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Autoreactive Tbet-Positive CD4 T Cells Develop Independent of Classic Th1 Cytokine Signaling during Experimental Autoimmune Encephalomyelitis

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Multiple sclerosis (MS) is a chronic autoimmune disease of the CNS that results in immune destruction of the myelin sheath (1, 2). Experimental autoimmune encephalomyelitis (EAE) is a commonly employed mouse model for MS and has been proven invaluable in dissecting the cellular and molecular components that mediate disease pathogenesis. Immunization of mice with the myelin oligodendrocyte glycoprotein (MOG)35–55 peptide induces a chronic form of EAE in which effector CD4 T cells play a central role. In addition, the fundamental requirement for CD4 T cells in mediating disease development has been demonstrated using the proteolipid protein relapsing-remitting model of EAE, as well as passive transfer of purified, myelin-specific CD4 T cells. Together, these data indicate that CD4 T cells are critical for the induction of EAE; however, which effector functions of the CD4 T cells drive the autoimmune inflammation remain ill defined.

CD4 T cells can be divided into distinct subsets based on their functional properties: Th1 cells produce IFN-γ, Th2 cells secrete IL-4, IL-5, and IL-13, Th17 cells make IL-17A, IL-17F, IL-21, and IL-22, and regulatory T cells are Foxp3 positive and produce IL-4, IL-5, and IL-13, Th17 cells make IL-17A, IL-17F, IL-21, and regulatory T cells are Foxp3 positive and produce IL-10 (3–6). It was originally proposed that Th1 cells were the pathogenic cell population during MS and EAE, as high levels of IL-12 and IFN-γ were found in the CNS, CD4 T cells producing IFN-γ were prevalent during disease, and adoptive transfer of Th1-polarized CD4 T cells conferred disease (7–10). Although these data suggested an important role for Th1 cells during disease, mice lacking IL-12 and IFN-γ signaling (IL-12p35−, IL-12Rβ2−, IFN-γ−, and IFN-γR−deficient mice) remained susceptible to EAE (11–14), bringing into debate the function of these cells during EAE and, more importantly, MS. Moreover, in recent years, an increasing amount of data has been published indicating a principle role for Th17 cells in mediating disease induction: IL-17-producing CD4 T cells are associated with the development of disease, and mice lacking factors coupled with Th17 differentiation (IL-23p19, retinoic acid-related orphan receptor γt, or IL-6) are resistant to EAE (12, 15, 16). However, neutralization of IL-17A did not completely abrogate disease, suggesting that other molecules contribute to disease pathogenesis (17). One such cytokine may be GM-CSF, as recent studies have demonstrated that expression of this cytokine by CD4 T cells is essential for the development of EAE (18, 19).

Th1 effector CD4 T cells represent a paradox during EAE, in addition to other chronic autoimmune inflammatory disorders; the cardinal Th1 cytokine, IFN-γ, is dispensable for disease, but the master Th1 transcription factor, Tbet, is required for the development of EAE (7, 14, 20–22). This highlights an unrecognized role for Tbet and potentially Th1 effector CD4 T cells...
REGULATION OF Tbet EXPRESSION DURING EAE

IFN-γ-expressing Th1 cells during autoimmunity that is independent of prototypic Th1-associated cytokine signaling. Collectively, our findings reveal a potentially pathogenic role of Tbet-expressing Th1 cells during autoimmunity that is independent of IFN-γ production.

Materials and Methods

Mice

The following mice were purchased from The Jackson Laboratory and/or were bred at the University of Alabama at Birmingham (UAB): C57BL/6 (wild-type [WT]), B6.129S6-Ifngtm1Ts (IFN-γ-deficient), B6.129S7-Stat1tm1Rds (STAT1-deficient), B6.129P2-Stat1tm1 (STAT1-deficient), and B6.129S1-I12β/12αtm1 (IL-12p35-deficient) mice. Ifngf12/12 bacterial artificial chromosome-In Tg (IFN-γ1B1) mice, which were previously described (23), were bred with IFN-γ-deficient mice in C57BL/6 background to generate IFN-γ-deficient IFN-γ1B1 mice (IFN-γ−/− × IFN-γ1B1). 129S6/SvEv-Stat1tm1Rds (STAT1-deficient) and 129SVE (WT) mice were purchased from Taconic Farms. All animals were bred and maintained according to Institutional Animal Care and Use Committee regulations.

EAE induction, clinical scoring, and anti–IFN-γ mAb treatment

EAE was induced in experimental mice between the ages of 6 and 12 wk by s.c. immunization with 200 μg Mycobacterium tuberculosis; Difco and i.p. pertussis toxin (50 μg; List Biological Laboratories) injections on days 0 and 2 (24). Disease scores were monitored daily with the following criteria: 0, no disease; 1, paralyzed tail; 2.0, hind limb paralyzed; 3.0, both hind limbs paralyzed; and 4.0, moribund. Anti–IFN-γ neutralizing mAb treatment was performed by i.p. injection of 100 μg anti–IFN-γ mAb (clone XM1G1.2; UAB hybridoma facility) to control C57BL/6- and IL12p35-deficient mice every 3 d from days 0–9 (25).

Mononuclear cell isolation and activation

Experimental mice were anesthetized and perfused with sterile PBS between 12 and 20 d after immunization depending on the disease severity. Brains, spinal cords, spleens, and inguinal lymph nodes were removed, and single-cell suspensions were prepared by mechanical disruption. Cells were prepared and cultured with RPMI 1640 with 10% FCS, 2 mML-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids, 1 μM sodium pyruvate, and 2.5 μM 2-ME (R10 media). Brain and spinal cord (CNS) mononuclear cells were purified by centrifugation over a 30/70% Percoll gradient and resuspended in R10 media. Mononuclear cells were left unstimulated, activated with 10 μM MOG35-55 peptide for 16 h, or restimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (750 ng/ml; Calbiochem) for 4 h. Monensin was added for the final 3 h of culture according to the manufacturer’s instructions (BD Pharmingen).

Surface, intracellular staining, and cell sorting

Standard cell-surface staining was performed on single-cell suspensions of spleen, lymph node, and CNS mononuclear cells with anti-CD4 PerCP-Cy5.5 (RM4-5; eBioscience) and anti-Thy1.1 FITC (OX-7; BD Pharmingen) (26). Cells were then either analyzed or fixed and permeabilized with permeabilization buffer (0.5% BSA-100 mM sodium phosphate; pH 7.4; 0.1% 0.1% IG1x; eBioscience) and stained intracellularly with anti–IFN-γ eFlour 450 (XM1G1.2; eBioscience), anti–IL-17A PE (TC11-18H10; BD Pharmingen), anti-Thet Alexa Fluor 647 (4B10; eBioscience), anti-IgG1x Alexa Fluor 647 (eBioscience), anti-TNF-α, anti–IL-2 PE-Cy7 (JES6-5H4; eBioscience), or anti–GM-CSF PE (MP1-22E9; eBioscience) according to the manufacturer’s instructions. Samples were acquired using an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star). For FACS sorting, pooled CNS mononuclear cells were stained with anti-CD4 eFlour 450 (RM4-5; eBioscience) and anti-Thy1.1 PE (OX-7; BD Pharmingen) and sorted using FACSaria and FACSaria II cell sorters (BD Biosciences) in the UAB Center for AIDS Research and UAB Comprehensive Arthritis, Musculoskeletal and Autoimmunity Center flow cytometry facilities, respectively.

RNA isolation, cDNA synthesis, and real-time PCR

RNA was extracted from the sorted CD4 T cells using the RNeasy Mini kit (Qiagen), and cDNA synthesis was performed with the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturers’ instructions. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and the primer pairs listed in Supplemental Table I, with β2-microglobulin as the housekeeping gene. Reactions were run in triplicate on the IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Relative gene expression was calculated according to the ΔΔ threshold cycle (Ct) method in which ΔΔCt = (ΔCt of IFN-γ-deficient cells – ΔCt of WT [IFN-γ-sufficient] cells). Statistics

Statistical significance was calculated by unpaired Student t test using Prism software (GraphPad). The p values ≤ 0.05 were considered significant unless specifically indicated otherwise in the text.

Results

The transcription factor Tbet is critical for IFN-γ production by CD4 T cells, as well as for the generation of Th1 effector CD4 T cells (5, 27). It has been shown that Tbet is necessary for the development of EAE and that CD4 T cell-intrinsic expression of Tbet is necessary to confer disease upon adoptive transfer (7, 20–22). To investigate what proportion of the effector CD4 T cells during disease express Tbet and if these cells represent a functionally distinct population of pathogenic effector CD4 T cells, we have used the MOG35-55 peptide immunization model of EAE in C57BL/6 mice (Fig. 1). At the peak of the disease symptoms (day 20), cells were isolated from secondary lymphoid tissues (spleens and inguinal lymph nodes) and the sites of active inflammation (the brains and spinal cords), and direct ex vivo intracellular Tbet staining was performed without restimulation to determine the frequency of Tbet-positive CD4 T cells. Within the spleens and lymph nodes, only a minor fraction of the CD4 T cells expressed Tbet; however, a significant proportion (>75%) of the CNS-infiltrating CD4 T cells stained positive for Tbet (Fig. 1B). These data demonstrate that Tbet-expressing effector CD4 T cells are present in the CNS during EAE.

The induction of EAE is associated with both IFN-γ-positive Th1 cells and IL-17-positive Th17 cells (1, 28), yet Tbet expression is primarily linked with development of Th1 effector cells, not Th17 cells. Because we observed that the majority of the CD4 T cells in the CNS of diseased mice were Tbet positive as well as produced IFN-γ and IL-17A, we sought to determine if these cells were of the Th1 or Th17 phenotype. To visualize the cytokine production profile of the Tbet-positive CD4 T cells, intracellular staining for IFN-γ, IL-17A, and Tbet was performed following a brief in vitro restimulation (Fig. 1C). As expected, three subsets of cytokine-producing CD4 T cells were detected: IFN-γ single-producing (SP) cells, IL-17A SP cells, and IFN-γ/IL-17A double-producing (DP) cells. Analysis of the Tbet levels within these individual subsets revealed a strong correlation between Tbet expression and IFN-γ production, as both the IFN-γ SP and IFN-γ/IL-17A DP cells were Tbet positive, whereas the IL-17A SP CD4 T cells demonstrated lower levels of Tbet staining. These data are consistent with published reports in which Tbet is an essential transcriptional regulator of IFN-γ production.
in CD4 T cells and Tbet expression is not required for IL-17A production; however, this molecule can be expressed by Th17 cells under certain conditions (4, 6, 29).

In addition to the cytokines IFN-γ and IL-17A, we also examined the ability of the Tbet-expressing CD4 T cells in the CNS to produce other effector cytokines. We observed that following polyclonal stimulation with PMA/ionomycin, between 25 and 50% of the Tbet-positive cells were capable of producing the proinflammatory cytokine TNF-α, and a fraction of these cells made GM-CSF and/or IL-2 (Fig. 1D). Similar trends were detected when cells were stimulated with the MOG35–55 peptide; however, the frequencies of cytokine-producing cells were reduced, particularly for IL-2 (data not shown). Taken together, these data indicate that Tbet expression is not only associated with IFN-γ production by CD4 T cells during EAE, but also additional proinflammatory cytokines that may contribute to disease pathogenesis.

The requirement for Tbet during EAE implies an important role for Th1 effector CD4 T cells in disease pathogenesis; nevertheless, the signature Th1 cytokine, IFN-γ, is not necessary for the development of the autoimmune state (7, 14, 20–22). Two potential explanations can reconcile these results: the induction of EAE in the absence of IFN-γ is mediated by a Tbet-independent mechanism, or Tbet-positive CD4 T cells promote disease even in the absence of IFN-γ via an IFN-γ-independent mechanism. To discriminate between these two possibilities, we examined the levels of Tbet within the effector CD4 T cells during EAE in IFN-γ-deficient and IFN-γR–deficient mice, both of which are devoid of IFN-γ signaling. Interestingly, a significant fraction (between 40 and 80%) of the CNS-infiltrating CD4 T cells in the IFN-γ-deficient and IFN-γR–deficient mice expressed Tbet, and the frequencies and numbers of these Tbet-positive cells were similar to those observed in the WT control mice (Fig. 2B, 2C, 2E, 2F, Supplemental Fig. 1). We also analyzed the effector cytokine profile of the CD4 T cells in the various cohorts of diseased mice. In keeping with previous findings that indicate IFN-γ can suppress the differentiation of Th17 cells (4, 6), we detected elevated frequencies of IL-17A–producing CD4 T cells in the CNS-associated tissues of the IFN-γ– and IFN-γR–deficient mice (Fig. 3). Taken together, there is a correlation between the presence of Tbet-positive effector CD4 T cells in the CNS and disease pathogenesis, even when the cardinal Th1 cytokine IFN-γ is not present.

Although the above data fit the explanation that Tbet-positive Th1 CD4 T cells function to promote autoimmune inflammation independent of IFN-γ secretion, it is unclear if Th1 effector CD4 T cells are present in the IFN-γ-deficient mice. Consistent with this concept, we noted that IFN-γ–producing CD4 T cells were still present during EAE in IFN-γR–deficient mice (Fig. 4), suggesting that these cells may function to drive disease separate from IFN-γ secretion. To investigate the possibility that Th1 cells arise in IFN-γ-deficient hosts, we have employed a unique IFN-γ reporter mouse model, the IFN-γ BI transgenic mouse, which has been previously used to study the fate of in vivo-generated Th1 effector CD4 T cells (23). These transgenic mice contain the Thy1.1 reporter molecule driven by the IFN-γ promoter on a bacterial artificial chromosome and mark IFN-γ–positive T cells with high fidelity (all Thy1.1+ T cells are IFN-γ+). The IFN-γ BI transgenic mice were bred onto the IFN-γ–deficient background, providing us with a system to identify Thy1 or Thy1-like CD4 T cells that could not produce IFN-γ. Following induction of EAE in IFN-γ BI and IFN-γ–deficient IFN-γ BI mice, we observed similar disease susceptibility between both cohorts (data not shown). Significantly, we found comparable frequencies of Thy1.1-positive
CD4 T cells in the inflammatory sites between both groups of mice (Fig. 5A, 5B), indicating the presence of Th1-like effector CD4 T cells during EAE in IFN-γ-deficient mice. The observation that effector CD4 T cells from both WT and IFN-γ-deficient mice express the IFN-γ reporter molecule during EAE led us to examine the Th1 properties of these cells. By intracellular staining, we confirmed that the Thy1.1+ cells in the WT mice were IFN-γ+, whereas the Thy1.1+ cells in the IFN-γ-deficient hosts were negative for this cytokine (Fig. 5C). In keeping with our earlier data, regardless of their ability to produce the cytokine IFN-γ, the Thy1.1+ CD4 T cells all expressed Tbet. To analyze additional genes associated with the Th1 lineage, we FACS sorted Thy1.1+ CD4 T cells from the pooled brains and spinal cords of WT and IFN-γ-deficient IFN-γBI mice and performed real-time PCR. Interestingly, the Thy1.1+ CD4 T cells from the IFN-γ-deficient mice retained expression of multiple Th1-associated genes, including CXCR3, IL-12Rβ2, STAT4, and IL-18R (Fig. 5D). These data further support the concept that Tbet-positive, Th1-like effector CD4 T cells are present in IFN-γ-deficient hosts and that these cells may contribute to disease pathogenesis independent of their ability to secrete the cardinal Th1 cytokine IFN-γ.

Our earlier data, as well as published reports, demonstrate the emergence of IFN-γ’IL-17’ DP CD4 T cells during EAE, as well as elevated levels of Th17 cells when disease is induced in IFN-γ-deficient hosts (Figs. 1C, 3). Therefore, we sought to determine if the IFN-γ reporter-positive CD4 T cells that developed in the IFN-γ-deficient mice exhibited properties of Th17 cells as well as Th1 cells. Intracellular IL-17A staining was performed on CD4 T cells from the brains and spinal cords of WT and IFN-γ-deficient IFN-γBI mice. In WT IFN-γ BI mice, a subset of Thy1.1+IL-17A+ cells was detectable that represented ~20% of the total Thy1.1+ cell population, a frequency that reflected the proportion of IFN-γ/IL-17A DP cells out of the total IFN-γ+ CD4 T cell population (Figs. 1C, 5C). However, when the IFN-γ-deficient mice were analyzed, we found a greater percentage of the Thy1.1+ CD4 T cells (~50%) also expressed the cytokine IL-17A. Nevertheless, a subset of IFN-γ reporter-positive cells did emerge in these hosts that was devoid of IL-17A expression. We extended this analysis by performing real-time PCR for several Th17-associated genes on FACS-sorted Thy1.1+ CD4 T cells from the CNS of WT and IFN-γ-deficient mice. In keeping with the increased proportion of IL-17A+ cells, we also observed modest elevations in the expression of the Th17-associated transcription factors retinoic acid-related...
orphan receptor $\gamma_T$ and aryl hydrocarbon receptor, as well as the cytokine IL-21 and IL-23R (Fig. 5E). Taken together, these data suggest that within the increased IL-17A population of cells seen in IFN-$\gamma$–deficient hosts, a subset of these cells may display features that are characteristic of the IFN-$\gamma$/IL-17A DP cells, not simply Th17 cells. Still, it does appear that a unique, Th1-like effector CD4 T cell population develops in the IFN-$\gamma$–deficient hosts.

It has been previously published that induction of Tbet expression in CD4 T cells during Th1 differentiation is dependent on IFN-$\gamma$ and STAT1 (30, 31); however, we found high levels of Tbet expression within CD4 T cells during EAE in the absence of IFN-$\gamma$ signaling (Fig. 2). We did observe a slight reduction in the mean fluorescence intensity (MFI) of the Tbet staining, suggesting lower levels of Tbet protein on a per-cell basis, but this did not reach statistical significance. Because IFN-$\gamma$ signaling via STAT1 has been shown to be important for Tbet upregulation in Th1 cells (31), we next analyzed Tbet levels in STAT1-deficient CD4 T cells during EAE in both B6 and 129S6 backgrounds. In the experiments using mice on the B6 background, the percentage of Tbet-positive cells, as well as the disease intensity, was comparable between B6 WT and STAT1-deficient mice (Fig. 6A, 6B, and data not shown). In 129S6 cohorts, even though the control mice exhibited mild disease, and the Tbet expression was not as high as in B6 control mice, the STAT1-deficient mice did develop exacerbated EAE, and high frequencies of Tbet-positive CD4 T cells were identified in the brains and spinal cords of diseased mice (Fig. 6B, Supplemental Table II). A slight diminution in the MFI
of the Tbet staining was detectable, suggesting that the IFN-γ/STAT1 pathway is not necessary for Tbet gene expression but may function to augment Tbet levels. These findings demonstrate a potential alternative pathway regulates Tbet induction during EAE.

In vitro differentiation of Th1 cells is achieved by stimulating naive CD4 T cells in the presence of IL-12; thus, we wanted to test if the induction of Tbet expression during EAE was the result of IL-12 signaling (3, 32). To examine the role of IL-12 in Tbet regulation during EAE, we immunized WT and IL-12p35–deficient mice with MOG 35–55 peptide and analyzed the CNS-infiltrating CD4 T cells at the peak of disease severity. These mice are known to develop EAE, as the IL-23 cytokine network is still intact (12). Staining for Tbet in the effector CD4 T cells revealed no differences in the percentages of Tbet-positive CD4 T cells as well as the MFI of Tbet staining between the control and IL-12p35–deficient mice, indicating IL-12 is not essential for Tbet expression during EAE (Fig. 6C, 6D). Still, it is possible that in the absence of IL-12, IFN-γ signaling acts in a compensatory manner to promote Tbet induction and vice versa. Therefore, we investigated how the upregulation of Tbet in CD4 T cells was influenced when both IFN-γ and IL-12 signaling were abrogated. Concurrent with EAE immunization, WT and IL-12p35–deficient mice were treated with anti–IFN-γ neutralizing mAb to block IFN-γ signaling. These treated mice developed EAE similar to control mice, as shown in Supplemental Table II. CD4 T cells isolated from the brains and spinal cords were analyzed for Tbet expression at the peak of disease. We did observe an increase in the frequency of IL-17A–producing CD4 T cells in the IL-12p35–deficient mice in which anti–IFN-γ mAb was administered, indicating that the neutralization protocol did have an impact in vivo (Supplemental Fig. 2). Interestingly, we did note a decrease in the percentage of Tbet-positive CD4 T cells in the brains of mice lacking IL-12 and IFN-γ signaling; however, this difference in Tbet staining was not observed in the spinal cords of immunized mice (Fig. 6E, 6F). So, although the absence of IL-12 and IFN-γ did have some impact on frequency of Tbet expressing CD4 T cells during EAE, it was not absolute. Therefore, other factors

FIGURE 5. The emergence of Th1 or Th1-like cells during EAE. A–C, EAE was induced in WT IFN-γ BI and IFN-γ–deficient IFN-γ BI (IFN-γ−/− × IFN-γ BI) transgenic mice, and IFN-γ reporter molecule (Thy1.1) expression was assessed 20 to 21 d later. A, Representative contour plots are gated on CD4 T cells, and the values indicate the percentage of Thy1.1-positive cells present in the gate. B, Combined data from two separate experiments are shown (n = 7). C, Cells from the BR and SC of WT and IFN-γ–deficient IFN-γ BI mice were stimulated for 4 h with PMA/ionomycin and subsequently stained for CD4 and Thy1.1 in conjunction with IFN-γ (left panel), Tbet (middle panel), or IL-17A (right panel). D–F, Thy1.1-positive CD4 T cells were FACS sorted from the CNS of WT and IFN-γ–deficient IFN-γ BI mice, and real-time PCR was performed for genes related to the (D) Th1, (E) Th17, and (F) Th2/ regulatory T cell lineages. Relative gene expression of IFN-γ–deficient compared with WT cells is shown as the ∆∆Ct value, in which ∆∆Ct = (ΔCt of the IFN-γ–deficient cells − ΔCt of the WT cells). Data were combined from three independent experiments, except the aryl hydrocarbon receptor (AHR) data, which are from two experiments.
besides the traditionally Th1-associated cytokines must regulate Tbet induction in vivo.

**Discussion**

During EAE, the major Th1 cytokine IFN-γ is not necessary for disease induction; however, the main Th1-associated transcription factor Tbet is, resulting in a conundrum regarding the role of Th1 cells during EAE (7, 14, 20–22). In this study, we demonstrate that Tbet-expressing CD4 T cells are present in the CNS in abundant frequencies during disease in WT as well as IFN-γ–deficient mice. These data, in conjunction with the upregulation of an IFN-γ reporter transgene by CD4 T cells genetically deficient in IFN-γ, suggest that Tbet-positive Th1 (or Th1-like) cells contribute to EAE independent of IFN-γ-deficient mice. This is consistent with data published by Yang et al. (7), who reported that IFN-γ–deficient CD4 T cells from the CNS of mice with active EAE upregulate Tbet following an overnight stimulation with MOG35–55 peptide. Tbet is known to regulate the expression of many genes, both in a positive and negative manner, such as IL-12Rβ2, CXCR3, osteopontin, SOCS1, and SOCS3 (31, 33–35). Therefore, Tbet may be important for the upregulation of pathogenic genes necessary for disease, or Tbet may be critical for the downmodulation of suppressive genes that prevent the onset of inflammation.

Induction of Tbet expression during in vitro Th1 differentiation has been shown to be IFN-γ and STAT1 dependent (30, 31); however, in this report, we reveal that upregulation of Tbet within effector CD4 T cells during EAE is independent of both IFN-γ and STAT1 signaling. Moreover, we show that Tbet expression during EAE does not require IL-12, a robust Th1-potentiating cytokine. TCR signaling and NFAT are known to be important for Tbet upregulation; however, this pathway alone is unable to cause Tbet expression (W. Yeh and L.E. Harrington, unpublished observations). These data highlight the differences observed between the in vitro and in vivo studies regarding Tbet regulation and indicate that other factors besides the notable Th1-associated proinflammatory cytokines are likely to mediate Tbet expression during EAE. One possibility is that Tbet upregulation is actively suppressed by molecules such as TGF-β, masking the positive impact of various molecules to turn on Tbet expression (29, 36).
inflammatory tissues during disease, as opposed to <20% of the CD4 T cells in the secondary lymphoid tissue. This differential expression of Tbet by the CNS-infiltrating CD4 T cells may reflect the preferential recruitment of Tbet-positive CD4 T cells to the inflammatory site or the upregulation of Tbet upon entry into the inflammatory area. Tbet was highly expressed in CD4 T cells that produced IFN-γ, both the IFN-γ SP and IFN-γ/IL-17A DP cells; however, lower levels of Tbet were detected in the IL-17A SP cells. Interestingly, within the IL-17A SP cells, there are both Tbethi and Tbetlo populations, suggesting the Th17 cells in the CNS during EAE are heterogeneous. Tbet has been demonstrated to suppress IL-17A production in certain circumstances (4, 6, 29); hence, the expression of Tbet by the IL-17A SP as well as IFN-γ/IL-17A DP cells is quite interesting. Previously, it has been shown that in vitro-polarized Th17 cells can transition into Th1 cells upon repeated stimulation, and this evolution requires Tbet (37). It is tempting to speculate that these Tbet-positive, IL-17A SP or IFN-γ/IL-17A DP cells found in the CNS during EAE are in the process of converting from a Th1 phenotype into a Th1 phenotype. Future studies will be necessary to decipher if this is the case and if this progression is essential for the onset of EAE. Recently, Herota et al. (38) published a report supporting this hypothesis. This article demonstrated that during EAE, genetically marked IL-17A–positive CD4 T cells convert into IFN-γ-producing cells overtime. Alternatively, another report has shown that Th17 cells polarized in the absence of TGF-β coexpress Tbet and IL-17A, and MOG-specific TCR transgenic CD4 T cells activated under these conditions were able to confer EAE upon adoptive transfer into Rag-deficient hosts (29). It is possible that the Tbet-positive CD4 T cells we have identified in the CNS resemble these in vitro-differentiated cells and have undergone a similar developmental program in vivo.

Our data in this report also raise interesting questions regarding the effector CD4 T cell populations that develop in IFN-γ–deficient mice undergoing EAE. Using a novel strain of IFN-γ reporter mice, the IFN-γ–deficient IFN-γ BI mice, we demonstrate that a subset of CD4 T cells turn on the IFN-γ promoter in the CNS during EAE, irrespective of their ability to produce the actual cytokine. We have termed these cells Th1-like effector CD4 T cells because they appear to retain many of the characteristics of traditional Th1 cells. Interestingly, we noted two distinct subsets of cells within this population of Thy1.1 reporter-positive CD4 T cells: those that express the Thy1.1 reporter alone and those that coexpress the Thy1.1 reporter molecule in conjunction with IL-17A. We propose that the cells that coexpress the Thy1.1 and IL-17A closely resemble the IFN-γ/IL-17A DP cells seen in the CNS of WT mice, whereas the CD4 T cells that are Thy1.1 positive but do not express IL-17A are similar to the IFN-γ SP cells. Hence, the increased levels of Th17 cells observed in the IFN-γ–deficient hosts are likely more heterogeneous in nature than previously believed. Future studies will need to be performed to determine how each of these cell populations contribute to the pathogenesis associated with EAE in the IFN-γ–deficient hosts and if these cell populations are also present in other chronic inflammatory disorders.

Overall, in this report, we demonstrate that the majority of effector CD4 T cells present in the CNS during EAE express Tbet, even under circumstances in which IFN-γ–producing CD4 T cells are not present due to genetic deletion of the Ifng gene. This suggests a potential pathogenic function for Th1 CD4 T cells during EAE that is separate from the secretion of the cytokine IFN-γ. Interestingly, the development of this autoimmune Tbet-positive CD4 T cell population is independent of the canonical Th1-inducing cytokines IFN-γ and IL-12. Hence, what molecules regulate Tbet expression in vivo and what genes downstream of Tbet drive EAE represent prospective therapeutic targets for MS and other chronic inflammatory diseases.

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Disclosures

The authors have no financial conflicts of interest.

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