

T-bet is essential for encephalitogenicity of both Th1 and Th17 cells

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The extent to which myelin-specific Th1 and Th17 cells contribute to the pathogenesis of experimental autoimmune encephalomyelitis (EAE) is controversial. Combinations of interleukin (IL)-1 β , IL-6, and IL-23 with transforming growth factor β were used to differentiate myelin-specific T cell receptor transgenic T cells into Th17 cells, none of which could induce EAE, whereas Th1 cells consistently transferred disease. However, IL-6 was found to promote the differentiation of encephalitogenic Th17 cells. Further analysis of myelin-specific T cells that were encephalitogenic in spontaneous EAE and actively induced EAE demonstrated that T-bet expression was critical for pathogenicity, regardless of cytokine expression by the encephalitogenic T cells. These data suggest that encephalitogenicity of myelin-specific T cells appears to be mediated by a pathway dependent on T-bet and not necessarily pathway-specific end products, such as interferon γ and IL-17.

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Abbreviations used: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; NS, nonsense.

Experimental autoimmune encephalomyelitis (EAE) has been used as a model for multiple sclerosis (MS) for over four decades. Our understanding of EAE and the immune cells that mediate the pathology of the disease has grown tremendously during this time. However, as the field of immunology evolves, many of the fundamental observations in EAE are questioned and revisited to further our understanding of EAE and ultimately enhance our understanding of MS. Although EAE was initially induced by immunization with myelin proteins emulsified in CFA, it can also be induced by adoptive transfer of myelin-specific CD4 $^+$ Th1 cells into naive recipient mice (McDonald and Swanson, 1988; Ando et al., 1989; Waldburger et al., 1996; Yura et al., 2001; Lovett-Racke et al., 2004; Gocke et al., 2007). The observation that myelin-specific CD4 $^+$ Th1 cells were sufficient to induce EAE focused MS research on these IFN- γ -producing T cells in MS patients. Although myelin-specific T cells were found in both MS patients and healthy individuals, which raised questions as to the relevance of these cells in MS patients, it does appear that myelin-specific T cells from MS patients are more likely to have a Th1 phenotype (Olsson et al., 1990; Sun et al., 1991; Voskuhl et al., 1993; Pelfrey

et al., 2000). Subsequently, several studies demonstrated that although healthy individuals had myelin-specific T cells, these cells were naive, whereas MS patients had activated and memory myelin-specific T cells, indicating that these cells had been previously activated (Allegretta et al., 1990; Lovett-Racke et al., 1998; Burns et al., 1999). In addition, a clinical trial with an altered peptide ligand from myelin basic protein (MBP), which was intended to down-regulate myelin-specific T cells, actually exacerbated disease in several MS patients, which was associated with increased frequency of MBP-specific T cells that produced IFN- γ , suggesting that MS is mediated by myelin-specific Th1 cells (Bielekova et al., 2000).

As a result, several studies focused on IFN- γ as the pathogenic molecule in EAE and MS. Surprisingly, IFN- γ -deficient mice and mice given antibodies to neutralize IFN- γ were still susceptible to EAE (Lublin et al., 1993; Ferber et al., 1996; Heremans et al., 1996; Willenborg et al., 1996). The number of myelin-specific CD4 $^+$ T cells was expanded in these mice,

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which may have occurred because of the loss of regulatory cells that were dependent on IFN- γ (Chu et al., 2000). However, several studies that specifically suppressed IFN- γ in the myelin-specific T cells before transfer into recipient mice demonstrated that altering the signaling pathway that results in IFN- γ production in CD4 $^{+}$ T cells decreases the encephalitogenic capacity of these cells (Racke et al., 1994; Racke et al., 1995; Lovett-Racke et al., 2004). In addition, STAT4 and T-bet, transcription factors in the Th1 cell differentiation pathway, have been shown to be critical for EAE induction (Chitnis et al., 2001; Bettelli et al., 2004; Lovett-Racke et al., 2004; Nath et al., 2006). Together, these data suggest that the differentiation pathway that generates Th1 cells may be important in encephalitogenicity, but the downstream production of IFN- γ by myelin-specific T cells may not be critical.

More recently, a new phenotype of CD4 $^{+}$ T cells was described in EAE that appears to be pathogenic. Stimulation of myelin-specific T cells from myelin-immunized mice with IL-23 promotes the expansion of T cells that express IL-17 (Langrish et al., 2005). Transfer of these myelin-specific IL-17-producing T cells, called Th17 cells, into naive mice induced EAE. In addition, microarray analysis of MS tissues indicates that IL-17 is present in MS lesions (Lock et al., 2002). We and others have shown that Th17 cells are found in the central nervous system (CNS) of mice after adoptive transfer of myelin-specific Th1 cells; however, the Th17 cells are derived from the recipient and not the transferred Th1 cells (Gocke et al., 2007; Lees et al., 2008). In addition, IL-17-deficient mice developed EAE, although disease severity is reduced and disease course altered (Komiyama et al., 2006; Haak et al., 2009). These studies have raised the question as to whether the pathogenic T cells in EAE and MS are actually Th1 or Th17 cells, or whether both populations contribute to the disease.

Several studies have defined cytokines, primarily IL-6 and TGF- β , which can induce the differentiation of CD4 $^{+}$ T cells into Th17 cells in vitro (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). However, it has not been demonstrated whether pure populations of myelin-specific Th17 cells can induce EAE, because previous studies have used lymphocytes from immunized mice that have been skewed in vitro to enhance either IFN- γ or IL-17 expression, but not exclude the other population (McGeachy et al., 2007; Stromnes et al., 2008). In the present study, we use TCR transgenic T cells specific for MBP Ac1-11 to study the differentiation of Th17 cells and their ability to induce EAE. In addition, we characterized the pathogenic autoreactive T cells in both induced and spontaneous EAE.

RESULTS

IL-6 + TGF- β fail to generate encephalitogenic Th17 cells

Several recent studies have defined cytokines that induce the differentiation of Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). It has also been demonstrated that myelin-specific T cells from immunized mice can be restimulated with IL-23 to enhance IL-17 production. These IL-23-driven IL-17-producing T cells can induce

EAE, often with atypical clinical signs, when transferred into recipient mice (Langrish et al., 2005; McGeachy et al., 2007; Stromnes et al., 2008). However, it has not been shown whether myelin-specific Th17 cells generated in vitro with IL-6 + TGF- β can induce EAE. Naive TCR transgenic splenocytes from a mouse that expresses TCR genes $V\alpha 2.3/V\beta 8.2$ that recognize MBP Ac1-11 were differentiated in vitro with peptide plus IL-6 + TGF- β or IL-12. Flow cytometric analysis was used to determine the percentage of CD4 $^{+}$ T cells producing IFN- γ and IL-17 under Th1-inducing (IL-12) and Th17-inducing (IL-6 + TGF- β) conditions. Differentiation of TCR transgenic T cells with IL-12 resulted in ~13% of CD4 $^{+}$ T cells expressing IFN- γ , and >40% of CD4 $^{+}$ T cells differentiated with IL-6 + TGF- β expressed IL-17 (Fig. 1 A). To determine if the Th17 cells generated by IL-6 + TGF- β were encephalitogenic, Th17 and Th1 cells generated in vitro were transferred into naive B10.PL recipients. Although all of the mice that received Th1 cells developed EAE, as previously observed (Gocke et al., 2007), none of the mice that received the Th17 cells developed disease (Fig. 1 B). In addition, no CNS-infiltrating mononuclear cells were recovered from the mice receiving the Th17 cells. Pure populations of naive TCR transgenic T cells acquired by cell sorting of CD4 $^{+}$ CD62L $^{+}$ cells were also differentiated in vitro with IL-6 + TGF- β , but these cells also failed to transfer EAE to recipient mice (unpublished data). It was possible that the Th17 cells were pathogenic but unable to access the CNS in the absence of Th1 cells. However, cotransfer of myelin-specific Th17 cells with suboptimal numbers of myelin-specific Th1 cells failed to induce EAE (Table I). Although pertussis toxin is not necessary to transfer EAE with the TCR transgenic Th1 cells, perhaps pertussis toxin was necessary for trafficking of Th17 cells to the CNS. However, i.p. injection of pertussis toxin on days 0 and 2 with transfer of Th17 cells also failed to induce EAE (Table I).

Th17 cells differentiated with IL-6 + TGF- β do not have a stable phenotype

Because IL-6 + TGF- β were capable of differentiating the TCR transgenic T cells into Th17 cells but failed to generate encephalitogenic T cells, we investigated the possibility that IL-23 was critical in the differentiation of pathogenic Th17 cells, because IL-23 had been shown to enhance the expansion of myelin-specific Th17 cells from immunized mice (Langrish et al., 2005). It was also possible that primary differentiation was not sufficient to generate highly encephalitogenic Th17 cells and that restimulation with antigen may be required. Therefore, TCR transgenic T cells were differentiated with MBP Ac1-11 in the presence of IL-6 + TGF- β in the presence or absence of IL-23. These cells were transferred into naive B10.PL mice and only one mouse in the IL-6 + TGF- β + IL-23 group developed EAE (Table I). After the primary differentiation in vitro, the Th17 cells were rested, restimulated with MBP Ac1-11, and transferred into recipient mice. All of the mice developed an early onset, severe EAE (Table I). However, analysis of the cytokine profile of

the transferred T cells after secondary stimulation showed that there were actually three distinct populations of T cells generated during the restimulation: Th1 cells (37%), Th17

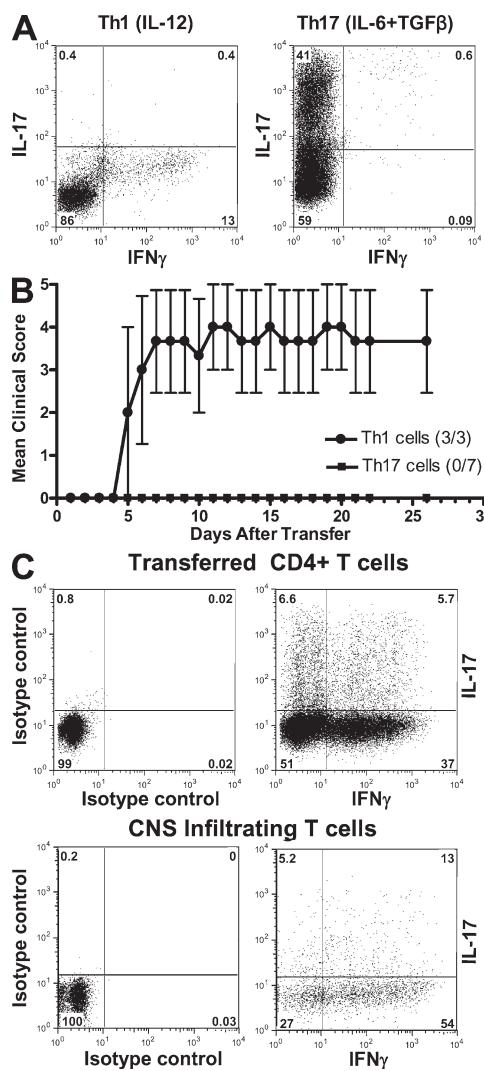


Figure 1. Th17 cells differentiated in vitro with TGF- β and IL-6 do not transfer EAE. Naive V α 2.3/V β 8.2 transgenic splenocytes were differentiated in vitro with MBP Ac1-11 plus TGF- β + IL-6 or IL-12 for 3 d. (A) Cells were harvested and analyzed by flow cytometry. Cells were gated on CD4+ T cells, and intracellular IL-17 and IFN- γ were analyzed. Data are representative of three independent experiments (percentages are shown). (B) Cells harvested from A were adoptively transferred into wild-type B10.PL recipient mice. Data are representative of six independent experiments (means \pm SEM). (C) Naive V α 2.3/V β 8.2 transgenic splenocytes were differentiated in vitro with MBP Ac1-11 and TGF- β + IL-6 for 3 d. The Th17 cells were rested for 4 d and restimulated with MBP Ac1-11 in the absence of exogenous cytokines for 2 d. The cells were analyzed for intracellular cytokines by flow cytometry (gated on CD4+ T cells; top) or transferred into naive B10.PL mice (Table I). CNS mononuclear cells were isolated from recipient B10.PL mice on day 7 after adoptive transfer (bottom). Flow cytometry was used to evaluate IFN- γ - or IL-17-producing T cells. Cells were gated on CD45 and V β 8 TCR-positive cells. Data are representative of two independent experiments (percentages are shown).

cells (6.6%), and a population that expressed both IFN- γ and IL-17 (5.7%; Fig. 1 C). Thus, restimulation of the Th17 cells with antigen induced IFN- γ production, making it unclear whether the IL-17- or IFN- γ -producing cells caused the disease. This also suggests that Th17 cells may not have a stable phenotype, which was also observed with the OVA-specific OT-II TCR transgenic T cells (Lee et al., 2009). Therefore, the CNS-infiltrating cells, gated on CD45 and V β 8 (expressed by the TCR transgenic T cells), were analyzed by flow cytometry. Although three populations of V β 8+ T cells were identified in the CNS as defined by cytokine production, the majority (54% single positive and 13% double positive) of CNS-infiltrating T cells produced IFN- γ (Fig. 1 C), suggesting that the severe disease was associated with an expansion of V β 8+ IFN- γ +producing cells in the CNS. In an effort to maintain the Th17 phenotype upon restimulation with antigen, IL-23 or IL-1 β were added during the restimulation with antigen, yet the cells still produced both IFN- γ and IL-17 and transferred disease similar to the cells restimulated with antigen only (Table I).

Role of IL-1 β and IL-23 in the differentiation of Th17 cells

Because IL-6 + TGF- β were capable of differentiating T cells into IL-17-producing T cells but not sufficient to generate encephalitogenic T cells during primary stimulation, we speculated that an alternative pathway may occur in vivo that generates encephalitogenic Th17 cells. Because cytokines such as IL-1 β and IL-23 had also been implicated in the differentiation of Th17 cells and EAE (Langrish et al., 2005; Sutton et al., 2006), we investigated whether combinations of IL-6, TGF- β , IL-1 β , and IL-23 might be required for the generation of encephalitogenic myelin-specific Th17 cells. Naive wild-type splenocytes, depleted of antigen presenting cells, were activated with plate-bound anti-CD3 + anti-CD28 with different combinations of these cytokines. As seen in Fig. 2 A, many combinations of these cytokines generated T cells capable of producing IL-17. However, in the absence of TGF- β , IFN- γ was also produced, suggesting that TGF- β may be critical for suppressing IFN- γ production, not necessarily inducing IL-17 production. Because differentiation with IL-1 β + IL-23 resulted in the greatest IL-17 production and IL-1 β + IL-23 + TGF- β had high IL-17 and low IFN- γ , we investigated whether these cytokine combinations may generate Th17 cells capable of inducing EAE. Splenocytes from naive TCR transgenic mice were differentiated in vitro with IL-6 + TGF- β , IL-1 β + IL-23, IL-1 β + IL-23 + TGF- β , or IL-12 and transferred into B10.PL mice. The mice that received the T cells differentiated with IL-12 or IL-1 β + IL-23 developed severe EAE (Fig. 2 B). As previously observed (Fig. 1 B), T cells differentiated with IL-6 + TGF- β did not transfer disease. However, the mice that received the T cells differentiated with IL-1 β + IL-23 + TGF- β did develop EAE (Fig. 2 B), albeit significantly milder cases than IL-12- or IL-1 β + IL-23-differentiated T cells. Analysis of the cytokines by flow cytometry at the time of transfer showed that T cells differentiated with IL-1 β + IL-23 + TGF- β were both Th1 (9.24%)

and Th17 (6.8%) cells, making it unclear whether the disease was mediated by IFN- γ – or IL-17–producing T cells (Fig. 2 C). We have shown previously that the IL-1R signaling pathway is critical for the production of IL-12 and the differentiation of encephalitogenic T cells (Deng et al., 2003), and thus it appears that IL-1 β is up-regulating IL-12 in antigen/APC-driven differentiation, which subsequently favors Th1 cell differentiation in the presence of IL-23. This experiment also illustrates that differentiation with anti-CD3 + anti-CD28 (Fig. 2 A) generates different T cell phenotypes than antigen/APC-driven T cell differentiation (Fig. 2 C). Although this may be in part caused by cytokines produced by the APCs, it may also be caused by differences in signaling cascades mediated by natural ligands that are not accurately recapitulated by antibody-mediated signaling (unpublished data).

It had also been suggested that in vivo–differentiated Th17 cells restimulated with IL-6 + TGF- β induce IL-10 and, therefore, these cells may be regulatory and not pathogenic (McGeachy et al., 2007). Therefore, IL-10 was analyzed by flow cytometry in these four differentiation conditions and did not correlate with encephalitogenicity (Fig. 2 C). In fact, differentiation with IL-6 + TGF- β generated the fewest IL-10-producing T cells. Analysis of disease severity in all four groups indicated that severity correlated with the number of IFN- γ -expressing CD4 $^+$ T cells and expression of T-bet, the transcription factor that regulates IFN- γ expression (Table S1). To verify that IL-6 + TGF- β was not promoting the differentiation of regulatory T cells, TCR transgenic cells differentiated with IL-6 + TGF- β were cotransferred with TCR transgenic

cells differentiated with IL-12. As expected, the cells differentiated with IL-6 + TGF- β did not induce EAE; however, they did not reduce the severity of EAE in Th1-mediated disease when cotransferred into mice, indicating that they do not have a regulatory phenotype (Fig. S1).

Multiple cytokine combinations promote the differentiation of Th17 cells but fail to generate encephalitogenic Th17 cells

To determine to what extent IL-1 β , IL-6, IL-23, and TGF- β contribute to the differentiation of Th17 cells and determine if any combination of these cytokines can generate encephalitogenic Th17 cells, Th17 cells were generated using all combinations of these cytokines, or neutralizing these cytokines with antibodies. Because all combinations in the absence of TGF- β could produce IFN- γ and TGF- β had been implicated as necessary for IL-17 production (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006), all combinations included TGF- β to increase the probability of generating pure Th17 cells. As seen in Table II and Fig. S2, the combination of all four cytokines generated the greatest number of IL-17–producing T cells and relatively few IFN- γ T cells, but these cells, as well as all of the other combinations, failed to induce EAE when transferred into recipient mice. It is interesting to note that although IL-1 β + IL-23 + TGF- β generated mildly encephalitogenic T cells in Fig. 2, differentiation with IL-1 β + IL-23 + TGF- β + anti-IL-6 failed to generate encephalitogenic T cells, suggesting that endogenous IL-6 was contributing to the development of encephalitogenic T cells. Analysis of T-bet by flow cytometry indicated that T-bet was decreased

Table I. Th17 cells differentiated with IL-6 + TGF- β are not encephalitogenic after primary stimulation

| T cell differentiation | Cytokines ^a | | Incidence of EAE | Mean day of onset | Mean clinical score on day of onset |
|--|------------------------|-------|------------------|-------------------|-------------------------------------|
| | IFN- γ | IL-17 | | | |
| Primary stimulation | | | | | |
| Th17 (IL-6 + TGF- β) | – | ++ | 0/28 (0%) | NA | NA |
| Th17 (IL-6 + TGF- β + IL-23) | + | ++ | 1/12 (8.3%) | NA | NA |
| Th17 (IL-6 + TGF- β) + PTX ^b | – | ++ | 0/7 (0%) | NA | NA |
| Th17 (IL-6 + TGF- β) + Th1 ^c | – | ++ | 0/4 (0%) | NA | NA |
| Th17 (IL-6 + α IL-12/IFN- γ /IL-4) | – | + | 17/17 (100%) | 7.6 | 2.2 |
| Th17 (IL-6 + α IL-12/IFN- γ /IL-4/TGF- β) | – | + | 4/5 (80%) | 6.3 | 1.5 |
| Secondary stimulation^d | | | | | |
| Th17 (IL-6 + TGF- β) | +++ | +++ | 10/13 (76.9%) | 6.2 | 2.8 ^e |
| Th17 (IL-6 + TGF- β + IL-23) | +++ | +++ | 6/7 (85.7%) | 8 | 2.3 |
| Th17 (IL-6 + TGF- β → IL-23) ^f | +++ | +++ | 3/5 (60%) | 11.3 | 1.3 |
| Th17 (IL-6 + TGF- β → IL-1 β) ^f | +++ | +++ | 4/5 (80%) | 11 | 2.3 |

NA, not applicable.

^aCytokine production was measured by ELISA at 72 h in culture during differentiation or following restimulation with MBP Ac1-11 only for secondary stimulation. +, <1 ng/ml; ++, 1–4 ng/ml; +++, >4 ng/ml.

^bMice were administered 200 ng pertussis toxin (PTX) on days 0 and 2 after transfer of Th17 (IL-6 + TGF- β) cells into mice.

^cMice were injected with 5×10^6 Th17 (IL-6 + TGF- β) cells plus 10^6 Th1 (IL-12) cells.

^dTh17 cells were rested for 4 d after differentiation and restimulated with MBP Ac1-11 only before transfer into naive B10.PL mice.

^eThe rapid onset and severity of the disease required that the mice be euthanized within 48 h of disease onset, and therefore, only the mean clinical score at the time of disease onset could be determined.

^fTh17 cells (IL-6 + TGF- β) were rested for 4 d after differentiation and restimulated with MBP Ac1-11 + IL-23 or IL-1 β before transfer into naive B10.PL mice.

>50% when IL-6 was neutralized (unpublished data). These data suggest that in vitro conditions that include TGF- β , which are capable of producing CD4 $^+$ T cells that express high levels of IL-17, are probably not the same conditions that generate encephalitogenic Th17 cells in vivo.

Myelin-specific T cells in spontaneous EAE are dependent on T-bet for encephalitogenicity

To enhance our understanding of the characteristics of pathogenic myelin-specific T cells, we phenotyped the myelin-specific T cells in mice with spontaneous EAE. Splenocytes from TCR transgenic mice that developed spontaneous EAE

were cultured with MBP Ac1-11, MBP Ac1-11 + TGF- β , or MBP Ac1-11 + IL-6 + TGF- β . Flow cytometric analysis demonstrated that T-bet levels were quite high (34%) in the splenocytes that received MBP Ac1-11 and inhibited in the splenocytes that had TGF- β ± IL-6 (Fig. 3 A). Interestingly, none of the stimulation conditions generated significant amounts of IFN- γ , although IL-17 was detected in the presence of IL-6 + TGF- β (Fig. 3 A). To determine if encephalitogenicity in myelin-specific T cells from mice with spontaneous EAE is dependent on T-bet, the splenocytes were transfected with siRNA-Tbet or siRNA-nonsense (NS) before activation with MBP Ac1-11 (and nontransfected,

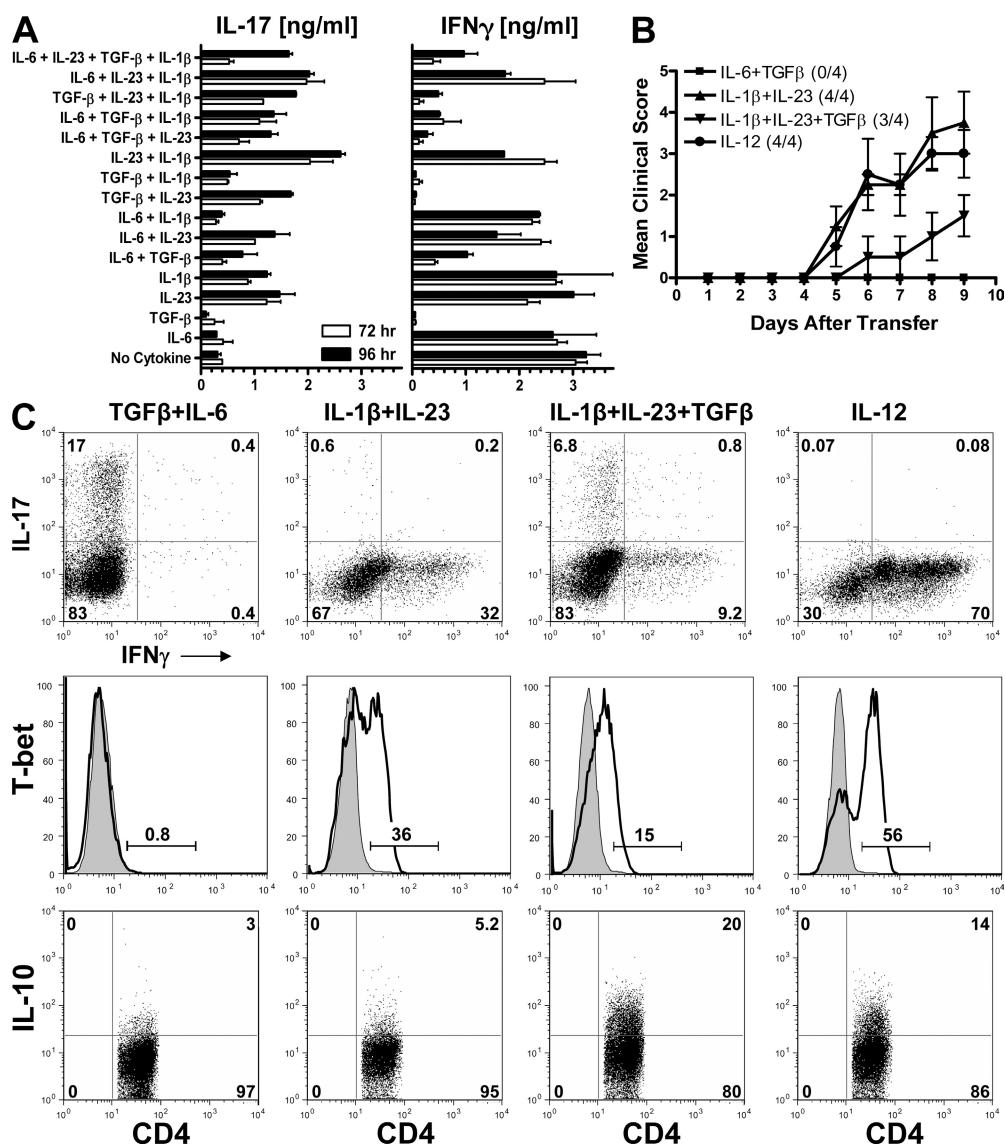


Figure 2. Encephalitogenicity correlates with IFN- γ production and T-bet expression. (A) Naive splenocytes from wild-type B10.PL mice were activated in vitro with anti-CD3/CD28 plus different combinations of cytokines for 72–96 h. ELISA was performed to detect IL-17 and IFN- γ secretion. Data are representative of three independent experiments (means \pm SEM). (B) Naive Va23/V β 8.2 transgenic splenocytes were differentiated in vitro with MBP Ac1-11 plus different combinations of cytokines for 3 d and transferred into naive B10.PL mice. Data are representative of two independent experiments (means \pm SEM). (C) Cells from B were harvested and flow cytometry was used to evaluate IFN- γ , IL-17, IL-10, and T-bet (white, T-bet; gray, isotype control) expression in CD4 $^+$ T cells before transfer. Data are representative of two independent experiments (percentages are shown).

irradiated wild-type B10.PL splenocytes) and transferred into naive B10.PL mice. The majority of mice receiving the siRNA-NS-transfected cells developed EAE, whereas the mice receiving the siRNA-Tbet-transfected cells failed to develop disease (Table III). These data suggest that encephalitogenicity of myelin-specific effector T cells that arise in spontaneous EAE is dependent on T-bet for pathogenicity but not necessarily IFN- γ , which is a downstream product of T-bet transactivation.

Role of T-bet in IL-17 expression by myelin-specific T cells

We had previously demonstrated that inhibition of T-bet with siRNA in the adoptive transfer model of EAE prevents the development of Th17 cells in the CNS (Gocke et al., 2007). However, we did not know if T-bet affected IL-17 production in myelin-specific effector T cells. We had previously found that draining lymph nodes from B6 mice immunized with MOG35–55/CFA express both Th1 and Th17 cells, as well as a small population of T cells that express both IFN- γ and IL-17 (unpublished data). To determine if T-bet plays a role in IL-17 production in T cells generated in vivo, B6 mice were immunized with MOG35–55/CFA and administered siRNA-Tbet or siRNA-NS via the tail vein to determine if T-bet affected the phenotype of the myelin-specific T cells that differentiated in vivo. The draining lymph nodes were removed 12 d later and stimulated with MOG35–55. Flow cytometric analysis demonstrated that the number of IFN- γ -expressing cells was reduced by 65% and that there were no detectable IL-17-producing T cells from the mice treated with siRNA-Tbet (Fig. 3 B). These data indicate that inhibition of T-bet suppresses the differentiation and/or expansion of both Th1 and Th17 cells.

Because myelin-specific TCR transgenic T cells differentiated with IL-6 + TGF- β do not transfer EAE (Fig. 1 B), but restimulation of these cells with antigen only generates highly encephalitogenic T cells composed of three populations, IFN- γ ⁺, IL-17⁺, and IFN- γ /IL-17 double-positive T cells (Fig. 1 C), we determined the expression of T-bet in these three T cell populations. Naive myelin-specific TCR transgenic T cells were differentiated with MBP Ac1–11 + IL-6 + TGF- β , rested, and restimulated with MBP Ac1–11 only, as

described in Table I, or anti-CD3/CD28. The cells were analyzed by flow cytometry to determine the level of T-bet in the CD4 $^+$ T cells. The cells that produced IFN- γ or IL-17 only had similar levels of T-bet, yet the cells that expressed both IFN- γ and IL-17 had significantly higher levels of T-bet (Fig. 3 C). Although the percentage of IL-17⁺ T cells was higher with anti-CD3/CD28 restimulation, IFN- γ expression was similar with antigen/APC or anti-CD3/CD28 restimulation, indicating that the APCs were not responsible for the shift in cytokine production. In addition, the Th17 cells differentiated with antigen/APCs were transfected with siRNA-Tbet or siRNA-NS after the rest and before restimulation with MBP Ac1–11 and transferred into naive B10.PL mice. As expected, the mice that received the siRNA-NS-transfected cells developed severe EAE, whereas the mice that received the siRNA-Tbet-transfected cells had a significantly milder disease course ($P = 0.002$), suggesting that suppressing T-bet was beneficial in ameliorating EAE mediated by Th1 and Th17 cells (Fig. 3 D). Flow cytometric analysis was used to verify efficient silencing of T-bet (Fig. 3 E) and analysis of cytokine production demonstrated that the amount of IL-17 was not altered by inhibiting T-bet, and IFN- γ was only modestly reduced (Fig. 3 F). These data indicate that although cytokine expression was not significantly altered in the effector T cells by reduced T-bet expression, suppression of T-bet was able to ameliorate T cell effector function.

Encephalitogenic Th17 cells express T-bet

Because IFN- γ -deficient mice develop EAE and develop encephalitogenic Th17 and not Th1 cells, we examined whether the CNS-infiltrating T cells coexpressed IL-17 and T-bet. EAE was induced in B6/IFN- γ ^{−/−} mice by immunization with MOG35–55/CFA and the mononuclear cells were isolated from the CNS during acute disease. The cells were incubated overnight with or without MOG35–55, and PMA/ionomycin was added to both cultures for the final 5 h. IL-17 was produced by the CD4 $^+$ T cells and expression was enhanced in the cells that received antigen (Fig. 4 A), indicating that the IL-17-producing T cells were specific for the immunizing antigen. Similarly, T-bet was expressed by the CNS-infiltrating CD4 $^+$ T cells, and T-bet expression was

Table II. Th17 cells generated in vitro with TGF- β fail to induce EAE

| Differentiation conditions | IFN- γ ^a | IL-17 ^a | IL-10 ^a | EAE incidence |
|--|----------------------------|--------------------|--------------------|---------------|
| TGF- β + IL-1 β + IL-23 + IL-6 | 2.1 | 24 | 4.8 | 0/5 |
| TGF- β + IL-1 β + IL-23 + α IL-6 | 4.8 | 1.5 | 1.3 | 0/5 |
| TGF- β + IL-1 β + α IL-23 + IL-6 | 2.5 | 17 | 3 | 0/5 |
| TGF- β + α IL-1 β + IL-23 + IL-6 | 1.5 | 12 | 2.3 | 0/6 |
| TGF- β + α IL-1 β + α IL-23 + IL-6 | 1.7 | 16 | 1.7 | 0/6 |
| TGF- β + α IL-1 β + IL-23 + α IL-6 | 3.7 | 0.9 | 1.2 | 0/5 |
| TGF- β + IL-1 β + α IL-23 + α IL-6 | 6.6 | 1.6 | 1.6 | 0/5 |

Bolded terms indicate antibodies to a specific cytokine.

^aThe percentage of cytokine-positive CD4 $^+$ T cells as determined by intracellular flow cytometric analysis. ELISA was used to measure cytokine production and correlated with the percentage of cytokine-positive CD4 $^+$ T cells.

higher in the MOG35–55–stimulated cells (Fig. 4 B). Analysis of T-bet in the CD4⁺IL-17⁺ T cells demonstrated that T-bet expression was almost threefold higher in the antigen-

stimulated cells compared with the cells stimulated without antigen (Fig. 4 C, right), illustrating that T-bet was expressed by the MOG35–55–specific Th17 cells in the CNS. Interestingly,

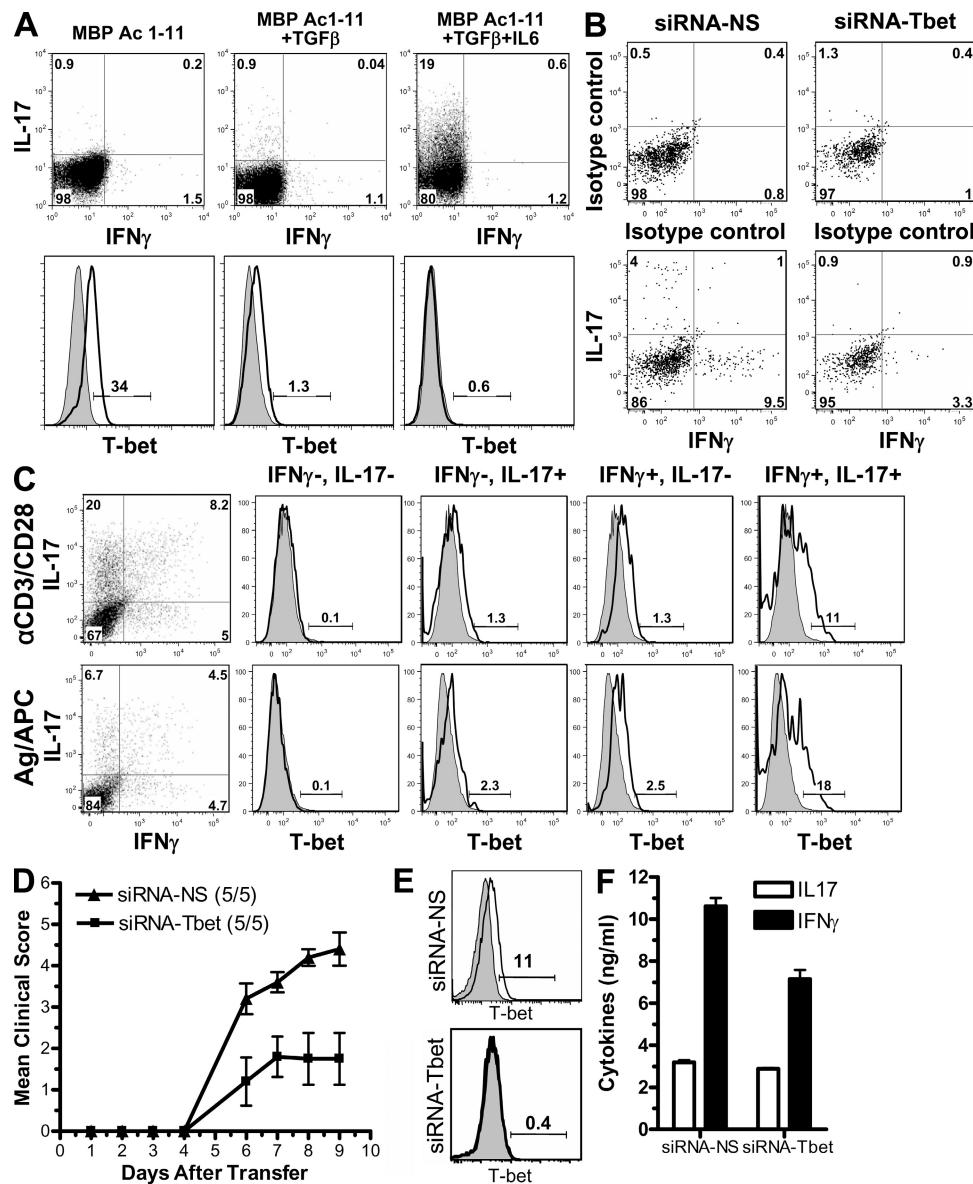


Figure 3. T-bet expression correlates with encephalitogenicity. (A) Splenocytes from a V α 2.3/V β 8.2 transgenic mouse with spontaneous EAE were activated in vitro with MBP Ac1-11, MBP Ac1-11 plus TGF- β , or MBP Ac1-11 plus TGF- β /IL-6 for 3 d. Flow cytometry was used to evaluate cytokine production and T-bet expression on CD4⁺ T cells. Data are representative of two independent experiments (percentages are shown). (B) B6 wild-type mice were injected with siRNA-Tbet or siRNA-NS at the time of immunization with MOG35–55/CFA. The lymph nodes were removed on day 11 after immunization and stimulated with MOG 35–55 for 3 d. Flow cytometry was used to evaluate cytokine production on activated CD4⁺ T cells and to confirm T-bet suppression that was >95% (not depicted). Data are representative of two independent experiments (percentages are shown). (C) Naive V α 2.3/V β 8.2 transgenic splenocytes were differentiated into Th17 cells in vitro with MBP Ac1-11 plus TGF- β /IL-6 for 3 d. Cells were rested and restimulated with MBP Ac1-11 in the presence of irradiated feeders or with 1 μ g/ml each of plate-bound anti-CD3/CD28. After 48 h, cells were analyzed by flow cytometry. Cells were gated on CD4 and T-bet expression was analyzed in each quadrant based on IL-17 and IFN- γ expression (gray, isotype control; white, T-bet). Data are representative of two independent experiments (percentages are shown). (D) Naive V α 2.3/V β 8.2 transgenic splenocytes were differentiated into Th17 cells in vitro with MBP Ac1-11 plus TGF- β /IL-6 for 3 d. Cells were rested for 4 d and transfected with siRNA-Tbet or siRNA-NS, followed by a 2-d restimulation with MBP Ac1-11 with non-transfected irradiated feeder cells. 5 \times 10⁶ cells per mouse were transferred into naive B10.PL mice. Data are representative of three independent experiments (means \pm SEM). (E) T-bet suppression was verified by flow cytometry. Data are representative of two independent experiments (percentages are shown). (F) ELISA was performed to detect IL-17 and IFN- γ secretion of the cells before transfer. Data are representative of two independent experiments (means \pm SEM).

there was no T-bet expression in the IL-17⁻ cells (Fig. 4 C, left), indicating that CD4⁺ T cells were not differentiating toward an IFN- γ ⁻ Th1 phenotype. To determine if T-bet was also expressed in IL-17⁺ T cells in the CNS in adoptive transfer EAE, TCR transgenic cells were differentiated into Th17 cells with IL-6 + TGF- β , rested, restimulated with antigen and transferred into naive mice. Similar to previous experiments (Fig. 1 C), IL-17⁺, IFN- γ ⁺, and double-positive T cells were found in the CNS cells. T-bet was observed in the CD4⁺IL-17⁺ and CD4⁺IFN- γ ⁺ T cells, indicating that T-bet was expressed in the IL-17⁺- and IFN- γ ⁺-infiltrating T cells (Fig. 4 D). The number of CD4⁺IL-17⁺IFN- γ ⁺ T cells was too low to accurately determine T-bet expression in this population.

Because T-bet-deficient mice do not develop EAE, yet IFN- γ ^{-/-} mice are EAE susceptible, we compared the Th17 cells in these two strains of mice. B6, B6/IFN- γ ^{-/-}, and B6/T-bet^{-/-} mice were immunized with MOG35-55 and the draining lymph nodes were removed on day 12. Because CNS-infiltrating cells could not be analyzed because T-bet^{-/-} mice do not develop EAE, flow cytometric analysis was performed on the lymph node cells. Both IFN- γ ^{-/-} and T-bet^{-/-} mice had significantly more activated MOG35-55-specific Th17 cells than the B6 control mice (Fig. 5 A). T-bet expression was higher in the CD4⁺ T cells from IFN- γ ^{-/-} mice compared with B6 mice and absent in T-bet^{-/-} mice (Fig. 5 B), which correlates with the observation that IFN- γ -deficient mice develop more severe EAE than wild-type mice. In addition, T-bet was expressed in the activated CD4⁺ Th17 cells (Fig. 5 C), illustrating that T-bet is expressed by Th17 cells differentiated in vivo that cause EAE. The fact that the draining lymph node cells from T-bet^{-/-} mice (EAE-resistant mice) express similar levels of IL-17 in the absence of T-bet as IFN- γ ^{-/-} mice (EAE-susceptible mice) indicates that T-bet is a critical factor for determining the encephalitogenicity of Th17 cells. In addition, the cells were transferred into wild-type B6 mice, and EAE was induced by the B6 and B6/IFN- γ ^{-/-} MOG35-55 T cells but not the B6/T-bet^{-/-} cells (Fig. 5 D), confirming the encephalitogenicity of the T-bet⁺ IL-17⁺ T cells but not the T-bet⁻ IL-17⁺ T cells.

IL-6 promotes the differentiation of encephalitogenic Th17 cells

To determine the cytokines that promote the differentiation of T-bet⁺ IL-17⁺ T cells, we analyzed T-bet expression by flow cytometry in TCR transgenic splenocytes differentiated with antigen/APCs and IL-6, IL-1 β , and/or IL-23 in the absence of TGF- β . CD4⁺ T-bet⁺ T cells were gated, and IL-17

and IFN- γ expression was analyzed. Although IFN- γ was observed under all cytokine conditions and is also seen with antigen-only differentiation, IL-17 was only observed with IL-6 differentiation (Fig. 6 A). Therefore, we compared IL-17 production by ELISA when the TCR transgenic cells were differentiated with IL-6 versus IL-6 + TGF- β and found that the amount of IL-17 was similar under both conditions (Fig. 6 B). To generate pure populations of Th17 cells that express T-bet, TCR transgenic splenocytes were differentiated with IL-6 ± TGF- β in the presence of neutralizing antibodies to IFN- γ , IL-12, and IL-4. Differentiation with IL-6 in the absence of IFN- γ , IL-12, and IL-4 generated IL-17⁺ IFN- γ ⁻ T cells that expressed T-bet (Fig. 6 C). IL-17 production was confirmed by ELISA (Fig. 6 D). The Th17 cells generated with IL-6 induced disease similar to Th1 cells (Fig. 6 E), suggesting that IL-6, in the absence of exogenous TGF- β , promotes the differentiation of encephalitogenic Th17 cells.

Because the amount of IL-17 was reduced in the absence of exogenous TGF- β and bovine serum may contain TGF- β that may contribute to the differentiation of Th17 cells with IL-6, the experiment was repeated with a neutralizing antibody to TGF- β included during the T cell differentiation. Using threefold higher amounts of anti-TGF- β than recommended by the manufacturer, we found that T-bet levels in the total CD4⁺ population were higher in the T cells differentiated with IL-6 + anti-IL-12/IFN- γ /IL-4 with anti-TGF- β than T-bet levels in the T cells differentiated with IL-6 + anti-IL-12/IFN- γ /IL-4 only (Fig. 7 A, top). However, the amount of IL-17 expressed by CD4⁺ T cells was very low by intracellular cytokine staining and decreased by 50% by ELISA in the presence of anti-TGF- β compared with IL-6 + anti-IL-12/IFN- γ /IL-4 only (Fig. 7 A, middle; and Fig. 7 B), indicating that TGF- β was contributing to the amount of IL-17 produced. Transfer of TCR transgenic T cells differentiated with IL-6 + TGF- β , IL-6 + anti-IL-12/IFN- γ /IL-4, and IL-6 + anti-IL-12/IFN- γ /IL-4/TGF- β demonstrates that omitting or neutralizing TGF- β -generated encephalitogenic T cells (Fig. 7 C). Neutralization of all active TGF- β in the cultures was confirmed by ELISA (unpublished data). Analysis of T-bet expression in the IFN- γ ⁺ and IL-17⁺ T cells demonstrates that T-bet is present in encephalitogenic T cell populations (Fig. 7 A, bottom). However, the low numbers of IL-17⁺ T cells in IL-6 + anti-IL-12/IFN- γ /IL-4/TGF- β differentiation precluded analysis of this population. There was no IFN- γ production, as measured by ELISA, by T cells differentiated with IL-6 under any condition in Fig. 7 (C and D) (not depicted). The amount of IL-17 produced, as measured by ELISA, in the presence of IL-6 clearly correlated with the

Table III. Encephalitogenicity in spontaneous EAE is dependent on T-bet

| | Incidence of EAE | Mean day of onset ^a | Maximum clinical score | Peak mean clinical score ^a |
|------------|------------------|--------------------------------|------------------------|---------------------------------------|
| siRNA-NS | 5/8 (62.5%) | 10.8 ± 1.1 | 2 | 1.1 ± 1 |
| siRNA-Tbet | 0/8 (0%) | NA | NA | NA |

NA, not applicable.

^aMean day of onset and peak mean clinical score were determined for the five mice that developed EAE.

amount of TGF- β present (Fig. 7 B), and encephalitogenicity correlated with T-bet expression (Fig. 7, A and C). The observation that differentiation with IL-6 + anti-IL-12/IFN- γ /IL-4/TGF- β generates encephalitogenic CD4 $^{+}$ T cells that

express low IL-17, no IFN- γ , and high T-bet is quite similar to our observation in spontaneous EAE in TCR transgenic mice in which T-bet levels are high yet cytokine production is undetectable (Fig. 3 A and Table III), supporting a critical

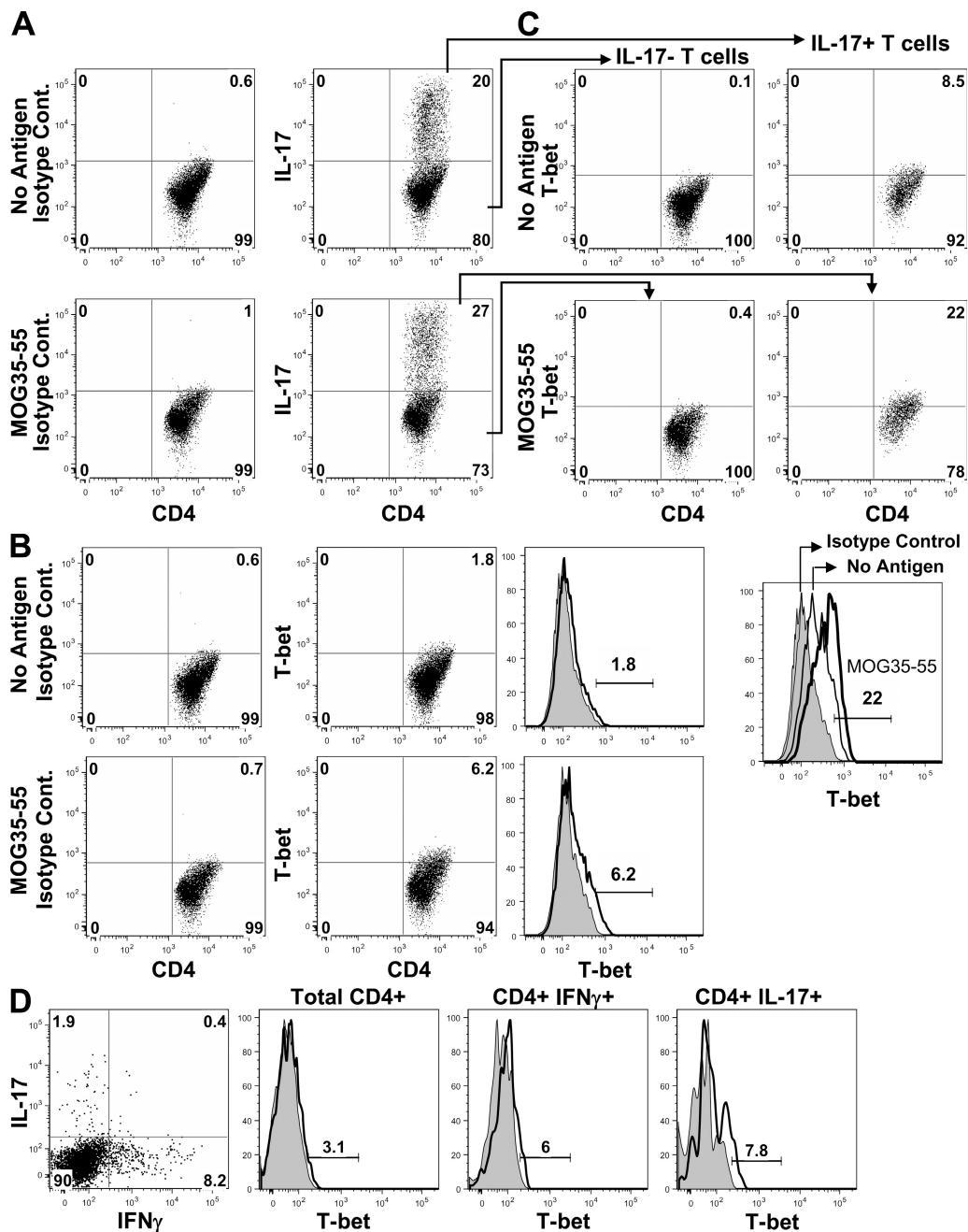


Figure 4. Myelin-specific T cells in the CNS of IFN- γ -deficient mice express IL-17 and T-bet. EAE was induced in B6/IFN- γ $^{-/-}$ mice by immunization with MOG35-55 emulsified in CFA. The CNS-infiltrating mononuclear cells were isolated from the brains and spinal cords of 14 mice 5 d after the mice began to develop signs of EAE. The cells were cultured overnight with or without 2 μ g/ml MOG35-55. PMA/ionomycin was added to all cells during the last 5 h of culture. Cells were gated on CD45 and CD4. (A) Intracellular IL-17 expression. (B) Intracellular T-bet expression (white, T-bet; gray, isotype control). (C) T-bet expression was analyzed in the CD4 $^{+}$ IL-17 $^{+}$ and CD4 $^{+}$ IL-17 $^{-}$ T cells in the absence or presence of MOG35-55 (white, T-bet; gray, isotype control). (D) Naive TCR transgenic splenocytes were differentiated with IL-6 + TGF- β , rested, restimulated with antigen, and transferred into naive B10.PL mice. Inflammatory cells were isolated in the CNS and gated on CD45 and CD4, and T-bet expression in the IL-17 $^{+}$ and IFN- γ $^{+}$ T cells was determined (white, T-bet; gray, isotype control). Data in A–D are representative of two independent experiments (percentages are shown).

role for T-bet in encephalitogenic T cells regardless of IFN- γ and IL-17 expression. Ex vivo analysis of these encephalitogenic IL-6-differentiated T cells found that the majority of CNS-infiltrating CD4 $^{+}$ T cells expressed neither IFN- γ or IL-17 (94%), but there was a small population expressing IFN- γ (5.2%), indicating that reexposure to antigen in vivo may influence cytokine expression (Fig. 7 D).

DISCUSSION

This study was initiated to determine the role of myelin-specific Th17 cells in EAE pathogenesis. Previous EAE studies had demonstrated that Th17 cells generated in vivo by immunization with myelin peptides in CFA could be expanded in vitro with IL-23 and induce EAE when transferred into naive recipi-

ents (Langrish et al., 2005; McGeachy et al., 2007; Stromnes et al., 2008). However, these studies never excluded the possibility that myelin-specific Th1 cells were also being transferred, and two studies actually show that IFN- γ is produced by the transferred populations (McGeachy et al., 2007; Stromnes et al., 2008). In addition, several studies had identified conditions that promoted the differentiation of Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006) yet had not determined if these differentiation conditions actually yielded encephalitogenic T cells. Using myelin-specific TCR transgenic mice that are housed in a specific pathogen-free environment, T cells were differentiated with TGF- β and combinations of IL-1 β , IL-6, and IL-23. Although several cytokine combinations generated myelin-specific T cells that expressed

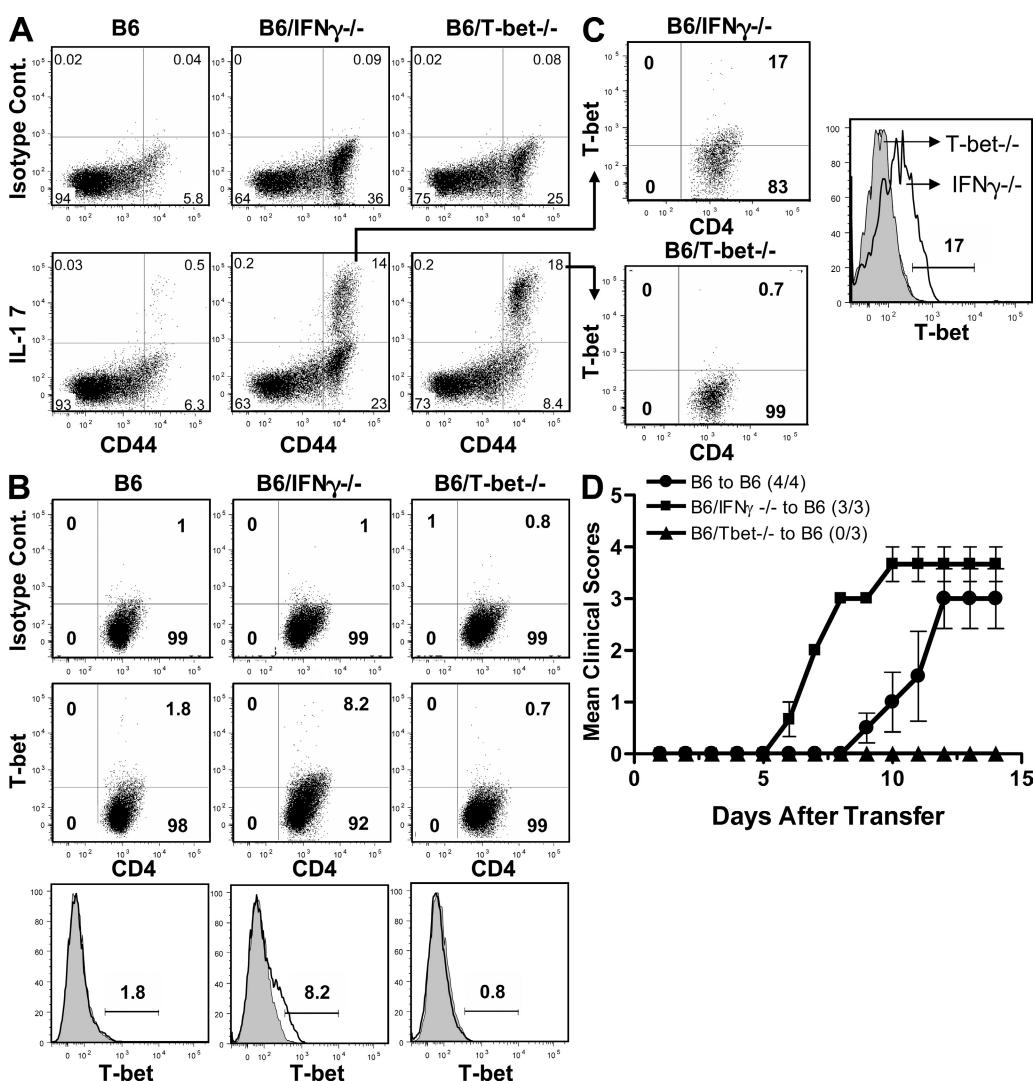


Figure 5. IFN- γ -/- and T-bet-/- mice generate myelin-specific Th17 cells, although EAE susceptibility differs. B6, B6/IFN- γ -/-, and B6/T-bet-/- mice were immunized with MOG35-55 emulsified in CFA. The draining lymph nodes of three mice per group were removed on day 12 and cultured with MOG35-55 for 3 d. (A) Cells were gated on CD4 and IL-17 expression was analyzed in the activated (CD44 hi) T cells (percentages are shown). (B) T-bet expression in the CD4 $^{+}$ T cells was analyzed (white, T-bet; gray, isotype control; percentages are shown). (C) The percentage of T-bet $^{+}$ cells was determined by gating on the CD4 $^{+}$ IL-17 $^{+}$ T cells. (D) Splenocytes from B6, B6/IFN- γ -/-, and B6/T-bet-/- mice immunized with MOG35-55 were stimulated in vitro with MOG35-55 and transferred into wild-type B6 mice and monitored for EAE development. Data are representative of two independent experiments (means \pm SEM).

high levels of IL-17 in the absence of IFN- γ , these Th17 cells failed to induce EAE. As others have shown (Langrish et al., 2005), we found that in vivo-differentiated Th17 cells that are expanded in vitro with IL-23 are highly encephalitogenic

(unpublished data), suggesting that myelin-specific Th17 cells contribute to disease pathogenesis. Thus, it appears that an alternative differentiation pathway generates encephalitogenic autoreactive Th17 cells in vivo.

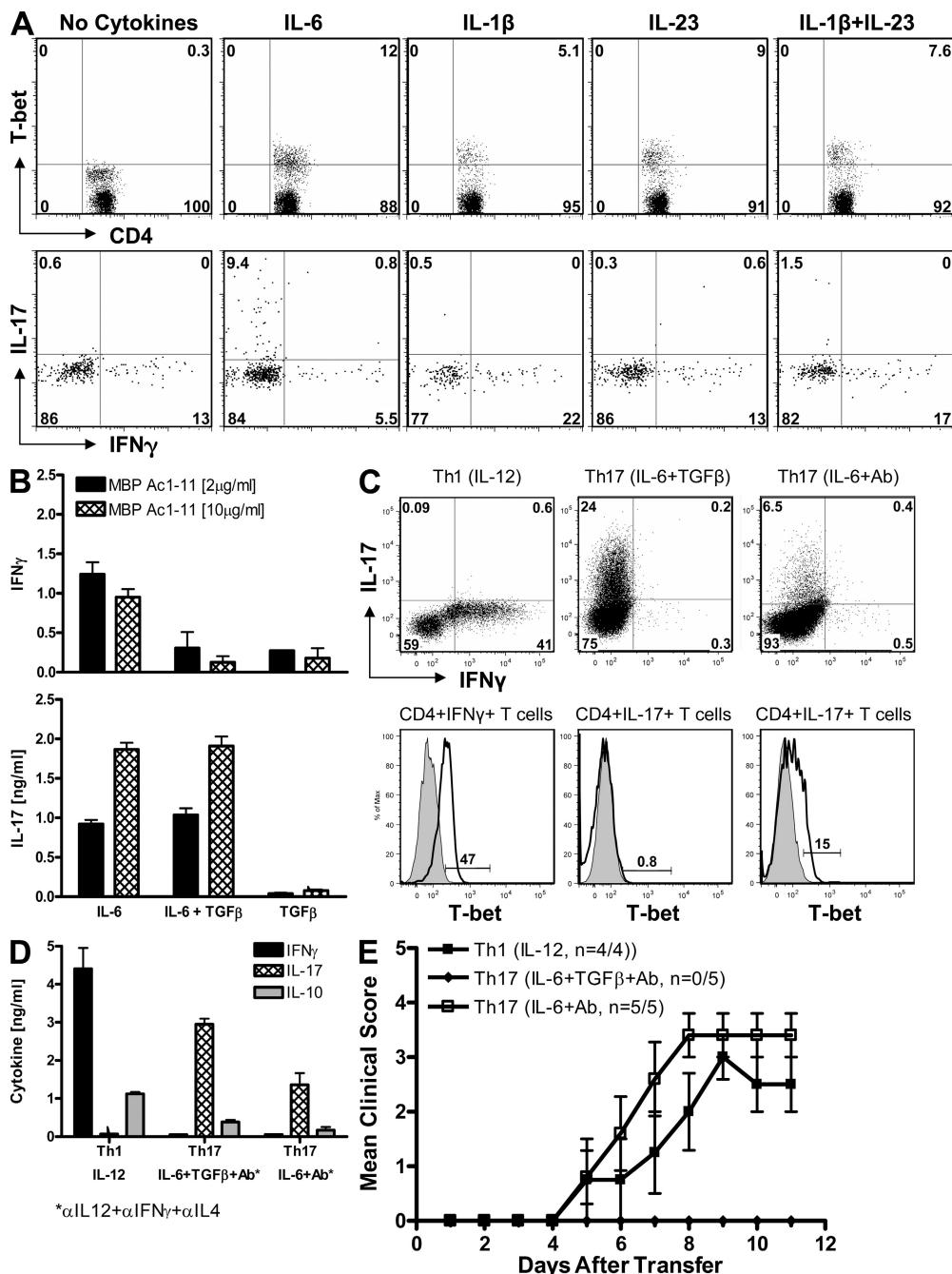


Figure 6. IL-6, in the absence of exogenous TGF- β , promotes the differentiation of encephalitogenic Th17 cells. (A) Naive TCR transgenic splenocytes were differentiated with MBPAc1-11 and IL-6, IL-1 β , IL-23, or IL-1 β + IL-23. Cells were gated on CD4 $+$ T cells and T-bet expression was analyzed. IFN- γ and IL-17 expression on CD4 $+$ T-bet $+$ cells was then analyzed (percentages are shown). (B) Naive TCR transgenic splenocytes were differentiated with IL-6, TGF- β , or both and cytokine production was determined by ELISA from the supernatants (means \pm SEM). (C) Naive TCR transgenic splenocytes were differentiated with IL-12 or IL-6 (\pm TGF- β) in the presence of neutralizing antibodies for IFN- γ , IL-12, and IL-4. Flow cytometry analyzed IL-17, IFN- γ , and T-bet expression in these cells (percentages are shown). (D) ELISA was used to measure cytokine expression from supernatants of cells in C (means \pm SEM). (E) The cells in D were transferred into naive B10.PL mice (5×10^6 cells per mouse) and monitored for development of EAE. Data are representative of three independent experiments (means \pm SEM).

Because myelin-specific Th17 cells differentiated in vitro with IL-6 + TGF- β were not pathogenic after primary differentiation, we speculated that they may be inefficient effector T cells until reexposure to antigen. However, restimulation

of Th17 cells generated in vitro resulted in decreased IL-17 production and increased IFN- γ production, suggesting that Th17 cells may not have a stable phenotype. In addition, these cells transferred disease, but it was not clear whether

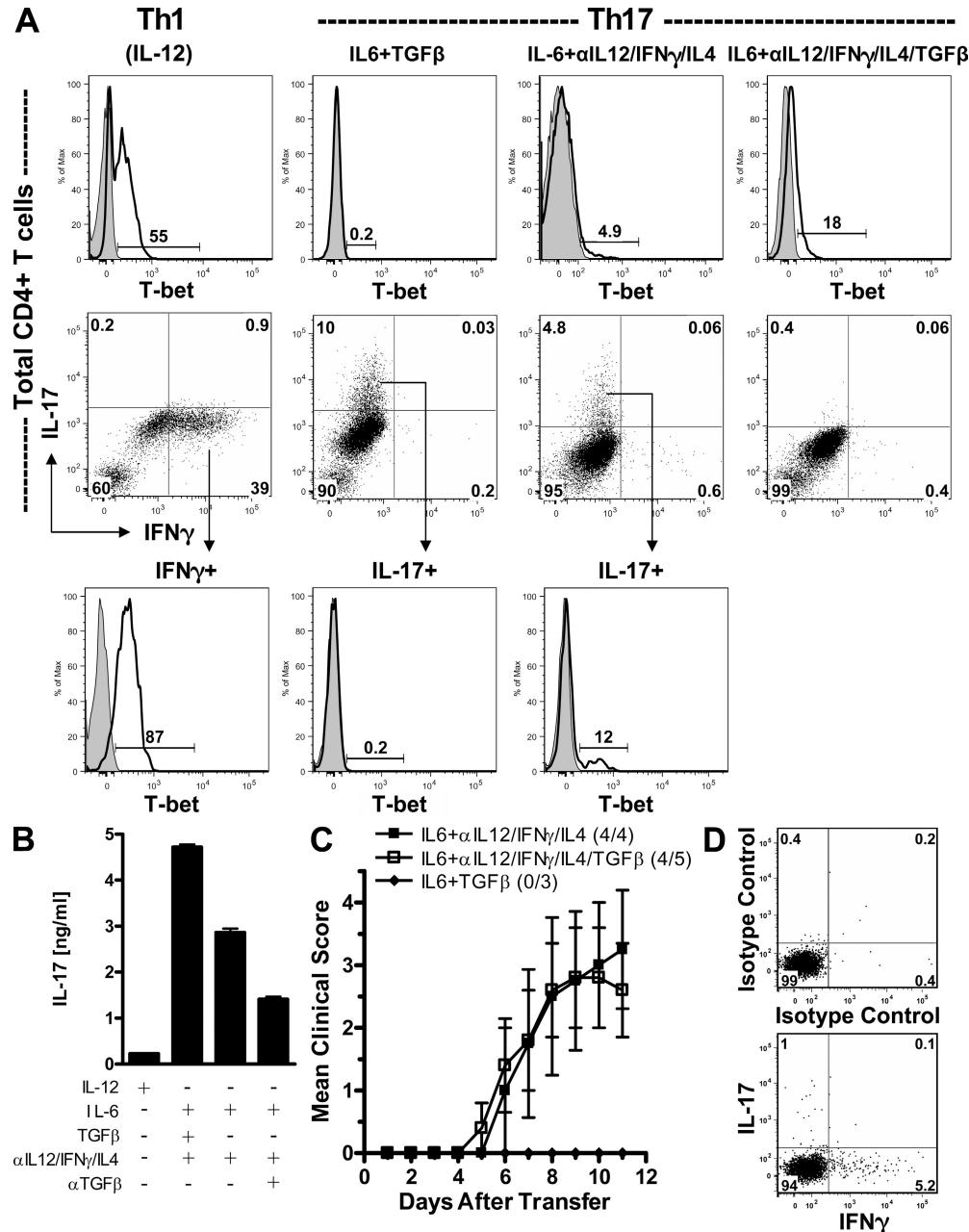


Figure 7. TGF- β contributes to IL-17 expression but not the encephalitogenic capacity of T cells differentiated with IL-6. Naive TCR transgenic splenocytes were differentiated with IL-12 (Th1), IL-6 + TGF- β + anti-IL-12/IFN- γ /IL-4, IL-6 + anti-IL-12/IFN- γ /IL-4, or IL-6 + anti-IL-12/IFN- γ /IL-4/TGF- β . (A) T-bet (white histogram; gray, isotype control) expression in total CD4 $^{+}$ T cells was determined by flow cytometry (top). IL-17 and IFN- γ expression in CD4 $^{+}$ T cells was determined (middle). IFN- γ and IL-17 were gated on and T-bet levels were determined in each population. Because the number of IL-17 $^{+}$ T cells was low with IL-6 + anti-IL-12/IFN- γ /IL-4/TGF- β differentiation, T-bet levels could not accurately be determined. (B) IL-17 levels from the supernatants from A were determined by ELISA. IFN- γ levels were undetectable in all IL-6 cultures (not depicted; means \pm SEM). (C) The T cell populations differentiated with IL-6 were transferred in to B10.PL (5×10^6 cells per mouse) and monitored for EAE development (means \pm SEM). (D) The CNS-infiltrating cells were isolated from the mice receiving the IL-6-differentiated (neutralizing other cytokines) T cells on day 12 after transfer. The cells were stimulated in vitro overnight with MBP Ac1-11, and intracellular IFN- γ and IL-17 were analyzed by flow cytometry. Data in A–D are representative of two independent experiments.

the disease was caused by IFN- γ –, IL-17–, or IFN- γ /IL-17–producing T cells because all were present in the transferred cells. Because identification of IFN- γ – and IL-17–producing cells requires intracellular cytokine staining, it is not possible to sort these cells and transfer each population into recipient mice to identify the pathogenic T cell population. However, we were able to isolate the T cells from the CNS of these mice after disease onset and found that the majority of the myelin-specific T cells in the CNS produced IFN- γ .

Because production of IL-17 was not sufficient for encephalitogenicity in our study but IFN- γ was consistently associated with disease, we analyzed other factors that may determine whether a myelin-specific CD4 $^{+}$ T cell population was encephalitogenic. One study suggested that IL-6 + TGF- β may not generate pathogenic Th17 cells, because myelin-specific T cells restimulated with these cytokines expressed IL-10 (McGeachy et al., 2007). In that study, myelin-specific T cells from immunized mice were restimulated *in vitro* with IL-6 + TGF- β ± IL-23 and analyzed for cytokine expression by flow cytometry, and it was found that the cells failed to transfer disease. However, cotransfer of these IL-10–producing cells with encephalitogenic T cells protected mice from EAE, suggesting that the cells were regulatory. In our study, we found no association between IL-10 production and encephalitogenicity in T cells undergoing primary differentiation with IL-6 + TGF- β . In fact, IL-10 expression was lowest in the T cells differentiated with IL-6 + TGF- β . Cotransfer of Th1 cells with Th17 cells differentiated with IL-6 + TGF- β did not protect mice from EAE (Fig. S1). In contrast, T-bet expression had the highest correlation with encephalitogenicity. Thus, we speculate that TGF- β , a known antagonist of T-bet expression (Gorelik et al., 2002; Park et al., 2005), may be responsible for minimizing the encephalitogenic capacity of myelin-specific Th17 cells generated *in vitro* with IL-6 + TGF- β .

T-bet is a transcription factor that was first associated with the differentiation of Th1 cells and IFN- γ production by CD4 $^{+}$ T cells (Szabo et al., 2000). We and others have found that T-bet is essential for the development of EAE (Bettelli et al., 2004; Lovett-Racke et al., 2004; Nath et al., 2006; Gocke et al., 2007) even though T-bet–deficient mice produce IL-17 (Harrington et al., 2005; Furuta et al., 2008; Phoon et al., 2008). In fact, several models of autoimmunity in which IL-17 has been implicated as a pathogenic cytokine are not inducible in T-bet–deficient mice (Neurath et al., 2002; Peng et al., 2002; Bettelli et al., 2004; Juedes et al., 2004; Nath et al., 2006; Wang et al., 2006). These observations suggest that the generation of autoreactive Th17 cells in the absence of T-bet is not sufficient to induce autoimmunity, even when the Th17 cells are differentiated *in vivo*. Myelin-specific T cells from T-bet–deficient mice fail to transfer disease to wild-type mice, confirming that T-bet is necessary for the generation of encephalitogenic T cells (Nath et al., 2006). Encephalitogenic T cells differentiated *in vitro* with IL-12 or IL-1 β + IL-23 expressed high levels of T-bet, whereas addition of TGF- β consistently reduced T-bet expression and encephalitogenic capacity. When T-bet levels were analyzed in mice with spon-

taneous EAE, T-bet expression was high, although these cells produced little, if any, IFN- γ or IL-17, suggesting that T-bet may be regulating other genes or pathways that are critical for encephalitogenicity, which may be unrelated to cytokine production. We have previously shown that suppression of T-bet inhibits IL-23R expression, and that T-bet binds the IL-23R promoter and plays a direct role in the transactivation of the IL-23R gene (Gocke et al., 2007). Historically, IL-23R was identified as a marker of memory Th1 cells (Oppmann et al., 2000; Parham et al., 2002), although the more recent literature suggests that the IL-23R pathway is critical for IL-17 production (Aggarwal et al., 2003; Langrish et al., 2005; Stromnes et al., 2008). These data were ascertained by studying mRNA or how T cells responded to IL-23. Unfortunately, there is no commercial IL-23R antibody available for flow cytometric analysis to definitively determine the cell populations that express this receptor and how the engagement of the IL-23R affects cytokine production and pathogenicity by Th1 or Th17 cells. However, it is clear that IL-23–deficient mice do not develop EAE, and thus, IL-23R engagement is critical in the pathogenesis of the disease (Cua et al., 2003). Therefore, it is possible that T-bet up-regulates IL-23R expression and that engagement of the IL-23R, whether on Th1 or Th17 cells, generates highly encephalitogenic CD4 $^{+}$ T cells. However, other genes such as osteopontin have also been shown to be regulated by T-bet, and osteopontin–deficient mice have a reduced susceptibility to EAE (Chabas et al., 2001; Jansson et al., 2002; Shinohara et al., 2005), necessitating a comprehensive analysis of genes transactivated by T-bet and their contribution to T cell encephalitogenicity.

The critical role of T-bet for encephalitogenicity was observed when siRNA was used to silence T-bet in highly encephalitogenic T cells that express both IFN- γ and IL-17, resulting in the reduced pathogenic potential of these myelin-specific T cells. In addition, administration of T-bet siRNA to mice immunized with myelin peptides in CFA reduces both IFN- γ and IL-17 production, suggesting that T-bet's role in T cell differentiation is not limited to Th1 cells. We confirmed this observation by analyzing myelin-specific T cells from the CNS of IFN- $\gamma^{-/-}$ mice that express both IL-17 and T-bet. In IL-23–driven IL-17–producing T cells from myelin-immunized mice, T-bet was also observed in these pathogenic IL-17–producing T cells (Chen et al., 2006). Analysis of the draining lymph nodes after active induction of EAE demonstrated that the Th17 cells in IFN- $\gamma^{-/-}$ mice express higher levels of T-bet than wild-type controls, which suggest that encephalitogenicity is associated with enhanced T-bet expression. The fact that the draining lymph node cells from T-bet $^{-/-}$ mice express similar levels of Th17 cells in the absence of T-bet as IFN- $\gamma^{-/-}$ mice indicates that T-bet is a critical factor for determining encephalitogenicity, not IL-17 expression. It is also interesting to note that systemic administration of T-bet siRNA in wild-type mice suppresses development of Th17 cells, whereas immunization of mice with a genetic deletion of T-bet results in a significant population of nonencephalitogenic Th17 cells. This suggests that mice incapable of generating

Th1 (*T-bet*^{-/-} and *IFN-γ*^{-/-} mice) promote Th17 cell differentiation but that wild-type mice in which *T-bet* is suppressed via siRNA do not favor Th17 cell differentiation, possibly because of Th1-related cytokines that are still present at low levels in the environment.

It was still unclear how *T-bet*⁺ IL-17⁺ encephalitogenic T cells could be generated if TGF-β was critical for development of Th17 cells. However, IL-6, in the absence of exogenous TGF-β and Th1-associated cytokines, promoted the differentiation of encephalitogenic Th17 cells that expressed *T-bet*. This confirmed that myelin-specific Th17 cells can induce EAE, but that *T-bet* must be expressed either by differentiation in a TGF-β-low environment or differentiation with IL-6 + TGF-β and reexposure to antigen in a TGF-β-low environment to allow for up-regulation of *T-bet*. The data in this study clearly define the necessity of *T-bet* in the development of autoimmune demyelinating disease, but they do not exclude the possibility that *T-bet*⁻ T cells contribute to pathogenesis. In addition, *T-bet* may be contributing to disease in multiple cell types, such as dendritic cells as observed in inflammatory arthritis (Wang et al., 2006), although this study focuses specifically on the role of *T-bet* in CD4⁺ T cells.

Although this study was initially designed to determine the role of myelin-specific Th1 and Th17 cells in EAE pathogenesis, we have found that pathogenesis is more closely associated with a transcription factor than cytokine production. We typically associate CD4⁺ T cell function with the cytokines that they produce. However, both *IFN-γ*- and IL-17-deficient mice develop EAE, although the disease course is altered (Ferber et al., 1996; Komiyama et al., 2006; Haak et al., 2009). In the case of autoimmunity, the function of autoreactive T cells may be mediated by a particular signaling pathway (i.e., *T-bet*) that programs the cells to be pathogenic, and not the end product of a pathway (i.e., cytokine production).

MATERIALS AND METHODS

Induction of EAE. B10.PL mice transgenic for the MBP Ac1-11-specific TCR chains Vα2.3 or Vβ8.2 were a gift from J. Goverman (University of Washington, Seattle, WA; Goverman et al., 1993). B10.PL, B6, B6/*IFN-γ*^{-/-}, and B6/*T-bet*^{-/-} mice were purchased from the Jackson Laboratory and bred in a specific pathogen-free animal facility at Ohio State University (OSU) Medical Center. All animal protocols were approved by the OSU Institutional Animal Care and Use Committee. EAE was induced in 8–10-wk-old B10.PL or B6 mice by s.c. injection over four sites in the flank with 200 µg MBP Ac1-11 or MOG35–55, respectively (Invitrogen) in an emulsion with CFA (Difco). 200 ng pertussis toxin per mouse in PBS was injected i.p. at the time of immunization and 48 h later. Mice were scored on scale of 0 to 6: 0, no clinical disease; 1, limp/flaccid tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5 quadruplegia or premonitory state; and 6, death.

EAE was also induced by adoptive transfer. Spleens from naive 5–10-wk-old Vα2.3/Vβ8.2 TCR transgenic mice were removed and single-cell suspensions were prepared. The naive status of the TCR transgenic T cells was verified by analysis of CD62 and CD44 by flow cytometry. Splenocytes were cultured in 24-well plates at 2 × 10⁶ cells/well with irradiated B10.PL splenocytes (6 × 10⁶ cells/well). Cells were activated with 2 µg/ml of MBP Ac1-11 and different combination of cytokines or neutralizing antibodies for cytokines to differentiate Th1 or Th17 cells. Cytokine and antibody concentrations were as follows: 0.5 ng/ml IL-12, 10 ng/ml IL-1β, 25 ng/ml

IL-6, 10 ng/ml IL-23, 1 ng/ml TGF-β, 4.5 µg/ml anti-IL-1β, 2 µg/ml anti-IL-6, 5 µg/ml anti-IL-23, 2 µg/ml anti-*IFN-γ*, 1 µg/ml anti-IL-12, 2 µg/ml anti-IL-4, and 0.35 µg/ml anti-TGF-β. After 72 h, the cells were washed with PBS and 5 × 10⁶ cells were injected i.p. into naive B10.PL mice. The mice were evaluated daily for clinical signs of EAE, as described in the previous paragraph.

Administration of siRNA in vivo and transfection with siRNA in vitro. Synthetic siRNA was purchased from Thermo Fisher Scientific, and stocks were prepared in the RNase-free H₂O at 160 µM and diluted to 50 µg/100 µl PBS (2 mg/kg/mouse) for i.v. administration via the tail vein. For in vitro transfection of Th17 cells, splenocytes from naive Vα2.3/Vβ8.2 TCR transgenic mice were cultured with 2 µg/ml of MBP Ac1-11 and TGF-β + IL-6 for 3 d to differentiate Th17 cells. The cells were rested for 4 d and transfected with siRNA-Tbet or siRNA-NS, as described previously (Lovett-Racke et al., 2004). After overnight transfection, the cells were washed and stimulated with 2 µg/ml of peptide in the presence of wild-type, nontransfected, irradiated splenocytes at a ratio of 1:5. For in vitro transfection of splenocytes from TCR transgenic mice with spontaneous EAE, the cells were transfected with siRNA overnight as previously described (Bettelli et al., 2006), washed, and activated with 2 µg/ml of MBP Ac1-11 in the presence of wild-type, nontransfected, irradiated splenocytes at a ratio of 1:5.

Cytokine ELISA. Supernatants were collected from TCR transgenic splenocytes cultured at 2 × 10⁶ cells/well with irradiated wild-type splenocytes (6 × 10⁶ cells/well) in 24-well plates. Purified anti-mouse primary antibodies (BD) were diluted in 0.1 M NaHCO₃ (pH 8.2) at 2 µg/ml. Immunolon II plates (Dynatech Laboratories) were coated with 50 µl of primary antibodies per well and incubated overnight at 4°C. The plates were washed twice with PBS/0.05% Tween 20. The plates were blocked with 200 µl of 1% BSA in PBS per well for 2 h. The plates were washed twice with PBS/Tween 20, and 100 µl of supernatants were added in duplicate. The plates were incubated overnight at 4°C and washed four times with PBS/Tween 20. Biotinylated rat anti-mouse secondary antibodies (BD) were diluted in PBS/1% BSA, 100 µl of 1-µg/ml biotinylated antibodies was added to each well, and plates were incubated at room temperature for 1 h. The plates were washed six times with PBS/Tween 20, and 100 µl avidin-peroxidase was added at 2.5 µg/ml and incubated for 30 min. The plates were washed eight times with PBS/Tween 20, and 100 µl ABTS substrate containing 0.03% H₂O₂ was added to each well. The plate was monitored for 10–20 min for color development and read at A₄₀₅. A standard curve was generated from the IFN-γ, IL-10, and IL-17 standards, and the concentration of IFN-γ, IL-10, and IL-17 in the samples was calculated.

Intracellular staining and flow cytometry. Flow cytometric analysis was performed to evaluate cytokine production and *T-bet* expression in splenocytes and CNS-infiltrating cells. CNS-infiltrating cells were prepared as previously described (Gocke et al., 2007). Splenocytes and/or CNS mononuclear cells were cultured with or without antigen. For the last 4–5 h of the incubation, 50 ng/ml PMA and 750 ng/ml ionomycin were added to cells. 1 µl/ml GolgiPlug was added to each well to block cytokine secretion 4 h before staining. Cells were collected, washed, and incubated with Fc block (BD) on ice for 15–20 min. The cells were washed and resuspended in staining buffer (1% BSA in PBS). The cells were incubated with mAbs to the cell-surface markers for 30 min at 4°C. After washing twice with staining buffer, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD) for 20 min at 4°C. Cells were stained for intracellular cytokines and *T-bet* with mAb diluted in PermWash solution for 30 min at 4°C. 80,000–100,000 live cell events were acquired on a FACSCalibur or FACSCanto (BD) and analyzed using FlowJo software (Tree Star, Inc.). PerCP-conjugated anti-CD45, allophycocyanin (APC)-conjugated anti-Vα2, FITC-conjugated anti-Vβ8, PE-conjugated anti-IL-17, APC-conjugated anti-*IFN-γ*, PerCP-conjugated anti-CD4, and APC-conjugated anti-IL-10 were purchased from BD. FITC-conjugated anti-*T-bet* was purchased from Santa Cruz Biotechnology, Inc.

Statistical analysis. A statistically significant difference in EAE clinical scores was considered to be P < 0.05 as determined by Mann-Whitney U test.

The analysis was performed on the raw data that included all clinical scores for each mouse for each time point in each group. The Mann-Whitney *U* test is nonparametric and therefore accounts for the fact that EAE scores are ordinal and not interval scaled.

Online supplemental material. Table S1 illustrates cytokine, T-bet and EAE data related to Fig. 2. Co-transfer of myelin-specific Th1 and Th17 cells (differentiated with IL-6 + TGF- β) is shown in Fig. S1 illustrating the IL-6 + TGF- β differentiated Th17 cells do not appear to regulate encephalitogenic Th1 cells. Cytokine expression of in vitro differentiation of myelin-specific T cells in presence of TGF- β and various combinations of IL-1 β , IL-6 and IL-23, or antibodies to neutralize these cytokines, is illustrated in Fig. S2. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20082584/DC1>.

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SUPPLEMENTAL MATERIAL

Yang et al., <http://www.jem.org/cgi/content/full/jem.20082584/DC1>

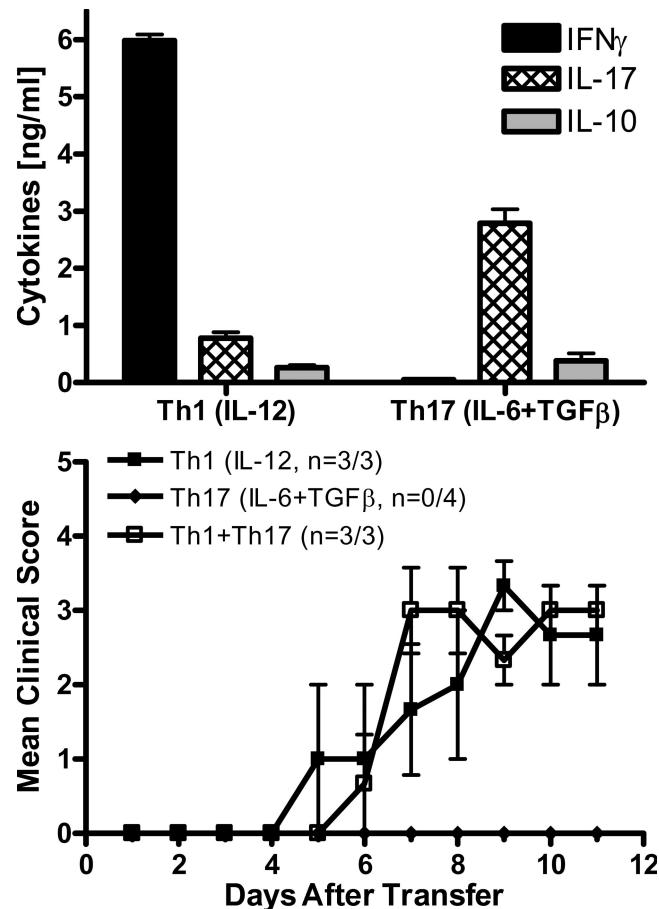


Figure S1. Th17 cells differentiated with IL-6 + TGF- β are not regulatory. TCR transgenic splenocytes were differentiated with IL-12 to generate Th1 cells or IL-6 + TGF- β to generate Th17 cells. Mice were injected i.p. with 5×10^6 Th1 cells, Th17 cells, or both cell types (means \pm SEM).

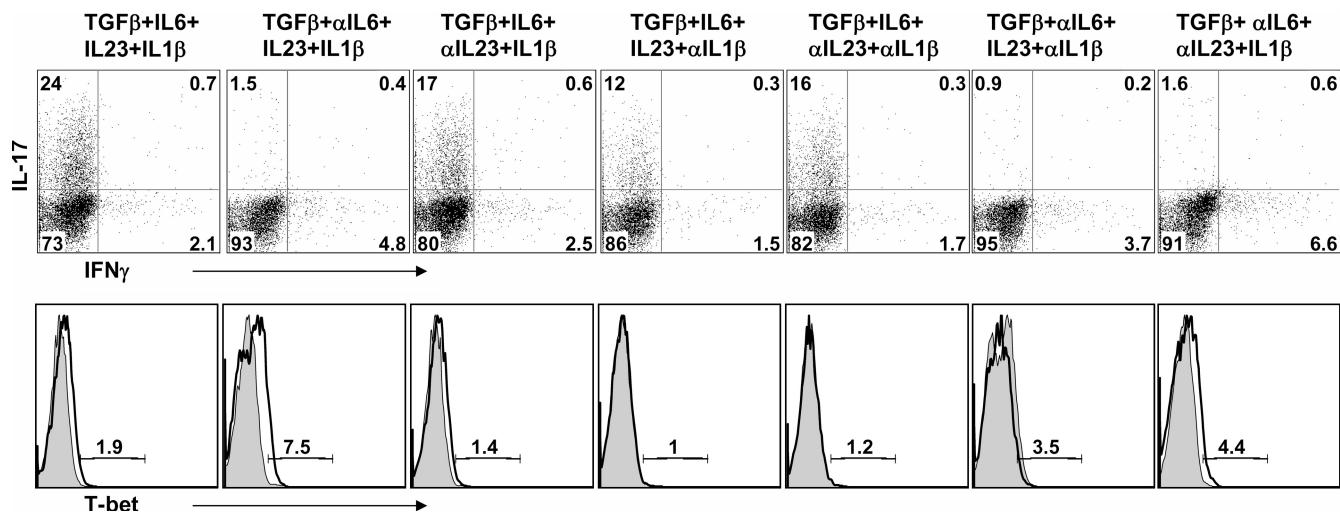


Figure S2. In vitro-generated Th17 cells with TGF- β are not encephalitogenic, regardless of cytokine conditions. Naive V α 2.3/V β 8.2 transgenic splenocytes were differentiated in vitro with MBPAc1-11 plus different combinations of cytokines for 3 d. Intracellular cytokine and T-bet expression (white, T-bet; gray, isotype control) was measured by flow cytometric analysis on CD4 $^{+}$ T cells. Data are representative of three independent experiments (percentages are shown).

Table S1. EAE severity correlates with IFN- γ and T-bet expression

| Differentiation conditions | IL-17 ^a | IFN- γ ^a | T-bet ^a | IL-10 ^a | EAE |
|-------------------------------------|--------------------|----------------------------|--------------------|--------------------|--------|
| IL-12 | 0.1 | 70 | 56 | 14 | Severe |
| IL-1 β + IL-23 | 0.6 | 32 | 36 | 5.2 | Severe |
| IL-1 β + IL-23 + TGF- β | 6.8 | 9.2 | 15 | 20 | Mild |
| IL-6 + TGF- β | 17 | 0.4 | 0.8 | 3 | None |

^aThe percentage of CD4 $^{+}$ T cells expressing this molecule determined by flow cytometric analysis.