Immunity, Vol. 21, 719–731, November, 2004, Copyright ©2004 by Cell Press

Silencing T-bet Defines a Critical Role in the Differentiation of Autoreactive T Lymphocytes

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Summary

As a means of developing therapies that target the pathogenic T cells in multiple sclerosis (MS) without compromising the immune system or eliciting systemic side effects, we investigated the use of T-bet-specific antisense oligonucleotides and small interfering RNAs (siRNA) to silence T-bet expression in autoreactive encephalitogenic T cells and evaluated the biological consequences of this suppression in experimental autoimmune encephalomyelitis, a model for MS. The T-bet-specific AS oligonucleotide and siRNA suppressed T-bet expression, IFNγ production, and STAT1 levels during antigen-specific T cell differentiation. In vitro suppression of T-bet during differentiation of myelin-specific T cells and in vivo administration of a T-bet-specific antisense oligonucleotide or siRNA inhibited disease. T-bet was shown to bind the *IFN*γ and *STAT1* promoters, but did not regulate the IL-12/STAT4 pathway. Since T-bet regulates IFNγ production in CD4+ T cells, but to a lesser extent in most other IFNγ-producing cells, T-bet may be a target for therapeutics for Th1-mediated diseases.

Introduction

Cytokines are the dominant factors directing the differentiation of T cells into Th1 and Th2 cells. Upon T cell receptor engagement of MHC/peptide complexes, two receptors, the IFNγ and IL-12 receptors, play key roles in the differentiation of naive T cells into Th1 cells. The IFNγ receptor activates a signaling pathway involving STAT1, while the IL-12 receptor activates a signaling pathway using STAT4. The differentiation of Th2 cells is mediated by IL-4, which induces the expression of the transcription factor, GATA3. GATA3 appears to be a master regulator in Th2 cells that transactivates the Th2 cytokine genes (*IL-4, IL-5, and IL-13*). More recently, the transcription factor T-bet has been found to be a key regulator of the *IFN*γ gene in Th1 cells. T-bet is a member of the T-box family of transcription factors that contains a highly conserved DNA binding domain, called the T-box, that binds to a core DNA sequence in promoter regions of a diverse set of genes. T-bet has been found to be expressed in Th1 cells, not Th2 cells (Szabo et al., 2000; Shier et al., 2000; Afkarian et al., 2002). However, ectopic expression of T-bet in Th2 lymphocytes results in IFNγ production and suppression of Th2 cytokines (Szabo et al., 2000), leading to speculation that T-bet may play a critical role in the differentiation of Th1 cells. In addition, T-bet-deficient mice fail to generate Th1 cells and spontaneously develop physiological and inflammatory changes characteristic of asthma, a Th2-mediated disease orchestrated by Th2 lymphocytes (Finotto et al., 2002). T-bet was originally believed to mediate its effects via the IL-12/STAT4 pathway (Szabo et al., 2000; Rengarajan et al., 2000), but more recent studies have demonstrated that its role in the production of IFNγ appears to be mediated by the IFNγ/STAT1 pathway (Mullen et al., 2001; Afkarian et al., 2002; Siebler et al., 2003). Although it was originally speculated that T-bet may be the master regulator of cytokine expression in Th1 cells, similar to GATA3 in Th2 cells, a subsequent study has questioned the precise role of T-bet in Th1 cells (Afkarian et al., 2002). Thus, the exact role of T-bet in the differentiation of naive antigen-specific T cells into Th1 cells has not been clearly established. Since many autoimmune diseases are mediated by Th1 cells, we investigated the role of T-bet and the genes it regulates in the differentiation of myelin-specific T cells into Th1 cells capable of inducing EAE.

EAE is an inflammatory, demyelinating disease of the central nervous system and is a model for the human disease multiple sclerosis (MS). EAE is induced in rodents by immunization with myelin proteins or peptides in complete Freund's adjuvant, or by the transfer of activated CD4+ myelin-specific Th1 lymphocytes into naive recipient animals. Shifting the phenotype of myelin-reactive T cells from a Th1 to a Th2 phenotype is beneficial in the treatment of EAE (Racke et al., 1994, 1995). However, many of the reagents, such as retinoids, PPAR agonists, and cyclophosphamide, that have been used to induce immune deviation affect many different cell types and signaling pathways, resulting in undesirable side effects. We have been investigating whether we can develop therapeutic agents that specifically target a gene to alter the encephalitogenic potential of autoreactive T cells and minimize the effects on other cell types and other signaling pathways within T cells.

To determine if suppression of T-bet may be beneficial in altering the development of encephalitogenic T cells, we developed an antisense oligonucleotide (AS oligo) and small interfering RNA (siRNA) specific for T-bet. AS oligos are small single-stranded DNA sequences that are usually complementary to the translation initiation site of the gene of interest. Hybridization to their target mRNA inhibits translation of the transcript by preventing the ribosomal complexes from binding to the now double-stranded translation initiation site or by promoting the degradation of the mRNA by RNase H. siRNAs silence genes utilizing an evolutionarily conserved mechanism of degrading mRNA complementary to any double-stranded RNA in a cell. RNA interference is thought to play a critical role in cellular responses to RNA viruses and in stabilizing the genome by sequestering repetitive

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Table 1. Sequences of Antisense Oligonucleotides and siRNAs

Gene	AS Sequence, 5′→3′	siRNA
T-bet	CTCCACGATGCCCATC	5′-UGAUCGUCCUGCAGUCUCUdTdT-3′ 3′-dTdTACUAGCAGGACGUCAGAGA-5′
Non-sense (NS)	CTATGTCATCCGCTCCAC	5′-CGAACGAGUACCGUACACUdTdT-3′ 3′-dTdTGCUUGCUCAUGGCAUGUGA-5′
GATA3	AGTCACCTCCATGTCCTC	
STAT1		5′-AUUCCAUCGAGCUCACUCAdTdT-3′ 3′-dTdTTAAGGUAGCUCGAGUGAGU-5′

sequences (Hannon 2002). These gene-silencing techniques offer several advantages over gene knockout and monoclonal antibody technologies for studying in vitro and in vivo roles of specific genes. First, mice that have a specific gene deleted may have altered the normal requirements for that protein during development, and the observed phenotypes may not be the same in a normal animal that has a gene suppressed in vitro or in vivo. Second, the gene is deleted from all cell types, which may make it difficult to determine the relevance of that gene in particular cell types that may play a role in the disease or pathway of interest. Third, monoclonal antibodies do not inhibit the expression of the protein, but they do interfere with the function of the protein, and their use is typically limited to membrane bound and secreted proteins, not intracellular proteins such as transcription factors. In the present study, we demonstrate that an AS oligo and siRNA specific for T-bet can be utilized to target T-bet both in vitro and in vivo to define the role of T-bet in the differentiation of autoreactive T cells and as a potential therapeutic target for Th1-mediated diseases.

Results

Generation of T-bet-Specific Antisense Oligonucleotides and Small Interfering RNA

To determine if suppression of T-bet may be beneficial in altering the development of encephalitogenic T cells, we developed an AS oligo and siRNA specific for T-bet (Table 1). Two control AS oligos were also generated, one specific for GATA3, which would have minimal effect or may enhance encephalitogenicity of T cells, and one containing a nonsense sequence (Finotto et al., 2001). The AS oligos were generated on a phosphorothioate backbone to increase their stability and minimize degradation. The siRNA specific for T-bet contains a 19 base sequence within the open reading frame generated with AA overhangs at the 5′ ends. The nonsense siRNA was a random sequence that had no significant sequence similarity to other genes.

We investigated whether these T-bet-specific nucleic acids were capable of suppressing T-bet expression in MBP Ac1-11-specific T cells. Splenocytes were isolated from B10.PL mice that were transgenic for the Vβ8.2 T cell receptor chain that recognizes MBP Ac1-11 when paired with a specific Vα2.3 T cell receptor chain (Goverman et al., 1993). Naive Vβ8.2 transgenic mice have a precursory frequency of MBP Ac1-11-specific T cells of 1 in 10^3–10^4 splenoctyes, this measure is more physiologically relevant in terms of the number of T cells in a population specific for a particular antigen compared to using the MBP Ac1-11-specific Vα2.3/Vβ8.2 transgenic splenocytes in which the vast majority of T cells recognize the peptide. The splenocytes were transfected with the AS oligos or siRNAs that had been bound to a transfection agent to maximize transfection into the cells. Transfected splenocytes were activated with MBP Ac1-11, and nuclear extracts were prepared. Western blot analysis with anti-T-bet demonstrated that T-bet protein expression was induced upon activation with MBP Ac1-11 and that this induction was inhibited in the activated splenocytes that were transfected with the siRNA and AS oligo specific for T-bet (Figures 1A and 1C). Densitometry of the T-bet and β-actin blots was performed, and the relative protein expression of T-bet was determined by normalizing the density of the T-bet bands to the β-actin bands (Figures 1B and 1D). The level of T-bet expression in mock-transfected TCR Vβ8.2 varies slightly between mice in the absence of antigen, as seen in Figure 1. Interestingly, when T-bet expression is detectable in the mock-transfected cells without antigen activation, as seen in Figures 1C and 1D, AS-Tbet can suppress the level of endogenous T-bet in the cells even in the absence of antigen.

To evaluate the efficiency of the transfection of the AS oligos and siRNAs into the primary splenocyte cultures, the AS oligos and siRNAs were labeled with fluorescein prior to splenocyte transfection. The cell suspensions were evaluated by fluorescent microscopy after transfection. The number of total cells versus the number of fluorescein-labeled cells showed that we consistently transfected >90% of the nonadherent splenocytes (Figure 2).

To verify that the inhibition of T-bet resulted in the suppression of IFNγ production, Vβ8.2 transgenic splenocytes were transfected with the T-bet-specific AS oligo and siRNA and activated with MBP Ac1-11, supernatants were collected, and IFNγ production was measured by ELISA. As seen in Figure 3A, IFNγ production was suppressed, but not totally inhibited, in the splenocytes that were transfected with the T-bet-specific nucleic acids, verifying that silencing T-bet significantly diminished IFNγ production. IFNγ production increases over time in the cells in which T-bet was silenced, but it is not known whether this IFNγ is expressed by CD4+ T cells or other immune cells in the splenocyte population. There was no IL-4 produced when T-bet was silenced during the primary activation (data not shown).

Silencing T-bet Alters the Encephalitogenicity of MBP-Specific T Cells

To determine if suppressing T-bet could alter the encephalitogenic capacity of MBP Ac1-11-specific T cells,

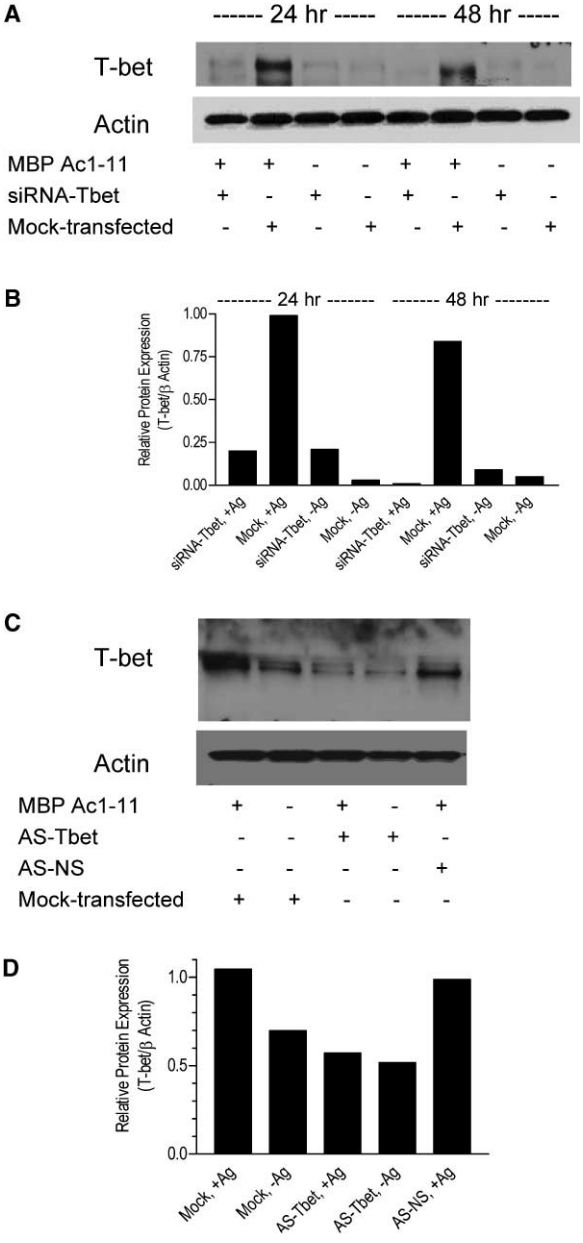

Figure 1. Suppression of T-bet Expression with siRNA and AS Oligo

(A–D) Splenocytes from a Vβ8.2 transgenic B10.PL mouse were transfected with an (A and B) siRNA specific for T-bet or an (C and D) AS oligo specific for T-bet. The cells were activated with MBP Ac1-11, and nuclear extracts were prepared at 24 and/or 48 hr postactivation. Protein levels were quantitated, and 30 μg for T-bet or 5 μg for β-actin was loaded per lane of an 10% SDS/PAGE gel. (A and C) The proteins were transferred to nitrocellulose membranes and probed with anti-T-bet or anti-β-actin. (B and D) Densitometry was performed, and relative T-bet expression was determined by normalizing the T-bet to β-actin levels.

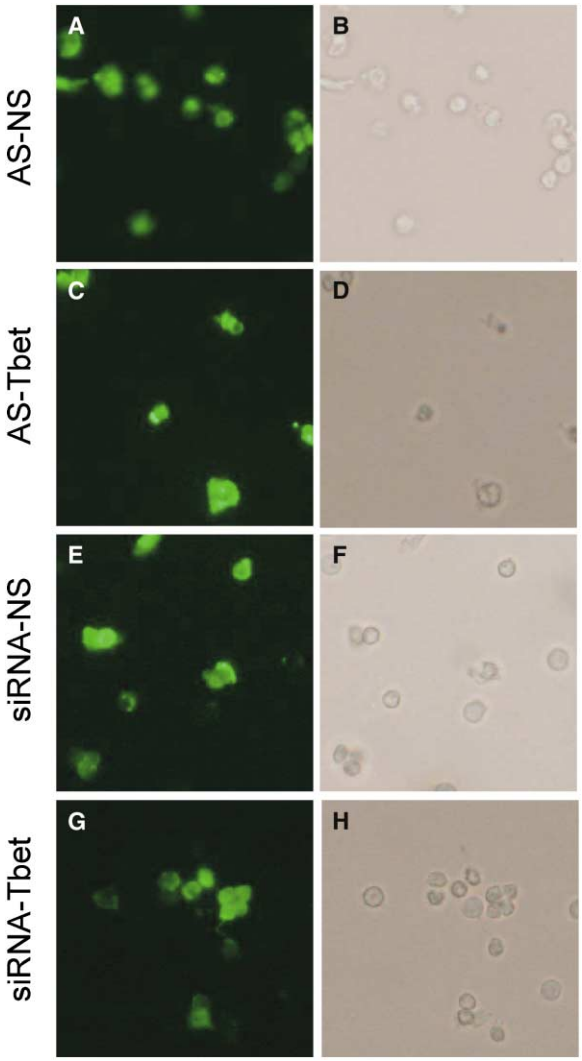

Figure 2. Primary Splenocyte Cultures Can Be Efficiently Transfected with AS Oligos and siRNAs

(A–H) AS oligos and siRNAs were labeled with fluorescein, bound to a transfection reagent, and then added to primary splenocyte cultures from a B10.PL mouse. After a 20 hr incubation, the nonadherent splenocytes were washed twice and examined by microscopy. The number of (A, C, E, and G) fluorescein-labeled cells, as well as the (B, D, F, and H) total number of cells, was counted in the same fields. The transfection efficiency was consistently >90%.

naive Vα2.3/Vβ8.2 transgenic splenocytes were transfected with AS-Tbet, siRNA-Tbet, and control nucleic acids prior to activation with MBP Ac1-11 in vitro and were transferred into wild-type B10.PL mice. Only 1 of 6 mice in the AS-Tbet group and 1 of 6 mice in the siRNA-Tbet group developed clinical signs of EAE (Figure 3B). In contrast, eight of ten mice in the control groups (nontransfected, siRNA-NS, AS-GATA3) developed EAE. This demonstrates that silencing T-bet in myelin-reactive T cells not only alters their cytokine production, but diminishes their pathogenic potential.

Since it had been previously demonstrated that AS oligos and siRNAs could be administered intravenously and exhibit specific biological effects in vivo (Shi et al., 1994; Finotto et al., 2001; Schlaak et al., 2001; Hadidi et al., 2002; Sorenson et al., 2003), the ability of these T-bet-specific nucleic acids to alter actively induced EAE was evaluated. B6 mice were given 20 or 50 μg siRNA-Tbet, AS-Tbet, AS-GATA3, or PBS via the tail vein at the time of immunization with MOG35-55/CFA. All of

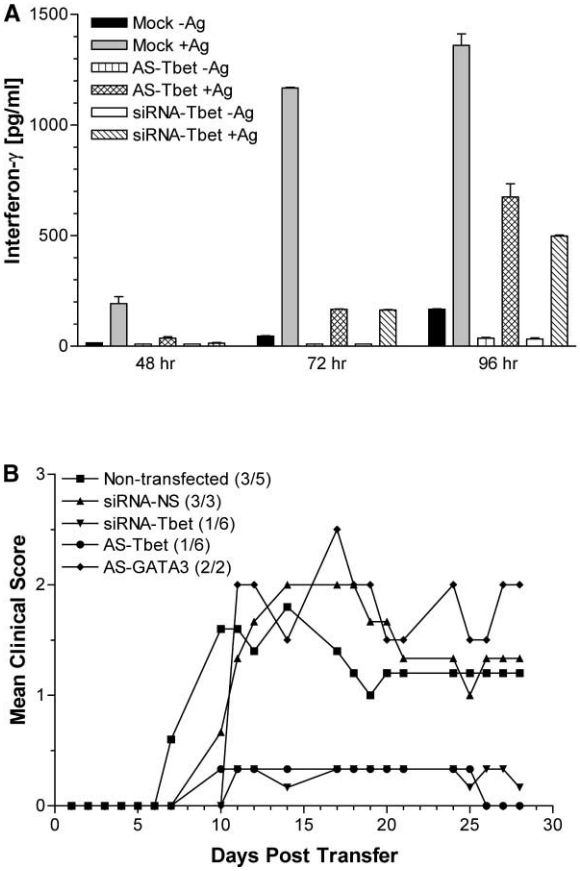

Figure 3. Silencing T-bet Alters the Phenotype of MBP Ac1-11-Specific T Cells

Splenocytes from a Vβ8.2 transgenic mouse were transfected with AS-Tbet or siRNA-Tbet, and after 24 hr, they were activated with Ac1-11.

(A) Supernatants from the cultured cells were collected at 24, 48, and 72 hr postactivation, and IFNγ levels were measured by ELISA.

(B) Vα2.3/Vβ8.2 transgenic splenocytes were transfected with siRNA-Tbet, siRNA-NS, AS-Tbet, AS-GATA3, or nothing prior to in vitro activation with Ac1-11, and after 72 hr, 10×10^6 cells were injected i.p. into B10.PL mice. The mice were monitored daily for clinical signs of EAE, and the siRNA-Tbet and AS-Tbet groups had a significantly reduced incidence of EAE compared to any of the control groups. (siRNA-NS versus siRNA-Tbet, $p < 0.0001$; AS-GATA3 versus AS-Tbet, $p < 0.0001$; siRNA-Tbet versus nontransfected, $p < 0.0001$; AS-Tbet versus nontransfected, $p < 0.0001$).

the PBS and AS-GATA3-treated mice developed EAE (Figure 4A), but disease was suppressed in the siRNA-Tbet- and AS-Tbet-treated mice in a dose-dependent manner (Figures 4B and 4C). To verify that i.v. administration of the T-bet-specific siRNA was suppressing T-bet in vivo, the draining lymph nodes were removed at 72 hr postimmunization/siRNA administration. Nuclear extracts were prepared from the cells, and Western blot analysis was used to determine the level of T-bet expression. As seen in Figure 4D, T-bet expression was suppressed >85% in the lymph node cells (LNC) from the mice that received the siRNA-Tbet compared to the mice that received the siRNA-NS, demonstrating that i.v. administration of siRNA-Tbet could effectively suppress T-bet expression in vivo. In addition, we examined the spontaneous proliferation rate in LNC by culturing these cells in the presence of ^{3}[H]-thymidine for 24 hr without antigen. The proliferation did not vary between the mice that received the control siRNA or the T-bet-specific siRNA (Figure 4E), indicating that silencing T-bet did not interfere with LNC responding to the immunization but did alter the phenotype of the responding cells.

Similarly, we examined LNC and splenocytes at day 13 postimmunization/siRNA or AS oligo treatment. None of the mice had developed clinical signs of EAE. At this time point, one would anticipate that the activated antigen-specific T cells would have migrated into the spleen. The level of T-bet in the splenocytes from the mice that received AS-Tbet or siRNA-Tbet was reduced by 60% and 98%, respectively, compared to the T-bet levels in the mice that received the AS-NS or siRNA-NS (Figure 4F). Antigen-specific IFNγ production and proliferation were also examined. As seen in Figure 4G, both the splenocytes and LNC from the AS-NS-treated mice produced significant amounts of IFNγ in response to the antigen. In contrast, there was no antigen-induced IFNγ production in the splenocytes of the AS-Tbet- or siRNA-Tbet-treated mice, and only a modest amount of IFNγ produced in LNC at 48 hr compared to the amount produced in LNC from the AS-NS-treated mice. LNC from the siRNA-Tbet-treated mice were not evaluated in this experiment because there were not enough cells recovered from the lymph nodes in these mice. However, in similar experiments not shown, there was no significant production of IL-4, IL-5, IL-10, or IFNγ in LNC or splenocytes in the mice that received siRNA-Tbet or AS-Tbet. At day 13, LNC from all of the groups showed antigen-specific proliferation, but only the splenocytes from the control mice demonstrated antigen-specific proliferation in the splenocyte population (Figure 4H), suggesting that the spleens of mice that had T-bet silenced had fewer antigen-specific T cells or that the phenotype of the antigen-specific T cells was different than the control-treated mice. Antigen-specific proliferation increased significantly in the splenocytes of the mice that developed EAE (data not shown). We also injected fluorescein-labeled AS oligos and siRNAs into mice i.v. to determine the tissue distribution of these small nucleic acids. Fluorescein-labeled cells were seen in the spleen, thymus, kidney, heart, and liver, but they were not seen in thigh muscle at 5 days postinjection (data not shown).

The Role of T-bet in the Differentiation of Encephalitogenic T Cells Can Be Bypassed by Exogenous IL-12

To determine the role that T-bet was playing in the differentiation of encephalitogenic T cells, we examined whether the differentiation of MBP Ac1-11-specific T cells in the presence of IL-12 altered the level of T-bet expression. Naive Vα2.3/Vβ8.2 transgenic splenocytes were activated with Ac1-11 in the presence or absence of IL-12. Nuclear extracts were prepared from activated cells, and the level of T-bet expression was determined by Western blotting. The splenocytes that were activated in the presence of IL-12 had similar levels of T-bet expression as the splenocytes that were activated with antigen only (Figure 5A). To confirm this observation,

splenocytes were activated in the presence or absence of IL-12 and were stained intracellularly with anti-T-bet. Flow cytometric analysis showed similar intracellular levels of T-bet in CD4+ T cells, regardless of the addition of IL-12 (Figure 5B). These observations suggest that T-bet expression is not altered by the presence of IL-12 and probably functions in a different segment of the Th1 differentiation pathway.

To further explore this observation, naive Vα2.3/Vβ8.2 transgenic splenocytes were transfected with AS-Tbet and siRNA-Tbet prior to activation with Ac1-11 in the presence of IL-12. Supernatants were collected from the cultured cells, and IFNγ levels were determined by ELISA, demonstrating significant IFNγ production in all transfection conditions (Figure 5C). At 72 hr postactivation, the splenocytes were transferred into B10.PL mice, and all cells activated in the presence of IL-12 could transfer disease into recipients, regardless of whether T-bet was silenced prior to activation (Figure 5D). These data indicate that T-bet functions independently of IL-12 and/or upstream of IL-12 in the differentiation of the encephalitogenic T cells.

T-bet Regulates the *IFNγ* and *STAT1* Genes

To determine what genes T-bet may directly regulate in the Th1 differentiation pathway, chromatin immunoprecipitation (ChIP) assays were utilized. Naive Vβ8.2 transgenic splenocytes were activated in vitro with MBP Ac1-11 and then fixed with formaldehyde to crosslink T-bet/DNA complexes. The cells were sonicated, and anti-T-bet was used to immunoprecipitate the DNA specifically bound to T-bet. The immunoprecipitated DNA was purified, and PCR amplification was used to identify promoter/enhancer regions of various genes that had been bound to T-bet. As seen in Figure 6A, the *IFNγ* could be amplified with the *IFNγ* promoter primer set #7, verifying that the *IFNγ* gene may be directly regulated by T-bet in these autoreactive T cells. Similarly, we immunoprecipitated DNA bound to *STAT1* from antigen-activated Vβ8.2 cells and amplified the *IFNγ* gene with two primer sets (Figure 6B). We also used *STAT1* and *STAT4* promoter/enhancer region-specific primers to determine if these genes may be regulated by T-bet. As expected, the *STAT4* gene was not amplified from the DNA precipitated with the T-bet antibody (data not shown). However, a primer set specific for the *STAT1* promoter region amplified a segment from T-bet-immunoprecipitated DNA (Figure 6C). Interestingly, this primer set amplified a sequence within the *STAT1* gene that contained a T-bet binding domain. This was a surprising observation because a previous study demonstrated that STAT1-deficient T cells could not express T-bet mRNA upon activation, suggesting that STAT1 was regulating the expression of T-bet (Afkarian et al., 2002). To determine if T-bet expression affected STAT1 protein expression, Vβ8.2 splenocytes were transfected with siRNA-Tbet, siRNA-NS, AS-Tbet, or AS-NS or were mock-transfected, and nuclear extracts were prepared after activation with MBP Ac1-11. A Western blot with anti-STAT1 was used to determine the level of STAT1 expression when T-bet was silenced. As seen in Figure 6D, STAT1 levels were decreased in the splenocytes in which T-bet had been silenced, suggesting that T-bet may be a transcription factor that upregulates STAT1 expression in Th1 cells. To verify this observation, T-bet expression was suppressed in splenocytes with siRNA-Tbet, and a chromatin immunoprecipitation assay was used to determine if the *STAT1* promoter could still be immunoprecipitated from activated splenocytes, as seen in Figure 6C. When the splenocytes were transfected with siRNA-NS, the *STAT1* promoter could be amplified in T-bet-specific immunoprecipitated DNA, but the *STAT1* promoter could not be amplified from the T-bet-specific immunoprecipitated DNA from the cells that had been transfected with siRNA-Tbet (Figure 6E). This confirms that T-bet directly binds the *STAT1* promoter and regulates STAT1 expression in activated splenocytes.

To determine if STAT1 may also regulate T-bet transcription in some type of feedback loop to optimize IFNγ production, a ChIP assay using anti-STAT1 to precipitate DNA/STAT1 complexes from activated Vβ8.2 splenocytes was performed. The 5′ end of the T-bet gene could not be amplified in these experiments (data not shown). However, due to the lack of data on the promoter/enhancer region of the T-bet gene, the primer sets that were used may not have been optimal. Therefore, we developed an siRNA specific for STAT1 and investigated whether reducing STAT1 expression altered T-bet expression. Naive Vβ8.2 splenocytes were transfected with siRNA-STAT1 and activated with MBP Ac1-11. Nuclear extracts were prepared and analyzed for STAT1, T-bet, and β-actin protein levels. As seen in Figure 6F, STAT1 expression in activated cells was reduced by 47% by the siRNA, which is significant given that STAT1 is constitutively expressed by a variety of cell types in the spleen. In addition, T-bet expression was reduced by 50% when the splenocytes were transfected with siRNA-STAT1, confirming the previously published observation that STAT1 regulates T-bet expression (Afkarian, et al., 2002).

Since it had been reported that IL-12Rβ2 mRNA was not upregulated in T-bet-deficient mice, suggesting that T-bet regulated the *IL-12Rβ2* gene (Afkarian et al., 2002), we investigated whether we could confirm this observation in our experimental system. First, we performed ChIP assays on activated Vβ8.2 cells with anti-T-bet and attempted to PCR amplify the promoter region of the IL-*12Rβ2* gene, but we were not successful (data not shown). Given that a negative result in this experiment does not preclude the possibility of T-bet regulating this gene, we analyzed IL-12Rβ2 protein expression in whole-cell lysates from Vα2.3/Vβ8.2 TCR transgenic splenocytes that had been transfected with AS-Tbet, siRNA-Tbet, or controls by Western blot, but there was very little change in IL-12Rβ2 expression when T-bet was silenced (data not shown). This was confirmed by ELISA in which the same whole-cell lysates were used as the antigen, and antibodies for IL-12Rβ2, T-bet, and STAT1 were used to determine the relative change in protein expression upon antigen activation. A ratio of approximately 1 was seen for IL-12Rβ2, which reflects no significant change in protein expression in the cells transfected with siRNA-Tbet or siRNA-NS (Figure 6G). In contrast, there was a 3-fold increase in STAT1 and T-bet expression in the cells transfected with siRNA-NS compared to the cells transfected with siRNA-Tbet,

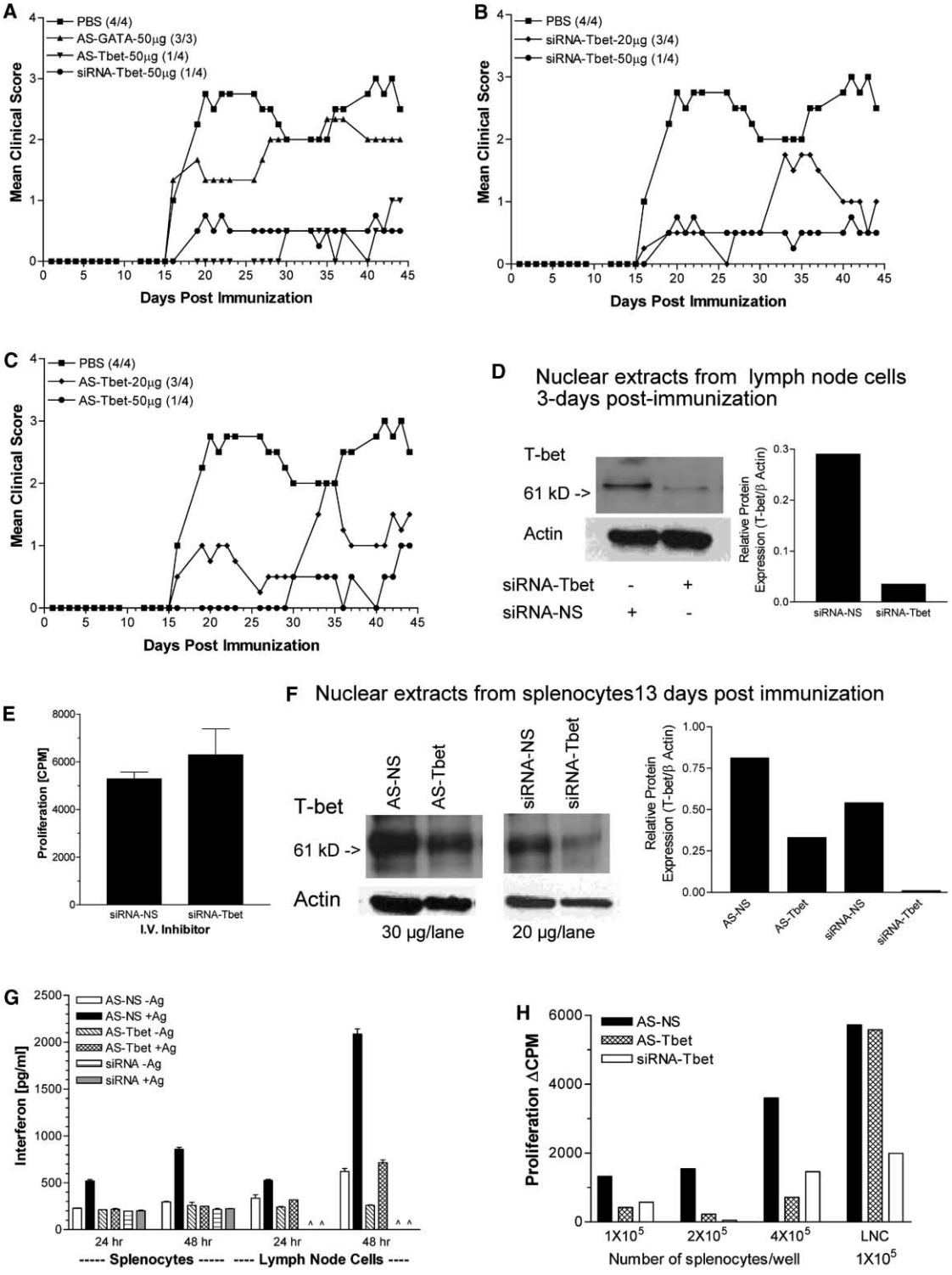

Figure 4. In Vivo Silencing of T-bet Suppresses the Incidence of Actively Induced EAE

(A–C) B6 mice were immunized with 200 μg MOG35-55/CFA, and AS-Tbet and siRNA-Tbet were given via the tail vein at the time of immunization. (A) AS-Tbet, siRNA-Tbet, AS-GATA3 (50 μg/mouse), or PBS were given i.v. (PBS versus AS-Tbet, $p < 0.0001$; PBS versus siRNA-Tbet, $p < 0.0001$; AS-GATA3 versus AS-Tbet, $p < 0.0001$; AS-GATA3 versus siRNA-Tbet, $p < 0.0001$). (B) B6 mice were given 20 or 50 μg siRNA i.v. (PBS versus siRNA-Tbet, 20 μg, $p < 0.0001$; PBS versus siRNA-Tbet, 50 μg, $p < 0.0001$; siRNA-Tbet, 20 μg versus siRNA-Tbet, 50 μg, $p = 0.0674$). (C) B6 mice were given 20 or 50 μg AS oligo i.v. (PBS versus AS-Tbet, 20 μg, $p = 0.0005$; PBS versus AS-Tbet, 50 μg, $p < 0.0001$; AS-Tbet, 20 μg versus AS-Tbet, 50 μg, $p = 0.0002$).

(D and E) The draining lymph nodes were removed from mice 3 days after they had been immunized and given 50 μg siRNA-NS or siRNA-Tbet. (D) Nuclear extracts were prepared from LNC, 30 μg protein was loaded per lane on a 10% SDS/PAGE gel, and Western blot analysis

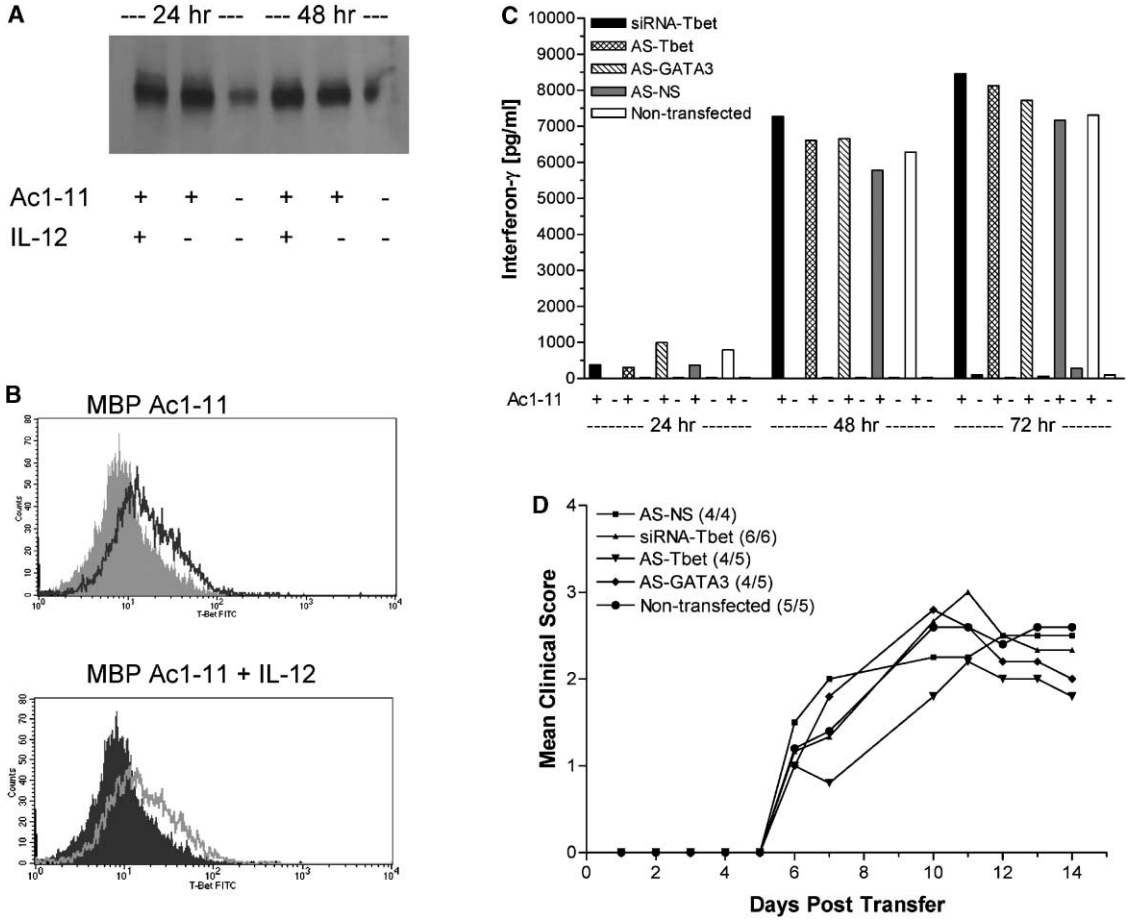

Figure 5. Differentiation of T Cells in the Presence of Exogenous IL-12 Is Not Dependent on T-bet

(A and B) Vα2.3/Vβ8.2 transgenic splenocytes were activated with Ac1-11 in the presence or absence of 1 ng/ml IL-12. (A) After a 24 or 48 hr stimulation, nuclear extracts were prepared, 30 μg protein was loaded per well, and Western blotting with anti-T-bet was used to determine the amount of T-bet expression. The background expression of T-bet in these nuclear extracts was higher than was seen in Vβ8.2 transgenic cells (Figure 1), because there are more spontaneously activated cells in the Vα2.3/Vβ8.2 mice. (B) The activated cells were stained intracellularly with anti-T-bet and were evaluated by flow cytometry.

(C and D) Vα2.3/Vβ8.2 transgenic splenocytes were transfected with AS-Tbet, AS-GATA3, AS-NS, siRNA-Tbet, or nothing 24 hr prior to activation with Ac1-11 in the presence or absence of 1 ng/ml IL-12. (C) The cells were cultured at 2×10^6 transfected cells/well with 6×10^6 irradiated, wild-type B10.PL splenocytes, supernatants were collected at 24, 48, and 72 hr postactivation, and ELISA was used to determine IFNγ production. (D) The transfected splenocytes that had been activated in the presence of IL-12 were injected i.p. into wild-type B10.PL mice at 10×10^6 cells/mouse, and clinical signs of EAE were monitored daily.

verifying that STAT1 and T-bet levels were significantly suppressed when the T-bet gene was silenced.

Discussion

This study demonstrates the significance of T-bet in the differentiation of Th1 cells in an antigen-specific system that has a pathogenic effect in vivo by using two gene-silencing techniques. We utilized MBP Ac1-11-specific TCR transgenic T cells that preferentially differentiate into Th1 cells upon activation with MBP Ac1-11. For the majority of in vitro experiments, splenocytes from mice that expressed only the Vβ8.2 chain of the transgenic TCR were used because the precursor frequency of

with anti-T-bet was performed. T-bet levels were normalized to β-actin levels. (E) LNC were plated at 1×10^5 cells per well in quadruplicate in 96-well plates, and 50 μCi ^{3}[H]-thymidine was added to each well without antigen. The cells were harvested at 24 hr, and the amount of ^{3}[H]-thymidine incorporation was determined.

(F–H) The draining lymph nodes and spleen were removed from mice 13 days after they had been immunized and given 50 μg AS-NS, AS-Tbet, or siRNA-Tbet. (F) Nuclear extracts were prepared from 15×10^6 splenocytes or LNC, either 20 (siRNA) or 30 (AS) μg/ml was loaded per lane of a 10% SDS-PAGE gel, and proteins were transferred to nitrocellulose and probed with anti-T-bet by Western blot. Normalization of the T-bet levels to β-actin levels quantitates the change in T-bet expression. (G) The splenocytes and LNC (4×10^6 cells/well) were cultured in 24-well plates with or without antigen. Supernatants were collected at 24 and 48 hr, and IFNγ levels were measured by ELISA. (H) Splenocytes and LNC from these mice were plated in quadruplicate in 96-well plates at three concentrations and were cultured with and without antigen. After 72 hr, 50 μCi ^{3}[H]-thymidine was added to each well, and the cells were harvested after an additional 18 hr incubation. The change in CPM between the antigen-stimulated and the no-antigen controls was determined.

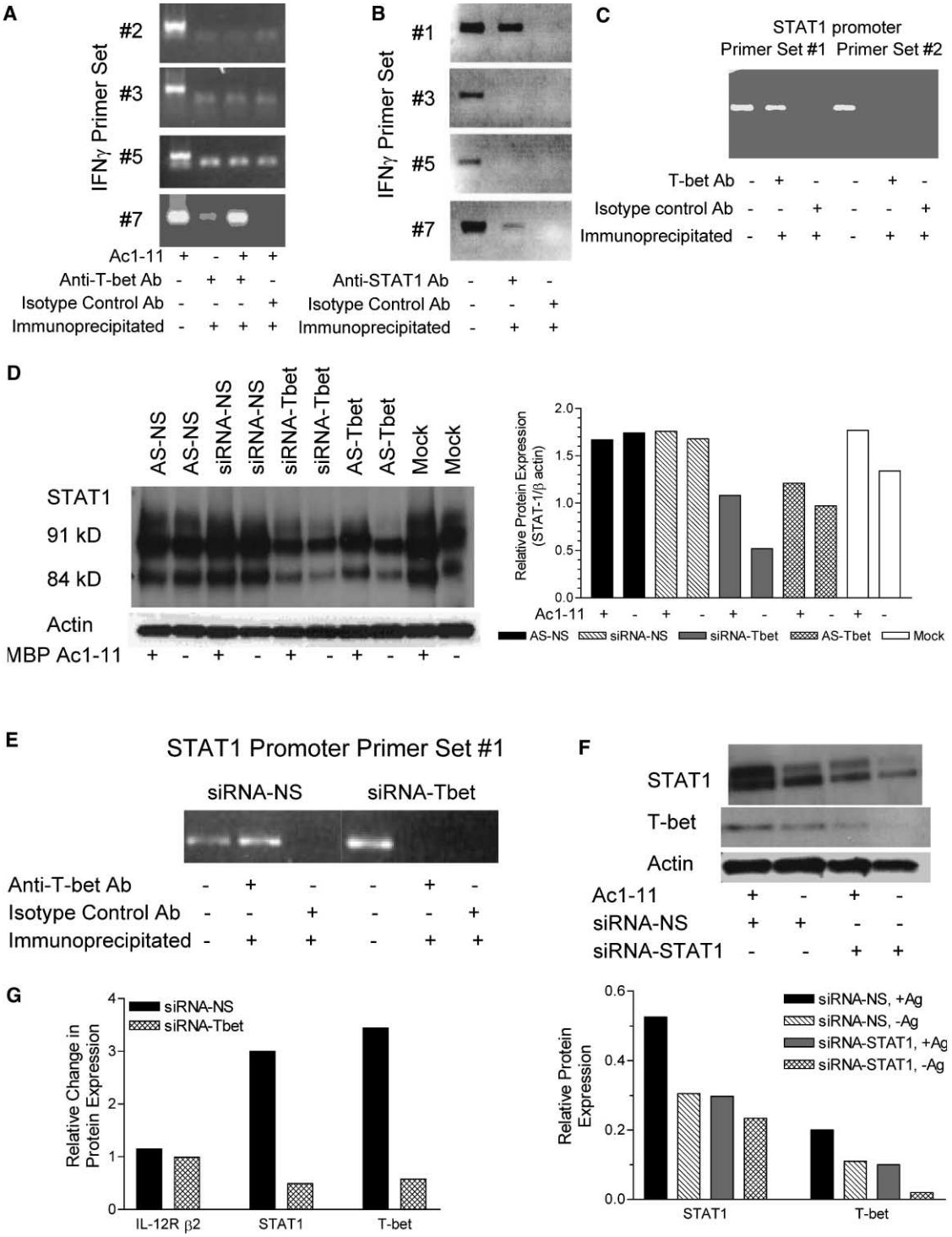

Figure 6. T-bet Regulates IFNγ and STAT1 Gene Expression during the Differentiation of Naive T Cells

Vβ8.2 transgenic splenocytes (15×10^6/condition) were cultured with and without Ac1-11 for 24 hr. The cells were then fixed with 1% paraformaldehyde, resuspended in RIPA buffer, and sonicated, and the supernatant containing the DNA/protein complexes was collected. Ten percent of the supernatant containing the total DNA was used as a positive control. The remaining supernatant was divided and immunoprecipitated with anti-T-bet antibody, anti-STAT1, or an isotype control antibody by using Protein A Sepharose beads. The immune complexes were eluted from the beads, and proteinase K was used to degrade the T-bet bound to the DNA. The DNA was purified by chloroform/ethanol precipitation, and multiple primers set within the promoter sequence of several genes were used to PCR amplify the gene of interest.

(A) DNA immunoprecipitated with anti-T-bet from Ac1-11-activated cells was used as the template in PCR reactions using eight primer sets for the IFNγ promoter region. One of the four primer sets (#7) shown specifically amplified the IFNγ gene.

(B) DNA immunoprecipitated with anti-STAT1 from Ac1-11-activated cells was used as the template in PCR reactions using four primer sets for the IFNγ promoter region. Two of the four primer sets (#1 and #7) specifically amplified the IFNγ gene.

MBP Ac1-11-specific T cells is 1 in 10^3–10^4 splenocytes, which is a more physiologically relevant number than the number of splenocytes from Vα2.3/Vβ8.2 TCR transgenic mice in which >90% of the splenic T cells respond to MBP Ac1-11. In addition, exogenous cytokines and antibodies to cytokines that are often added to T cell cultures to drive the differentiation of the cells were not used in most of these studies; thus, we could access the extent of T-bet's role in Th1 differentiation when the source of cytokines was limited to the antigen-presenting cells and responding T cells. T-bet expression in ovalbumin-specific T cell receptor transgenic cells differentiated in vitro demonstrated varied levels of T-bet expression under different Th1-inducing conditions (Afkarian et al., 2002). Since the primary source of IFNγ and IL-12 during the differentiation of Th1 cells in vivo is the antigen-presenting cells, NK cells, and CD8+ T cells, we thought that stimulation of splenocytes with antigen alone provided the most accurate assessment of T-bet's role in the differentiation of naive cells since all of these cell populations are present in primary splenocyte cultures. T-bet expression was induced upon activation of naive Vβ8.2 TCR splenocytes with MBP Ac1-11, as originally observed in T cell clones activated with PMA/ionomycin or anti-CD3 (Szabo et al., 2000). Both the AS oligo and siRNA specific for T-bet could effectively suppress T-bet protein expression in MBP Ac1-11-stimulated Vβ8.2 TCR transgenic splenocytes. IFNγ levels were substantially reduced in the splenocyte cultures that had been transfected with the T-bet-specific AS oligo or siRNA without any reciprocal induction of Th2 cytokines. The transfection efficiency in the nonadherent splenocytes was consistently >90%, indicating that there was not a preferential transfection of certain splenocyte subpopulations.

To determine if silencing T-bet could effectively suppress EAE induction in vivo, mice were given a single i.v. injection of AS-Tbet or siRNA-Tbet at the time of immunization, and this injection reduced the incidence of disease by as much as 75% depending on the dose. Ex vivo analysis of the draining LNC and splenocytes demonstrated suppressed T-bet expression and IFNγ production. At day 13, antigen-specific proliferation was seen in LNC, but it was dramatically reduced in the splenocytes of the AS-Tbet- and siRNA-Tbet-treated mice, suggesting that the number of antigen-specific cells in the spleen was reduced or had an altered phenotype resulting in altered trafficking or reduced proliferation capacity. In contrast to T-bet-deficient mice, no increase in Th2 cytokines was seen in mice that received a single dose of AS-Tbet or siRNA-Tbet.

The role of T-bet in experimental models of inflammatory diseases has been studied previously in model systems. T-bet deficiency was found to protect from Th1-mediated experimental colitis in an adoptive transfer model, and T-bet-deficient mice were found to be more susceptible to an actively induced Th2-mediated colitis (Neurath et al., 2002). This finding suggests that T-bet plays a critical role in these T cell-mediated inflammatory diseases. Similarly, Con-A-induced liver injury was ameliorated in T-bet-deficient mice (Siebler et al., 2003). However, T-bet-deficient Balb/c mice immunized with an insulin peptide develop diabetes-related phenotypes similar to wild-type Balb/c mice, indicating that T-bet does not play a critical role in this inflammatory model (Melanitou et al., 2003). It has been shown recently that T-bet-deficient mice are resistant to actively induced EAE and develop a Th2 phenotype (Bettelli et al. 2004). In contrast to these previous studies, we studied the role of T-bet in T-bet-competent mice by using gene-silencing techniques. Suppression of T-bet during the in vitro differentiation of myelin-reactive T cells diminished the capacity of these cells to cause EAE when transferred into naive mice. More importantly, in vivo administration of the siRNA or AS oligo specific for T-bet prevented the onset of actively induced EAE, suggesting that T-bet may be a viable target for therapeutic intervention in Th1-mediated diseases.

Although it was originally reported that T-bet may play a role in the IL-12/STAT4 signaling pathway in Th1 cells (Szabo et al., 2000), other studies have implicated T-bet in the transactivation of *IFN*γ via the IFNγ/STAT1 pathway (Mullen et al., 2001; Afkarian et al., 2002; Siebler et al., 2003). In the MBP Ac1-11-specific T cells, the level of T-bet expression was not increased when the cells were differentiated in the presence of IL-12. In addition, when T-bet expression was silenced in Ac1-11-specific T cells prior to antigen stimulation in the presence of IL-12, IFNγ production and the encephalitogenic capacity of the cells was not diminished, suggesting that T-bet was not necessary for the differentiation of Th1 cells when the IL-12/STAT4 pathway was directly induced. It is well established that the IL-12/STAT4 pathway plays a significant role in the differentiation of naive T cells into Th1 cells (Gately et al.,1998). However, the IL-12Rβ2 chain is dependent on the IFNγ/STAT1 signaling path-

(C) DNA immunoprecipitated with anti-T-bet antibody from Ac1-11-activated cells was used as the template in PCR reactions using three primer sets for the STAT1 promoter region. One of the two primer sets (#1) shown specifically amplified the STAT1 gene.

(D) Naive Vβ8.2 transgenic splenocytes were transfected with AS-NS, siRNA-NS, siRNA-Tbet, or AS-Tbet or were mock-transfected 24 hr prior to activation with Ac1-11. Nuclear extracts were prepared from the cells after a 24 hr activation, and 30 μg protein was loaded per lane on a 4%–20% gradient SDS/PAGE gel, transferred to nitrocellulose, and probed with anti-STAT1. Densitometry was used to normalize the STAT1 levels to the β-actin levels to quantitate the relative change in STAT1.

(E) Vβ8.2 transgenic splenocytes were transfected with siRNA-NS or siRNA-Tbet prior to activation. Anti-T-bet was used to immunoprecipitate the T-bet bound DNA from the cells and the STAT1 promoter was PCR amplified by using primer set #1.

(F) Vβ8.2 transgenic splenocytes were transfected with siRNA-NS or siRNA-STAT1 and were activated with MBP Ac1-11 for 48 hr, and nuclear extracts were prepared. Western blots were performed by using anti-STAT1, anti-T-bet, and anti-β-actin. Densitometry was used to normalize the STAT1 and T-bet levels to β-actin levels.

(G) Whole-cell lysates from MBP Ac1-11-activated Vβ8.2 splenocytes were used as the target antigen in an ELISA. Anti-IL-12Rβ2, anti-STAT1, and anti-T-bet were the primary antibodies. The relative change in protein expression was determined by subtracting the background and then determining the ratio of the OD of the antigen-activated cells to the OD of the no-antigen cells.

way, and this dependence establishes a dependence of the IL-12/STAT4 pathway on the IFNγ/STAT1 pathway. In this study, we found no evidence that T-bet directly regulated the expression of the *IL-12Rβ2* gene.

To determine what genes in the Th1 differentiation pathway may be directly transactivated by T-bet, chromatin immunoprecipitation (ChIP) assays were used. Using ectopic expression of T-bet, it has been shown that IFNγ and IL-12Rβ2 expression was upregulated in T-bet-expressing cells, but whether this effect resulted from directly transactivating these genes or transactivating other genes that subsequently regulate *IFNγ* and *IL-12Rβ2* gene expression was not addressed (Afkarian et al., 2002). To confirm that the *IFNγ* gene directly regulates T-bet in activated T cells as recently observed (Cho et al., 2003), Vβ8.2 transgenic splenocytes were activated, and the DNA/T-bet complexes were immunoprecipitated from fixed cells with anti-T-bet. An *IFNγ* promoter sequence was amplified by PCR, confirming that T-bet binds to the *IFNγ* promoter. Analysis of the 3500 bases upstream of the *IFNγ* gene translation initiation site identifies several potential T-bet binding sites based on the T-box consensus DNA binding sequence, emphasizing the potential of T-bet as a strong transactivator of the *IFNγ* gene. The T-bet-precipitated DNA was also used as a template for amplification of the *STAT1* and *STAT4* promoter sequences to determine if T-bet could potentially regulate either of these genes, which are known to play significant roles in the IFNγ receptor and IL-12 receptor signaling pathways, respectively. As anticipated, the *STAT4* promoter sequence was not amplified, but a *STAT1* promoter sequence was amplified. This *STAT1* promoter sequence contained a T-box DNA binding sequence, verifying the potential for this *STAT1* promoter sequence as a viable target for T-bet. This was a surprising observation in light of the previous finding that T-bet expression was absent in STAT1−/− cells, suggesting that STAT1 expression was necessary for T-bet expression (Afkarian et al., 2002). Suppression of T-bet with siRNAs resulted in suppressed STAT1, and suppression of STAT1 with a STAT1 siRNA resulted in suppression of T-bet, implying that STAT1 and T-bet may regulate each other.

Study of the the roles of the various cytokines, cytokine receptors, and transcription molecules in the generation of encephalitogenic Th1 cells using gene knockout mice and monoclonal antibodies has generated conflicting data. Mice lacking the IL-12 p40 subunit are protected from EAE, whereas mice lacking the IL-12 p35 subunit or the IL-12Rβ2 chain remain susceptible to EAE (Becher et al., 2002; Gran et al., 2002; Zhang et al., 2003). The myelin-reactive T cells in the IL-12Rβ2-deficient mice still produced significant amounts of IFNγ, although less than wild-type mice, suggesting that the IFNγ/STAT1 pathway was sufficient to activate encephalitogenic Th1 cells. IL-12 may not be a critical cytokine in EAE, but IL-23, which shares the p40 subunit with IL-12, may actually be mediating the effects originally attributed to IL-12 in EAE (Cua et al., 2003). STAT4-deficient mice are also protected from EAE, but it is uncertain whether this is due to an impaired signaling in the IL-12/STAT4 pathway in the T cells or due to the loss of STAT4 in antigen-presenting cells that produce IFNγ, which activates the IFNγ/STAT1 pathway (Chitnis et al., 2001). Since it was well established that myelin-specific CD4+ Th1 cells were the lymphocyte population that transferred EAE to naive recipient animals, it was not anticipated that IFNγ and IFNγ receptor-deficient mice would develop more severe EAE than wild-type mice (Ferber et al., 1996; Willenborg et al., 1996; Chu et al., 2000). Likewise, administration of anti-IFNγ to wild-type mice exacerbates EAE (Billiau et al., 1988; Duong et al., 1992, 1994; Lublin et al., 1993; Heremans et al., 1996). These mice have a massive expansion of myelin-specific CD4+ cells, suggesting that the complete loss of IFNγ results in a diminished capacity to regulate the autoreactive T cells (Chu et al., 2000) and reinforcing the notion that loss of a particular gene from all cell types can have unforeseen side effects. Although these observations indicate that IFNγ is not essential for the induction of EAE, they do not negate the fact that encephalitogenic T cells generated in vivo in wild-type mice produce significant amounts of IFNγ or the fact that in vitro suppression of IFNγ production during the stimulation of myelin-specific T cells reduces the encephalitogenic capacity of these cells (Olsson, 1992). Taken together, these studies would suggest that suppressing IFNγ production in the encephalitogenic Th1 cells, while preserving IFNγ expression in other cell types, may provide therapeutic benefit. Although T-bet is expressed in CD4+ Th1 cells, CD8+ T cells, NK cells, B cells, and dendritic cells upon activation, the requirement for T-bet for the expression of IFNγ varies significantly between cell types. In T-bet-deficient mice, IFNγ is not expressed by CD4+ T cells (Szabo et al., 2002). In contrast, CD8+ T cells and B cells were originally shown to express normal levels of IFNγ in T-bet-deficient mice (Szabo et al., 2002). However, IFNγ has more recently been shown to be reduced in CD8+ T cells during antigen-specific activation of TCR-transgenic splenocytes deficient in T-bet (Sullivan et al., 2003). Similarly, splenic dendritic cells and NK cells from T-bet-deficient mice have reduced IFNγ production (Lugo-Villarino et al., 2003; Szabo et al., 2002). Murine macrophages do not express T-bet, indicating that IFNγ transcription in these cells is regulated by other factors (Lugo-Villarino et al., 2003). Therefore, it seems that T-bet may be a target for suppressing the development of CD4+ Th1 cells, while maintaining some level of IFNγ production in other cell types that play a role in EAE. In addition, T-bet may affect other genes yet to be identified, directly or indirectly, that may alter the program of the T cells during activation, which may result in reduced encephalitogenicity.

Experimental Procedures

Mice and Induction of Experimental Autoimmune Encephalomyelitis

B10.PL and B6 mice were bred in a barrier animal facility at UT Southwestern Medical Center. B10.PL mice transgenic for the MBP Ac1-11-specific TCR chains Vα2.3 or Vβ8.2 were a gift from J. Goverman (Goverman et al., 1993). The Vα2.3 and Vβ8.2 transgenic mice were crossbred in a barrier animal facility at UT Southwestern Medical Center.

For adoptive transfer experiments, the spleens from Vα2.3/Vβ8.2 transgenic mice (5–10 weeks old) were removed, and single cell suspensions were prepared. The splenocytes were cultured in 24-well plates at 2×10^6 cells per well in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, HEPES buffer, 5 ×

10^{-5} M 2-ME, and nonessential amino acids. The cells were transfected with AS oligos and siRNAs as described below. Splenocytes from wild-type B10.PL mice were irradiated with 3000 rads and cultured in 24-well plates at 6×10^6 cells per well in RPMI 1640 supplemented as described above, except the fetal bovine serum concentration was increased to 10% and pencillin/streptomycin were added at 100 U/ml. MBP Ac1-11 peptide was added to the cultures at 2 μg/ml. After 24 hr of transfection of the transgenic splenocytes and 24 hr of incubation of the irradiated wild-type splenocytes with peptide, the transfected cells were washed with HBSS and were added to the irradiated wild-type splenocytes. The cells were incubated for an additional 72 hr, then the cells were washed with PBS twice, and 10×10^6 activated cells were injected i.p. into wild-type B10.PL mice. The mice were evaluated daily for clinical signs of EAE. The following scoring system was used: 0 = no signs of disease, 1 = limp tail, 2 = moderate hind limb weakness, 3 = severe hind limb weakness, 4 = hind limb paralysis, 5 = quadriplegia or moribund, 6 = death due to EAE (Racke et al., 1994).

For actively induced EAE, B6 mice were given 20 or 50 μg AS oligo or siRNA in 100 μl PBS via the tail vein. The mice were then immunized s.c. with 200 μg MOG35-55 peptide emulsified in CFA per mouse (Difco Laboratories) above the shoulders and flanks. A 200 ng dose of pertussis toxin (List Biological Laboratories) was given i.p. at the time of immunization, as well as 2 days postimmunization. The mice were monitored daily for clinical signs of EAE.

Antisense Oligonucleotides, siRNA, and Transfection

AS oligos generated on a phosphorothioate backbone were purchased from Life Technologies. The siRNAs were purchased as purified duplexes from Dharmacon RNA Technologies. The sequences are shown in Table 1.

For in vitro transfection of splenocytes, 2 μl TransIT-TKO transfection reagent (Mirus) was diluted in 50 μl serum-free/antibiotic-free RPMI 1640 media per well. After a 10 min incubation at room temperature, 1 μl 40 μM siRNA or 2 μl 20 μM AS oligo was added to 52 μl diluted transfection reagent. The AS oligos and siRNAs were incubated with the diluted transfection reagent at room temperature with gentle agitation for 30 min. The AS oligos or siRNAs were added to the Vβ8.2 transgenic splenocyte cultures containing 5×10^6 cells in 500 μl media per well of a 24-well plate and incubated overnight at 37°C. On the following day, the nonadherent cells were collected and washed with fresh media. The adherent cells in the plates were also washed once with fresh media. The nonadherent cells were resuspended in 2 ml media and placed back in their original wells. MBP Ac1-11 peptide was added at 2 μg/ml. For Vα2.3/Vβ8.2 transgenic splenocytes, 2×10^6 splenocytes were placed in each well of a 24-well plate. The transfection protocol was the same, except the cells were placed with wild-type splenocytes (6×10^6 cells/well) that had been irradiated and cultured with MBP Ac1-11 after the 24 hr transfection.

ELISA

For IFNγ, purified anti-mouse IFNγ (Pharmingen) was diluted in 0.1 M $NaHCO_3$ (pH 8.2) at 2 μg/ml. Immunolon II plates (Dynatech Labs) were coated with 50 μl primary antibody 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 1% BSA in PBS per well for 2 hr. The plates were then washed twice with PBS/Tween, and 100 μl supernatants was added in duplicate. The plates were incubated overnight at 4°C and washed four times with PBS/Tween. Biotinylated rat anti-mouse IFNγ (Pharmingen) was diluted in PBS/1% BSA, 100 μl of 1 μg/ml biotinylated antibody was added to each well, and plates were incubated at room temperature for 1 hr. The plates were washed six times with PBS/Tween, 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, and 100 μl ABTS substrate containing 0.03% H_2O_2 was added to each well. The plate was monitored for 15–20 min for color development and read at OD_{405}. A standard curve was generated from the IFNγ standards, and the concentration of IFNγ in the samples was calculated.

For IL-12Rβ2, STAT1, and T-bet, whole-cell lysates were generated from 2×10^6 Vα2.3/Vβ8.2 TCR transgenic splenocytes that have been activated with MBP Ac1-11 plus 6×10^6 irradiated feeder cells from wild-type B10.PL mice. The protein concentration was determined by using the BioRad Protein Assay. Immulon II plates were coated with 20 μg/ml protein in 0.1 M $NaHCO_3$ (pH 8.2) overnight at 4°C. The plates were washed twice with PBS/Tween and blocked with PBS/BSA for 2 hr, and anti-IL-12Rβ2, anti-STAT1, and anti-T-bet were added at 5 μg/ml into duplicate wells for 2 hr. The plates were washed six times with PBS/Tween, and a biotinylated secondary antibody was added at 2 μg/ml for 1 hr. The remainder of the assay was the same as the IFNγ ELISA. The relative change in each protein was determined by subtracting the background and determining the ratio of the OD of the activated cells to the OD of the no-antigen cells.

Preparation of Nuclear Extracts and Western Blotting

Splenocytes were transfected as described above. At 24 or 48 hr post-antigen activation, the cells were collected ($15–20 \times 10^6$ cells/condition), and nuclear extracts were prepared by using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce). In brief, the cells were spun down and resuspended in reagent CER I. The cells were vortexed for 15 s and incubated on ice for 10 min. CER II was added to the cells, and cells were vortexed for 5 s, incubated on ice for 1 min, and then vortexed again for 5 s. The tubes were then centrifuged for 5 min at $20,000 \times g$. The supernatants were removed, the reagent NER was added to the pellets, and the tubes were vortexed for 15 s and then incubated on ice for 10 min. The tubes were vortexed, incubated on ice three more times, and then centrifuged for 10 min at $20,000 \times g$.

Prior to the Western blot, the protein concentration of these extracts was determined by using the BioRad Protein Assay. The nuclear extracts were diluted in 10× SDS loading buffer and boiled for 3 min. Extracts were electrophorectically separated on 10% (or 4%–20%) SDS-PAGE gels and were transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBS. The primary antibody was usually diluted 1:100 in blocking buffer and was added to the membrane for 2 hr. The membrane was washed three times in TBS/Tween. The secondary antibody bound to HRP was diluted 1:1000 and was added to the membrane for 1 hr. The membrane was washed three times, and a chemiluminescent substrate (Santa Cruz Biotechnology, Inc.) was added for 1 min; the blot was then exposed to film for various times (0.05–10 min). T-bet antibody, STAT1 antibody, IL-12Rβ2 antibody, goat anti-mouse IgG-HRP, and anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology, Inc. The density of the bands was determined by using an AlphaImager (Alpha Innotech Corp.). The data was normalized by dividing the density of the T-bet or STAT1 band by the density of the β-actin band.

Chromatin Immunoprecipitation Assays

We used a modified version of the technique previously described (Dedon et al., 1991; Braunstein et al., 1993). Vβ8.2 transgenic splenocytes (10×10^6 per condition) were stimulated with antigen or no antigen for 24 hr. The cells were then fixed in media with a final concentration of 1% formaldehyde for 20 min at room temperature. The cells were then washed twice with PBS and resuspended in lysis buffer. The cells were pulse sonicated for 3 min to shear the DNA into 400–900 bp lengths. The supernatant containing the DNA/protein complexes was collected after centrifugation to remove the cell debris. An aliquot of the supernatant was set aside for a positive control in the PCR reactions, because all the DNA from the cells is contained in the supernatant. An antibody known to immunoprecipitate the transcription factor of interest, as well as an isotype control antibody, was added to the supernatant to bind the DNA/transcription factor complexes. All of the antibodies were the same as those used in the Western blot. After a 24 hr incubation at 4°C, protein A sepharose beads were added and incubated at room temperature for 2 hr. The beads were washed, and the immune-complexes were eluted from the beads with SDS/$NaHCO_3$. The formaldehyde cross-links from the immunoprecipitated DNA/protein complexes, as well as the total DNA/protein complexes, were reversed by adding 3 M NaOAc to a final concentration of 0.3 M and incubating at 65°C overnight. The DNA was precipitated with EtOH at –20°C. The remaining protein was degraded by Proteinase K digestion at 42°C for 2 hr. The DNA was purified by phenol:chloroform extraction,

followed by EtOH precipitation. The DNA was then used as the template in PCR reactions specific for the gene of interest. Multiple primer sets that cover the entire promoter/enhancer region of the genes of interest were used to minimize missing critical binding sites. IFNγ primer sets (5′-3′) were #1: CCATACGCAGACACCATTG (forward) and TAGAAACACGAGCTCTGGG (reverse), #2: TCTCTTGA GGTCCTCCATGC (forward) and GCACATTCTGCTACGCTTG (reverse), #3: GAACATAGAACGGTCCCCGT (forward) and TCCTGGTC TACAGAGTGAG (reverse), #5: CTGGGTCAAGATAACTGGG (forward) and TCAGCCAAAGGCTCAACCA (reverse), and #7: CACGTT GACCCTGAGTGAT (forward) and GAGGAAACTCTTGGGCTTC (reverse). The STAT1 primer sets were #1: CAGGATGGAGGTTCTC AACCTG (forward) and GTGAACGGATATCTGCAGCTCC (reverse), #2: GGAAGTGCTTGTGAGCTATC (forward) and CCATGCTAACCA TCTCTGCC (reverse), and #3: CAGTGGGTAGAAGGTCTTGCTG (forward) and AGTGCATTGGAAAGCTGG (reverse). The PCR conditions were 55°C annealing for 1 min, 72°C elongation for 1 min, and 94°C denaturing for 1 min for a total of 35 cycles.

Flow Cytometric Analysis of Intracellular T-bet

Vα2.3/Vβ8.2 TCR transgenic splenocytes were cultured at 5×10^6 cells/well in a 24-well plate with MBP Ac1-11 at 2 μg/ml, Ac1-11 + IL-12 at 1 ng/ml, or no antigen for 24 hr. The cells were stained with anti-CD4-PE for 30 min and were then permeabilized with 2× FACS Lysing Solution (Becton Dickinson) supplemented with 0.025% Tween 20 for 10 min. The cells were then stained with anti-T-bet-FITC (Pharmingen) for 30 min and were fixed with 4% paraformaldehyde. Cells were gated on viable CD4+ lymphocytes, and the data was analyzed by using Cell Quest software.

Acknowledgments

We thank Dr. Thomas Kodadek for helping establish the chromatin immunoprecipitation assay in our laboratory. We also thank Dr. Nitin Karandikar for assisting us with the intracellular staining and flow cytometry. This study was supported by grants from the Yellow Rose Foundation, Once Upon A Time…, and the National Institutes of Health (R01 NS037513 and K24 NS044250).

Received: February 17, 2004
Revised: September 20, 2004
Accepted: September 29, 2004
Published: November 16, 2004

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