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J Immunol 2007; 178:1341-1348; doi: 10.4049/jimmunol.178.3.1341
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T-bet Regulates the Fate of Th1 and Th17 Lymphocytes in Autoimmunity

Anne R. Gocke,* Petra D. Cravens,* Li-Hong Ben,* Rehana Z. Hussain,* Sara C. Northrop,* Michael K. Racke,2*† and Amy E. Lovett-Racke*

IL-17-producing T cells (Th17) have recently been implicated in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), an animal model for the human disease multiple sclerosis. However, little is known about the transcription factors that regulate these cells. Although it is clear that the transcription factor T-bet plays an essential role in the differentiation of IFN-γ-producing CD4⁺ Th1 lymphocytes, the potential role of T-bet in the differentiation of Th17 cells is not completely understood. In this study, therapeutic administration of a small interfering RNA specific for T-bet significantly improved the clinical course of established EAE. The improved clinical course was associated with suppression of newly differentiated T cells that express IL-17 in the CNS as well as suppression of myelin protein-specific Th1 autoreactive T cells. Moreover, T-bet was found to directly regulate transcription of the IL-23R and, in doing so, influenced the fate of Th17 cells, which depend on optimal IL-23 production for survival. We now show for the first time that suppression of T-bet ameliorates EAE by limiting the differentiation of autoreactive Th1 cells, as well as inhibiting pathogenic Th17 cells via regulation of IL-23R. The Journal of Immunology, 2007, 178: 1341–1348.

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Received for publication August 10, 2006. Accepted for publication October 31, 2006.

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1 These studies were supported, in part, by U.S. Public Health Service National Institutes of Health Grants NS37513 and NS44250 and National Multiple Sclerosis Society Grant RG2969-B-7 (to M.K.R.). A.E.L.-R. is a National Multiple Sclerosis Society Harry Weaver Neuroscience Scholar.

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3 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; siRNA, small interfering RNA; MBP, myelin basic protein; siRNA-NS, non-sense siRNA; siRNA-T-bet, T-bet-specific siRNA; ChIP, chromatin immunoprecipitation; EAM, experimental autoimmune myocarditis.

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The Journal of Immunology

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease mediated by myelin-specific CD4⁺ Th1 lymphocytes. The disease can be induced in mice by immunization with various myelin proteins or peptides emulsified in CFA or by the transfer of activated myelin-specific CD4⁺ Th1 lymphocytes into naive recipients. Both clinically and histopathologically, EAE is similar to multiple sclerosis (MS), yet the etiology of MS remains unknown. To develop therapies that target encephalitogenic T cells, we have been using small interfering RNA (siRNA) as a mechanism to suppress particular proteins essential to CD4⁺ Th1 cells. One protein that we have targeted is the transcription factor T-bet, which has been found to be a key regulator of the proinflammatory immune response (2). T-bet is a member of the T box family of transcription factors and binds to a core DNA sequence in the promoter regions of a diverse set of genes. T-bet has been found to be expressed in Th1 cells, but not Th2 cells, and ectopic expression of T-bet in Th2 lymphocytes results in IFN-γ production and suppression of Th2 cytokines (2–5). We found previously that silencing T-bet could prevent the development of EAE (5). Similarly, T-bet-deficient mice are resistant to the disease (6).

Recently, IL-23, which is produced by macrophages and dendritic cells, was also shown to be essential for the development of EAE (7). IL-23, a member of the IL-12 family, is a heterodimeric cytokine comprised of the p40 subunit of IL-12 and a specific p19 subunit. In addition, IL-23 has been shown to play an important role in the maintenance of a unique population of T cells known as Th17 cells (8, 9). This population of T cells, which appears to develop in the presence of TGF-β and IL-6, depends on IL-23 for growth and survival (10–12). Th17 cells are believed to be pathogenic in EAE, but the transcription factors necessary for their development are still undefined. Although it is clear that T-bet plays an essential role in the differentiation of CD4⁺ Th1 lymphocytes (2, 5, 13), the potential role of T-bet in the IL-23/IL-17 pathway is not well understood. Recently, it was shown that Th17 cells can develop in the absence of T-bet and STAT1 in vitro, but T-bet is important for continued IL-17 production in the presence of IL-23 (14). To investigate the additional mechanisms by which suppression of T-bet ameliorates EAE and to examine the ability of T-bet to regulate the IL-23/IL-17 pathway, T-bet-specific siRNA was administered to mice with established EAE induced by adoptive transfer of activated Th1 lymphocytes. EAE was ameliorated in these mice, and this therapeutic benefit was mediated in part by suppression of Th17 lymphocytes in the CNS and by limiting development and effector functions of autoreactive Th1 lymphocytes.

Materials and Methods

Induction of EAE and in vivo administration of siRNA

Spleens from 129/SvJba/s.Cg-Tcralpha10/Tcrbeta8.2 TCR-transgenic mice (5–10 wk old) (15) were removed and single-cell suspensions were prepared. Splenocytes were cultured in 24-well plates at 2 × 10⁶ cells/well (5). Cells were activated with myelin basic protein (MBP) Ac1-11 (2 μg/ml), rIL-12 (0.5 ng/ml), and irradiated B10.PL splenocytes (8 × 10⁶ cells/well). 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 previously described (5). Synthetic siRNA (5) was purchased from Thermo Fisher Scientific and stocks were prepared in the manufacturer’s buffer at 160 μM and diluted to 50 μg/100 μl of PBS (2 mg/kg/mouse) for i.v.
administration via the tail vein. A Mann-Whitney U test was used to determine the statistical significance of the treatment.

Transfection with siRNA, preparation of lysates, and Western blotting

Splenocytes were transfected in vitro, and nuclear extracts were prepared as previously described (5). Similarly, splenocytes were isolated from mice treated with siRNA and nuclear extracts were prepared. For preparation of total cell lysates, splenocytes were transfected with siRNA and activated with MBP Ac1-11 for 48 h or isolated from treated mice. Cells were collected, spun down, and resuspended in SDS-lysis buffer. Cells were lysed on ice for 30 min and spun down to remove cell debris. Total cell lysates were also made from the brains of mice using a tissue homogenizer and lysing with SDS-lysis buffer. Protease inhibitors (aprotinin, leupeptin, and pepstatin) were added to all lysates at the time of preparation.

The protein concentration of all lysates was determined by using the Bio-Rad protein assay. The lysates were diluted in 5× SDS loading buffer and boiled for 3 min. Lysates were electrophoretically separated on 4–20% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Western blotting and densitometry were performed as previously described (5) using the following Abs: T-bet, GATA3, IL-12, actin, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, goat anti-rat IgG-HRP, and rabbit anti-goat IgG-HRP (Santa Cruz Biotechnology) and IL-23R, IL-23p19, and IL-17 (R&D Systems).

ELISA

Cytokine expression was determined in the supernatants of splenocytes cultured at 4 × 10⁶ splenocytes/ml in 24-well plates activated with MBP Ac1-11 (2 μg/ml). IFN-γ and IL-4 ELISA were performed as previously described (16). IL-17 ELISA was performed using the mouse IL-17 DuoSet (R&D Systems).

Chromatin immunoprecipitation (ChIP) assays

The ChIP assay has been described in detail using a T-bet Ab from Santa Cruz Biotechnology (5). The primer set for the T-bet site for PCR amplification of the IL-23R was as follows: forward, GCCGGACTTTAAC CTACTAAAGCC and reverse, CACTTATGTCAGAGCTCTGACT. PCR conditions were 55°C for 1 min, 72°C for 1 min, and 94°C for 30 cycles using previously described PCR mixes (5).

Overexpression of T-bet in EL4 cells

Full-length human T-bet was inserted into the pCS2 vector. Transient transfection of EL4 cells was performed using the Amaxa Nucleofection System. Two million cells per transfection were reprogrammed in nucleofection solution L. Setting C-009 was used for nucleofection with the standard protocol. Cells were transfected with a pCS2-T-bet expression construct or an empty vector control. RT-PCR was performed at 24 h posttransfection to examine the effect of T-bet overexpression on mRNA expression levels for the endogenous target gene IL-23R.

Flow cytometry

The following mAb were used: PerCP-conjugated anti-CD45 mAb, allophycocyanin-conjugated anti-Var2 and FITC-conjugated anti-V98, PE-conjugated anti-IL-17, PE-conjugated anti-IFN-γ (BD Pharmingen), PE-Cy5-conjugated anti-CD4, and PE-Cy7-conjugated anti-CD3 (eBioscience). Brains, spinal cords, and spleens were harvested from mice. Tissues were processed through a 70-μm nylon mesh cell strainer. Splenocytes were treated with RBC lysing buffer (Sigma-Aldrich). CNS cells from all mice in each experimental group were pooled and processed using a modification of a previously described protocol (17). In brief, CNS cells were washed twice in 37% Percoll and CNS mononuclear cells were isolated by centrifugation at 2118 × g for 15 min at 22°C over a 30/70% Percoll gradient. The interphase cells were collected, washed with ice-cold 0.5% BSA/PBS, and resuspended in complete RPMI 1640. CNS mononuclear cells and splenocytes (2.5 × 10⁶ cells/ml) were cultured for 18 h with MBP Ac1-11 (2 μg/ml). For the last 4–5 h of the incubation, PMA (50 ng/ml) and ionomycin (750 ng/ml) were added to cells cultured in the absence of Ag. GolgiPlug (1 μM/ml) was added to each well. For flow cytometry, cells were collected, washed, and resuspended in staining buffer (4% FCS and 0.1% sodium azide in PBS). Fc receptors were blocked with anti-CD16/32, and the cells were incubated with mAbs for 30 min at 4°C. After washing twice with staining buffer, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4°C. Cells were stained for intracellular cytokines with mAb diluted in PermWash solution for 30 min at 4°C. Cells were washed, resuspended in staining buffer, and fixed in 1% paraformaldehyde. Fifty thousand to 100,000 events were acquired on a FACScalibur or LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Results

Amelioration of EAE by silencing T-bet

To determine whether suppression of T-bet following disease manifestation may be clinically effective in EAE, siRNA specific for T-bet (siRNA-T-bet) was administered i.v. to mice with clinically established disease. Naive Vv23/Nv8.2 TCR-transgenic splenocytes, which recognize MBP peptide Ac1-11, were differentiated in vitro with MBP Ac1-11 plus IL-12 and transferred into naive B10.PL mice. Lymphocytes differentiated in this manner have a Th1 phenotype, producing significant amounts of IFN-γ, but no detectable IL-4 or IL-17 (Fig. 1a). Two days after the onset of clinical signs of EAE, siRNA-T-bet or siRNA-NS (non-sense) was administered i.v. The disease appeared to stabilize in the siRNA-T-bet-treated group, but progressed in the siRNA-NS-treated group (Fig. 1b). A second dose of siRNA was administered 2 wk later, because the effect was not sustained in mice treated with a single dose (data not shown). By day 40, the siRNA-T-bet-treated mice showed a remarkable improvement in their clinical scores, whereas the control mice continued to deteriorate (p < 0.001), demonstrating that suppression of T-bet was beneficial in the treatment of EAE.

Clinical improvement correlates with reduced T-bet and IFN-γ

To verify that T-bet was suppressed in vivo, the spleen was removed from siRNA-treated mice at various time points, and T-bet expression was measured in nuclear protein extracts. After the first dose of siRNA-T-bet (day 18), T-bet expression in the splenocytes was significantly reduced compared with the siRNA-NS mice (Fig. 1c). In contrast, GATA3, a Th2-specific transcription factor, was up-regulated. T-bet remained suppressed in the siRNA-T-bet-treated mice 3 wk after the second siRNA treatment (day 44). On day 37, GATA3 levels were increased, but by day 44, there was no significant difference in GATA3 levels between the two groups of mice.

Because T-bet regulates IFN-γ expression in CD4⁺ T cells, Ag-induced IFN-γ expression was measured in the splenocytes. IFN-γ levels were reduced at day 18, and as late as day 60 in the siRNA-T-bet-treated group compared with the controls (Fig. 1d). Because GATA3, which is necessary for optimal IL-4 production, was increased in the siRNA-T-bet-treated mice, we evaluated whether there was an increase in IL-4 in response to MBP Ac1-11. However, there was no significant IL-4 expression in splenocytes from any of the mice, regardless of treatment or time point (data not shown), suggesting that a shift to a Th2 phenotype in the MBP Ac1-11-specific T cells was not responsible for the clinical improvement. The presence of IL-17 could not be detected in splenocytes from any of the experimental animals by Western blot or ELISA (data not shown).

Regulation of IL-23R by T-bet

Because we were unable to detect IL-17 in the experiments described above, we sought to examine whether T-bet could play a role in the IL-23/IL-17 pathway upstream of IL-17 induction. Therefore, the promoter region of the IL-23R, which is expressed on activated or memory T cells (18), was examined and found to contain a T box consensus sequence 3243 bases upstream of the ATG start codon, as well as four STAT binding sites (Fig. 2a), indicating that T-bet could potentially regulate IL-23R. Because it has previously been shown that mice deficient in IL-23 were resistant to EAE (7), IL-23R may be critical in EAE pathogenesis.
To determine whether T-bet binds directly to the IL-23R promoter, ChIP assays were performed in splenocytes that were transfected with siRNA-NS or siRNA-T-bet before activation with MBP Ac1-11. DNA that was specifically bound by T-bet was subsequently used as the template in PCR. Primer sets were designed around the T-bet site, as well as the STAT sites (negative controls), to verify that T-bet-immunoprecipitated DNA was specifically binding to the T-bet site and not to other transcription factor sites. The IL-23R promoter sequence containing the T-bet site was specifically amplified from the siRNA-NS-transfected cells, but not from the siRNA-T-bet-transfected cells, verifying the direct binding of T-bet to the IL-23R promoter (Fig. 2b).

It has been suggested that the detected binding of a transcription factor at a promoter site by ChIP assay does not necessarily result in an obligate functional role for that transcription factor in the regulation of the associated gene (19). Therefore, it is necessary to analyze both the ability of a transcription factor to bind to a promoter region by ChIP assay and to evaluate the ability of this binding to result in a functional consequence. This can be done by examining target gene expression in response to overexpression or absence of the transcription factor (19). To confirm that T-bet was regulating the functional expression of the IL-23R, T-bet was overexpressed in EL4 cells using the pCS2 expression vector, resulting in a significant increase in IL-23R mRNA (Fig. 2c). These data, along with the binding detected via ChIP assay, suggest that T-bet directly transactivates the IL-23R gene.

To determine whether silencing T-bet inhibits IL-23R protein expression, splenocytes from a mouse with actively induced EAE were transfected in vitro with siRNA-NS or siRNA-T-bet and activated with Ag. Immunization of mice with MBP Ac1-11/CFA generates both IFN-γ- and IL-17-producing autoreactive T cells in the periphery, and, thus, these splenocytes were used as a source of IL-23R-expressing Th17 cells (data not shown). Western blot analysis demonstrated a 45% reduction in IL-23R at day 18, and...
IL-23R was undetectable by day 44 in the siRNA-T-bet mice (Fig. 3b). Because IL-23R engagement can drive IL-17 expression (8, 9), we analyzed IL-17 production by ELISA following MBP Ac1-11 activation in vitro from splenocytes of siRNA-NS and siRNA-T-bet-treated mice at multiple time points and found no IL-17 production (data not shown). In addition, IL-17 expression was analyzed by Western blot analysis using the same whole-cell lysates that were used to evaluate IL-23R expression ex vivo, but no IL-17 was observed (data not shown). Because IFN-γ was expressed by the Ag-specific splenocytes (Fig. 1d), but IL-17 could not be detected, we wanted to determine whether this observation was due to the differential expression of IL-12 and IL-23 in the spleen. IL-12 p70 expression was the same in the siRNA-T-bet- and siRNA-NS-treated mice (Fig. 3b). However, there was no IL-23 p19 expression, suggesting that there was no detectable IL-23 present in the spleen to drive IL-17 expression in mice that develop EAE by transfer of Th1 lymphocytes.

FIGURE 2. T-bet regulates the transcription of IL-23R. a, Promoter region of the IL-23R gene is shown, illustrating the T-bet and STAT consensus sequence sites. Arrows, The regions covered by the primer sets. b, Splenocytes from a V/J88.2 TCR-transgenic mouse were transfected with siRNA-T-bet or siRNA-NS and a ChIP assay was performed with an Ab specific for T-bet. DNA bound to T-bet was purified and used as a template for PCR. The IL-23R gene was specifically amplified in cells transfected with siRNA-NS, but this binding was abolished in cells transfected with siRNA-T-bet (b, lanes 3 and 7). Lanes 1 and 5, Input DNA from each sample. An isotype control Ab was used in lanes 2 and 6, whereas lanes 4 and 8 represent nonactivated TCR splenocytes. c, T-bet was overexpressed in EL4 cells and mRNA expression was determined by PCR. All results shown are representative and were repeated a minimum of two times.

FIGURE 3. Silencing T-bet in vivo suppresses IL-23R and IL-17 expression. a, Splenocytes from a B10.PL mouse in which EAE was induced by immunization with MBP Ac1-11/CFA were used as a source of Ag-specific Th17 cells. We found that MBP Ac1-11-specific Th17 cells are prevalent in the periphery following immunization (data not shown). The splenocytes were transfected in vitro with siRNA-T-bet or siRNA-NS and activated. Western blot analysis was used to determine the level of T-bet and IL-23R expression. Splenocytes were isolated from EAE-affected B10.PL mice treated with siRNA-T-bet or siRNA-NS at 18 and 44 days posttransfer. Western blot analysis was performed for IL-23R, IL-12 p70, and IL-23 p19 expression. Recombinant IL-23 p19 was run as a positive control and is shown in the rectangle. IL-17 expression was also evaluated, but was not detectable. Densitometry was performed and relative IL-23R expression was determined by normalizing to actin. Each lane is representative of a different mouse. c, Brains were isolated from EAE-affected B10.PL mice treated with either siRNA-T-bet or siRNA-NS at day 18. Western blot analysis was used to determine IL-23R, IL-17, IL-12 p70, and IL-23 p19 expression. Densitometry was performed and relative IL-23R and IL-17 expression was determined by normalizing to actin. Each lane is representative of a different mouse. Insufficient protein was recovered from the spinal cords for evaluation. All results shown are representative and were repeated a minimum of two times.
In vivo suppression of T-bet results in decreased IL-23R and IL-17 in the CNS

Because it is possible that IL-17 is driving EAE pathogenesis in the CNS, IL-23R and IL-17 expression were analyzed in the brains of EAE-affected mice treated with siRNA-NS or siRNA-T-bet. IL-23R and IL-17 were observed in the brains of control mice, but expression was significantly reduced in the siRNA-T-bet-treated mice (Fig. 3c), suggesting that T-bet is regulating IL-23R expression and affecting the expression of IL-17 in the CNS. It had previously been shown that IL-23 is expressed in the CNS by activated microglia in mice with EAE (20). Analysis of IL-12 p70 and IL-23 p19 expression revealed similar levels between the groups of mice (Fig. 3c), indicating that the reduced IL-17 in the siRNA-T-bet-treated mice was not due to lack of IL-23 in the CNS. Unfortunately, we and others have been unable to analyze IL-23R expression on subpopulations of T cells because there is no Ab sufficient for flow cytometric analysis (24).

Characterization of IFN-γ- and IL-17-producing lymphocytes in the CNS

Because IL-17-producing lymphocytes in the CNS of mice with EAE have not been well characterized, we initially examined whether IL-17-producing lymphocytes were present during the acute phase of the disease. The lymphocytes from the spleen and CNS of four B10.PL mice with severe EAE induced by the transfer of MBP Ac1-11-specific Th1 cells were analyzed by four-color flow cytometry. The lymphocytes were activated in vitro with PMA/ionomycin to enhance cytokine expression of all activated cells or MBP Ac1-11 to specifically activate the transferred lymphocytes. Cells were stained for CD45 to differentiate the infiltrating hematopoietic cells from CNS-derived mononuclear cells. Cells were also stained for the Vα2 and Vβ8 TCR chains to identify the transferred Th1 cells and examined for expression of IFN-γ or IL-17. Splenocytes and CNS-derived lymphocytes were gated on CD45 and then on Vβ8. The expression of Vα2+ cells within the CD45+ Vβ8+ (Fig. 4, a–l) and CD45+ Vβ8− (Fig. 4, m–x) T cell populations that express IFN-γ or IL-17 is shown. IFN-γ-expressing T cells were found in all populations examined. In contrast, IL-17-producing T cells were only found in the CD45+ Vβ8+ Vα2+ lymphocytes from the CNS that were stimulated with PMA/ionomycin (Fig. 4c), suggesting that these cells were not specific for MBP Ac1-11. Because there was no IL-17 expression in response to MBP Ac1-11 (Fig. 4, i and n), no IL-17 was expressed by the adoptively transferred Vα2+ Vβ8+ lymphocytes found in the CNS (Fig. 4, o and u), and no IL-17-producing lymphocytes were found in the spleen (Fig. 4, f, l, r, and x), the IL-17-producing lymphocytes appear to be a newly differentiated lymphocyte population found only in the CNS.

To determine the effect of in vivo administration of siRNA-T-bet on the IFN-γ- and IL-17-producing lymphocytes, multiparameter flow cytometry was used. In addition to CD45, Vα2, and Vβ8 staining, CD3 and CD4 were also used to identify the IFN-γ- and IL-17-producing lymphocytes. CNS mononuclear cells were isolated 22 days after the transfer of Th1 lymphocytes and 13 days after receiving siRNA. The siRNA-NS mice (n = 3, EAE score, 2) had CD4+ lymphocytes that expressed IFN-γ (25%) and IL-17 (2.2%) (Fig. 5a). The percentage of CD4+ cells that expressed IFN-γ in the siRNA-T-bet mice (n = 3, EAE score, 1.3) was only modestly decreased (20.3%), but the number of IL-17-producing lymphocytes was at background levels (0.7%). In addition, IFN-γ expression by the MBP Ac1-11-specific Vα2+ Vβ8+ CD4+ T cells was decreased >70% in the siRNA-T-bet-treated mice at day 22 (Fig. 5b). The percentage of CD4+ T cells expressing IFN-γ in the CNS dramatically declined by day 43 (13 days after the second dose of siRNA), regardless of TCR usage (Fig. 5c). The number of IL-17-producing T cells was too few to evaluate at day 43 in these mice with mild EAE. We consistently found that the number of Th17 cells in the CNS of EAE-affected mice was highest at the acute phase of disease as shown in Fig. 4 and decreased over time as shown in Fig. 5. However, the number of Th17 cells in the CNS was usually undetectable in the siRNA-T-bet-treated mice at all time points.

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**FIGURE 4.** Th17 cells differentiate early in the target organ. CNS lymphocytes (left panels) and splenocytes (right panels) were isolated from B10.PL mice (n = 4, EAE score, 3.5) with severe EAE within 2 days of disease onset. All CNS cells from each experimental group were pooled. Cells were incubated with and without MBP Ac1-11 for 18 h. Cells cultured in the absence of MBP and without MBP Ac1-11 for 18 h. Ac1-11 were then stimulated for 4 h with PMA/ionomycin. Brefeldin A was added to all cells for the final 4 h of culture. Four-color flow cytometry was used to evaluate IFN-γ or IL-17 production. Cells were gated on CD45 and then on Vα2, or IL-17, whereas the lower two panels (m–x) show the number of Vα2+ cells within the CD45+ Vβ8+ population that express these cytokines. Results shown are representative of two independent experiments.
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Discussion

This study provides evidence that the IL-23R and the subsequent expansion of IL-17-producing T cells are regulated by T-bet and demonstrates the benefit of silencing T-bet for treatment of an autoimmune disease. We show that EAE-affected mice treated with siRNA specific for T-bet were able to recover significantly from disease. Clinical improvement correlated with reduced T-bet expression, reduced IFN-γ production, and a reciprocal increase in the Th2 transcription factor GATA3 in splenocytes. These data indicate that silencing T-bet, even after EAE is induced, can alter pathogenesis and reduce clinical signs of EAE.

Analysis of other genes that may be regulated by T-bet in encephalitogenic T cells identified IL-23R as a potential candidate and, subsequently, shed light on the significant clinical improvement observed in EAE. It has been shown that mice deficient in IL-23 are resistant to disease, indicating a critical role for IL-23 in the pathogenesis of the EAE (8). Moreover, T-bet-deficient mice have been shown to be resistant to EAE (6), and we recently demonstrated that silencing T-bet at the time of EAE induction reduced the incidence and severity of the disease, indicating that T-bet is critical for the differentiation of autoreactive T cells (5). Therefore, because T-bet can regulate the IL-23R gene and, in doing so, limit IL-23 engagement and affect the fate of pathogenic Th17 cells, loss of T-bet can result in decreased encephalitogenicity, incidence, and severity of disease.

Although we were unable to detect measurable levels of IL-17 expression in the spleen of mice with EAE induced by adoptive transfer, we were able to detect IL-17 in the CNS of EAE-affected mice. When T-bet was silenced, we observed a subsequent decrease in both IL-23R expression and IL-17 expression in the CNS. Recent studies have shown that pathogenic IL-23-driven IL-17-producing cells can invade the CNS and promote the development of chronic CNS inflammation associated with autoimmunity (21). Indeed, microarray analysis demonstrated IL-17 expression in the plaques of MS brains (22) and increased IL-17-expressing monocytic cells present in the cerebrospinal fluid of MS patients (21). Using bone marrow chimeric mice in which the p40 subunit common to IL-12 and IL-23 was absent in the CNS, it was shown that IL-23 produced by resident microglia and infiltrating macrophages in the CNS was critical to the clinical onset of EAE, but not for chronic CNS inflammation, suggesting that IL-23R engagement in the CNS is critical to the pathogenicity of infiltrating myelin-specific T cells (20). In addition, IFN-γ was found to enhance IL-23 p19 expression in primary microglia cultures, supporting the notion that the infiltrating Th1 cells in EAE may mediate IL-23 p19 production in the CNS (18).

An interesting possibility has been raised that IL-12-driven Th1 cells and IL-23-driven Th17 cells are related and arise from the same T-bet-expressing precursor (23). In this situation, T-bet would play a central role in regulating both IL-12-driven IFN-γ production and IL-23-driven IL-17 production, both of which have been shown to be important in EAE. However, two recent studies have concluded that Th17 cells are derived via a lineage distinct from Th1 cells (24–25). In our study, the IL-17-producing T cells in the CNS were found to be a distinct population from the autoreactive Th1 cells. Initial studies also suggested that Th17 cells are generated most effectively in vitro when IL-23 is present and IFN-γ and IL-4 are neutralized (24–25). However, we found Th17 cells differentiated in the CNS of EAE-affected mice in the presence of IFN-γ-producing Th1 cells. This is in accordance with a recent study in which both T-bet+/+ and T-bet−/− pathogenic CD4+ T cell lines produced elevated levels of IFN-γ or IL-4, respectively, but also secreted significant and comparable amounts of IL-17 (26). Interestingly, one model of autoimmunity, experimental autoimmune myocarditis (EAM), has been found to have increased disease severity in T-bet-deficient mice (26). Increased numbers of Th17 cells and decreased numbers of IFN-γ−/− CD8+ T cells in the hearts of T-bet-deficient mice led the authors to conclude that T-bet is a negative regulator of EAM. The differentiation and effector functions of T cells that are generated in T-bet-deficient mice may vary from the normal mechanisms that occur in wild-type mice that may partially explain why Th17 cells are expanded in T-bet-deficient mice, but not in normal mice in which T-bet is suppressed by RNA interference. The observation that most models of inflammatory autoimmune diseases, such as EAE, type 1 diabetes, Crohn’s disease, atherosclerosis, and arthritis are dependent on T-bet, suggests that the pathogenic immune mechanisms in EAM are unique (5, 6, 27–30). However, in both EAE and EAM, both Th1 and Th17 cells coexist in the target organ, suggesting that IFN-γ may not inhibit Th17 cells.

FIGURE 5. Silencing T-bet suppresses IFN-γ and IL-17 production by CNS lymphocytes. Cells were prepared as described in Fig. 4 for multiparameter flow cytometry to characterize the IFN-γ- and IL-17-producing T cells in the CNS following siRNA-T-bet treatment. Two additional markers were included in this analysis, CD3 and CD4. The gates shown were determined by the IgG isotype control staining in which the background was 1%. a, Cytokine expression was analyzed in the CD45+CD3+CD4+ cells at day 22 (13 days after siRNA treatment, n = 3 for each group). b, Cytokine expression in the MBP Ac1-11-specific CD45+CD3+CD4+Vα2+Vβ8+ lymphocytes was analyzed at day 22 (n = 4 for siRNA-NS and n = 3 for siRNA-T-bet). No IL-17 was observed in this population. c, Cytokine expression was analyzed in the CD45+CD3+CD4+ cells at day 43 following two siRNA treatments. Results shown are representative of two independent experiments.
in vivo (26, 31). It has also been speculated that IFN-γ-deficient mice develop severe EAE because of an enhanced myelin-specific Th17 cell population (17, 24). However, if IL-17-producing T cells are the primary encephalitogenic cells and their production is induced in T-bet-deficient mice, this contradicts the observation that T-bet-deficient mice, which express little IFN-γ, are actually resistant to EAE induction. Several recent articles indicate that TGF-β in the presence of IL-6 is critical for the differentiation of Th17 cells, and that IL-23 is important for the growth and function of these cells (10–12). Resident CNS cells have the capacity to produce IL-23, IL-6, and TGF-β (7, 32–33), which could contribute to the differentiation and expansion of this unique Th17 cell population that we see in the CNS. In addition, Bettelli et al. (11) demonstrate that the balance in the TGF-β-induced development of Th17 cells and CD4+CD25+ regulatory T cells is dependent upon the presence or absence of IL-6, respectively. Because the number of CD4+-infiltrating cells in the CNS of siRNA-T-bet-treated mice was often higher than that of controls (Fig. 5c), the therapeutic benefit may be partially mediated by enhanced development of regulatory T cells.

One concern about the suppression of T-bet and the up-regulation of GATA3 is the potential to predispose the mice to the development of Th2-mediated diseases, such as asthma, because T-bet-deficient mice develop both physiological and inflammatory features characteristic of asthma (34). In addition, in vivo suppression of GATA3 with an antisense oligonucleotide protects mice from the development of asthma (35). To address this concern, the lungs of mice treated with siRNA specific for T-bet were evaluated by immunohistochecmistry (data not shown) and there was no evidence of inflammation, suggesting that the level of T-bet suppression and the transient increase in GATA3 were not sufficient to predispose the mice to Th2-mediated disease.

A previous study demonstrated that IL-23-driven autoreactive Th17 cells were capable of inducing EAE by the adoptive transfer of low numbers of cells (17), but the phenotype of the T cells in the CNS was not determined in these mice. In the present study, EAE was induced by adoptive transfer of classical MBP-specific Th1 lymphocytes, and Th17 cells could not be found in the periphery of these mice. However, low numbers of IL-17-producing cells were observed in the CNS of these mice, suggesting that Th17 cells may be participating in disease pathogenesis. Thus, it appears that classical Th1 cells can at least participate in the initial inflammatory phase of EAE and that IL-17-producing T cells are generated rapidly in the CNS and may be critical to target organ damage. The recent observation that T-bet is required for optimal IL-17 production in the presence of IL-23 (14), taken together with the mechanistic data shown in this report, supports the conclusion that one mechanism by which T-bet modulates IL-23/IL-17-mediated inflammation is by directly regulating IL-23R. Moreover, the orphan nuclear receptor RORγt was shown to be required for the differentiation of Th17 cells, and RORγt-deficient mice had reduced EAE severity (31). Therefore, both RORγt and T-bet appear to be required for EAE and appear to play distinct, yet complementary, roles in the development of Th17 cells. This study demonstrates that T-bet is an important regulator of the proinflammatory immune response and explains why silencing T-bet can be both protective and therapeutic in autoimmune disease.

Disclosures

The authors have no financial conflict of interest.


