific. Among IFN-γ⁺ and IL-17⁺ T cells, 19.4 ± 5.7 and 24.8 ± 6.3% were MOG<sub>38-49</sub>/I-Ab<sup>+</sup>, respectively. However, there was a significant enrichment (42 ± 5.6%) of MOG-specific T cells from mice with EAE were sorted and activated 2 d later with irradiated IL-12b<sup>+</sup> splenic feeder cells as well as anti-CD3 in the presence or absence of IL-23. IL-17 and IFN-γ intracellular staining was performed 7 d later. One representative experiment of two is shown. **<i>p</i> ≤ 0.01. **<i>p</i> ≤ 0.001.
CNS of mice with EAE and their activation in the absence of IL-23 led to the partial loss of either IL-17 or IFN-γ expression (Fig. 1F). In contrast, stimulation of IL-17+IFN-γ− T cells in the presence of IL-23 resulted in the appearance of IL-17+IFN-γ+ cells. It also significantly increased the percentage of IL-17+IFN-γ− T cells and stabilized them (Fig. 1F). Therefore, IL-23 likely maintains IL-17+IFN-γ+ T cells present in target tissues under inflammatory conditions.

IL-23 enhances Th17 cell plasticity and the generation of IL-17+IFN-γ+ T cells IL-12 and IFN-γ are negative regulators of RORγt, IL-17, and GM-CSF; it was therefore striking to observe the existence of an Ag-specific population expressing all three cytokines concomitantly. Our data would suggest that IL-23 might be important for the generation of Th cells coexpressing IL-17, IFN-γ, and GM-CSF. To address this question, we chronically stimulated differentiated Th17 cells in the presence of IL-23. This led to the emergence of Th17 cells, which produced IFN-γ and concomitantly expressed T-bet and RORγt (Fig. 2A, 2B). Of note, GM-CSF expression was markedly increased in IL-17+IFN-γ+ T cells compared with IL-17-single producing cells (Fig. 2C). This phenomenon was specific to IL-23 because IL-17+IFN-γ− T cells were absent from IL-23R−/− mice (Fig. 2A, 2B, Supplemental Fig. 2C). Next, we analyzed the requirement of IL-23 for the generation of these cells in vivo. Because IL-23R knockout (KO) mice are protected from EAE, we analyzed the percentage of cytokine-producing cells in the peripheral immune organs 10 d after immunization. There was no difference in the number of IFN-γ− T cells between wild-type (WT) and IL-23R KO mice (Fig. 2D). However, IL-23R KO mice displayed a significant decrease in the percentage of IL-17+IFN-γ− and IL-17+IFN-γ+ T cells (Fig. 2D). Thus, chronic exposure of Th17 cells to IL-23 leads to the induction of IL-17+IFN-γ− (GM-CSF+) T cells in vitro and in vivo. It is tempting to speculate that IL-17+IFN-γ− T cells emerge to a higher extent in the target tissues during autoimmune disease development where they are constantly exposed to their autoantigen in an IL-23-rich cytokine environment.

IFN-γ production by IL-17+ T cells is independent of bona fide Th1-specific transcription factors The ontogeny and factors controlling IL-17+IFN-γ+ T cells are not known. Because these cells express T-bet and IFN-γ (Figs. 1A, 2A, 2B), and T-bet has been described as a critical factor for Th17 cell pathogenicity (7, 13), we next determined whether Th1-specific transcription factors were required for their differentiation in response to IL-23 in vitro. We differentiated naive T cells lacking IFN-γ−driving transcription factors into Th17 cells and expanded these cells with IL-23. Importantly, IL-17+IFN-γ− cells were present whether T cells originated from WT, STAT1 KO, STAT4 KO, or T-bet KO mice (Figs. 3A–C). Eomes did not compensate for T-bet deficiency because the loss of both transcription factors did not hamper the generation of IL-17+IFN-γ+ T cells (Fig. 3D), nor did STAT4 nor STAT1 in the T-bet KO mice, which was assessed by phosphorylation of STAT4 or STAT1 in WT

![FIGURE 2](http://www.jimmunol.org/)  
**FIGURE 2.** IL-23 induces IL-17+IFN-γ+ Th17 cells in vitro. (A) Intracellular cytokine staining for IL-17 and IFN-γ in Th17 cells from WT and IL-23R−/− mice stimulated for two rounds in the presence or absence of IL-23. (B) Frequency of RORγt+IL-17+ and T-bet+IFN-γ+ cells among splenic CD4+ T cells was determined by intracellular cytokine staining. The graph represents cumulative data of five to seven mice per group from two independent experiments (mean ± SEM).  

![FIGURE 3](http://www.jimmunol.org/)  
**FIGURE 3.** Th1-associated transcription factors are not required for the IL-23-dependent induction of IL-17A+IFN-γ+CD4+ T cells. Naive CD4+ T cells from STAT1−/− (A), STAT4−/− (B), Tbx21−/− (C), and Tbx21−/−/Eomes−/−CD4+ (D) and WT control mice were primed as Th17 cells, restimulated with IL-23 two more times, and analyzed. In (A)–(D), numbers in each quadrant indicate the percentage of total, viable CD4+ T cells. (E) Summary (mean) of all priming experiments. Error bars denote ± SEM. Data are from two or three experiments per group.
versus T-bet–deficient Th17 cells cultured with IL-23 (Supplemental Fig. 2D, 2E). This indicates that Th17 cells can express IFN-γ after chronic stimulation with IL-23 in the absence of transcriptional factors that regulate IFN-γ production by Th1 cells such as T-bet, Eomes, STAT1, and STAT4 (Fig. 3E). These data argue against the model that IL-23 is upstream of T-bet as previously described (13).

Selective deficiency of T-bet in T cells does not prevent EAE development and CNS-infiltrating IFN-γ^−^IL-17^+^ T cells

Our data raise the following questions: Can the emergence of IL-17^IFN-γ^-^ T cells in response to IL-23 be recapitulated in vivo in the absence of T-bet? Furthermore, can T-bet–deficient Th17 cells induce EAE? To address these questions, we immunized mice in which T-bet was specifically deleted in T cells (T-bet^fl/flCD4^Cre^). In contrast to T-bet KO mice (14), T-bet^fl/flCD4^Cre^ mice were not resistant to the development of EAE. However, these mice developed EAE with delayed onset (16.75 ± 4.1 versus 12.7 ± 4.1) and lower incidence (66.0 versus 91.7%) compared with control mice (7, 13, 15) because we see enhanced IFN-γ and T-bet expression in IL-17^IFN-γ^-^ T cells compared with IL-17^+^ T cells, but our data that address the selective defect of T-bet in T cells argue against a direct role of T-bet in the pathogenicity of Th17 cells and its absolute requirement for the development of EAE. Our results further suggest that IFN-γ expression is regulated differently in Th1 versus Th17 cells. Interestingly, T-bet was recently shown to interact with the transcription factor Runx1 and to inhibit Runx1-mediated transactivation of Rorc (23). It is therefore possible that the association between T-bet and Runx1 does not occur in IFN-γ^−^IL-17^+^ Th17 cells, which allows for coexpression of T-bet and Rorγt and continuation of the Th17 differentiation program. Future experiments are needed to determine the mechanisms by which T-bet affects the function of Rorγt in T cells that produce both IL-17 and IFN-γ.

In summary, our study highlights the plasticity of pathogenic Th17 cells present in the CNS during EAE and the importance of IL-23 in this process. It further establishes the pathogenic potential of IL-17^IFN-γ^-^ T cells in EAE by demonstrating that MOG-specific, GM-CSF^+^ T cells are selectively enriched in this population. Finally, this study demonstrates that Th17 cells can induce CNS autoimmunity independently of T-bet. Collectively, these results have important implications for the development of drugs to target Th17 cells, their effector cytokines, and their functions.

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**Disclosures**

The authors have no financial conflicts of interest.

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