Chapter 3
T-bet: A Critical Regulator of Encephalitogenic T Cells

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Abstract T-bet is a transcription factor that regulates CD4 Th1 cell differentiation and mice deficient in T-bet fail to develop experimental autoimmune encephalomyelitis, demonstrating its critical role in the generation of immune-mediated demyelinating disease. More importantly, silencing T-bet in a model for multiple sclerosis (MS) demonstrates that it is a viable therapeutic target. T-bet has been found to correlate with disease activity and therapeutic efficacy, suggesting that it may also be a biomarker for MS. Defining the role of T-bet in generating encephalitogenic T cells and their effector functions may provide insight into the mechanisms that underlie the pathology of MS lesions.

3.1 Introduction

T-bet was originally cloned and characterized in 2000 by Laurie Glimcher's laboratory in an effort to dissect the regulatory pathway that ultimately led to the development of Th1 cells (Szabo et al. 2000). Under the hypothesis that the *IL2, IFNg*, and *TNFb* genes would all be regulated by a common transcription factor, which would result in Th1 cells, since the expression of these cytokines defines Th1 cells, at least one hybrid system utilizing an IL-2 promoter–reporter construct was used to identify mRNA that was differentially expressed in Th1 cells relative to Th2 cells. Sequence homology to the T box family of transcription factors was the basis for the name T-bet, *T b*ox expressed in *T* cells. T-bet is a 530 amino acid protein encoded by the *Tbx21* gene on chromosome 17 in humans and chromosome 11 in mice, and it contains a 189 amino acid T box DNA-binding domain. T-bet was found to directly regulate the expression of IFNγ and repress the expression of Th2 cytokines (Szabo et al. 2000; Finotto et al. 2002; Lovett-Racke et al. 2004; Hwang et al. 2005; Jenner et al. 2009). Since myelin-specific Th1 cells were capable of

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inducing experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS), and Th1 cells were found in the central nervous system (CNS) of MS patients, a tremendous amount of research has focused on the role of Th1 cells and associated signaling pathways as a means to understand the pathophysiology of MS (McDonald and Swanborg 1988; Ando et al. 1989; Waldburger et al. 1996; Yura et al. 2001; Lovett-Racke et al. 2004; Gocke et al. 2007; Yang et al. 2009). T-bet, a Th1-associated transcription factor, has been found to be a critical factor in the development of encephalitogenic CD4 T cells in EAE, as well as an effective therapeutic target (Lovett-Racke et al. 2004; Bettelli et al. 2004; Gocke et al. 2007; Yang et al. 2009). Furthermore, T-bet has been identified as a potential biomarker for disease activity and therapeutic efficacy in MS patients (Nath et al. 2004; Frisullo et al. 2006; Peng et al. 2006; Frisullo et al. 2007; Drulovic et al. 2009; Iorio et al. 2009; Kleiter et al. 2010; Frisullo et al. 2011). Thus, T-bet provides insight into the mechanisms by which CD4 T cells mediate CNS lesion development, as well as a potential therapeutic target for the treatment of MS.

3.2 Characterization of the Immune Response in Multiple Sclerosis

MS is an old disease whose pathology was not formally articulated until the nineteenth century when Jean-Marie Charcot described CNS lesions associated with episodic neurological deficits.

> On histological sections, multiple sclerosis lesions contain perivascular inflammation and demyelination. Plaques occur anywhere within the white matter of the central nervous system. The most frequently affected sites are optic nerves, brainstem, cerebellum and spinal cord. Lesions in these areas often correlate with clinical systems. In the cerebral hemispheres, periventricular distribution of plaques is often seen. When plaques are adjacent to the cortex, subcortical myelinated nerves are often spared. Plaques located near the gray matter may spread into the gray matter, including deep nuclei and the cortex. Axons are spared within the initial lesions, but are later destroyed (Charcot 1968; translated from French).

Although tremendous research has been done to understand the pathophysiology of MS, Charcot's initial description is still as accurate a description of the MS lesions as any published since that time. In addition, the components of the lesions as delineated by Charcot, inflammation, demyelination, and axonal destruction, have been the basis of most research on MS. Since inflammation appears to be the predecessor of the demyelination and axonal damage, characterization of the inflammatory response has been a priority in MS research. As our understanding of immunology has evolved over the past decades, our understanding of the inflammatory response in the CNS of MS patients has also evolved, yet the cause of the disease remains unknown and our ability to modify the disease course limited.

3.3 CD4 T Cell Lineages

The hallmark observation that CD4 T cells can express distinct cytokine profiles and play different roles in protective immunity has shed light on the differential role of CD4 T cell lineages in health and disease. The original observation that effector CD4 T cells primarily expressed the signature cytokines IFNγ or IL-4, known as T-helper 1 (Th1) or T-helper 2 (Th2) cells, respectively, prompted interest in how these two lineages develop (Mosmann et al. 1986). T cell receptor (TCR) binding to MHC/peptide complexes (signal 1) and CD28 interaction with CD80/CD86 (signal 2) are the initial activation signals required for the activation of T cells via antigen-presenting cells (APCs; Fig. 3.1; June et al. 1990). However, cytokines produced in the microenvironment provide critical signals that determine the lineage commitment of CD4 T cells. If the environment is rich in IFNγ and IL-12, CD4 T cells differentiate into Th1 cells (Figs. 3.1 and 3.2). All CD4 T cells express the interferon receptor, which when engaged, results in the phosphorylation of the transcription factor STAT1 and translocation to the nucleus (Fig. 3.2a; Afkarian et al. 2002). STAT1 contributes to the expression of T-bet, the master regulator of Th1 cells (Fig. 3.2b–c; Lovett-Racke et al. 2004). There is a positive feedback loop between STAT1 and T-bet that promotes the Th1 phenotype and induces the expression of the IL-12 receptor β2 chain (IL12Rβ2). When the IL12Rβ2 chain pairs with the constitutively expressed IL12Rβ1 chain, this allows IL-12 signaling to occur. IL-12 receptor signaling causes phosphorylation of STAT4, translocation to the nucleus, and binding to the *Ifng* gene promoter (Fig. 3.2d; Jacobson et al. 1995). In conjunction with STAT1 and T-bet, STAT4 induces expression of IFNγ and lineage commitment to a Th1 phenotype (Fig. 3.2e). Since Th1 cells express IFNγ and IFNγ initiates the differentiation of Th1 cells, the microenvironment will continue to favor the differentiation of naive CD4 T cells into Th1 cells, and thus an immune response to a particular antigen is typically dominated by a specific CD4 T cell lineage. Similarly, CD4 Th2 cells are generated by IL-4 receptor signaling that causes phosphorylation of STAT6, translocation to the nucleus, and binding to the *gata3* gene promoter (Hsieh et al. 1992; Hou et al. 1994; Zheng and Flavell 1997). GATA3 is the master transcriptional regulator of Th2 cells that promotes the expression of IL-4 as well as other Th2-associated cytokines such as IL-5 and IL-13.

More recently, a unique population of CD4 T cells known as Th17 cells has been identified as another lineage defined by the expression of IL-17 (Yao et al. 1995). IL-6 appears to be the critical cytokine for the differentiation of naive CD4 T cells into Th17 cells (Veldhoen et al. 2006; Bettelli et al. 2006; Mangan et al. 2006; Acosta-Rodriguiz et al. 2007; Wilson et al. 2007; Yang et al. 2008a, 2008b; Yang et al. 2009; Ghoreschi et al. 2010). IL-6 receptor signaling causes the phosphorylation of STAT3, which in coordination with the transcription factor RORγt induce IL-17 expression (Fig. 3.2g–h). However, other cytokines have been implicated to enhance Th17 commitment in conjunction with IL-6, namely TGFβ in murine T cells (Figs. 3.1 and 3.2g) and TGFβ, IL-1β, and IL-21 in human T cells. Myelin-specific Th1 and Th17 cells are present in mice with EAE and MS patients, and both populations appear to mediate CNS pathology (Traugott and Lebon 1988; McDonald and

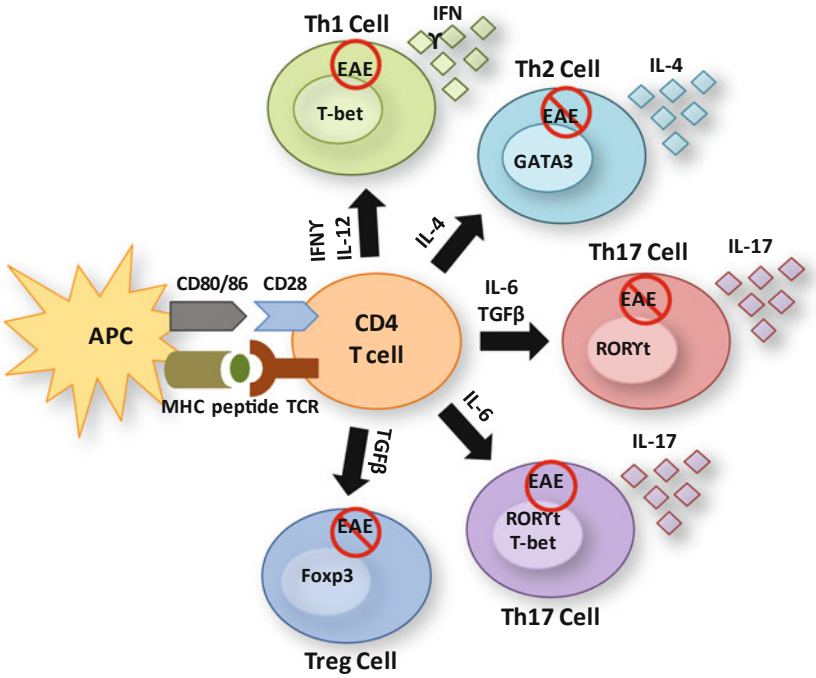

Fig. 3.1 Differentiation of CD4 T cells into effector T cells that mediate central nervous system (CNS) demyelination. Naive CD4 T cells engage antigen-presenting cells (APC) to become activated. Both MHC/peptide engagement with the T cell receptor (TCR) and costimulation mediated by CD80/86 (B7.1/B7.2) with CD28 is required to initiate T cells activation. These activated T cells have the capacity to differentiate into different T cell lineages depending on the cytokines in the local environment. IFNγ and IL-12 promote the differentiation of Th1 cells, which are primarily regulated by the transcription factor T-bet, express IFNγ, and can mediate experimental autoimmune encephalomyelitis (EAE). Th2 cells depend on IL-4 activation via the transcription GATA3 and typically are associated with EAE resistance. Th17 cells can be generated in the presence of IL-6 in the presence or absence of TGFβ; however, only Th17 generated in the absence of TGFβ are capable of causing EAE due to the negative regulation of T-bet by TGFβ. CD4 T cells can also develop into a regulatory population that limits the activation and effector functions of other CD4 T cells

Swanborg 1988; Ando et al. 1989; Waldburger et al. 1996; Yura et al. 2001; Lock et al. 2002; Lovett-Racke et al. 2004; Gocke et al. 2007; Kroenke and Segal 2007; Tzartos et al. 2008; Yang et al. 2009).

CD4-positive T cells may also develop into a regulatory population that plays a vital role in controlling and dampening of effector T cells to maintain the balance between protection and potential immune-mediated pathology (Kumar et al. 1996; Hori et al. 2003). CD4-regulatory T cells (Tregs) can develop in the thymus or in the periphery. TGFβ signaling is believed to be the critical cytokine signaling required for Treg development, which induces the expression of Foxp3, the transcription factor required for Treg development and function (Fig. 3.1; Marie et al. 2005). These four dominant populations of CD4 T cells provide molecular signals to other cells of the

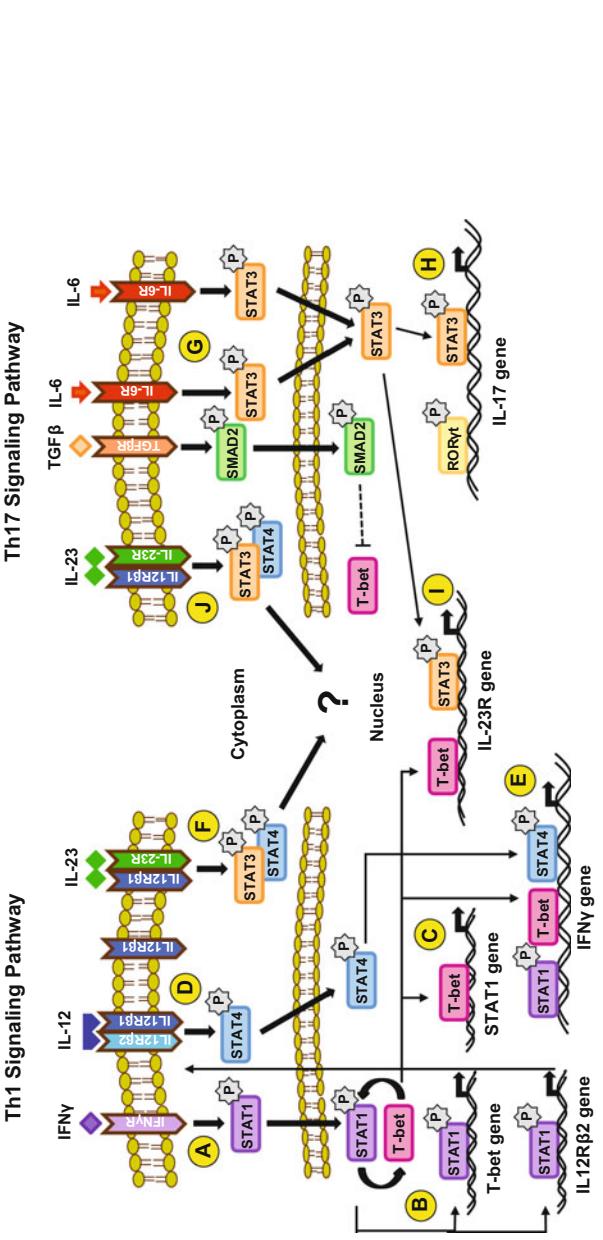

Fig. 3.2 Th1 and Th17 cells have the capacity to cause immune-mediated demyelinating disease via signaling pathways dependent on T-bet. Both Th1 and Th17 cells can cause experimental autoimmune encephalomyelitis (EAE) and both populations are present in multiple sclerosis (MS) lesions. (**a**) Th1 cells differentiate due to the initial signaling via the IFNγ receptor resulting in STAT1 phosphorylation and translocation to the nucleus whereby T-bet and STAT1 regulate each other's expression in a positive feedback loop and induce expression of the IL-12 receptor β2 chain (**b**). T-bet regulates STAT1 and IL-23 receptor expression (**c**). IL-12 receptor signaling can proceed (**d**), resulting in STAT4 phosphorylation and translocation to the nucleus, which ultimately leads to the activation of the IFNγ gene (**e**). The IL-23 receptor is upregulated during T cell differentiation (**f**), at least partially due to T-bet, and this is necessary for critical signals required for acquisition of an encephalitogenic phenotype. (**g**) IL-6 signaling during T cell activation favors Th17 development via phosphorylation of STAT3, a transcription factor that coordinates with RORγt to regulate IL-17 gene transcription (**h**). Phosphorylation STAT3 also contributes to the expression of the IL-23 receptor gene, making transcription factors commonly associated with both Th1 and Th17 cells necessary for induction of the IL-23 receptor (**i**). However, it is unknown what critical function is induced by IL-23 receptor signaling

immune system, which coordinate an immune response to protect us from pathogens and cancer. The effectiveness of our immune system is evident in that the vast majority of us live long and healthy lives. However, approximately 5 % of the population suffers from some form of autoimmunity and since autoimmunity is mediated by adaptive immune responses, a tremendous amount of research has focused on the role of CD4 T cells in the onset and progression of autoimmune diseases. Although the pathology of autoimmune diseases may be caused by auto-antibodies or cytotoxic CD8 T cells, CD4 T cells play vital roles in the development of antibodies and CD8 T cells, making CD4 T cells a potential common link among all autoimmune diseases.

3.4 Role of CD4 T Cells in Experimental Autoimmune Encephalomyelitis

EAE has been used as a model for MS research since the 1960s. The model originated from the observation that a small number of individuals who received the rabies virus vaccine, a live-attenuated vaccine grown in the CNS of rabbits, developed encephalomyelitis. Investigation into the cause of the postvaccine encephalomyelitis led to the discovery that the CNS material that contaminated the vaccine could induce a hypersensitivity reaction that resulted in an immune-mediated encephalomyelitis and the onset of symptoms reminiscent of MS (Rivers et al. 1933). EAE is typically induced in rodents by subcutaneous immunization of myelin proteins or peptides emulsified in Complete Freund's Adjuvant (CFA). However, adoptive transfer of myelin-specific CD4 Th1 cells into naive recipient mice will also result in EAE development (McDonald and Swanborg 1988; Ando et al. 1989; Waldburger et al. 1996; Yura et al. 2001; Lovett-Racke et al. 2004; Gocke et al. 2007; Yang et al. 2009), supporting the hypothesis that CD4 T cells are the primary mediator of this model autoimmune disease.

Since myelin-specific Th1 cells were sufficient to induce EAE in mice, research on the role of T cells in MS focused on Th1 cells. Several studies that reduced IFNγ in myelin-specific T cells prior to transfer to recipient mice found that changing the signaling pathways, which are necessary for Th1 cell differentiation, diminishes the encephalitogenic capacity of these myelin-specific CD4 T cells (Racke et al. 1994; Racke et al. 1995; Lovett-Racke et al. 2004). Furthermore, mice deficient in STAT4 and T-bet, two transcription factors that play vital roles in the Th1 differentiation pathway, were resistant to EAE induction, supporting the hypothesis that EAE is mediated by Th1 cells (Chitnis et al. 2001; Bettelli et al. 2004; Nath et al. 2006). To determine whether IFNγ was a potential therapeutic target, EAE experiments in mice deficient in IFNγ signaling were performed. Contrary to expectations, mice deficient in IFNγ were susceptible to EAE (Ferber et al. 1996). In addition, systemic treatment of mice with IFNγ-neutralizing antibodies did not suppress EAE (Lublin et al. 1993; Heremans et al. 1996; Willenborg et al. 1996). Together, these studies indicate that IFNγ is not essential for the development of encephalitogenic T cells in mice; however, molecules in the Th1 differentiation pathway are necessary.

The observation that CD4 Th1 cells were sufficient to cause EAE, yet IFNγ was dispensable, prompted the investigation of other cytokines in the development and function of encephalitogenic T cells. Since IL-12 was a key cytokine necessary for Th1 cell differentiation, the components of IL-12 and the IL-12 receptor were analyzed using mice genetically deficient in each subunit of the IL-12 cytokine and receptor. IL-12 is a heterodimer composed of a p40 and p35 subunit. Mice deficient in the IL-12p40 were resistant to EAE, yet mice deficient in IL-12p35 remained susceptible to EAE (Becher et al. 2002; Gran et al. 2002). Since IL-12p40 was also a component of IL-23 (Oppmann et al. 2000), EAE was evaluated in mice deficient in the IL-23-specific subunit, p19. IL-23p19-deficient mice were protected from EAE induction, similar to the IL-12p40-deficient mice, indicating that IL-23 signaling was necessary for EAE development (Cua et al. 2003). Culturing of myelin-specific T cells isolated from immunized mice with IL-23 promoted the expansion of myelin-specific IL-17-producing T cells (Langrish et al. 2005). In addition, transfer of these IL-23-expanded T cells into naive mice resulted in EAE, leading to speculation that Th17 cells were actually the primary encephalitogenic CD4 T cell population in EAE and perhaps MS. However, mice deficient in IL-17 remained susceptible to EAE, although the disease may be less severe, suggesting that IL-17 is also not necessary for the development of encephalitogenic T cells (Komiyama et al. 2006; Haak et al. 2009). These data indicate that IL-23, an APC-produced cytokine, is necessary for the development of encephalitogenic T cells. Yet, IFNγ and IL-17, the cytokines that define Th1 and Th17 cells, are not essential.

So what molecules expressed by myelin-specific CD4 effector T cells are required for encephalitogenicity? Use of genetic knock-out mice has shown that GM-CSF (Ponomarev et al. 2007; Codarri et al. 2011; El-Behi et al. 2011), STAT4 (Chitnis et al. 2001), and T-bet (Bettelli et al. 2004; Nath et al. 2006) are required for T cell encephalitogenicity. GM-CSF, a cytokine expressed by numerous cell types including macrophages, endothelial cells, and fibroblasts, is expressed by both Th1 and Th17 cells. However, STAT4 and T-bet are transcription factors that are typically associated with Th1 cells, making it unclear why deletion of these genes would result in EAE resistance if Th17 cells are sufficient to induce EAE. Suppression of T-bet with an siRNA in mice found that not only was EAE inhibited, but both Th1 and Th17 cells failed to differentiate indicating that T-bet was affecting both encephalitogenic T cell populations (Gocke et al. 2007; Yang et al. 2009). Thus, the contribution of T-bet in regulating genes in encephalitogenic T cells must extend beyond IFNγ and Th1-associated genes.

3.5 Role of T-bet in Regulating Gene Expression in CD4 T Cells

T-bet was originally described as the "master" regulator of Th1 cell differentiation because it directly regulates the expression of the IFNγ in CD4 T cells (Szabo et al. 2000). In naive CD4 T cells, the *Ifng* gene is epigenetically repressed by histone 3 lysine 4 trimethylation (H3K27me3; Fig. 3.3). Upon T cell activation and differentiation into a Th1 phenotype, T-bet physically associates with the *Ifng* promoter. This

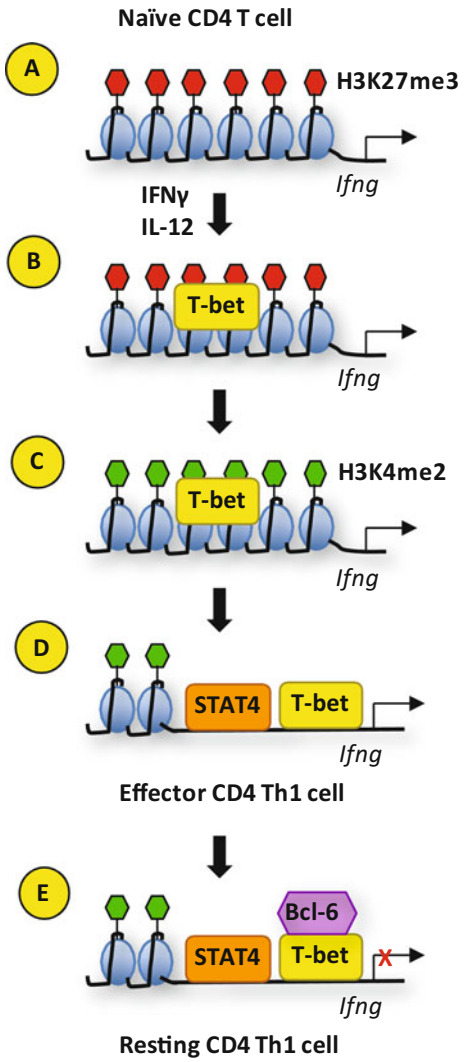

Fig. 3.3 T-bet is the critical transcription factor for the regulation of the IFNγ gene. **a** IFNγ gene is epigenetically repressed via H3K27me3 in naive CD4 T cells. **b** IFNγ and IL-12 receptor signaling induces T-bet, which recruits H3K27 demethylase complexes to the *Ifng* loci to reverse the epigenetically repressed state. **c** T-bet recruits a permissive H3K4 methyl transferase to establish a permissive epigenetic state via H3K4me2. **d** Chromatin remodeling allows Th1-associated transcription factors to bind and promote IFNγ production and the Th1 phenotype. **e** T-bet recruits the transcriptional repressor Bcl-6 to the *Ifng* loci late in Th1 cell differentiation to limit IFNγ production and return Th1 cells to a resting state

causes recruitment of H3K27 demethylase complexes to the *Ifng* loci to reverse the epigenetically repressed state (Lewis et al. 2007; Miller et al. 2008). T-bet subsequently recruits a permissive H3K4 methyl transferase to establish a permissive epigenetic state via H3K4me2, resulting in chromatin remodeling which allows T-bet and STAT4 to bind to the *Ifng* promoter. Typically within 3 days of activation, a naive CD4 T cell will express IFNγ and commit to a Th1 lineage. However, T-bet also plays a repressive role in Th1 cells by recruiting Bcl-6 to the *Ifng* loci late in Th1 cell differentiation (Oestreich et al. 2011). This results in reducing IFNγ production and return to a resting state of Th1 cells, probably to limit the effector function of Th1 cells, minimize tissue damage, and reduce the potential for autoimmunity.

T-bet-deficient mice are resistant to EAE development (Bettelli et al. 2004; Lovett-Racke et al. 2004; Nath et al. 2006), while IFNγ-deficient mice remain susceptible to EAE (Ferber et al. 1996). This suggests that T-bet must regulate genes other than *Ifng* that are necessary for EAE development. Since it had been shown that suppressing T-bet not only reduced Th1 cells but also Th17 cells in mice with EAE, T-bet may be regulating gene(s) common to both Th1 and Th17 cells (Gocke et al. 2007; Yang et al. 2009). Using chromatin immunoprecipitation assays, as well as overexpression of T-bet in cell lines, T-bet was found to positively regulate the expression of the *Il23r* gene (Gocke et al. 2007). It had previously been observed that IL-23 and IL-23 receptor-deficient mice were resistant to EAE suggesting that IL-23 receptor signaling is a vital component of EAE regardless of whether it is mediated by Th1 or Th17 cells (Zhang et al. 2003; Cua et al. 2003). Prior to the discovery of Th17 cells, it had been shown that IL-23 receptor was expressed on activated T cells that were largely of a Th1 phenotype (Oppmann et al. 2000; Parham et al. 2002), so it is feasible that IL-23 may affect both Th1 and Th17 cells. In addition, IL-23 had been shown to promote the expansion of encephalitogenic Th17 cells (Langrish et al. 2005), while neutralizing IL-23 can ameliorate EAE (Chen et al. 2006). It was also shown that IL-23 produced by microglia and CNS-infiltrating macrophages was necessary for the onset of EAE, indicating that myelin-specific T cells required IL-23 receptor expression to mediate demyelination in the CNS (Becher et al. 2003). Using primary microglia cultures, it was found that IFNγ enhanced IL-23 expression, supporting the hypothesis that CNS-infiltrating Th1 cells may contribute to expansion of Th17 cells in the CNS via their production of IFNγ. Similarly, Th17 cells are found in the CNS of mice with EAE induced by adoptive transfer of myelin-specific Th1 cells (Gocke et al. 2007; Lees et al. 2008). Thus, T-bet appears to be critical for EAE due to its role in upregulating the expression of the IL-23 receptor during T cell activation and differentiation.

It had been shown that CD4 T cell lineage commitment is determined by the interaction of T-bet and GATA-3. T-bet represses Th2 cell differentiation by a tyrosine kinase-mediated interaction with GATA-3 (Hwang et al. 2005). Since encephalitogenic CD4 T cells could be both Th1 and Th17, this led to the intriguing question as to what was the role of T-bet in encephalitogenic Th17 cells, beyond IL-23 receptor expression. As previously mentioned, silencing T-bet in wild-type mice with EAE results in fewer Th17 cells. However, induction of EAE in T-bet-deficient mice results in a significant population of myelin-specific Th17 cells, yet these T-bet- deficient mice fail to develop EAE (Yang et al. 2009). This suggests that T-bet is critical in the development of encephalitogenic Th17 cells, but not in the development of CD4 T cells that are capable of expressing IL-17. Several studies showed that naive murine T cells could be differentiated into Th17 cells with IL-6 and TGFβ (Fig. 3.1; Veldhoen et al. 2006; Bettelli et al. 2006; Mangan et al. 2006). However, these differentiation conditions failed to generate Th17 cells that could induce EAE when transferred into wild-type mice, suggesting that the in vivo conditions that generate encephalitogenic T cells must be different (Yang et al. 2009; Ghoreschi et al. 2010). It was subsequently shown that differentiation of naive T cells with IL-6, in the absence of TGFβ and cytokines that promote Th1 or Th2 cells, generates Th17 cells capable of transferring EAE (Fig. 3.1; Yang et al. 2009; Ghoreschi et al. 2010). This observation

was consistent with a previous study that found that TGFβ is a negative regulator of T-bet (Gorelik et al. 2002; Park et al. 2005). Therefore, Th17-inducing conditions that included TGFβ-generated T cells failed to express T-bet. Although these T cells expressed significant amounts of IL-17, they were T-bet-negative and lacked a critical element of an encephalitogenic T cell. However, encephalitogenic T cells from wild-type mice had T-bet-positive Th17 cells, and Th17 cells differentiated in vivo with IL-6 in the absence of TGFβ also expressed T-bet, indicating that T-bet was a critical factor in encephalitogenic T cells regardless of whether they expressed a Th1 or Th17 phenotype (Yang et al. 2009).

T-bet has been implicated in the regulation of other molecules associated with the Th1 phenotype and encephalitogenicity. For example, T-bet has been shown to be required for optimal migration of Th1 cells into tissues. T-bet appears to regulate the binding of Th1 cells to P-selectin, an adhesion molecule expressed on inflamed endothelium (Lord et al. 2005). T-bet has also been found to regulate the chemokine receptor CXCR3, which is highly expressed on encephalitogenic Th1 cells (Qin et al. 1998; Sallusto et al. 1998; Beima et al. 2006). This led to speculation that CXCR3 may be vital to T cell trafficking to the CNS and thus, T-bet may be critical due to its role in upregulating CXCR3 expression on CD4 T cells. Although CXCR3 inhibitors have been shown to slightly diminish EAE severity (Kohler et al. 2008; Ni et al. 2009; Sporici and Issekutz 2010), CXCR3-deficient mice remain susceptible to EAE (Liu et al. 2006; Muller et al. 2007) indicating that CXCR3 is not critical to EAE development and, therefore, it is not an essential element regulated by T-bet. This indicates that the diminished trafficking capacity of T-bet-deficient CD4 T cells is probably mediated by a mechanism independent of CXCR3.

CD80/86 on APCs interaction with C28 on T cells is necessary to activate naive T cells in conjunction with TCR engagement (Fig. 3.1). However, memory and effector T cell populations do not require this costimulation signal via CD28, making it possible for memory and effector T cells to function in a rapid and precise manner (Dubey et al. 1996; Yi-qun et al. 1996; Lovett-Racke et al. 1998). More recently, it was discovered that there were other costimulatory molecules, such as inducible costimulator (ICOS) expressed on memory and effector T cells that contribute to their effector function. Inhibition of ICOS after the induction of EAE attenuated the disease (Rottman et al. 2001; Sporici et al. 2001). Ex vivo analysis of the CNS of these mice showed that the myelin-specific CD4 T cells became activated, produced IL-2, and trafficked to the CNS. However, IFNγ production was diminished and enhanced the apoptosis of memory T cells. T-bet was found to bind the ICOS promoter to regulate ICOS expression in conjunction with the transcription factor, NFATc2 (Tan et al. 2008). Since T-bet is not expressed in naive T cells and only expressed following T cell differentiation, the induction of ICOS in memory and effector T cells is temporally consistent with T-bet expression. In human T cells, ICOS was found to dramatically enhance the expansion of IFNγ + IL-17 + T cells, which have been found to have enhanced proinflammatory effector function in EAE and other diseases (Paulos et al. 2010). Interestingly, these cells also express high levels of T-bet, suggesting that T-bet may be enhancing the pathogenic potential of IFNγ + IL-17 + T cells via ICOS costimulation.

In 2001, a large-scale sequencing of cDNA libraries acquired from CNS lesions from MS patients found enhanced expression of osteopontin, a cytokine previously associated with bone formation (Chabas et al. 2001). Rats with EAE were also found to have enhanced osteopontin expression in their CNS. Although mice deficient in osteopontin develop EAE, the disease is less severe, had fewer relapses, and diminished proinflammatory cytokine expression (Chabas et al. 2001; Jansson et al. 2002). T-bet was found to at least partially regulate osteopontin expression, and T-bet-dependent osteopontin expression is necessary for optimal differentiation of Th1 cells (Shinohara et al. 2005). Osteopontin was subsequently found to initiate recurrent relapses and enhance neurological deficits in mice with EAE via enhanced survival of myelin-specific T cells (Hur et al. 2007). T-bet had originally been considered a transcription factor, critical in the initial differentiation of CD4 T cells, but this study indicates that T-bet function in effector and memory T cells via ICOS and osteopontin may be just as important in demyelinating autoimmunity. This was validated in treatment studies of EAE with an siRNA specific for T-bet in which disease was virtually halted in mice when T-bet was suppressed (Gocke et al. 2007). Furthermore, osteopontin levels are elevated in the CSF and serum of MS patients, and elevated osteopontin in the CSF appears to be correlated with relapses (Bornsen et al. 2011; Wen et al. 2012), reminiscent of elevated T-bet levels in MS patients (Frisullo et al. 2009; Kleiter et al. 2010).

T cell Ig- and mucin-domain-containing molecule 3 (Tim-3) was found to be expressed by Th1 cells and blockade of Tim-3 in EAE results in enhanced disease severity, suggesting that Tim-3 plays a role in limiting the effector function of Th1 cells (Monney et al. 2002; Khademi et al. 2004). Tim-3 was also found to be expressed on resident CNS cells in rats with EAE, suggesting that it may contribute CNS autoimmunity in the target organ as well as the infiltrating T cells (Gielen et al. 2005). T cell clones from the CSF of MS patients found that Tim-3 and T-bet were reduced compared to clones derived from control subjects, and that the MS patient T cells had enhanced production of IFNγ suggesting that Tim-3 inversely correlates with IFNγ expression (Koguchi et al. 2006). Using an siRNA specific for Tim-3, T cell proliferation and IFNγ production were reduced, demonstrating the Tim-3 expression plays a significant role in Th1 cell function. CSF T cell clones with low Tim-3 levels also demonstrated resistance to inhibitor signals via CTLA-4, suggesting that their enhanced effector functions may be partially due to diminished regulation (Koguchi et al. 2006). Blockade of Tim-3 ex vivo in MS patients' CD4 T cells did not alter IFNγ production as it did in control subjects, demonstrating a defect in Tim-3 immunoregulation (Yang et al. 2008a, 2008b). Interestingly, glatiramer acetate or IFNβ treatment reversed the functional defect. Thus, dysregulated expression of Tim-3 in MS patients may contribute to disease pathology, and current immunomodulatory drugs may be beneficial at normalizing immune dysregulation mediated by Tim-3. T-bet was found to contribute to the expression of Tim-3 in Th1 cells (Anderson et al. 2010). Since Tim-3 plays a role in dampening Th1 responses, it appears that T-bet-regulated expression of Tim-3 plays a role in not only inducing Th1 responses, but also regulating them in a manner that limits Th1 effector functions. Since MS patients appear to have diminished Tim-3 expression in effector/memory

T cells, the balance of protective adaptive immune responses may be tipped such that proinflammatory T cells go unregulated for extended periods of time resulting in autoimmune tissue damage.

Together, these studies demonstrate that T-bet regulates the expression of several genes that play different roles in T cells' differentiation and effector functions. T-bet may be an effective therapeutic target in EAE due to its ability to modulate different mechanisms in T cells that are required for development, trafficking, and pathogenesis in immune-mediated demyelinating disease.

3.6 Relevance of T-bet in Multiple Sclerosis

It is clear that T-bet is an essential factor in encephalitogenic T cells in mice. Yet, its role in MS is much more difficult to assess. However, changes in T-bet levels have become a common measure of therapeutic potential of drugs evaluated in EAE and MS patients. T-bet levels in peripheral T cells are enhanced in MS patients during relapses relative to remission and are elevated in MS patients compared to healthy controls (Frisullo et al. 2006; Kleiter et al. 2010). In addition, pSTAT1, the transcription factor that is initially activated during Th1 cell differentiation (Fig. 3.1a) is also upregulated. Since pSTAT1 contributes to the expression of T-bet (Afkarian et al. 2002; Lovett-Racke et al. 2004), it is not surprising that T-bet and pSTAT1 levels have a positive correlation. In addition, T-bet and pSTAT1 levels correlated with active lesions in the CNS as determined by MRI, suggesting that T-bet may be a marker of disease activity in MS. Glucocorticoids, which have anti-inflammatory properties, have been used successfully to treat MS exacerbations for decades. Ex vivo analysis of T cells from MS patients treated with glucocorticoids found that T-bet and pSTAT1 were reduced, as well as IFNγ production by peripheral lymphocytes (Frisullo et al. 2007), indicating that T-bet may be a potential biomarker of therapeutic efficacy.

During pregnancy, MS patients have a reduced exacerbation rate compared to pre- and postpregnancy (Korn-Lubetzki et al. 1984). The mechanisms that underlie this protection probably lie in the immune-tolerant state that is induced to protect the fetus. Interestingly, relapse rates in women postdelivery are often enhanced but typically return to prepregnancy levels within 1 year. In pregnant MS patients, T-bet levels are reduced, consistent with an anti-inflammatory or tolerant state (Iorio et al. 2009). However, in MS patients who have a relapse after delivery or have new active lesions, T-bet and pSTAT1 levels are enhanced, as well as IFNγ and IL-17 levels. In addition, there is an increase in CD4+ CD25+ Foxp3+- regulatory T cell population during pregnancy that may be suppressing the T-bet + T cells. Again, this study indicates that T-bet may be a marker of disease activity in MS.

For the past two decades, IFNβ has been used as a therapy for relapsing–remitting MS. In a study to determine whether Th1 or Th17 cells were altered due to IFNβ therapy, IFNγ and T-bet mRNA levels were reduced at 1-year postinitiation of IFNβ therapy, while IL-17 and RORγt (the Th17-associated transcription factor) were

unchanged (Drulovic et al. 2009). Analysis of IFNβ responders versus nonresponders found that reduced T-bet levels correlated with a favorable clinical response to IFNβ treatment, further supporting the role of T-bet as a critical molecule in MS pathogenesis and as a potential indicator of therapeutic efficacy.

More recently, T-bet expression has been evaluated in clinical trials of new potential therapies for MS. Statins, 3-hydroxy-3-mehtylglutaryl coenzyme-A reductase inhibitors that have traditionally been used to reduce cholesterol, have been found to have immunomodulatory effects. Due to their widespread use and good safety record, they have been evaluated in MS as potential therapies. In clinical trials, ex vivo analysis revealed that MS patients on statins had reduced T cell proliferation, reduced proinflammatory cytokine expression and reduced T-bet expression, as well as significant clinical benefit to the patients (Peng et al. 2006).

Although there is evidence that T-bet levels correlate with disease activity and treatment efficacy in MS patients, it is still unclear whether T-bet is aberrantly expressed in MS patients, perhaps making them more susceptible to proinflammatory responses and the development of autoimmunity. MS patients have an increased incidence of other Th1-mediated autoimmune diseases such as diabetes, thyroiditis, and psoriasis, suggesting that they may possess a bias toward robust Th1 responses that may make them susceptible to Th1-mediated autoimmune diseases (Roquer et al. 1987; Karni and Abramsky 1999; Sloka 2002; Annunziata et al. 2003; Nielsen et al. 2006). A comprehensive analysis of miRNA expression in naive CD4 T cells of MS patients suggested that MS patients may have an inherent propensity to differentiate into Th1 cells due to loss of miRNA-mediated regulation of genes in the Th2 pathway (Guerau-de-Arellano et al. 2011). Since miRNAs negatively regulate gene expression, enhanced miRNA expression will result in decreased target gene expression. It was found that MS patients have enhanced expression of miR-27, miR-128, and miR-340 in CD4 T cells, resulting in decreased expression of Bmi1, GATA3, and IL-4. Since GATA3 and T-bet compete for lineage commitment during activation of naive T cells, reduced GATA3 leads to a dominant Th1 response. This study suggests that T-bet may be aberrantly expressed in CD4 T cells of MS patients due to miRNA-mediated suppression of Th2-associated molecules.

Several studies found that T-bet and IFNγ expression were also regulated by miRNA (Ma et al. 2011; Steiner et al. 2011; Smith et al. 2012). IFNγ induces the expression of miR-29, as well as T-bet, in CD4 T cells (Smith et al. 2012). T-bet directly regulates the expression of IFNγ, committing the CD4 T cell to the Th1 lineage. However, miR-29 subsequently binds to the mRNA of T-bet and IFNγ, demonstrating that a negative feedback loop exists as an additional mechanism to maintain a proper balance of IFNγ and to dampen effector Th1 responses appropriately following an inflammatory response. In resting memory, CD4 T cells of MS patients, T-bet, and miR-29 levels are increased, compared to control subjects (Smith et al. 2012). However, activation of MS patient memory CD4 T cells results in a significant decrease in miR-29 levels indicating that the feedback loop between miR-29 and T-bet/IFNγ is dysregulated in MS patients. The diminished capacity of miR-29 to regulate T-bet levels may be another mechanism that may promote chronic inflammation in MS patients.

3.7 Is T-bet a Valid Therapeutic Target for Multiple Sclerosis?

The T-bet-regulated gene that has been studied the most extensively is IFNγ. The contradictory data on the role of IFNγ in EAE and MS suggest that care must be taken on how therapies may systemically alter IFNγ levels. So is T-bet a valid therapeutic target in light of the data of EAE when IFNγ is suppressed? The observation that IFNγ-deficient mice remained susceptible to EAE (Ferber et al. 1996), and often had a more severe disease course than wild-type controls, led to caution about using drugs that may dramatically effect IFNγ levels. The EAE data in IFNγ-deficient mice and mice treated with antibodies to neutralize IFNγ suggested the IFNγ may play a positive regulatory role in the disease course (Lublin et al. 1993; Ferber et al. 1996; Heremans et al. 1996; Willenborg et al. 1996). However, there were two small clinical trials in MS patients that may suggest that inhibiting IFNγ in humans may be beneficial. In the 1980s, it was recognized that cytokines regulate immune responses and it was hypothesized that IFNγ may be beneficial in normalizing the aberrant immune responses observed in MS patients. IFNγ was administered to MS patients, resulting in an increased number of clinical exacerbations in the patients (Panitch et al. 1987). Subsequently, it was observed that IFNγ induces apoptosis of human oligodendrocytes, the cells that make myelin, and IFNγ expression in MS lesions colocalizes with apoptotic oligodendrocytes (Vartanian et al. 1995; Baerwald and Popko 1998), suggesting that IFNγ may play a role in the demyelination observed in the CNS of MS patients. These observations led to a clinical trial in which an IFNγ-neutralizing antibody was administered to MS patients, resulting in a clinical benefit (Skurkovich et al. 2001). Due to the contradictory data between mice and humans, systemic blockade of IFNγ has not continued in humans. However, the beneficial observations in humans suggest that the Th1 pathway is a viable therapeutic target.

When T-bet is genetically deleted or when T-bet is suppressed systemically with siRNA, mice are resistant to EAE induction, supporting T-bet as an effective therapeutic target (Bettelli et al. 2004; Lovett-Racke et al. 2004). Moreover, administration of a T-bet siRNA after the onset of EAE suggests that T-bet is a potential therapeutic target for established autoimmune demyelinating disease (Gocke et al. 2007). This is a critical point since we cannot predict who will develop MS and, therefore, must develop therapeutics that can prevent the development of autoreactive T cells, as well as suppress the effector/memory T cells that are already mediating lesion formation in MS patients. Furthermore, in MS patients treated with nonspecific immunomodulatory drugs, T-bet is reduced in patients who have clinical benefit (Drulovic et al. 2009).

Although T-bet is expressed in most immune cells, its role in EAE appears to be primarily mediated by CD4 T cells. In addition, T-bet regulates IFNγ in CD4 T cells, but does not appear to regulate IFNγ expression extensively in other immune cells (Szabo et al. 2000; Szabo et al. 2002). Therefore, suppression of T-bet does not result in systemic loss of IFNγ production and any positive effects of IFNγ that may be mediated by other immune cells would not be dramatically affected by suppression of T-bet. Thus, targeting T-bet may have far fewer potential side effects compared to global suppression of IFNγ.

T-bet and GATA3, the Th2- associated transcription factor, physically associate and compete to dominate lineage-specific genes in order to generate Th1 or Th2 cells (Hwang et al. 2005; Jenner et al. 2009). There is concern that suppression of T-bet may make individuals susceptible to Th2-mediated diseases, since GATA3 will not be as tightly regulated in the absence of T-bet. T-bet-deficient mice develop lung pathology consistent with allergic asthma (Finotto et al. 2002), a classic Th2-mediated disease. However, in mice treated with a T-bet siRNA, no lung pathology was observed, suggesting that diminishing T-bet expression, as opposed to complete abrogation of T-bet, may not have the same outcome (Lovett-Racke et al. 2004). The observation that MS patients have a decreased incidence of allergy and asthma is evidence that their immune responses may be biased away from Th2 responses (Bergamaschi et al. 2009; Pedotti et al. 2009). Thus, reducing T-bet expression in MS patients may simply normalize immune responses such that proinflammatory T cell effector functions are reduced, but not such that Th2- mediated diseases are a real threat.

3.8 Conclusion

MS is a complex disease that involves both inflammation and neurodegeneration. Since the inflammation appears to precede pathology and neurological deficits, a tremendous amount of research has been devoted to understanding the inflammatory process that is occurring at the site of lesion formation. In this quest, effector CD4 T cells have been found to be at the center of the immune response that results in demyelination and axonal damage. Why and how CD4 T cells become programmed to enter the apparently healthy CNS and mediate damage is unclear. However, T-bet is one molecule that appears to be associated with the capacity of CD4 T cells to cause CNS injury. Due to its relatively limited expression in humans, it may be a viable therapeutic target for MS. In addition, defining the role of T-bet in generating encephalitogenic T cells and their effector functions provides insight into the mechanisms that underlie the pathology of MS lesions.

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