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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-17A+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.

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/6 (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-betΔ10,CD4Cre, and Tbx21−/−, eomesodermin (Eomes)Δ10,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/6 background. All animals were bred and maintained under specific pathogen-free conditions.
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 (FACS Aria; 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-Ab/MOG38–49 tetramer was obtained through the 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).

**EAE induction**

EAE was induced by s.c. immunization of mice into the flanks with an emulsion of MOG35–55 peptide (100 μg) emulsified in CFA supplemented with 4 mg/ml Mycobacterium tuberculosis extract H37Ra (Difco). Additionally, the animals received 200 ng pertussis toxin (List Biological Laboratories) i.p. on days 0 and 2. Clinical signs of EAE were assessed according to the following score: 0, no signs of disease; 1, loss of tail tonicity; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb and forelimb paralysis.

**Statistical analysis**

Statistical analysis was conducted with GraphPad Prism software. The p values were calculated with a Student paired t test. A p 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 MOG38–49/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 CNS at the peak of disease, with limited expression of GM-CSF and few IL-17+IFN-γ- T cells (Fig. 1A, Supplemental Fig. 1A). Remarkably, CNS cytokine secretion increased by 77-fold for IL-17+IFN-γ+ T cells (Fig. 1A). CNS-infiltrating T cells are further characterized by higher T-bet, retinoic acid–related orphan receptor (Ror)γt, IL-23R, and GM-CSF expression and lower TGF-B3 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.
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-deficient mice (Fig. 2A, 2B, Supplementary 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 T 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 T cells.

**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+ Th17 cells propagated with IL-23 for three rounds of stimulation. Data are from at least three independent experiments (mean ± SEM). (C) Expression of GM-CSF among WT IL-17+IFN-γ+ and IL-17+IFN-γ+ Th17 cells propagated with IL-23 for three rounds of stimulation. Data are from at least three independent experiments (mean ± SEM). (D) WT and IL-23R−/− mice were immunized with MOG35–55 emulsified in CFA. At day 10, the proportion of IL-17+, IFN-γ+, and GM-CSF+ 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.** 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−/−CD4Cre (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 (Supplementary 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+ Th1 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 CD4 T cells (T-betfl/flCD4Cre). In contrast to T-bet KO mice (14), T-betfl/flCD4Cre mice were not resistant to the development of EAE. 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 (Fig. 4A, quadrant indicated the percentage of total, viable CD4+ T cells. (B) Intracellular cytokine staining of IL-17 and IFN-γ in CNS-infiltrating CD4+ T cells. Numbers in each quadrant indicate the percentage of total, viable CD4+ T cells. (C) Quantification of cytokine-producing cells in the CNS of T-betfl/fl and T-betfl/fl CD4Cre mice. Data are representative of two independent experiments with at least six mice per group (mean ± SEM).

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.

References


Supplemental Figure 1

A

B

C

D

E

Ax0

Ax1

Ax17

AxTh

Events (% of max)

Ax0

Ax1

Ax17

AxTh

Events (% of max)

Ax0

Ax1

Ax17

AxTh

Events (% of max)
Supplemental Figure 2

A

B

C

D

E
Supplemental Data

Supplemental Figure 1. Characterization of Th1 and Th17 cells in vitro and in vivo

(A) Intracellular cytokine staining for IL-17 and GM-CSF in WT CD4+ T cells isolated from LN and CNS of mice at the peak of EAE. Numbers in quadrants indicate percent cells in each throughout. (B) Expression of T-bet, Rorc, GM-CSF, IL-23R and TGF-β3 gene transcripts is determined by quantitative RT-PCR analysis on CD4+ CD25- effector T cells isolated from the spleen or CNS of mice with EAE and presented relative to the expression of β-actin. One representative out of two experiments is shown. (C) Intracellular cytokine staining for IL-17 and IFN-γ in Th1 and Th17 cells differentiated in vitro. (D) Quantitative RT-PCR analysis of T-bet, Rorc, IL-23R and GM-CSF mRNA on naïve CD4+ T cells differentiated for 48 hrs as in C. (E) Expression of IL-23R in in vitro differentiated Th0, Th1 and Th17 cells.

Supplemental Figure 2. Gene expression signature of IL-17+ IFN-γ+ Th17 cells and their requirement for STAT1 and STAT4 in absence of T-bet

Relative expression of GM-CSF, T-bet, Rorc (A) and CXCL3, Granzyme B and Perforin (B) mRNAs in comparison to β-actin among cytokine-producing CD4+ T cells in the CNS at the peak of EAE. Data are from 2-4 independent experiments with 4-6 mice per group (mean ± SEM). (C) Frequency of IL-17+ IFN-γ+ T cells after Th17 cell priming and consecutive restimulation with or without IL-23. Data are from three independent experiments (mean ± SEM). Flow-cytometric analysis of STAT3 and STAT4 (D) and STAT1 (E) phosphorylation by Th17 cells from WT, STAT1KO, STAT4KO and Tbet^{flfl} CD4^{Cre} mice, expanded in the presence of IL-23 for 7 days; untreated cells (dashed line),
IL-23 or IL-12/IFN-γ treated cells (solid line). Data are representative of 3 independent experiments.