Clarifying the Critical Factors for Th1 and Th17 Pathogenicity in an Animal Model of CNS-Targeted Autoimmune Disease

by

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DEDICATION

I have chosen to dedicate the rest of my life to my son David and my husband Seth.
This body of work is dedicated to my parents.

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Introduction

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Immune surveillance permits a host to identify and sequester invading pathogens or foreign materials. Defense against a spectrum of pathogenic microbial infections requires the activity of a diverse repertoire of immune cells, which can be broadly divided into innate and adaptive components. Innate immune cells directly sense and ingest pathogens and produce pro-inflammatory cytokines and antimicrobial agents, including TNF-α, IL-1, IL-6, nitric oxide (NO), antimicrobial peptides, and reactive oxygen species (ROS). Innate immune cells respond to conserved pathogen-associated molecular patterns expressed on the surface of bacteria and create a hostile environment for infection, either by production of proinflammatory cytokines or chemokines that recruit more inflammatory cells. Innate immune cells include myeloid cells, including monocytes, macrophages, granulocytes, and dendritic cells, as well as innate lymphoid cells. In contrast to the non-specific innate response that recognizes non-specific pathogens,

adaptive immunity results in an antigen-specific response and the formation of memory, allowing a long-lasting, highly efficient response to subsequent infection. Adaptive immune cells include T lymphocytes (CD4+ and CD8+ cells) and B lymphocytes, which express unique antigen recognition receptors on their cell surface. The receptor expressed on T cells is referred to as the T cell receptor (TCR). Ideally, the TCR is specific to peptides unique to foreign pathogens presented by APCs in the context of a major histocompatibility complex (MHC) molecule. Briefly, APCs phagocytose pathogens or their components, process them internally to form a peptide-MHC complex, and present the complex on the APC surface. A T cell expressing a unique TCR that recognizes that particular peptide interacts with the peptide MHC complex as well as costimulation molecules expressed by the activated APC to activate the T cell itself. Activated T cells produce effector cytokines and chemokines to promote inflammatory cell activation and recruitsment to the site of infection. CD8+ T cell TCRs are triggered by peptides that are complexed with MHC Class I (MHC I) molecules, which are expressed by all cells other than erythrocytes. CD8+ T cells directly kill cells that present cognate antigen in the context of MHC I. CD4+ T cells are triggered by peptides complexed with MHC Class II molecules, which are exclusively expressed by “professional” APCs, and include dendritic cells, macrophages, and B cells. CD4+ T cells are considered T helper cells (Th), as they function as potent innate immune cell activators and contribute to B cell maturation and antibody class switching.

Ligation of the TCR with the peptide-MHC complex, in combination with co-stimulatory molecule interactions and cytokines expressed by APCs, expands and differentiates naïve T cells along different lineages toward the formation of effector and memory cells. While effector cells produce high levels of proinflammatory cytokines at the site of infection, CD4+ and CD8+ memory T cells have a lower threshold for activation than naïve cells and are poised to expand rapidly and execute effector functions upon re-encounter with cognate antigen. Antigen-experienced memory T cells can be divided into effector memory and central memory based on their cytokine function and expression of activation and homing molecules on their surface. Central memory T cells reside in secondary lymphoid tissues, produce low amounts of cytokines, and proliferate following antigen exposure. Conversely, effector memory cells, which may develop from central memory cells, are found in non-lymphoid tissue, produce large amounts of pro-inflammatory effector cytokines, and proliferate poorly. Both memory T cell populations within CD4+ and CD8+ lineages require less co-stimulation from APCs and innate cytokines, and thereby, induce a more rapid and effective memory response upon subsequent exposure to the previously encountered antigen [1]. Following reactivation, T cells rapidly produce cytokines with pleiotropic effects that are best-suited for the defense against a particular pathogen, including induction of chemokines and adhesion molecules and stimulation of myeloid cells. CD4+ T cells differentiate into functionally distinct subsets that dictate the nature of that inflammatory response.

CD4+ Th lineages

T cell polarization is driven by cytokines produced by activated innate and B cells [2]. APCs respond to different types of stimuli via receptors that recognize particular pathogen-associated molecular patterns, including the bacterial surface protein lipopolysaccharide and virally associated double-stranded RNA. The cytokines produced by the APCs and other innate cells create an environment that, when encountered by activated CD4+ T cell, can promote T cell polarization toward effector function. Divergence toward separate CD4+ T cell populations leads to highly effective and specific pathogen clearance following activation and rechallenge. CD4+ T cell lineages are characterized by the cytokines required for their polarization, the transcription factors that coordinate their development and function, their hallmark effector cytokines produced, and the environment that results from their activity.

Th1 and Th2 cells

Mossman and Coffman originally described two unique CD4+ populations as T helper 1 (Th1) and Th2 [3]. Th1 cells are marked by their expression of IFN-γ, while Th2 cells express IL-4, IL-5, and IL-13. Following clonal expansion, Th1 and Th2 cells maintain hallmark cytokine production upon rechallenge long-term, demonstrating that they are divergent and stable lineages. Th1 cells develop following exposure to IFN-γ and IL-12p70 (IL-12), which are expressed by innate cells in response to specific pathogen-associated molecular patterns, such as extracellular bacteria [4]. IL-12 is produced by macrophages and dendritic cells

and is a heterodimer made of the IL-12p35 and IL-12p40 subunits. IL-12 signals through the IL-12 receptor (IL-12R), a heterodimer formed by IL-12Rβ1 and IL-12Rβ2 [5]. IL-12R is expressed following IFN-γ receptor engagement on CD4+ T cells in combination with TCR stimulation with antigen complexed with MHCII and co-stimulatory molecule interactions [6]. Signaling via the IFN-γ receptor promotes the activity of T-bet, a transcription factor considered to be the "master" transcription factor for Th1 cells directly regulating the expression of IL-12Rβ2 [4, 7, 8]. IL-12R engagement promotes the phosphorylation of signal transducer and activator of transcription (STAT) 1 and STAT4 to further enhance the Th1 program [9]. IFN-γ then acts as the defining cytokine and effector molecule for Th1 and functions to activate macrophages, monocytes, and other inflammatory cells to promote phagocytosis, lysosomal degradation, oxidative species production, and pathogen clearance.

Th2 cells, on the other hand, are defined by IL-4 production and are polarized following exposure to IL-4 and antigen presentation by basophils as well as other APCs. Th2 cells express the transcription factor GATA3 to promote their transcriptional program. The signature cytokine expressed by Th2 cells is IL-4. [10-14].

Following the description of Th1 and Th2 cells, studies comparing the two cell types determined that different effector CD4+ T cell lineages were more or less effective at responding to certain antigens, owing to the cytokine milieu produced

following antigen encounter. For example, *Leishmania major* infection is controlled by Th1 cells in an IFN-γ-dependent manner. Mouse strains such as C57BL/6, which naturally mount a robust Th1 response, are able to clear and survive *L. major* infection. Other strains, such as BALB/c, have a bias toward Th2 differentiation, cannot properly clear the infection and thus are susceptible to disease [15, 16]. Conversely, parasites including helminths are known to stimulate Th2 responses and require IL- 4 production by CD4+ T cells for their clearance. Th2 cells and their signature cytokines have also been found in allergic and atopic settings. These studies and others established a paradigm whereby divergent and stable CD4+ T cell subsets, defined by their polarizing environments, transcriptional programs, and effector cytokine production mediate immune responses to clear specific types of pathogens.

Th17 Cells

More recently, several other Th lineages have been described. The most comprehensively understood pro-inflammatory CD4+ T cell subset outside of the Th1/Th2 lineages is T helper 17 (Th17) cells, named for their expression of IL-17A and other IL-17 isoforms [17, 18]. IL-17 binds to its receptor on inflammatory cells like neutrophils to promote their activation and recruitment. Th17 development is promoted by IL-6, TGF-β, IL-21, IL-1, and IL-23, which are produced by innate cells following exposure to microbes including extracellular bacteria. IL-23 is similar to IL-12 in that both cytokines share the IL-12p40$^{-/-}$ subunit. IL-23 however expresses a unique IL-23p19 subunit. Control of IL-23p19

and IL-12p35 are tightly controlled at the transcriptional level to ensure proper activation of either Th17 or Th1 cells, respectively. The transcriptional program of Th17 cells is coordinated by STAT3 phosphorylation and ROR-γt, the key regulator of the Th17 transcription. ROR-γt promotes the expression of IL-17, which then promotes recruitment and activation of innate proinflammatory cells and expression of other cytokines, chemokines, and antimicrobial factors [19-21]. Due to its effectiveness at regulating extracellular bacterial infections, Th17-mediated immunity has been shown to be critical at mucosal and barrier sites [23].

Other T cell Populations

Regulation of the inflammatory and immune response following infection is crucial for preventing collateral damage to the host. Moreover, the regulatory response is required for suppression of autoimmunity. CD4+ regulatory T cells (Tregs) are found systemically and can be rapidly recruited to sites of infection. The exact mechanism of Tregs is unclear; however they are known to suppress proliferation and cytokine production of activated CD4+ Th cells. Tregs produce IL-10 and other soluble factors to promote a suppressive environment in certain settings and express surface molecules, such as CTLA-4, to regulate the activation of APCs [24]. Treg transctiption is considered to be controlled by the transcription factor FoxP3 and Tregs express no Th1, Th2, or Th17 hallmark cytokines, nor IL-2.

Several other types of CD4+ T cell populations have been described, but are less defined or outside of the scope of this dissertation. These include Th9 cells, which are found in settings of parasitic helminth infection. Additionally, T follicular helper cells (Tfh) promote antibody production within B cell follicles. Tfh cells express IL-21, but most importantly they function to support B cells by providing T cell help at germinal centers, allowing for proper B cell activation, maturation, and antibody-mediated immune function [24].

Classification of different CD4+ effector populations into distinct lineages is convenient for determining factors that effectively mediate the immune response against various pathogens. However, recent data has demonstrated that in certain environments, T cells become unstable and acquire functions associated with other lineages.

CD4 Th Plasticity

A feature of several of the Th lineages that lie outside of the classic Th1/Th2 paradigm is the relatively newly described feature of plasticity, particularly in the Th17 subset. Th17 cells have been shown to down regulate IL-17 and begin to exhibit properties of other Th lineages, including Th1 and Tfh cells, while Th1 and Th2 cells are typically considered to be stable and maintain their signature cytokine and transcriptional program [25]. This is perhaps due in part to epigenetic regulation of histone modification signatures in critical Th lineage genes. Wei et al. demonstrated that in Th17 cells, the loci encoding Th17-

associated genes like *rorc* and *il17* are poised in a “permissive” histone methylation state, with histone ^{3}H trimethylated at lysine 4 (H3K4me3), which allows DNA to become less tightly bound to the nucleosome and promotes gene expression [26]. Similarly, in Th1 cells, the *tbx21* and *ifng* loci, which encode the Th1 factors T-bet and IFN-γ, respectively, include permissive H3K4me3 signatures. However, in Th17 cells, histone modifications at the *tbx21* and other lineage-associated loci are “bivalent,” or contain both repressive H3 trimethylation at lysine 27 (H3K27me3), as well as activating H3K4me3 modifications. This is thought to contribute to the plasticity seen in Th17 cells, since Th1 and Th2 cells demonstrate fewer bivalent histone modification signatures at their associated loci, possibly allowing for their stability [26].

Th17 plasticity was first demonstrated *in vitro* using populations of highly polarized IL-17-producing T cells. Following a primary stimulation in the presence of TGF-β and IL-6, Th17 cells rechallenged in the presence of IL-23 or IL-12 downregulated IL-17 and upregulated IFN-γ at the population level [27]. This was shown to be T-bet- and STAT4-dependent. Furthermore, transfer of Th17-polarized cells resulted in the accumulation of IFN-γ- producing T cells at sites of inflammation in a colitis model [27]. Other groups have demonstrated Th17 plasticity *in vivo* in the context of tumor and diabetes models [28]. In 2011, Hirota *et al.* developed a fate mapping IL-17A reporter mouse to track the fate of cells that at any time expressed IL-17. They demonstrated that CD4+ T cells that expressed IL-17A could down regulate IL-17 and up regulate IFN-γ *in vivo* [29]. These cells were termed “exTh17”. Indeed, at sites of inflammation, a high

frequency of IFN-γ-producing CD4+ T cells, originally thought to be Th1 cells based on signature cytokine production, were determined to be exTh17 cells. The development of exTh17 cells was attributed to their T-bet expression and was dependent on IL-23 signaling [29].

These observations raised the following questions: first, is the conversion of a Th17 cell to an exTh17 critical (or detrimental) for Th17-mediated immunity; and second, can IFN-γ-producing Th1 cells can promote inflammation independently of initial Th17 programming? More recent studies determined using IL-17A fate-mapping reporter mice that Th17 cells are not only capable of becoming IFN-γ producers, but can also develop a Tfh-like phenotype in the Peyer's patches and are critical for IgA responses in the gut [30]. Several other examples of CD4 plasticity have been described, including in Treg and Tfh lineages, though the function and relevance of those cells is somewhat controversial [31-36]. In any case, the observation that a CD4+ effector cell lineage can convert into another brings into question the contribution of either cell type separately.

CD4+ T Cells in Autoimmune Disease

CD4+ T cells and other adaptive immune cells are obviously critical for clearance of foreign pathogens, but they can become pathogenic in the setting of autoimmunity. Typically, during the process of negative selection in the thymus, CD4+ T cells with receptors that recognize host-derived (self) antigens are identified and eliminated. However, many individuals have auto-reactive T cells

that escape the thymus. If self-reactive cells are allowed to escape, they either remain ignorant to their self-antigens or are tightly regulated by Tregs and other suppressive mechanisms that induce tolerance. However, if these regulatory mechanisms fail, or if self-antigens are presented in the context of danger, autoimmunity can occur [37]. During T cell-mediated autoimmunity, self-reactive T cells see their cognate auto-antigen presented by MHC molecules and mediate a proinflammatory immune response mediated by macrophages, DCs, and other cells that results in immune complex formation, tissue damage, and illness. Autoimmune diseases can be systemic, as in the case of systemic lupus erythematosus, in which antibodies specific for widespread antigens from DNA and nucleoproteins induce disease. Autoimmune disease can also be tissue-specific and target a single organ or tissue-type, as in Type I Diabetes, in which T cells react against insulin-producing pancreatic islet cells to cause diabetes, or as in multiple sclerosis, described in detail below. Although the exact cause of most autoimmune diseases is unknown, several studies have detailed the presence of autoreactive CD4+ T cells in healthy individuals. It can then be assumed that in the context of autoimmune disease, these cells are either improperly regulated or improperly activated by cytokines in their environment and in turn express factors to promote inflammation. Understanding the factors that are required for CD4+ T cell pathogenicity in autoimmune disease, either in the signals required for their generation or the signals they produce to promote their pathogenic effector functions, could offer insight into identification of possible therapeutic targets.

Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS with genetic and environmental associations. Familial studies as well as geographic and ethnic population studies have demonstrated increased risk for disease among close family members of MS patients as well as in certain ethnic demographics [38]. MS is characterized by demyelinating lesions in the brain and spinal cord. Demyelination describes the loss of myelin, the layer of lipids and proteins that insulate neuronal axons. Demyelination of axons during MS leads to inefficient transmission of neuronal signals and reduced control of motor and cognitive functions. These inflammatory lesions vary widely in space, time, and cellular composition, and their location impacts the clinical manifestation of disease [42]. Symptoms are highly diverse in category and severity, within and across patients, and include spasticity, pain, vision loss, fatigue, and paralysis [43].

Current dogma states that MS is an autoimmune disease. The presence of inflammatory lesions in the CNS in the absence of infection, as well as the relative effectiveness of immunomodulating drugs, is suggestive of autoimmunity. Further, Genetic studies in MS patients have identified immune molecules, especially HLA-DR (encoding specific human MHC II loci) and IL-7 receptor alleles as being strongly associated with MS [39]. These factors also suggest the involvement of autoreactive CD4+ T cells. Current models of MS propose that myelin-specific T cells are primed in the periphery by unclear mechanisms and

gain access into to the CNS parenchyma. Once there, they activate resident and recruited inflammatory cells, including microglia, macrophages, monocytes, and granulocytes, to express mediators such as TNF-α, reactive oxygen species, and IFN-γ [40, 41]. The broad heterogeneity of lesions, symptoms, and clinical manifestations makes effective treatments elusive and suggests the involvement of diverse inflammatory mechanisms.

Again, as in most autoimmune diseases, it is the activation rather than existence of self-reactive T cells that will induce MS. Several studies have determined that healthy individuals have myelin-reactive T cells and yet are spared disease [44]. Understanding the processes controlling autoreactive CD4+ T cell encephaliotogenicity, including T cell activation, homing to the CNS, and the factors produced to promote damage, will result in effective therapies that could be tailored to control any stage of MS.

Experimental Autoimmune Encephalitomyelitis

The most widely used model for CNS-targeted autoimmune demyelination is the rodent model of experimental autoimmune encephalomyelitis (EAE). Used as a model for human multiple sclerosis, EAE has been used to analyze the properties of pathogenic T cells [45]. EAE requires peripherally expanded myelin-antigen-specific effector CD4+ T cells to infiltrate the CNS, typically resulting in an ascending paralysis in C57Bl/6 mice. Scoring rubrics are used to quantify disease severity over time. The disability that develops in these mice is

correlated with immune cell infiltration into the CNS parenchyma and a loss of myelin, similar to the pathology seen in MS. Induction of EAE in different inbred strains of mice can induce various clinical courses which reflect different phenomenology of MS. The immunization of C57Bl/6 mice induces a chronic ascending paralysis, while SJL mice display relapsing-remitting forms of EAE. In other mouse strains, EAE presents with more ataxia and imbalance than paralysis, which is thought to result from leukocyte infiltration into specific regions of the CNS [46-51]. EAE, in any form, offers a model to investigate the priming of T cells, their homing to the CNS, and how those cells induce damage upon crossing the blood brain barrier.

EAE can be induced in mice by either active immunization or adoptive (passive) transfer of myelin-reactive T cells. In active immunization, naïve mice are immunized with neuroantigen in the presence of adjuvant. In C57Bl/6 models, mice are immunized with a peptide fragment from myelin oligodendrocyte protein (MOG_{35-55}) with complete Freunds adjuvant (CFA) and pertussis toxin to induce EAE typically within 12-14 days. Active immunization models are used to determine disease properties within a single host, including the contribution of the innate response to adjuvant and factors that promote disease development and abrogation. In adoptive transfer models, CD4+ T cells are recovered from either immunized mice or naïve mice with a TCR specific for myelin antigen. CD4+ cells are polarized and expanded in culture with recombinant cytokines and antigen and transferred into naïve hosts. This model is useful to determine

the requirements for any particular component of disease during the priming, polarization, or effector phase. Furthermore, transfer of encephalitogenic CD4+ Th cells into naïve mice eliminates the potentially confounding effects of the adjuvant on the host inflammatory and immune response. In either case, EAE is a useful model to determine the critical properties of the CD4+ T cells which mediate disease.

Th Effector Phenotypes in EAE

Active immunization and adoptive transfer models of EAE have significantly contributed to our understanding of the relevance of CD4+ T cell lineages in autoimmune diseases like MS [52, 53]. If MS is mediated by autoreactive CD4+ T cells, it seems apparent that determining the critical CD4+ T cell lineage for MS initiation or progression could ultimately lead to better therapeutic targets. What is less apparent is whether EAE and MS are mediated by a single CD4+ T cell lineage and which molecules or properties are absolutely critical for individual subsets to promote encephalitogenicity.

Historically, it was believed that EAE and MS were Th1-mediated. A high frequency of IFNγ-producing T cells are found in the CNS and periphery of mice with actively-induced EAE, and patients with MS have high levels of IFN-γ in lesions located in CNS lesions and IFN-γ-producing myelin reactive T cells in the periphery [54-57]. Furthermore, in EAE, culturing myelin-specific CD4+ T cells in the presence of IL-12 prior to transfer, thereby promoting Th1 development and expansion, confers encephalitogenicity [58]. However, paradoxically, mice

deficient in IFN-γ, the IFN-γ receptor, or IL-12p35 are highly susceptible to EAE, though IL-12p40-deficient mice are completely resistant [58-61].

Following the discovery of Th17 cells, the contribution of Th1 cells was reconsidered. IL-17A is expressed in the blood and cerebrospinal fluid of MS patients, as well as by CD4+ and CD8+ T cells in active MS lesions [62-64]. Mice deficient in IL-17A and/or IL-17F fail to develop severe EAE by active immunization, though they are not completely resistant [65, 66]. The contribution of Th1 cells to EAE was partially clarified when in 2000, Oppmann *et al.* demonstrated that IL-12p40 forms a heterodimer with IL-23p19 to form IL-23 [67]. This discovery led to two important observations: first, IL-23p19-deficient mice were completely resistant to EAE and second, IL-23 promotes Th17 terminal differentiation and pathogenicity [68-70]. Indeed, several groups have since demonstrated that the pathogenicity Th17 cells in EAE is critically tied to IL-23 exposure rather than the intensity of IL-17 production. Polarization with TGF-β and IL-6 alone can promote ROR-γt and IL-17 production, but encephalitogenicity is not conferred until Th17 cells are exposed to IL-23 [71-73]. Thus, in the absence of either of the IL-23 subunits--but not IL-12p35--Th17 cells could not expand in the CNS and mediate disease [68, 70].

These data suggested that IL-23-driven Th17 cells, and not IL-12-driven Th1 cells, were the critical mediators of EAE. However, it could not be denied that Th1 cells, or at least factors involved in Th1 differentiation and function,

contributed heavily to EAE development. Furthermore, if Th17 cells drive MS, what could explain the previously mentioned presence of Th1 cells in patients with MS. Mice deficient in T-bet, the master regulator of Th1 transcription, and STAT4, a regulator of IL-12 signaling on Th1 cells, were shown to be relatively resistant to EAE [74, 75]. Polarization of cells with IL-12 to promote Th1 responses – and suppress IL-17 production—can promote EAE in adoptive transfer models [58]. Furthermore, mice deficient in hallmark Th17 molecules RORγt and IL-17 are not completely resistant to EAE [21, 65, 66].

In 2008, Kroenke *et al.* demonstrated that myelin antigen-specific CD4+ T cells polarized with either IL-12 or IL-23 to promote IFN-γ or IL-17 production, respectively, could induce EAE via similar and distinct pathways [76]. Despite inducing EAE with similar incidence, severity, and time from onset to peak, the two models appeared to promote different cytokine and chemokine profiles and histological features. Mice that received IL-23-polarized cells had higher proportions of granulocytes in the CNS at peak disease, while IL-12-polarized recipients had a higher proportion of inflammatory monocytes and macrophages. Furthermore, IL-12 and IL-23-mediated EAE responded differently toTh1- or Th17-associated cytokine neutralizing antibody administration. These data demonstrated that Th1-mediated EAE and Th17-mediated EAE appeared to be distinct models of CNS-targeted autoimmune disease. These models have since been useful in determining the factors required for pathogenicity in one population *versus* the other and, ostensibly, better delineate the two effector

populations. Further studies have suggested that some patients with MS may have a Th1 or Th17 bias, which could significantly alter response to treatment as well as suggest a mechanism of better-targeted therapies [77-80].

These studies raised important questions about reported data from IL-23-deficient systems: if IL-12 polarization can promote Th1 encephalitogenicity, why are IL-23-deficient mice completely resistant to EAE? IL-23R-deficient mice appear to have normal Th1 development and function in response to *T. gondii* infection, which is typically cleared by the Th1 response, so why are these functional Th1 cells unable to induce EAE [70]? Unfortunately, adoptive transfer studies of Th1 and Th17 cells could not eliminate the possibility of IL-23 expression in IL-12-mediated transfers during either the *in vivo* priming phase, the *in vitro* polarization, or by the host following transfer. Therefore it is unclear if unmeasured IL-23 exposure is critical for IL-12-polarized Th1-mediated EAE. Furthermore, as previously described, Th17 cells polarized in the presence of IL-23 are pathogenic and plastic in autoimmune disease. The conversion from an IL-17-producer to an IFN-γ-producer is also promoted by IL-12 *in vitro* and requires T-bet. This raises the question of whether IL-12-polarized cells could simply be expanded exTh17s cells, and whether true Th1 cells play any role at all in EAE. Lastly, IL-23 is absolutely required for Th17 cell pathogenicity in EAE. Is it possible that the IL-23-driven epigenetic reprogramming that converts a Th17 cell to an exTh17 cell is required for CD4+ T cell encephalitogenicity?

Research Focus and Outline

Collectively, these data outline a possible model: IL-23 signaling on Th17 cells promotes T-bet expression, which, via transcriptional and epigenetic reprogramming and the support of continued IL-23 or IL-12 exposure, induces Th17 cells to become pathogenic exTh17 cells. This model would explain the requirement for both IL-23 and T-bet, two factors which were previously considered to belong to distinct lineages, and bring them together to promote a single powerful effector population. This model would also eliminate the contribution of *bona fide* Th1 and stable Th17 cells in EAE, and undermine the discoveries that IL-12- and IL-23-polarized cells induce EAE with distinct properties. Furthermore, it would suggest that in this particular model of autoimmunity, any IL-17 producing CD4 T cell is simply on a developmental pathway to become an IFN-γ producer, and Th1, Th17, and exTh17 cell differentiation and involvement would require reexamination in other models. This model can be explored by asking two questions: can IL-12 promote EAE independently of IL-23, and is Th17 cell plasticity absolutely required for Th17 cell encephalitogenicity. These questions are addressed with the following aims:

Aim 1, Chapter 2: Demonstrate that IL-12 polarized cells can induce EAE in the complete absence of IL-23. This novel finding will fortify the belief that *bona fide* IL-12-polarized IL-23-independent Th1 cells do have a place in CNS autoimmunity and that their contributions are distinct from Th17 cells.

AIM 2, Chapter 3: Demonstrate that stable T-bet-deficient Th17 cells can induce EAE following adoptive transfer, and remain stable following the onset of

disease. These data will demonstrate that Th17 cells do not require the property of plasticity to be pathogenic. However, stable Th17 cells are less potent at inducing EAE.

Aim 3, Chapter 4: Examine if the diminished potency of T-bet deficient Th17 cells is related to a deficiency in homing to the CNS.

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CHAPTER II

IL-23 is dispensable in an IL-12-driven model of EAE

Introduction

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS). Inflammatory cells gain access to the CNS, drive multifocal lesion formation and cause damage to the myelin sheath that insulates axons. MS is a heterogeneous disease with considerable diversity in the clinical course, symptoms, and therapeutic responsiveness to disease-modifying therapies [1]. Despite the efforts of numerous research groups, validated biomarkers have yet to be identified that are predictive of relapse rate, disability progression or therapeutic response profiles [2-4].

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune demyelinating disease in laboratory animals that simulates many of the clinical and histopathological features of MS. EAE can be induced via active immunization of susceptible inbred mouse strains with myelin antigens emulsified with Freund's Complete Adjuvant (CFA). Alternatively, it can be induced by the adoptive transfer of primed and polarized myelin-antigen specific CD4+ T cells into naïve syngeneic hosts. In most adoptive transfer models of EAE, it is

necessary to stimulate primed CD4+ T cells *in vitro* with antigen in the presence of either recombinant IL-12 or IL-23 for several days prior to transfer, as exposure to either IL-12 or IL-23 is crucial for the acquisition of encephalitogenic properties by CD4+ T cells. However, the relative roles of IL-12 and IL-23 in EAE and MS, and the degree to which the molecules that are induced downstream of their signaling overlap or are independent, are matters of controversy. A great deal of research is currently focused on elucidating the mechanisms by which encephalitogenic T cells traffic to the CNS, penetrate the blood-brain-barrier (BBB), become reactivated in the target organ, recruit other leukocytes to nascent lesions and inflict damage to myelin and axons.

IL-12 and IL-23 are structurally similar cytokines that are produced by myeloid cells to drive CD4+ T cell polarization [5]. IL-12 and IL-23 are heterodimers that share a common subunit, the IL-12p40 (p40) chain. The unique chains of IL-12 and IL-23 are the IL-12p35 (p35) subunit and the IL-23p19 (p19) subunit, respectively. Expression of the p35 and p19 subunits is tightly controlled on the transcriptional level, while expression of p40 is more promiscuous. Myeloid cells produce bioactive IL-12 and IL-23 in response to ligation of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), or ligation of costimulatory molecules such as CD40 on their cell surface. Both IL-12 and IL-23 signal through receptors that are also heterodimers and share a common chain, IL-12Rβ1 subunit, complexed with IL-12Rβ2 and IL-23R, respectively. The IL-12 and IL-23 receptors are expressed on conventional CD4+ and CD8+ T cells, as

well as on innate lymphocytes including NK cells and NK T cells. IL-12 induces expression of the transcription factor T-bet and the signature cytokine IFN-γ resulting in the differentiation of Th1 cells [6]. Conversely, IL-23 induces expression of the transcription factor ROR-γt and stabilizes the phenotype of Th17 cells.

IFN-γ-producing CD4+ T cells are found at high frequency in the CNS of mice with EAE as well as in MS lesions. Hence, it was initially concluded that that myelin-reactive Th1 cells are the primary effector cells in autoimmune demyelinating disease. However, it was subsequently discovered that C57BL/6 mice deficient in critical Th1-associated factors like IFN-γ, IL-12p35, or the IL-12Rβ2 subunit are susceptible to EAE following active immunization with MOG_{35-55} in CFA [7-10]. Conversely, C57BL/6 mice deficient in the IL-23p19 subunit or the IL-23R receptor subunit were absolutely resistant to EAE induced using the same protocol. This led to the revised theory that IL-23 modulated Th17 cells initiate the disease process [11-13]. The paradox raised by these collective data appeared to be resolved by the realization that Th17 cells are plastic [14-16]. In a number of experimental systems, IL-17 producing CD4+ T cells become unstable and upregulate the Th1 transcription factor T-bet [17-19]. This leads to the expression of IFN-γ and downregulation of IL-17, resulting in conversion into exTh17 cells. Using fate-mapping mice, it was recently shown that a high frequency of the IFN-γ producing cells infiltrating the CNS of C57BL/6 mice immunized with MOG_{35-55} in CFA did at one time express iL-17 and are, in fact,

exTh17 cells [14]. ExTh17 cells are as of yet virtually indistinguishable from canonical Th1 cells, which ostensibly do not require IL-23 for their development. Interestingly, exposure of IL-6 and TGF-β-polarized Th17 cell lines to IL-12 during rechallenge has also been shown to catalyze their conversion to the exTh17 phenotype, on a population level, *in vitro* [20]. Currently, published adoptive transfer models of IL-12-driven EAE do not consider the contribution of unmeasured levels of IL-23 in the donor, culture, or host before or after culture. Therefore, it is questionable whether the required role of IL-23 is to promote plastic Th17 cell development, and whether transferred iL-12-polarized IFN-γ-producing CD4+ T cells are actually IL-23-polarized exTh17 cells.

We and others have described divergent characteristics of IL-12 and IL-23 polarized myelin-reactive T cells in adoptive transfer models of EAE [12, 21]. Several groups have used the IL-12/IL-23 adoptive transfer EAE model to determine the roles of individual cytokines, chemokines, and other factors in disease induced by each lineage, thereby establishing the degree to which the respective pathogenic pathways overlap. The discovery of exTh17 cells and the observation that IL-23, but not IL-12, is required in active immunization models of EAE, raise the question of whether “true” *bona fide* IL-12-polarized Th1 cells contribute to EAE pathogenicity in any context. Understanding the contribution of Th1 cells *versus* extTh17 cells has translational relevance, as it is currently unclear if the clinical heterogeneity of MS patients reflects differences in the phenotype of dominant effector cell populations.

To further investigate these issues, we have developed IL-23- and IL-12-independent adoptive transfer protocols. We hypothesized that the relative importance of stable Th17 cells, exTh17 cells and bona fide Th1 cells is context dependent. Here, we polarize IL-12p40- and IL-23R-deficient MOG_{35-55}-specific CD4+ T cells with IL-12 to generate highly polarized Th1 cells and transfer them into either IL-12p40-deficient or wild-type hosts. We demonstrate for the first time that IL-12-polarized Th1 cells can promote EAE in the complete absence of IL-23 or its receptor. We characterize this novel IL-23-independent model of EAE in comparison to disease induced by stable Th17 cells. We find that EAE induced by *bona fide* Th1 cells is actually more severe then that induced by highly polarized and stable Th17 cells. We find that Th17 effector cells do not convert into ex-Th17 cells but remain stable following transfer into IL-12-deficient hosts. This suggests that expression of IL-23 in the donor, culture, and host is insufficient to drive Th17 cell plasticity. We also find that IL-23-independent Th1 cells promote monocyte accumulation in the CNS by a partially CCL2-dependent pathway. Using multiplex cytokine analysis, we demonstrate that highly polarized IL-12 and IL-23-independent T cells invoke distinct inflammatory profiles. To assess translational relevance, we measure IL-23 and IL-12 expression in the serum of MS patients, rather than IFN-γ and IL-17, to define populations that produce relatively high levels of IL-12 or IL-23. We then compared the expression of IL-12 and IL-23-associated chemokines to assess global cytokine and chemokine expression patterns

Materials and Methods

Mice: 8-16-week old IL-12p40$^{-/-}$, IL-12Rβ2$^{-/-}$ and IL-23R$^{-/-}$ mice werebred in specific pathogen-free conditions in the University of Michigan animal housing facilities. Wild-type C57BL/6 CD45.1 congenic mice were obtained from NCI Fredrick and held in specific pathogen-free conditions. All protocols were approved by the University of Michigan Committee on Use and Care of Animals.

Immunization and T cell polarization: Mice were subcutaneously immunized with an emulsion of complete Freund's adjuvant (CFA, Difco) and 100μg myelin oligodendrocyte glycoprotein peptide 35-55 MEVGWYRSP-FSRVVHLYRNGK (MOG35-55, Biosynthesis). 10-14 days later, draining lymph nodes were harvested and homogenized into a single cell suspension. Cells were cultured for 96h in the presence of MOG35-55 and polarized under Th1-polarizing conditions with 6ng/mL rmIL-12 (R&D Systems) and anti-mouse IL-4 (clone A11B11) or Th17-polarizing conditions with 8ng/mL rmIL-23 (R&D Systems), 10ng/mL rmIL-1α (PeproTech), 10ug/mL anti-mouse IL-4 and 10ug/mL anti-mouse IFNγ (clone XMG1.2). In some experiments, 5ug/mL anti-IL-23p19 (eBioscience) was added to Th1-polarizing conditions.

Induction of EAE: After 96 hours of culture, the proportion of CD4+ cells was calculated using flow cytometry. 2x10^{6} IL-12p40$^{-/-}$ CD4+ cells were injected into IL-12p40$^{-/-}$ mice. IL-23R$^{-/-}$ and IL-12Rβ2$^{-/-}$ CD4+ cells were purified (>95%) using MACS and 3x10^{6} were injected into wild type CD45.1 hosts. Mice were monitored daily for signs of disease as described previously.

Flow Cytometry: Intracellular staining was performed prior to adoptive transfer to assess CD4+ cell polarization. A subset of cells were stimulated with PMA (50ng/mL) and ionomycin (2μg/mL) in the presence of brefeldin A (10ug/mL) for 6 hours. Following disease onset, myeloid and T cell populations and cytokine production were measured using surface and intracellular flow cytometry in the spleens and CNS. Spleens were homogenized and passed over a 70μm filter. Brains and spinal cords were homogenized using an 18g needle and syringe and digested in a solution of Collagenase A (1mg/mL, Roche) and DNase1 (1mg/mL, Sigma-Aldrich). Mononuclear cells were enriched using a 30/70 percoll gradient (GE Healthcare).

Immunofluorescence: Mice were perfused 18 days after T cell transfer with Tyrode's solution followed by 4% paraformaldehyde in phosphate buffered saline (PBS). The spinal column was removed, postfixed for 24 hours in paraformaldehyde, and cryoprotected for 24 hours in 30% sucrose in PBS. The spinal cord was isolated and frozen in OCT medium. 20 um sections were treated with 10 mM sodium citrate (pH 6.0, 70° C) for 15 minutes to increase antigen retrieval and incubated in blocking buffer (5% normal goat serum and 0.1% Triton X-100 in PBS) for 1 hour. Sections were incubated overnight in primary antibody diluted in blocking buffer, and after PBS washes, sections were incubated for 2 hours in secondary antibody diluted in blocking buffer. Primary antibodies included syrian hamster anti-CD3e (1:70, BD), rat anti-MBP (1:300, Millipore), and mouse anti-SMI32 (1:2000, Covance). Secondary antibodies included AlexaFluor 488-conjugated goat anti-rat IgG, AlexaFluor 594-conjugated

goat anti-mouse IgG, AlexaFluor 647-conjugated goat anti-syrian hamster IgG (1:300, Life Technologies). Nuclei were labeled with DAPI (1:1000) for 5 minutes. Images were acquired on a Nikon A1 confocal microscope.

Multiplex Protein Array: PBS-perfused brains and spinal cords were harvested at peak disease. Tissue was homogenized with an 18G needle in PBS and protease inhibitor. Homogenate supernatants were analyzed using a Milliplex Mouse 32-plex kit. Data was analyzed using Luminex Software.

MS Patient Sample Collection and Analysis: Blood samples were taken monthly from MS patients not undergoing active treatment and divided into peripheral blood mononuclear cells for gene expression analysis and plasma for serum protein analysis. RNA was reverse-transcribed and analyzed for transcription factors (using somethign???) and normalized to four housekeeping genes. Serum protein levels were measured with a Milliplex Human Cytokine Kit and analyzed using Luminex software. Values are averages of 12 monthly visits.

Statistical Analysis: Data were analyzed using Graphpad Prism software. P-values were calculated using unpaired, two-tailed Mann-Whitney T-Tests.

Results

Th1 cells can induce EAE independently of IL-23

According to the current dogma, CD4+ T cells must be exposed to IL-23 become encephalitogenic. We have previously shown that myelin-specific CD4+ T cells stimulated with IL-12 and antigen express molecules typically associated with Th1 cells, including IFN-γ, IL-12R, and T-bet, but not Th17-associated factors. These cells induce EAE following transfer into naïve hosts. However, these adoptive transfer models of "Th1"-mediated EAE via the transfer of IL-12-modulated CD4+ T cells have not considered the possibility that those donor cells are exposed to IL-23 during priming *in vivo* and convert to ex-Th17 cells during culture with IL-12.

We questioned whether, under some circumstances, Th1 cells could induce EAE without the influence of IL-23 during the priming and effector phase. To investigate those questions and compare IL-23-independent Th1 cells with IL-12-independent Th17 cells, we immunized mice deficient in IL-12p40 (which lack both IL-12 and IL-23) with MOG_{35-55} in CFA. Draining lymph node cells were obtained 10-14 days later and cultured with antigen and recombinant IL-12 or IL-23 to promote Th1 and Th17 cell differentiation, respectively. This experimental design allowed us to compare IL-12- and IL-23-modulated effector cells on an identical genetic background, primed in the same microenvironment with the same immunogenic stimuli. Flow cytometric analysis following culture

demonstrated that myelin-reactive CD4+ T cells were highly polarized along their respective lineages. Specifically, IL-12p40$^{-/-}$ Th1-polarized CD4+ cells contained a high percentage of IFN-γ^{+}IL-17^{-} single producers and very few IFN-γ^{+}IL-17^{+} double producers or IFN-γ^{-}IL-17^{-} single producers. Conversely, IL-12p40$^{-/-}$ Th17-polarized cells lines contained a high percentage of IFN-γ-IL-17^{+} single producers and very few IFN-γ^{+}IL-17^{+} double producers or IFN-γ^{+}IL-17^{-} single producers (figure 2.1a). IL-23-polarized IL-12p40$^{-/-}$ Th17 cells also produced significantly higher levels of GM-CSF, consistent with previous reports that IL-23 preferentially promotes GM-CSF production by CD4+ T cells [22, 23].

We next transferred polarized IL-12p40$^{-/-}$ Th1 or Th17 cells into naïve CD45.1 congenic IL-12p40$^{-/-}$ hosts. We found that IL-12p40$^{-/-}$ Th1 cells induce EAE in IL-12p40$^{-/-}$ hosts at an incidence that is actually higher than that induced by IL-12p40-/- Th17 cells (100% *versus* 65%) (figure 2.1b). These results were reproduced when IL-12p35$^{-/-}$ Th17 cells were transferred into IL-12p35$^{-/-}$ hosts and IL-23p19$^{-/-}$ Th1 cells were transferred into IL-23p19$^{-/-}$ hosts (data not shown). At peak disease, we recovered spinal cord mononuclear cells for analysis of cytokine production via flow cytometry. CNS infiltrating Th1-polarized IL-12p40$^{-/-}$ donor cells maintained a high frequency of IFN-γ+IL-17- CD4+ cells, and low frequencies of IFN-γ+IL-17+ and IFN-γ-IL-17- producers (figure 2.1c). Surprisingly, Th17-polarized IL-12p40$^{-/-}$ donor CD4+ T cells maintained a high frequency of IFN-γ-IL-17+ producing T cells and low frequencies of IFN-γ+IL-17+ and IFN-γ+IL-17- producers, similar to their profile immediately prior to transfer.

These data suggest that IL-12p40$^{-/-}$ CD4+ T cell exposure to IL-23 *in vitro* is not sufficient for exTh17 development; rather, Th17 plasticity requires at least an early or continued exposure to IL-12 or IL-23 or both following immunization and adoptive transfer. Irrespective of the lineage of donor cells, IL-12p40$^{-/-}$ host CD4+ T cells produced little to no IL-17 or IFN-γ following disease onset (data not shown).

Using a complimentary approach, IL-12Rβ2$^{-/-}$ and IL-23R$^{-/-}$ mice were primed with MOG_{35-55} in the presence of CFA. IL-23R$^{-/-}$ lymph node cells were cultured under Th1 conditions and IL-12Rβ2$^{-/-}$ lymph node cells were cultured under Th17 conditions. These experiments eliminate the possibility that myelin-reactive effector T cells are inadvertently exposed *in vitro* to small quantities of IL-12 or IL-23 contaminants in media prior to adoptive transfer. Following polarization, IL-23R$^{-/-}$ CD4+ T cells had a higher frequency of IFN-γ^{+}IL-17^{-} CD4+ T cells than IL-12Rβ2$^{-/-}$ Th17 cells and a lower frequency of IFN-γ^{-}IL-17^{+} cells (figure 2.1d). IL-23R$^{-/-}$ CD4+ T cells had few IFN-γ^{-}IL-17^{+} or IFN-γ^{+}IL-17^{+} cells. Polarized cells were CD4+-purified (>92% purity) and transferred into wild-type CD45.1+ hosts.

We found that IL-23R$^{-/-}$ Th1 cells and IL-12R$^{-/-}$ Th17 cells induced EAE with comparable incidence, kinetics and mean peak severity (figure 2.1e). Shortly following clinical onset, mononuclear cells were harvested from the CNS, stained intracellularly and analyzed by flow cytometry to assess cytokine expression.

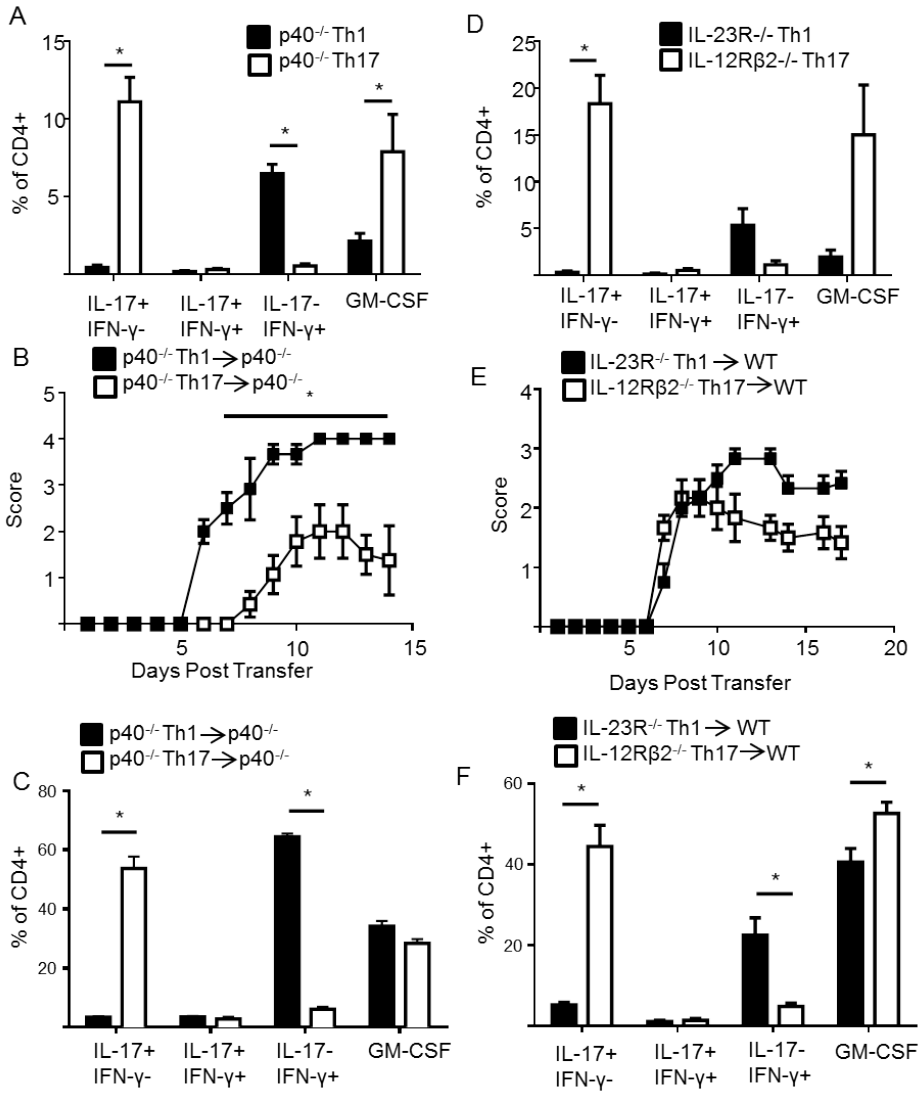

Figure 2.1: Th1 and Th17 cells do not require IL-23 and IL-12, respectively, for the acquisition of encephalitogenic properties. (A-C) IL-12p40$^{-/-}$ (p40$^{-/-}$) mice were immunized with MOG_{35-55} emulsified in CFA. Lymph nodes were harvested and cultured with antigen and Th1 polarizing conditions or Th17 polarizing conditions. A) After four days of culture, a subset cells were stimulated with PMA and ionomycin in the presence of brefeldin A and analyzed for cytokine expression with flow cytometry. Frequencies indicate cytokine-positive cells within CD4+ gate. B) IL-12- or IL-23-polarized IL-12-40$^{-/-}$ T cells were injected into naïve IL-12p40$^{-/-}$ hosts. Hosts were monitored daily for signs of EAE. C) Following EAE onset, mononuclear cells were harvested from the spinal cords of sick mice and analyzed for intracellular cytokine expression. Data shown represents frequencies of cytokine-producing cells within the CD4+and CD45.1 (donor) gate. (D-F) IL-23R$^{-/-}$ and IL-12Rβ2$^{-/-}$ mice were immunized as described and lymph node cells were cultured with antigen and Th1 or Th17 conditions, respectively. D) Flow cytometry was used to determine the frequencies of cytokine-positive cells. Frequencies indicate cytokine-positive cells within CD4+ gate. E) CD4+ cells were purified from IL-23R Th1 or IL-12Rβ2$^{-/-}$ Th17 cultures and injected into naïve wild-type CD45.1 congenic hosts (WT). F) Following disease onset, mononuclear cells were recovered from the spinal cords of sick mice. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A and analyzed for CD4+ cytokine expression using flow cytometry. Data shown represents frequencies of cytokine-producing cells within CD4+ CD45.2 (donor) gate.

Donor CD4+ T cells were stable, as IL-23R$^{-/-}$ CD4+ cells maintained a higher frequency of IFN-γ^{+}IL-17^{-} cells and donor IL-12Rβ2$^{-/-}$ CD4+ cells maintained a higher frequency of IFN-γ^{-}IL-17^{+} cells. As seen in the IL-12p40$^{-/-}$ transfers, despite exposure of IL-12Rβ2$^{-/-}$ CD4+ T cells to IL-23 following immunization, culture, transfer, and disease progression, IL-12Rβ2$^{-/-}$ Th17 cells remained stable (figure 2.1f), suggesting that Th17 plasticity may be dependent on IL-12, and not IL-23, in these models.

Highly polarized Th1 cells trigger the formation of monocyte-rich CNS infiltrates

We next analyzed the cellular composition of CNS infiltrates in Th1 *versus* Th17 recipients. Mice that received Th1-polarized IL-12p40$^{-/-}$ cells had higher frequencies and absolute numbers of CD45^{hi}CD11b^{+}Ly6c^{+}Ly6g^{-} monocytes in the brain and spinal cord infiltrates, and significantly lower frequencies and absolute numbers of CD45^{hi}CD11b^{+}Ly6c^{+}Ly6g^{+} granulocytes in the brain when compared to IL-12p40$^{-/-}$ mice that received Th17-polarized IL-12p40$^{-/-}$ (figure 2.2a). We also observed similar frequencies of monocytes and significantly lower frequencies of granulocytes in the spleens. Similarly, at peak disease, recipients of IL-23R$^{-/-}$ Th1 cells had significantly higher numbers and proportions of monocytes in the brain relative to recipients of IL-12Rβ2$^{-/-}$ Th17 cells. However, there were no significant differences between monocyte and granulocyte frequencies in spinal cord or spleen (figure 2.2b). Differences in observed

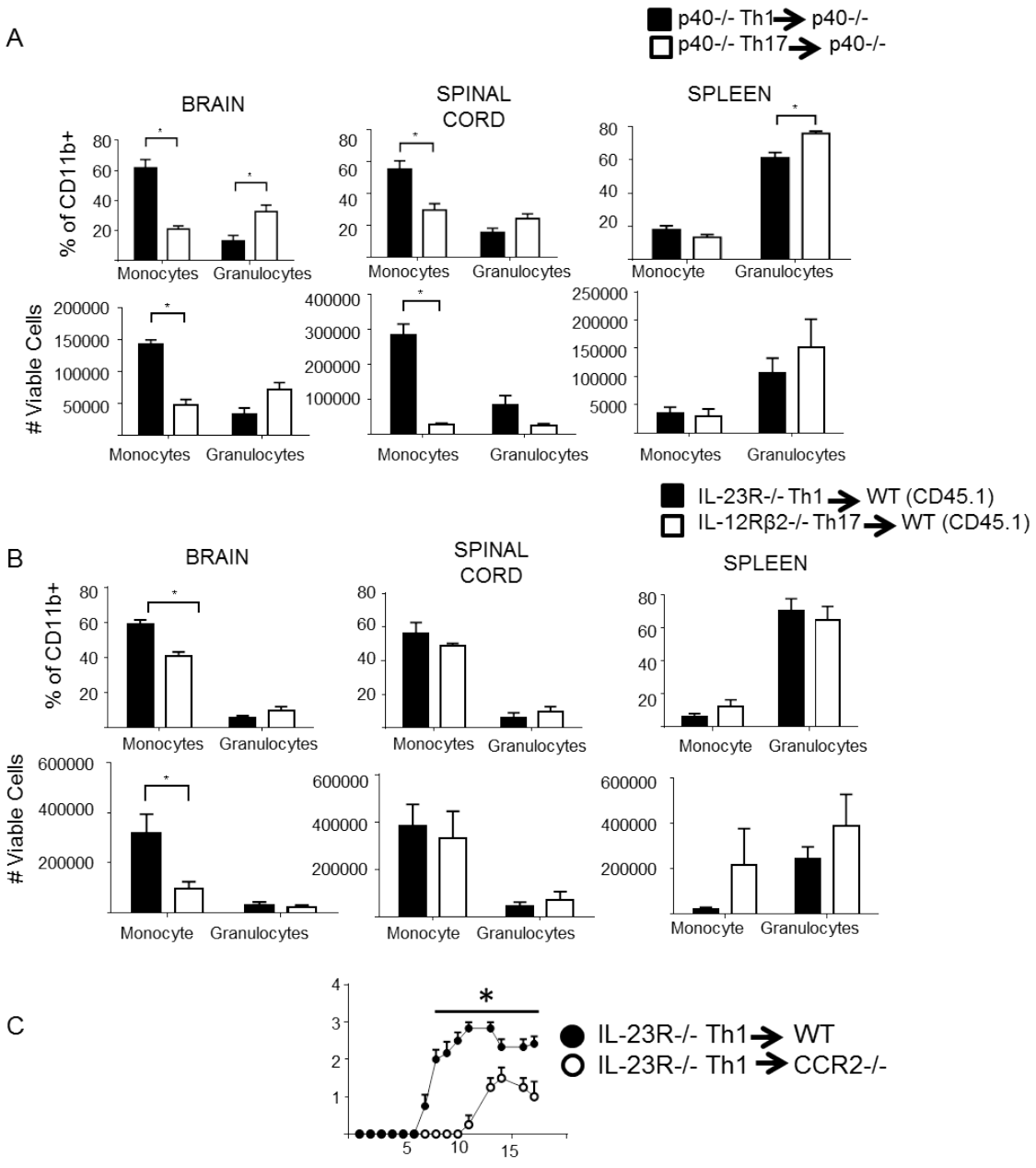

Figure 2.2. IL-23-independent Th1 cells promote enhanced monocyte recruitment to the CNS. (A) MOG_{35-55}-specific IL-12p40$^{-/-}$ (p40-/-) Th1 or Th17 cells were adoptively transferred into p40-/- hosts as previously described. (B) MOG_{35-55}-specific IL-23R$^{-/-}$ Th1 or IL-12Rβ2$^{-/-}$ Th17 cells were adoptively transferred into CD45.1 wild-type hosts. After disease onset, mononuclear cells were harvested from the CNS and spleen. Myeloid populations were analyzed using flow cytometry. Frequencies are based on CD11b+ CD45^{hi} cells. (C) MOG_{35-55}-specific IL-23R$^{-/-}$ Th1 cells were transferred into wild-type or CCR2$^{-/-}$ mice and monitored daily for signs of EAE. (*p<.05)

granulocyte and monocyte frequencies in the IL-12p40 transfer models compared to the cytokine receptor knockout transfer models be explained with compensatory effects of wild-type host cells on myeloid cell recruitment in the latter models. Furthermore, there may be requirements for IL-12p40 signaling on other cell populations that would be lost in IL-12p40$^{-/-}$ mice, possibly altering the cellular composition during disease. The observation that the cellular composition of CNS infiltrates differ between EAE induced by highly polarized and stable Th1 *versus* Th17 effectors is consistent with our previously published observations which describe similar phenomena using wild-type donors and hosts [21]. To determine whether monocyte recruitment is critical for the pure Th1-driven disease, we transferred IL-23R$^{-/-}$ Th1 cells into mice deficient in CCR2. CCR2 is enriched on monocytes relative to neutrophils and it binds to CCL2, which is expressed at sites of inflammation. CCR2 function is required for normal monocyte recruitment to the CNS during EAE [24, 25]. In the absence of CCR2 in host mice, IL-23R$^{-/-}$ Th1 cells induce a significantly milder and delayed disease relative to wild type hosts (figure 2.2c).

Chemokine and cytokine profiles in the CNS reflect the cellular composition of CNS infiltrates

Differences in cellular composition in the CNS of IL-12p40$^{-/-}$ hosts likely reflect differences in cytokine and chemokine profiles in mice with highly polarized models of Th1- or Th17-mediated EAE. At peak disease, supernatants from

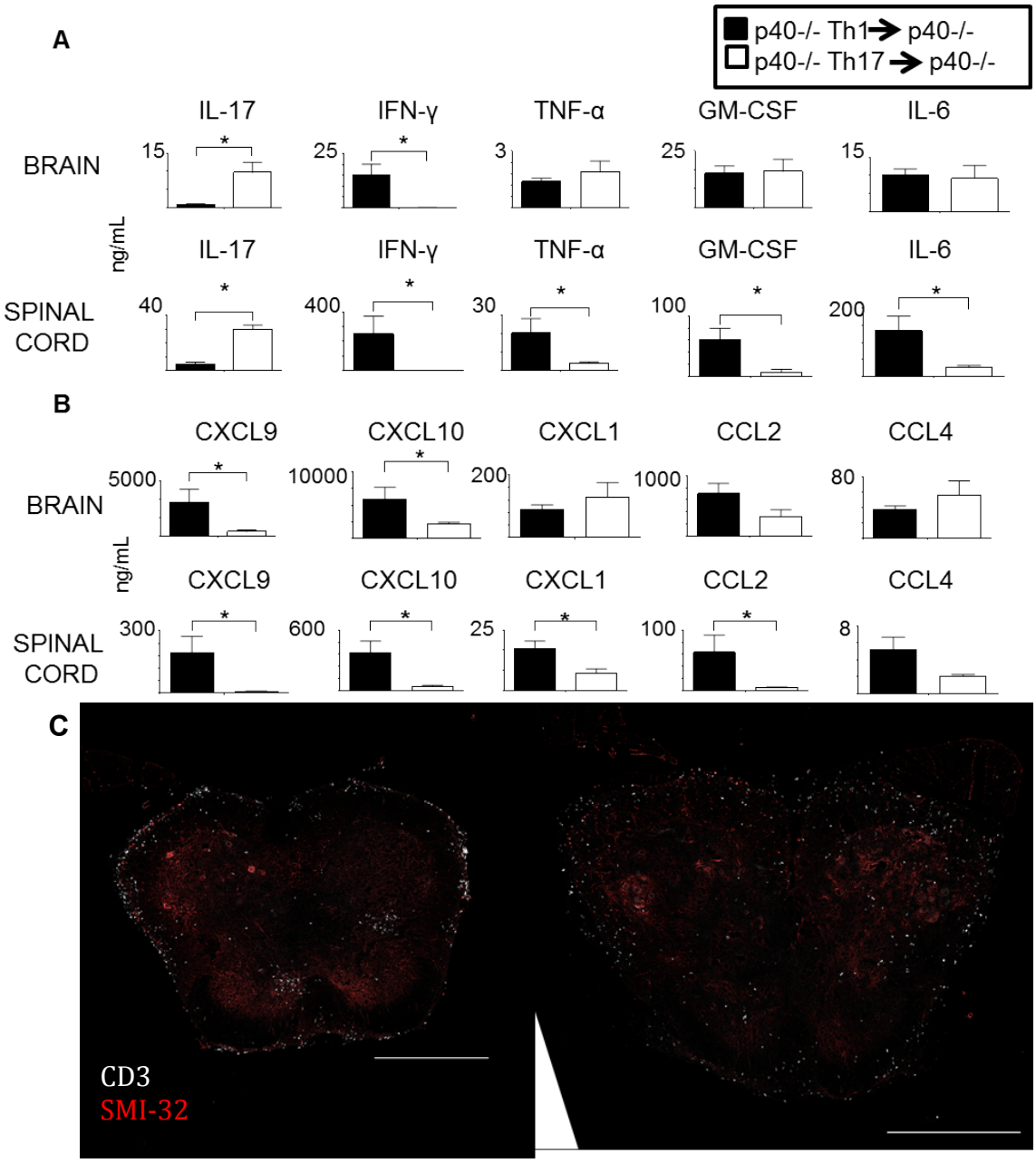

Figure 2.3. Highly polarized Th1 and Th17 cells induce different protein profiles and infiltration patterns in the CNS. IL-12p40$^{-/-}$ Th1 and Th17 cells were adoptively transferred into IL-12p40$^{-/-}$ mice as described previously. Following disease onset, brains and spinal cords were homogenized in the presence of protease inhibitors. Supernatants were isolated and analyzed using Luminex multiplex protein assay to measure (A) cytokines and (B) chemokines from a mouse inflammation panel. (C) IL-23R$^{-/-}$ Th1 cells or IL-12Rβ2$^{-/-}$ Th17 cells were adoptively transferred into wild-type mice to induce EAE. Following disease onset, frozen sections were prepared for immunofluorescence and labeled with anti-CD3 and SMI-32. (*p<.05).

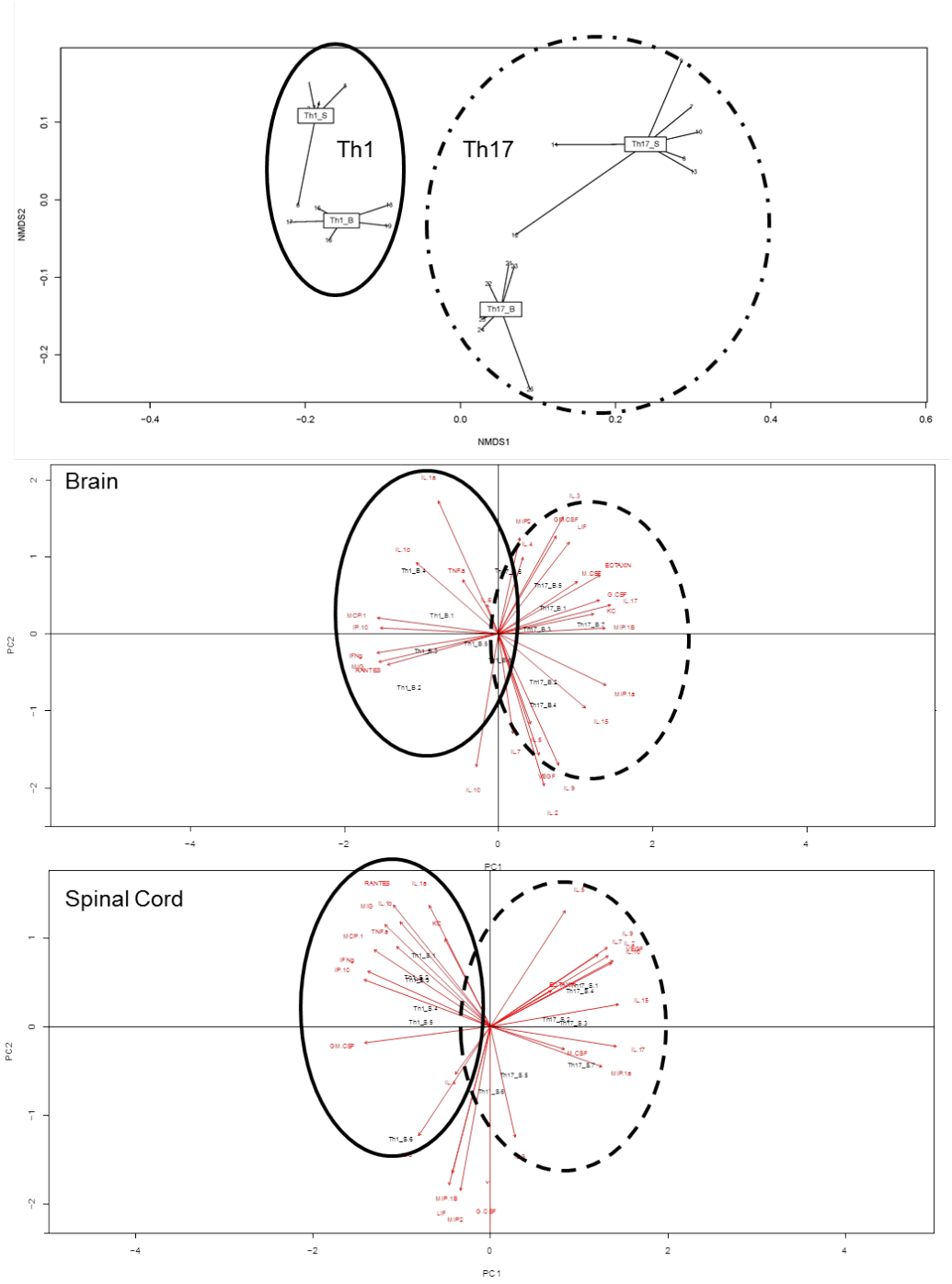

Figure 2.4. Inflammatory Response in the CNS induced by highly polarized Th1 or Th17 cells. IL-12p40$^{-/-}$ Th1 and Th17 cells were adoptively transferred into IL-12p40$^{-/-}$ mice as described previously. Following disease onset, brains and spinal cords were homogenized in the presence of protease inhibitors. Supernatants were isolated and analyzed using Luminex multiplex protein assay. Non-metric multidimensional scaling was performed (top) and principle component analysis (PCA) was used to determine the individual parameter contribution to formed groups (middle and bottom).

CNS tissue homogenates were isolated from IL-12p40$^{-/-}$ recipients of either Th1 or Th17 IL-12p40$^{-/-}$ cells. Using a multiplex protein array, protein levels of several cytokines and chemokines were analyzed. As expected, we found higher levels of IL-17 and lower levels of IFN-γ in the both the brain and spinal cord of Th17 recipients relative to Th1 recipients (figure 2.3a). In fact, we typically found extremely low levels of IFN-γ in both the brain and spinal cord in the Th17 recipients, and most samples had an IFN-γ concentration below the limit of detection. In spinal cords, but not brains, of Th1 recipient mice, we found higher levels of TNF-α, IL-6, and surprisingly, GM-CSF (figure 2.3a). Higher levels of these proinflammatory cytokines may underlie the increased disease severity or incidence in the Th1 IL-12p40$^{-/-}$ transfers. We also observed higher levels of CXCL9 and CXCL10, which are chemokines known to be expressed in the CNS in response to IFN-γ (figure 2.3b) [26-28]. CCL2 levels were also relatively high in the Th1 recipients, consistent with our observation that “pure “ Th1 cells induce monocyte-rich infiltrates (figure 2.3b). Surprisingly, CXCL1, which binds to CXCR2 expressed by neutrophils, was also elevated in Th1 recipients (figure 2.3b). Overall, these multiplex data suggest that IL-12p40$^{-/-}$ Th1 cells promote an enhanced inflammatory cytokine and chemokine profile in the CNS of IL-12p40$^{-/-}$ hosts relative to analogous Th17 transfers, likely owing to their potency to induce disease. Therefore, despite the complete absence of functional IL-23 in these adoptive transfer models of EAE, IL-12-polarized Th1 cells were potent inducers of inflammation. Furthermore, IL-23 polarization *in vitro* in the absence of IL-12

was insufficient to mount a similar response in IL-12p40$^{-/-}$ Th17-modulated disease.

In order to assess the histological infiltration patterns in our highly polarized Th1 and Th17-mediated transfers, lumbar spinal cord sections from wild-type mice that had received IL-23R$^{-/-}$ Th1 cells or IL-12Rβ2$^{-/-}$ Th17 cells were analyzed with immunofluorescence. In both groups, CD3+ cells associated with SMI-32, a marker of neuronal damage. Infiltration patterns in the parenchyma of mice that received IL-23R$^{-/-}$ Th1 were characterized as being localized on the periphery of the spinal cord, while IL-12Rβ2$^{-/-}$ Th17 recipients were marked with characteristic finger-like projections (figure 2.3c). Again, this is consistent with our previous wild-type adoptive transfer models of IL-12- and IL-23-mediated disease [21].

While two-dimensional analysis of the serum levels of cytokines and chemokines was informative to determine relative expression on a per-protein basis, the advantage of a high-throughput multivariate assay such as a Luminex assay is to assess global protein expression patterns within the host and attempt to differentiate between pathologic groups. To accomplish such an assessment, we applied non-metric multidimensional scaling (NMDS) to the multiplex protein array data acquired from analysis of IL-12p40$^{-/-}$ Th1 and Th17 brain and spinal cord homogenates (figure 2.4, top). NMDS is an ordination technique that groups observations in space based on multivariate similarity. The more similar two mice were in global cytokine/chemokine production, the closer they would group in the

NMDS plot. We expected that recipient mice receiving Th1 and Th17 polarized T cells would form distinct groups if pathogenesis was different in these transfer models. Indeed, recipient mice formed distinct groups based on Th1 and Th17 polarizations. Furthermore, brains and spinal cords within Th1 or Th17 groups were also distinct. Next, we used principle component analysis (PCA) as a second ordination technique to visualize the relative contributions of individual cytokines to the differences between Th1 and Th17 groups within each the brain (figure 2.4, middle) and spinal cord (figure 2.4, bottom). In agreement with the individual cytokine plots referenced in figure 2.3, the PCA demonstrated an inverse relationship between IL-17A and IFN-γ ,as they are nearly opposing vectors in the PCA biplots and were associated with Th1 and Th17 recipients, respectively. CXCL10 (IP-10), CXCL9 (MIG) and CCL2 (MCP-1) also associated with the Th1 group, while CXCL1 (KC) and G-CSF were associated with the Th17 group. In contrast, factors like GM-CSF and IL-1 are thought to promote Th17 responses, though they were found to be more important in the Th1 group in the spinal cord. This may help explain the increased potency of IL-12-polarized relative to IL-23-polarized IL-12p40$^{-/-}$ cells, though the mechanisms have yet to be determined. Overall, these data indicate that adoptive transfer of Th1 or Th17 IL-12p40$^{-/-}$ cells induces distinct expression of cytokines and chemokines, resulting in two novel mutually exclusive disease groups in EAE

Group	Cytokine	*p*-value	r	R squared
IL-12-Skewed (IL-12 correlation)	IL-17A (serum)	0.15	0.74	0.55
	IFN-γ (serum)	0.17	0.72	0.51
	IL-17A (MBP)	**0.02**	**-0.93**	**0.87**
	IFN-γ (MBP)	0.06	-0.85	0.73
	GM-CSF	0.01	0.91	0.95
	TNF-α	0.93	-0.06	0.003
	CXCL10	0.17	-0.72	0.52
	CCL2	0.56	-0.36	0.13
	CXCL5	0.97	0.02	0.0007
	CXCL1	0.54	0.37	0.14
IL-23-Skewed (IL-23 correlation)	**IL-17A (serum)**	**0.014**	**0.51**	**0.26**
	IFN-γ (serum)	0.74	-0.07	0.005
	IL-17A (MBP)	0.91	-0.03	0.0007
	IFN-γ (MBP)	0.95	0.01	0.0002
	GM-CSF	0.07	0.38	0.14
	TNF-α	0.92	-0.02	0.0005
	CXCL10	0.33	-0.21	0.05
	CCL2	0.97	-0.008	5.832e-005
	CXCL5	0.91	-0.02	0.0005
	CXCL1	0.9248	0.02085	0.0004346

Table 2.1. Correlation of IL-12 and IL-23 serum concentration with proinflammatory cytokines and chemokines within IL-12-skewed and IL-23-skewed MS patients, respectively.

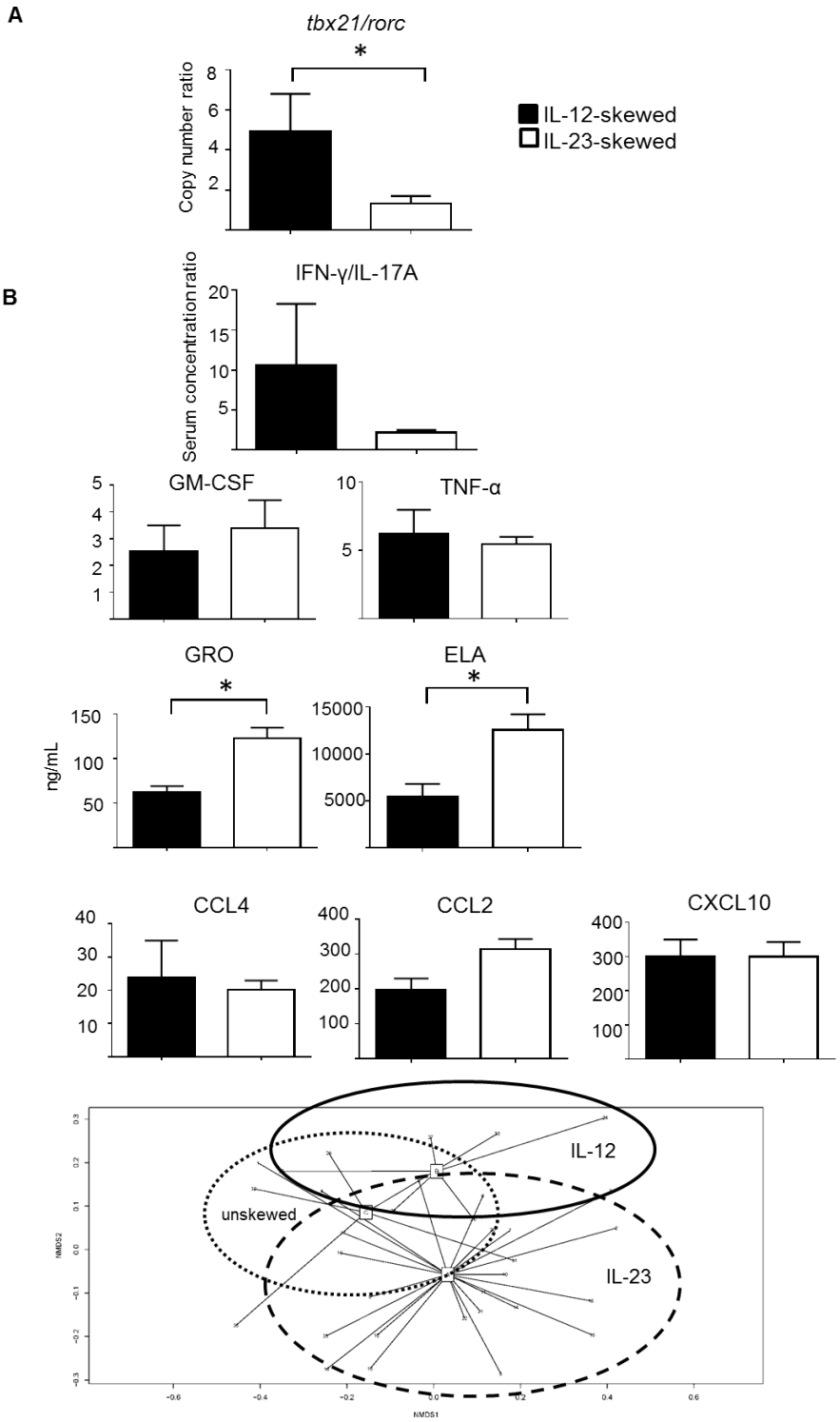

Figure 2.5. Inflammatory Response in the periphery of MS patients suggests IL-12 and IL-23 skewed groups. Blood samples from untreated MS patients was drawn monthly and processed for (A) RNA, (B) ELIspot and (C) multiplex protein array analysis. IL-12-skewed and IL-23-skewed patients were compared for (A) relative expression of T-bet and Ror-γt, (B) MBP-specific IL-17 and IFN-γ, and (C) serum protein concentration. D) Data from protein arrays in IL-12- and IL-23-skewed and unskewed groups were analyzed using NMDS. (** p<.05)*

Analysis of Serum Protein Levels from MS Patients Suggests Grouping Based on IL-12 or IL-23 Expression

Thus far, we have demonstrated that IL-23-independent Th1 cells and IL-23-independent Th17 cells mediate distinct forms of EAE. Translating these data to MS research is challenging, as MS is highly heterogeneous and complex relative to inbred mouse models of EAE. Several groups have analyzed datasets from human studies in an attempt to categorize groups of patients who exhibit patterns of cytokines, clinical course, and response to therapy with the goal of identifying biomarkers and more specific therapeutic targets [29]. A number of studies have grouped MS patients based on relative IFN-γ and IL-17 production as a proxy of Th1- or Th17-mediated disease. However, these classifications do not take into account the contribution of exTh17 cells, and as of yet there are no markers in humans or mice to distinguish between exTh17 cells from Th1 cells. Therefore, we collected data from MS patients not undergoing active treatment to determine whether IL-12 and IL-23 expression would correlate with cytokine and chemokine profiles seen in highly polarized Th1 and Th17 adoptive transfer models of EAE. Serum and PBMCs were collected monthly and analyzed using a multiplex protein array and qPCR. Using data from the protein array, groups were defined as being “IL-23-skewed” if the twelve-month average serum concentration of IL-23 was at least two-fold higher than IL-12, “IL-12-skewed” if the serum concentration of IL-12 was at least two-fold higher than IL-23. Those that fell outside either definition were considered “unskewed”. Within these definitions,

71% of patients were IL-23-skewed and 14% were IL-12-skewed, and 14% of patients were unskewed. To determine whether the expression level of classically Th1- or Th17-associated cytokines or chemokines correlated with IL-12 or IL-23 expression in those respective skewed groups, we used regression to determine *r* values, R squared values, and *p*-values (Table 2.1). Within the IL-12-skewed group, only IL-17A produced in response to myelin basic protein correlated negatively ($p=0.02$, $r= -0.93$) with IL-12 serum concentration. Within the IL-23-skewed group, serum concentrations of IL-17A were positively correlated ($p=0.014$), though these data poorly fit the model ($R^2=.26$).

We also analyzed Th1 and Th17-associated chemokines and cytokines to directly compare their expression levels between IL-12-skewed and IL-23-skewed patients. Patients in the IL-12-skewed groups had a higher ratio of *tbx21* to *rorc* gene expression than those in the IL-23-skewed group (figure 2.5a). We found no difference between IL-12- and IL-23-skewed patients in IL-17, IFN-γ, GM-CSF, TNF, CCL4, CCL2, or CXCL10 expression based on serum protein levels. Expression of GRO and neutrophil elastase (ELA) was significantly higher in IL-23-skewed patients (figure 2.5b).

Although two-dimensional cytokine data from skewed human populations did not recapitulate the highly polarized data from our adoptive transfer experiments, we wanted to determine whether NMDS analysis would demonstrate global differences in cytokine and chemokine among MS patients grouped by IL-12 and

IL-23 expression, similar to the data seen in mice. Based on this analysis, IL-12-skewed patients (A) formed a distinct group from IL-23-skewed patients (B), whereas unskewed patients were intermediate (figure 2.5d). This result is consistent with data from mouse experiments to support the hypothesis that IL-12 and IL-23 expression levels could define global cytokine profiles and differentiate between MS patient populations.

Discussion

We questioned whether IL-12 and IL-23 could induce autoimmune disease via truly independent pathways. While we and others have proposed distinct mechanisms driving IL-12-mediated and IL-23-mediated EAE, these mechanisms did not consider the concept that IL-23 exposure leads to Th17 cell encephalitogenicity and plasticity. The discovery of Th17 cell plasticity suggested the possibility that IL-23 and IL-12 may have cooperative roles to promote disease via a singular pathway. Here, we demonstrated that IL-23 and IL-12 polarization could promote CD4+ T cell encephalitogenicity independently of one another. To our knowledge, this is the first report of EAE development in the complete absence of IL-23. Surprisingly, we determined that IL-23-independent Th1 cells were more encephalitogenic than IL-12-independent Th17 cells, demonstrating that exposure to IL-23 is not sufficient for Th17 cell encephalitogenicity. Ongoing studies will examine the role of IL-12 in Th17 cell polarization and potency in EAE.

We next compared the properties of these two highly polarized and mutually exclusive models of EAE. We described the distinct cellular and cytokine profiles of IL-12- and IL-23-independent adoptive transfer models of EAE. Following transfer, IL-12-independent Th17 cells were stable and did not exhibit Th17 cell plasticity, which could be associated with the reduced potency of IL-12-independent Th17 cells. Furthermore, it suggests that IL-12, rather than IL-23, may play a significant role in Th17 cell plasticity *in vivo.* Relative to IL-12-

independent Th17-mediated adoptive transfer models of EAE, IL-23-independent Th1 models promote a typical but highly polarized Th1-associated cytokine response, as well as enrichment in monocyte accumulation and concurrent increases in monocyte homing chemokine expression in the CNS. We also concluded that IL-23-independent Th1 transfer models of EAE are partially dependent on the monocyte homing chemokine CCL2. We observed higher levels of proinflammatory cytokines in the CNS of recipients of IL-12p40$^{-/-}$ Th1 cells compared to Th17 cells, which could also be associated with the enhanced potency seen in the IL-23-independent Th1 cells. Differences between cytokine and chemokine levels are even more striking when analyzed holistically to determine global patterns.

These highly polarized mouse models described the divergent and mutually exclusive roles of Th1- and Th17-associated factors without considering IFN-γ and IL-17A expression *a priori.* We applied this mechanism translationally to determine whether relative IL-12 and IL-23 serum levels would be indicative of distinct proinflammatory mechanisms. We grouped patients based on IL-12 and IL-23 expression and compared cytokine profiles between those groups. We found that on a per-cytokine basis, there were not differences between IL-12 and IL-23-skewed patient populations. However global cytokine and chemokine expression analysis across an entire dataset determined that groups based on relatively higher IL-12- or IL-23 expression were indeed distinct from each other, and groups with similar levels of IL-12 and IL-23 were intermediate.

While these data were less striking relative to the observations seen in mice, we feel that analyzing the relative levels of IL-12 and IL-23 to determine whether distinct patient populations exist is a sensible method. Data from mouse models published here and elsewhere have suggested that in the consideration of the dispensability of IFN-γ and IL-17 during EAE and the phenomenon of Th17 cell plasticity, developing translational data which defines patients by IFN-γ and IL-17 levels may not be informative.

Differences between features of highly polarized Th1 and Th17-mediated EAE are informative in determining the mechanism of action and particular factors promoted by either cell type. Future studies attempting to understand monocyte recruitment, for example, could be facilitated by comparing the monocyte-rich highly polarized Th1 adoptive transfer models of EAE to analogous Th17 models. Furthermore, using IL-12- or IL-23-independent adoptive transfer models, intrinsic properties of these highly polarized CD4+ T cell subsets can be measured. Future experiments involve determining Th1- or Th17-specific homing, survival, and activation properties.

The heterogeneity of multiple sclerosis and the individuals affected by it has made MS research challenging. Understanding cellular and molecular differences across patient groups could allow researchers to target therapies in patients with particular profiles. Here, we defined patient groups in ways that

reflect our mouse models to determine whether differences emerge similar to those seen in the mice. Though the data based on patient groups based on IL-12 and IL-23 expression were only striking when considering the entire cytokine and chemokine dataset, our data represents the global differences between patient populations. Datasets with more patients and measured parameters will refine the reported data here. However, we feel that defining groups of MS patients based on IL-12 and IL-23 levels is a reasonable method for defining different populations.

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Chapter III

Highly polarized Th17 cells induce EAE via a T-bet-independent mechanism

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Summary

In the MOG_{35-55} induced EAE model, autoreactive Th17 cells that accumulate in the central nervous system (CNS) acquire Th1 characteristics via a T-bet dependent mechanism. It remains to be determined whether Th17 plasticity and encephalitogenicity are causally related to each other. Here we show that IL-23 polarized $Tbet^{-/-}$ Th17 cells are unimpaired in either activation or proliferation, and induce higher quantities of the chemokines RANTES and CXCL2 than wildtype (WT) Th17 cells. Unlike their WT counterparts, $Tbet^{-/-}$ Th17 cells retain an IL-17^{hi}IFN-$\gamma^{neg\text{-}lo}$ cytokine profile following adoptive transfer into syngeneic hosts. This population of highly polarized Th17 effectors is capable of mediating EAE, albeit with a milder clinical course. It has previously been reported that the signature Th1 and Th17 effector cytokines, IFN-γ and IL-17, are dispensable for the development of autoimmune demyelinating disease. The current study demonstrates that the "master regulator" transcription factor, T-bet, is also not universally required for encephalitogenicity. Our results contribute to a growing

body of data showing heterogeneity of myelin-reactive T cells and the independent mechanisms they employ to inflict damage to CNS tissues, complicating the search for therapeutic targets relevant across the spectrum of individuals with multiple sclerosis.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T-cell-mediated autoimmune disease of the central nervous system (CNS), widely used as an animal model of multiple sclerosis (MS). Despite substantial progress in elucidating pathogenic pathways that drive EAE, the mechanisms employed by autoreactive T cells to initiate inflammatory demyelination and, hence, the effector functions that are critical for their encephalitogenicity, are largely unknown. We and others have previously shown that IL-12-polarized Th1 and IL-23-polarized Th17 cells specific for the same myelin antigen are independently capable of inducing EAE following adoptive transfer into naïve syngeneic hosts [1, 2]. Surprisingly, full blown disease occurs in the absence of the signature Th1 and Th17 cytokines, IFN-γ and IL-17A/F, either alone or in combination [3-5]. More recently, the master regulatory transcription factor, T-bet, was identified as a critical molecule in the programming of encephalitogenic Th17 as well as Th1 cells [6]. T-bet was originally described as a driver of Th1 differentiation via direct activation of the IFN-γ gene and upregulation of the IL-12 receptor β2 chain [7, 8]. Gocke et al. [9] subsequently reported that T-bet could also promote the differentiation of autoimmune effector Th17 cells by inducing IL-23 receptor expression.

Several laboratories have established a role for T-bet in the plasticity of Th17 cells, particularly in their acquisition of Th1-like characteristics to become so called “ex-Th17” cells [10-12]. Fate mapping experiments using IL-17 reporter mice demonstrated that the majority of $CD4^+$ T cells infiltrating the CNS of C57BL/6 mice actively immunized with a peptide of myelin oligodendrocyte glycoprotein (MOG_{35-55}) are ex-Th17 cells [13, 14]. This observation has led some investigators to speculate that the plasticity of myelin-reactive Th17 cells is causally related to their acquisition of encephalitogenic properties. If they are correct then T-bet would be critical for the development of EAE based on its role in facilitating the transition of myelin reactive Th17 cells into ex-Th17 cells. In the current study we directly assess the requirement of T-bet expression in IL-23 polarized, myelin-reactive T cells for the adoptive transfer of EAE. We find that, unlike their WT counterparts, autoreactive T-$bet^{-/-}$ cells resist conversion to an ex-Th17 phenotype upon in vitro or in vivo reactivation. Moreover, these stable Th17 cells trigger the accumulation of myeloid cells in the spleen and CNS, thereby retaining the ability to induce EAE in WT as well as RAG2-deficient hosts.

Materials and Methods

Mice: 8-12-week-old C57BL/6 wild-type, CD45.1 congenic, T-bet$^{-/-}$ and RAG2$^{-/-}$ mice were obtained from the Jackson Laboratory and housed in microisolator cages under specific pathogen-free conditions. T-bet$^{-/-}$ and RAG2$^{-/-}$ mice were subsequently bred in our facility. All animal protocols were approved by the University Committee on Use and Care of Animals.

Induction of EAE: Mice were injected subcutaneously with 100 µg MOG35-55 MEVGWYRSP-FSRVVHLYRNGK (Biosynthesis) in complete Freund's adjuvant (Difco). For induction of EAE by active immunization, inactivated Bordetella pertussis toxin was administered intraperitoneally on days 0 and 2.

For induction of EAE by adoptive transfer, draining lymph nodes were harvested 10-14 days post-immunization, homogenized, and passed through a 70-µm cell strainer (BD Falcon). LNCs were cultured in vitro with MOG_{35-55} (50 µg/ml) under conditions favorable to the generation of Th17 cells (rmIL-23, 8 ng/mL; rm IL-1α, 10 ng/ml; anti-IFN-γ (clone XMG1.2), 10 µg/mL; anti-IL-4 (clone 11B11), 10 µg/mL). 2 x 10^6 $CD4^+$ T cells were injected intraperitoneally, and mice were observed daily for signs of EAE as described previously [15].

Flow cytometry: Spinal cords were harvested at peak disease, homogenized in DNase (1 mg/mL) and collagenase A (2 mg/mL) and incubated for 30 min at 37 °C. Mononuclear cells were isolated over a 30/70% Percoll gradient (GE

Healthcare). Splenocytes were passed through a 70-µm cell strainer, ACK lysed and washed twice prior to analysis. For intracellular staining, cells were stimulated with PMA (50 ng/mL) and ionomycin (2µg/mL) in the presence of brefeldin A (10 µg/mL) for 6 h or with MOG_{35-55} for 24 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin prior to incubation with flourochrome-conjugated antibodies. Flow cytometry was performed using a BD FacsCanto II.

ELISA: Splenocytes were cultured with or without MOG_{35-55} (50 mg/ml) in a 96 well plate (2 x 10^6 cells/ well). Supernatants were collected at serial time points and analyzed by sandwich ELISA according to the manufacturer's protocol (eBioscience). In some experiments, cell culture supernatants were analyzed using luminex protein array according to the manufacturer's instructions (Millipore).

ELISPOT assay: The frequency of antigen-specific cytokine producers was determined following culture for 24 h in 96-well filtration plates (Millipore), with or without 50 µg/ml MOG_{35-55}. Antibodies from eBioscience were: anti-IL-17 (TC11-18H10), biotinylated anti-IL-17 (TC11-8H4), IFN-γ (AN18) and biotinylated anti-IFN-γ (R4-6A2). Streptavidin–alkaline phosphatase (Southern Biotech) and an alkaline phosphatase substrate kit (Vector Laboratories) were used to identify trapped cytokine. Spots were counted using the CTL ImmunoSpot Analyzer (Cellular Technology) with ImmunoSpot software, and the number of spots in the medium-only wells subtracted to generate the data shown.

Statistical analysis: Statistical analyses were performed using GraphPad Prism statistical analysis software. Group differences were analyzed by unpaired, two-tailed Students t test. P-values of 0.05 or less were considered significant.

RESULTS

MOG_{35-55} immunized T-bet$^{-/-}$ mice generate a Th17 biased response and succumb to EAE

The master transcription factor, T-bet, has been implicated in the pathogenesis of EAE and MS [16-19]. We revisited the role of T-bet in EAE by comparing the clinical courses of C57BL/6 T-bet$^{-/-}$ and WT mice following subcutaneous immunization with an emulsion of MOG_{35-55} in CFA and intraperitoneal injection of inactivated *Bordetella pertussis* toxin. Ninety percent of T-bet$^{-/-}$ mice succumbed to moderate to severe EAE, although disease onset was slightly delayed compared with that of their WT counterparts (Fig. 1A). Examination of cytokine expression by CNS mononuclear cells pooled from representative mice in each group, and by splenocytes harvested from individual mice at peak EAE, revealed skewing towards an IL-17^{+}IFN-γ^{-} profile in the T-bet$^{-/-}$ cohort (Fig. 1B and C). Splenocytes from immunized T-bet$^{-/-}$ mice produced significantly higher levels of IL-17 and lower levels of IFN-γ than splenocytes from WT mice in response to *in vitro* challenge with MOG_{35-55} (Fig. 1D).

IL-23 polarized T-bet$^{-/-}$ Th17 cells are phenotypically stable *in vitro* and *in vivo*

Collectively, these data suggested that in the absence of T-bet, inflammatory demyelination was mediated by myelin-reactive Th17 cells that resist conversion

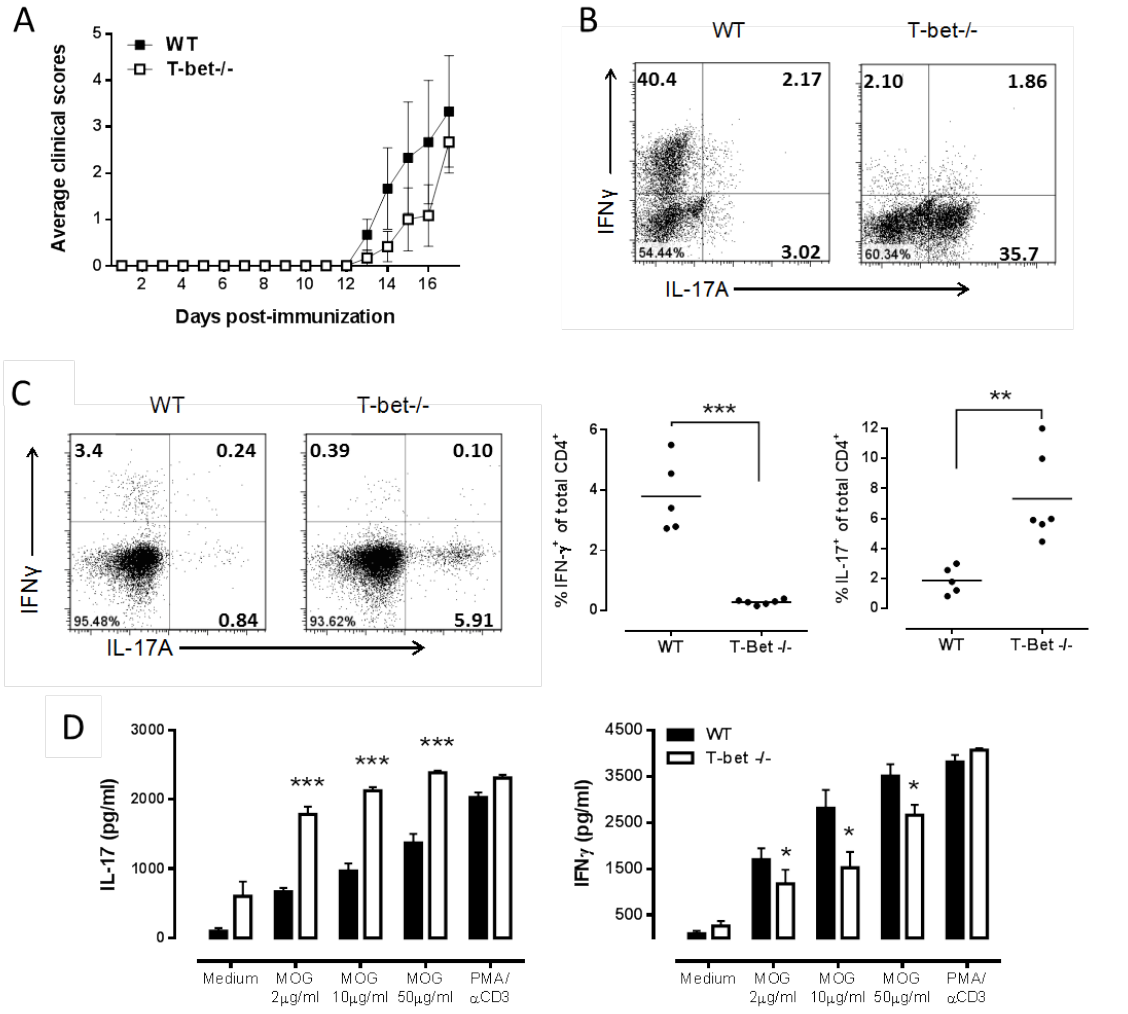

Figure 3.1. MOG_{35-55} primed T-bet$^{-/-}$ mice mount an enhanced Th17 response and succumb to EAE. EAE was induced in WT and T-bet$^{-/-}$ C57BL/6 mice via immunization with MOG_{35-55} in CFA and administration of inactivated pertussis toxin. (A) WT (closed squares) and T-bet$^{-/-}$ (open squares) mice were rated on a daily basis for degree of neurological disability by an examiner blinded to genotype. Clinical scores for each cohort were averaged over two experiments with a total of 8-10 mice/group. (B) Mononuclear cells and (C) splenocytes from the CNS were harvested at peak EAE. CNS mononuclear cells, pooled together from mice in each group, or splenocytes harvested from individual mice, were analyzed by intracellular staining and flow cytometry following stimulation with PMA and ionomycin for 6 h. Representative flow cytometry plots are gated on CD4$^+$ T cells. Each symbol represents an individual mouse and data shown are representative of three independent experiments. Bar represents mean. (D) Splenocytes obtained from MOG_{35-55}-immunized WT (closed bars) and T-bet$^{-/-}$ (open bars) mice were cultured with either MOG_{35-55} at the indicated concentrations, PMA and anti-CD3, or medium only. Supernatants were collected at 96 hours and subjected to sandwich ELISA to measure cytokine levels. The data are shown as mean/+SEM of 5 mice per groups and are from one experiment representative of four experiments. *** P<0.001;** P<0.01; * P<0.05 compared to WT control, unpaired, two-tailed Student's t test.

to ex-Th17 cells. In support of this hypothesis, MOG-primed T-bet$^{-/-}$ CD4^{+} T cells predominantly exhibited an IL-17^{+}IFN-γ^{-} profile following a 96 h culture with antigen plus recombinant IL-23 and IL-1β, while a significant percent of WT CD4^{+} T cells cultured under the same conditions were IL-17^{-}IFN-γ^{+} (Fig. 2A). IL-23 polarized T-bet$^{-/-}$ CD4^{+} T cells maintained a Th17 phenotype upon secondary challenge with MOG_{35-55} alone (Fig 2B and C). Multiplex bead immunoassays revealed increased levels of RANTES and CXCL2 in supernatants from primary cultures of T-bet$^{-/-}$ Th17 cells by comparison with WT Th17 cells (Fig 2D). Conversely, the concentration of the IFN-γ-induced chemokine CXCL9 was relatively low in supernatants from T-bet$^{-/-}$ Th17 cell cultures. T-bet$^{-/-}$ cells also expressed GM-CSF at a lower frequency than WT cells during primary culture (Fig. 2A). However, T-bet$^{-/-}$ and WT Th17 cells secreted comparable quantities of GM-CSF upon secondary challenge (Supporting Information Information Fig. 1). T-bet$^{-/-}$ and WT Th17 cells produced similar quantities of other cytokines and chemokines implicated in EAE pathogenesis, including IL-1α, IL-6, and G-CSF (Fig 2D). The majority of T-bet$^{-/-}$ Th17 cells upregulated activation markers and proliferated in response to antigen to a similar extent as their WT counterparts (Fig. 2E), indicating that their failure to acquire Th1 characteristics was not a consequence of insufficient antigen presentation or TCR engagement. The fact that a relatively high percentage of T-bet$^{-/-}$ cells expressed a CD44^{+}CD69^{+}CD25^{+}CD62L^{neg} profile could reflect a less differentiated state [20].

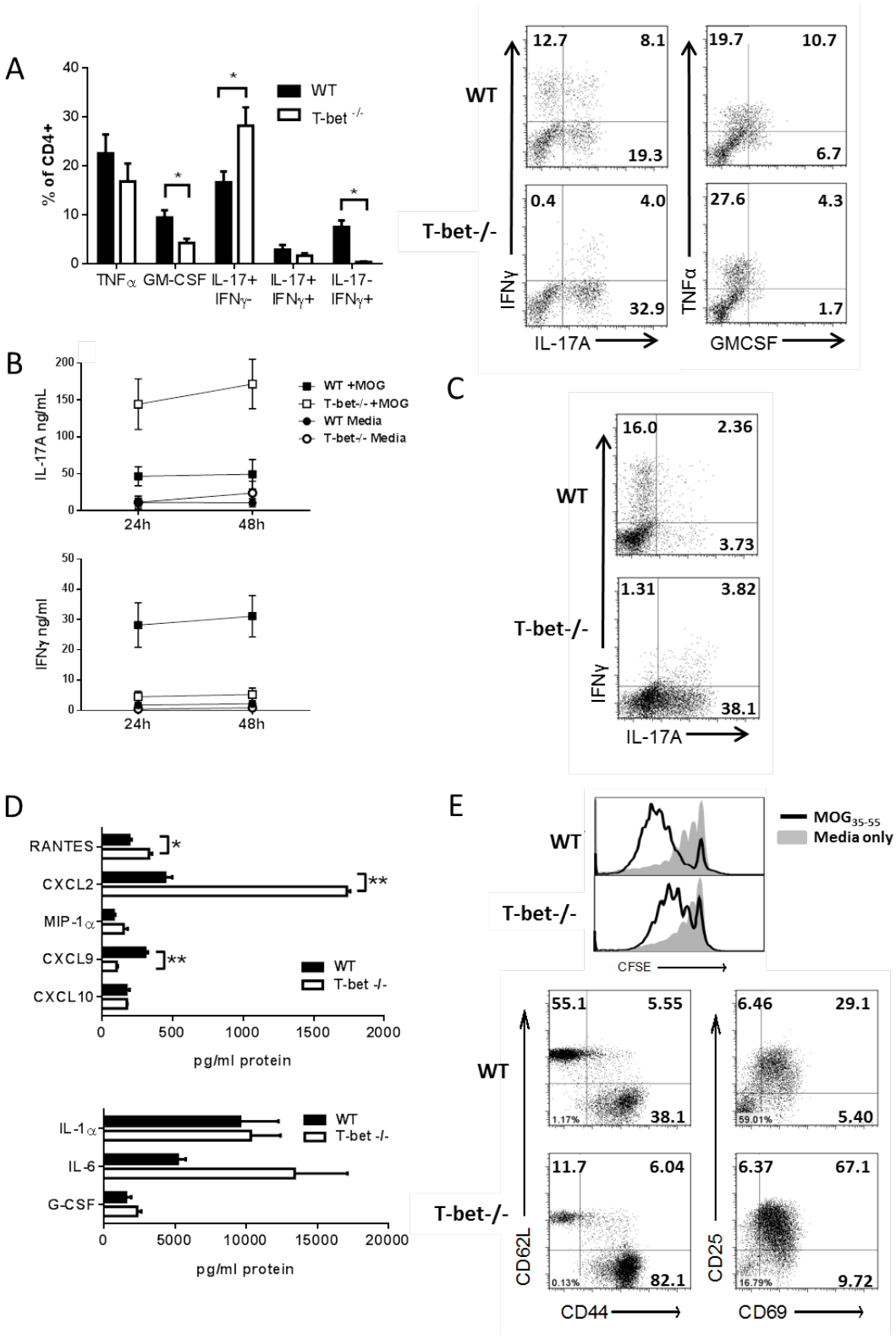

Figure 3.2. IL-23 polarized T-bet−/− Th17 cells are stable in vitro (legend on next page).

Figure 3.2. IL-23 polarized T-bet−/− Th17 cells are stable in vitro. WT and T-bet−/− mice were immunized with $MOG_{35–55}$/CFA. Ten to 14 days later, single cell suspensions of draining LN cells were cultured with $MOG_{35–55}$ in the presence of IL-23, IL-1α, anti-IFN-γ, and anti-IL-4. (A) At 96 h cells were harvested, washed, and stimulated with PMA and ionomycin for 6 h prior to for intracellular staining and flow cytometry. The percentages of WT (black bars) and T-bet−/− (white bars) CD4+ T cells that expressed cytokines as indicated were averaged over seven experiments with 4–6 donors/group/experiment (left). Representative flow cytometry plots are gated on CD4+ T cells. (B) Following 96 h primary culture, LN cells were washed and restimulatedwith T-cell-depleted syngeneic splenocytes pulsedwithMOG35–55. Supernatants collected 24 and 48 h later were analyzed by ELISA. Data are shown as mean + SEM of three samples from three independent experiments. (C) LN cells from primed mice were cultured for 96 h in the presence of MOG35–55 and Th17 polarizing factors after which they were rested in the absence of antigen or recombinant cytokines for an additional 96 h. They were then washed and restimulated with T-cell-depleted splenocytes and MOG35–55. Cells were harvested 24 h later for intracellular staining followed by flow cytometry. Representative flow cytometry plots are gated on CD4+ T cells. (D) Supernatants from cells cultured for 96 h as in (A) were analyzed by the Luminex protein assay. Data are shown as mean + SEM of two samples from two experiments. (E) CFSE dilution (top) and activation status (bottom) of cells cultured as in (C). Cells were stained with CFSE prior to rechallenge with MOG35–55 (solid line) or media alone (shaded) and analyzed by flow cytometry 6 days later. Activation marker expression was assessed 24 h after rechallenge. Histograms are gated on CD4+ T cells. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared to WT controls, unpaired two-tailed Student's *t*-test.

We next compared the stability of MOG-primed, IL-23 polarized T-bet$^{-/-}$ and WT CD4^{+} CD45.2^{+} T cells *in vivo* following transfer into naïve CD45.1 congenic hosts. Spleens harvested from the recipients of T-bet$^{-/-}$ donor cells contained a higher frequency of MOG_{35-55}-specific IL-17 producers and a lower frequency of MOG_{35-55}-specific IFN-γ producers than spleens from recipients of WT donor cells (Fig. 3A). These stable T-bet$^{-/-}$ Th17 cells induced EAE in 85-90% of hosts, although disease severity was reduced compared with recipients of WT cells (Fig. 3B). IL-23 polarized T-bet$^{-/-}$ Th17 cells did not express FoxP3 and did not mitigate EAE severity when co-transferred with WT Th17 effectors (data not shown).

FACS analysis of spinal cord mononuclear cells at peak disease indicated that the majority of infiltrating CD45.2^{+} T-bet$^{-/-}$ donor cells were IL-17^{+}IFN-γ^{-}, while the majority of infiltrating CD45.2^{+} WT donor cells were IL-17^{-}IFN-γ^{+} (Fig. 3C).

<u>Tbet$^{-/-}$ Th17 cells transfer EAE to hosts lacking endogenous B or T lymphocytes</u>

Although T-bet$^{-/-}$ donor cells were enriched for the CD4^{+} T-cell subset prior to transfer, we entertained the possibility that immunocompetent host T cells had been activated by contaminating donor APCs bearing MOG_{35-55}/ class II complexes. Therefore, we repeated the adoptive transfer experiments using RAG2$^{-/-}$ recipients. Consistent with the results obtained in immunocompetent

hosts, RAG2$^{-/-}$ mice were susceptible to disease induced by IL-23 polarized T-bet$^{-/-}$ donor cells (Fig 3D). At peak disease, a very high percent of the T-bet$^{-/-}$ cells that had accumulated in the CNS of RAG2$^{-/-}$ recipients were IL-17^{+}IFN-γ^{-} (Fig. 3E and F). Similarly, the frequency of IL-17^{+}IFN- γ^{-} T-bet$^{-/-}$ cells was significantly higher than that of WT donor Th17 cells in the spleen (Fig. 3F). IFN-γ single-producing T-bet$^{-/-}$ cells were virtually undetectable either in the spleen or the CNS. In contrast, IL-17^{-}IFN- γ^{+} cells were more numerous than IL-17^{+}IFN- γ^{-} cells among the WT donor population in both the periphery and the CNS. Spleens of RAG2$^{-/-}$ mice that received T-bet$^{-/-}$ donor cells were disproportionately enlarged, primarily due to a local expansion of myeloid cells (Fig 3G, right panel). There was no difference in the absolute numbers of CD4^{+}CD3^{+} T cells, granulocytes or monocytes infiltrating the spinal cords of T-bet$^{-/-}$ or WT hosts (Fig 3G, left panel).

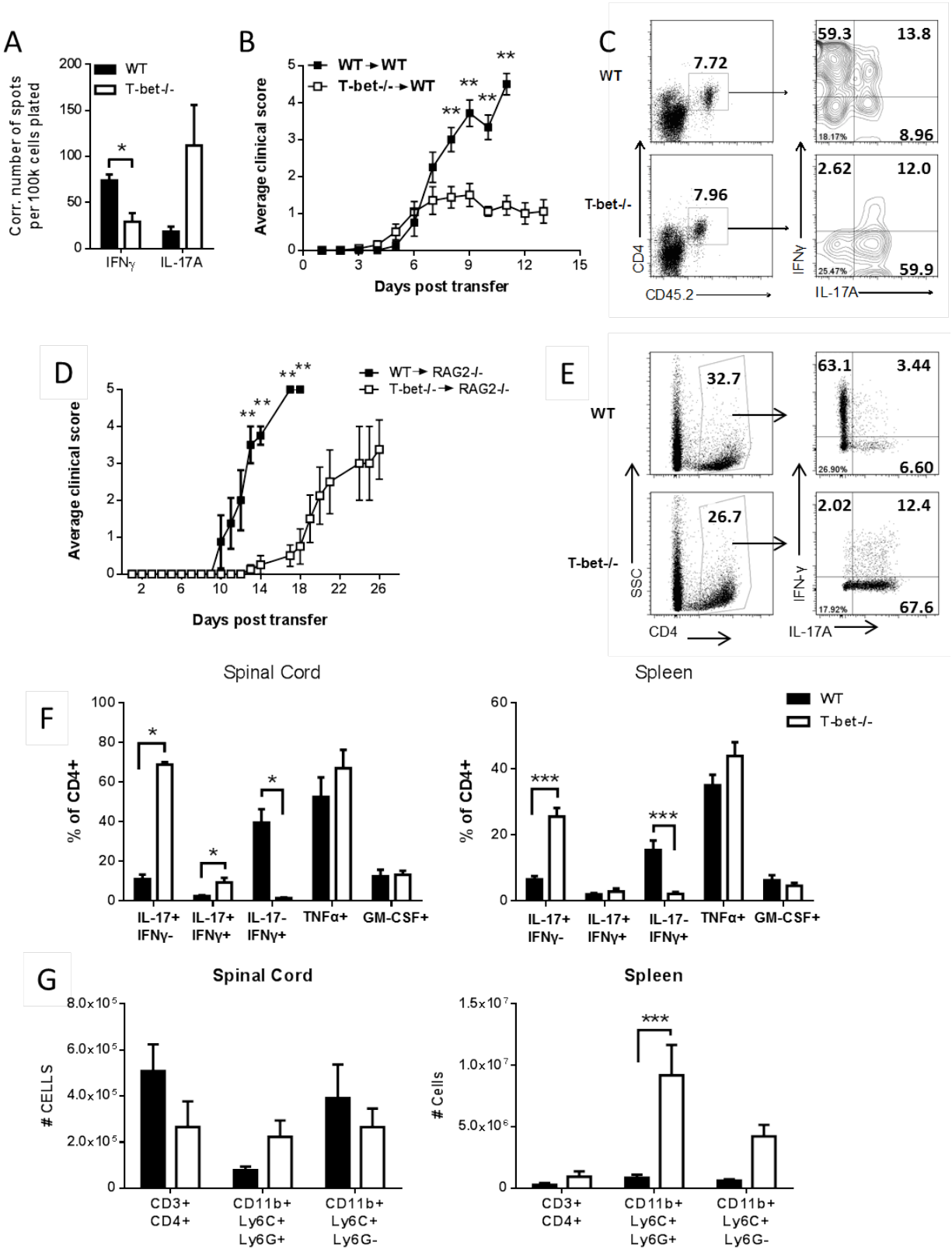

Figure 3.3. IL-23 polarized T-bet−/− Th17 cells are encephalitogenic (legend on next page).

Figure 3.3. IL-23 polarized T-bet−/− Th17 cells are encephalitogenic. (A–C) IL-23 polarized T-bet−/− and WT CD4+ T cells were adoptively transferred into naïve CD45.1 congenic hosts (5 × 106 CD4+ T cells/recipient). (A) Splenocytes harvested from hosts at peak disease were subjected to IL-17 and IFN-γ ELISpot assays. Background spots (detected in wells that were not pulsed with antigen) were subtracted to generate the Ag-specific data shown. (B) Adoptive transfer recipients of WT (closed symbols) or T-bet−/− (open symbols) MOG-reactive Th17 cells were rated on a daily basis for degree of neurological disability. The data shown are from one experiment with 4–6 mice/group representative of three performed. (C) CNS mononuclear cells obtained at peak disease were analyzed for intracellular cytokine expression by flow cytometry following stimulation with PMA and ionomycin for 6 h. Representative flow cytometry plots are gated on CD4+CD45.2+ donor T cells. (D–G) WT (closed symbols) or T-bet−/− (open symbols) Th17 cells were transferred into na¨ıve RAG2−/− hosts (5 × 106 CD4+ T cells/recipient). (D) Recipients were monitored on a daily basis and scored for degree of neurological disability. (E, F, G) CNS mononuclear cells and (F, G) splenocytes were harvested at peak disease and analyzed for (E, F) intracellular cytokine expression and (G) surface marker expression by flow cytometry. Representative flow cytometry plots for intracellular cytokine expression are gated on CD4+ donor T cells. Monocytes were defined as CD11bposLy6Cint-hiLy6Gneg. Granulocytes were defined as cD11bposLy6CintLy6Gpos. (D, F, G) Data are shown as mean + SEM of four mice per group from one experiment representative of three performed. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared to WT controls, unpaired two-tailed Student's *t*-test.

Discussion

MS is a heterogeneous disease characterized by diversity in both the clinical course and in responsiveness to individual therapeutic agents. At present, no biomarkers have been identified that can guide the selection of an optimal disease modifying regimen. Strategies to manage MS are complicated by the observation that distinct myelin-reactive Th-cell subsets can induce inflammatory demyelination via independent cellular and molecular pathways [1]. Therefore it is not surprising that signature Th1 and Th17 cytokines are dispensable for the manifestation of EAE [3-5]. The identification of a molecule that is critical for encephalitogenicity, irrespective of Th effector phenotype, would serve as an ideal therapeutic target. The transcription factor T-bet has been proposed as a candidate therapeutic target in MS, based on its non-redundant roles in Th1 differentiation and in Th17 plasticity. However, in the current study we show that IL-23 polarized myelin-reactive Th17 cells can mediate autoimmune demyelination without expressing T-bet or converting into Th1 ("ex-Th17") cells. Consistent with our findings, Duhen et. al. [21] recently reported that T-bet deficiency confined to $CD4^+$ T cells does not confer resistance against EAE induced by active immunization with MOG peptide emulsified in CFA. We found that stable T-$bet^{-/-}$ Th17 cells maintain the capacity to produce GM-CSF, and induce augmented production of CXCL2, each of which has been implicated in EAE pathogenesis [15, 22-24]. In ongoing studies we are investigating whether compensatory upregulation of these factors drives the accumulation of myeloid

cells ($Ly6G^+$ granulocytes in particular) in the spleens of the recipients of T-$bet^{-/-}$ Th17 donor cells. Engagement of alternative chemokine/cytokine pathways could underlie the preserved encephalitogenicity of myelin-reactive T-$bet^{-/-}$ Th17 cells.

We consistently found that MOG-specific T-$bet^{-/-}$ Th17 cells induce a milder course of EAE than their WT counterparts. This could be due to reduced production of the pro-inflammatory factor GM-CSF, as we observed in primary cultures of T-$bet^{-/-}$ and WT $CD4^+$ T cells (Fig. 2A). However, we detected similar frequencies of GM-CSF^+ cells among T-$bet^{-/-}$ and WT donor cells harvested from the CNS and peripheral lymphoid tissues of adoptive transfer recipients with EAE (Fig. 3F and data not shown). Furthermore, MOG-primed, Th17 polarized T-$bet^{-/-}$ and WT cells produce comparable amounts of GM-CSF upon secondary challenge (Supplementary Information Fig. 1). The diminished potency of T-$bet^{-/-}$ donor cells could also be secondary to a failure to express adhesion molecules, such as P-selectin ligand, and chemokine receptors, such as CXCR3, that facilitate efficient CNS trafficking [25]. The delay in clinical onset that we observed following adoptive transfer of T-$bet^{-/-}$ effectors into $RAG2^{-/-}$ hosts (Fig.1 D) is consistent with that hypothesis. Finally, our experiments revealed differences in the composition of myeloid cells that were mobilized and recruited by T-$bet^{-/-}$ versus WT effector cells (Fig. 3G and data not shown) which could be responsible for differences in EAE severity. Each of the above possibilities is currently under investigation in our laboratory.

In conclusion, the current study contributes to a growing body of data that demonstrates that multiple parallel immunopathogenic pathways can potentiate autoimmune neuroinflammation, and it suggests that disease modifying therapies might need to be customized based on immune profiling.

Acknowledgements

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CHAPTER IV

Diminished Potency of T-bet-Deficient Th17 Cells Suggests Impaired Homing to Sites of Inflammation

Introduction

As previously mentioned, T-bet is known to be critical for the transcriptional programming of Th1 cells, and recently several groups have raised questions about its possible contribution to Th17 development, especially during EAE. IL-23 is critical for Th17 but not Th1 cell encephalitogenicity [1-3]. Several groups have also shown that IL-23 polarization promotes T-bet expression by Th17 cells, which thereby promotes their conversion to exTh17 cells [1, 3, 4]. This may suggest that IL-23- and T-bet-driven plasticity is a critical feature of encephalitogenic Th17 cells. Indeed, early studies determined that T-bet-deficient mice are relatively resistant to disease following active immunization [5]. However, recent data, including published research presented in this dissertation, have demonstrated T-bet expression and Th17 plasticity are not absolutely required for the acquisition of encephalitogenicity by Th17 cells [6-9].

We demonstrated that T-bet expression and Th17 cell plasticity are not critical for Th17 encephalitogenicity. However T-bet deficiency attenuates the potency of IL-23 polarized Th17 cells. Hence, following transfer into $Rag2^{-/-}$ and wild-type mice, IL-23-polarized T-bet-deficient CD4+ cells were less potent at inducing EAE than

their wild-type counterparts. We hypothesized that the diminished potency of T-bet$^{-/-}$ Th17 cells could be a result of the following possibilities: T-bet$^{-/-}$ Th17 cells were not proliferating or surviving appropriately; T-bet$^{-/-}$ Th17 cells were not promoting innate and resident cell effector function in the CNS or elsewhere; T-bet$^{-/-}$ Th17 cells became regulatory or suppressive or promote a suppressive environment; or T-bet$^{-/-}$ Th17 cells became trapped somewhere outside of the CNS and could not effectively induce disease.

With regard to the role of T-bet in proper CD4+ T cell homing, the role of T-bet has been previously described in the expression of chemokine and integrin expression on Th1 cells. T-bet deficient NK and Th1 cells have reduced expression of the sphingosine-1-phosphate receptor 5 (S1P15), which promotes egress from lymph nodes [10]. T-bet-deficient Th1 cells also demonstrate reduced binding to P-selectin by regulating the post-translational modifications of P-selectin ligand [11]. T-bet also regulates the expression of CXCR3, a chemokine receptor which binds CXCL9, CXCL10, and CXCL11, which are expressed in the CNS of mice with EAE and MS patients [12-14]. Though some groups have proposed that diminished CXCR3 expression explains the reduction in EAE severity in T-bet-deficient CD4+ Th1 cells, data from our lab has suggested that CXCR3 is not critical for, nor does it promote, Th1 or Th17 trafficking to the CNS during EAE in wild-type mice [7, 15].

To investigate the factors of T-bet-deficient Th17 cells which promote their potency, we developed a co-transfer model of EAE induction. We primed wild-type and T-bet$^{-/-}$ mice and polarized lymph node cells with IL-23. We then transferred cells into RAG2$^{-/-}$ hosts. RAG2$^{-/-}$ mice received either wild-type cells alone, T-bet$^{-/-}$ cell alone, or equal numbers of wild-type and T-bet$^{-/-}$ cells. We demonstrate that T-bet-deficient Th17 cells accumulated less in the CNS but not the periphery relative to wild-type Th17 cells, though their proliferation and survival appears to be similar. We conclude that several trafficking molecules are significantly altered in T-bet-deficient Th17 cells relative to WT in the same environment. This suggests that the defects seen in T-bet-deficient Th17 cells are a cell-intrinsic result of impaired trafficking.

Materials and Methods

Mice and EAE Induction: C57Bl/6 (CD45.1+) mice (B6.SJL-Ptprca Pepcb/BoyJ) were purchased from The Jackson Laboratory and used as wild-type controls in co-transfer experiments. T-bet-/- mice were bred and maintained at the University of Michigan under specific pathogen-free conditions as previously described. Wild-type (CD45.1+) and T-bet-/- mice were immunized and lymph node cells were polarized in the presence of IL-23 and antigen. Following polarization, 2x106 CD4+ wild-type and/or 2x106 T-bet-/- cells were transferred into RAG2-/- hosts. Mice were monitored daily for signs of disease.

Flow Cytometry: At peak EAE, CNS, spleen, blood cells were harvested as described. Cells were stained with fluorescently labeled antibodies specific for the indicated antigens. For in vivo proliferation analysis, mice were treated with 1mg BrdU. After 14-16 hours, BrdU incorporation was measured using FITC BrdU Flow Kit (BD Biosciences). Apotosis was measured using the Annexin V Staining Kit (ebioscience).

Data analysis and Statistics: Flow cytometry data was analyzed with FlowJo software (Treestar). Statistics were analyzed using GraphPad Prism Software (GraphPad Software) and p-values were determined using a t test.

Results

We have previously demonstrated that following polarization with IL-23, transferred antigen-specific T-bet$^{-/-}$ Th17 cells are stable in the CNS and induce a less severe disease in wild-type and RAG2$^{-/-}$ hosts [8]. To investigate the mechanism by which T-bet promotes Th17 cell encephalitogenicity, we compared disease severity in RAG2$^{-/-}$ wild-type, T-bet$^{-/-}$, or co-transfer recipients. Wild-type donor cells were from C57Bl/6 mice that express CD45.1 to differentiate them from T-bet$^{-/-}$ CD45.2 cells. Mice that received wild-type cells alone developed severe EAE while those that received T-bet-/- cells alone developed a delayed and and milder disease course, as previously demonstrated. Mice that received both wild-type and T-bet-/- cells developed EAE with a similar disease course to those mice that received wild-type cells (figure 4.1). T-bet$^{-/-}$ Th17 did not suppress EAE either by action of T-bet$^{-/-}$ Th17 cells themselves of the environment they promote (figure 4.1).

We compared the migration, survival, and migration properties of wild-type and T-bet$^{-/-}$ CD4+ T cells following adoptive transfer model. Mice were sacrificed either following onset of EAE in recipients of wild-type cells (day 6-10 post-transfer) or following onset of EAE in recipients of T-bet$^{-/-}$ cells (day 11-14 post-transfer). We tracked transferred cells throughout the host using markers for CD4+ T cells and wild-type (CD45.1) and T-bet$^{-/-}$ (CD45.2) donor cells. Recipients of wild-type cells did not typically survive long enough to be examined

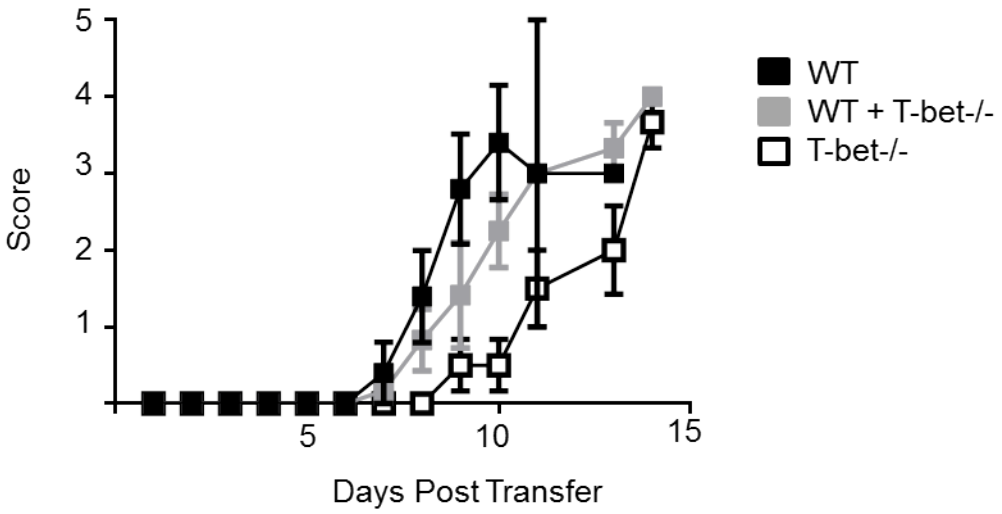

Figure 4.1. Co-transfer of T-bet$^{-/-}$ Th17 cells with wild-type (WT) Th17 cells does not suppress or delay WT-mediated disease. Wild-type (WT) or T-bet-/- mice were immunized with MOG_{35-55} and complete Freund's adjuvant (CFA). 10-14 days later, lymph node cells were recovered and polarized in the presence of antigen, rIL-23, rIL-1α, anti-IFN-γ (XMG1.2) and anti-IL-4 (A11B11). After 4 days, $2x10^6$ CD4+ cells were transferred into RAG2$^{-/-}$ hosts. Mice were monitored daily for signs of EAE

at the later time point. Co-transfer recipients and recipients of T-bet$^{-/-}$ Th17 cells alone were taken at both time points if possible. Following disease onset, infiltrating cells from the CNS were recovered and proportions and absolute numbers of CD45.1+ and CD45.2+ cells were analyzed. Recipients of both wild-type and T-bet$^{-/-}$ cells had typically half as many T-bet$^{-/-}$ CD4+ cells in the brain and spinal cord as wild-type cells (Figure 4.2a and b). Interestingly, those recipients had similar or higher proportions and numbers of T-bet$^{-/-}$ CD4+ cells in the spleen. At later time points, when recipients of T-bet$^{-/-}$ cells had developed disease, a similar phenomenon was observed, indicating that once WT CD4+ cells had infiltrated the CNS, they would continue to outcompete T-bet$^{-/-}$ CD4+ cells, and T-bet$^{-/-}$ cells were not simply delayed in their accumulation in the CNS (Figure 4.2a, b).

The difference seen between the frequency of wild-type and T-bet$^{-/-}$ CD4+ T cell accumulation in the CNS, but not the periphery, could be explained by differences in proliferation or apoptosis rates of T-bet$^{-/-}$ CD4+ T cells. We previously demonstrated that following rest and rechallenge in vitro, T-bet$^{-/-}$ CD4+ Th17 cells proliferated more slowly in response to antigen [8]. However, *in vivo* BrdU incorporation studies demonstrated proliferation rates were similar between wild-type and T-bet$^{-/-}$ cells in the CNS following disease onset (figure 4.3a). Next, apoptosis rates were measured using AnnexinV staining of cells from the CNS. Similar frequencies of AnnexinV+ cells were seen in wild-type and T-bet$^{-/-}$ CD4+ T cells in the CNS (figure 4.3b).

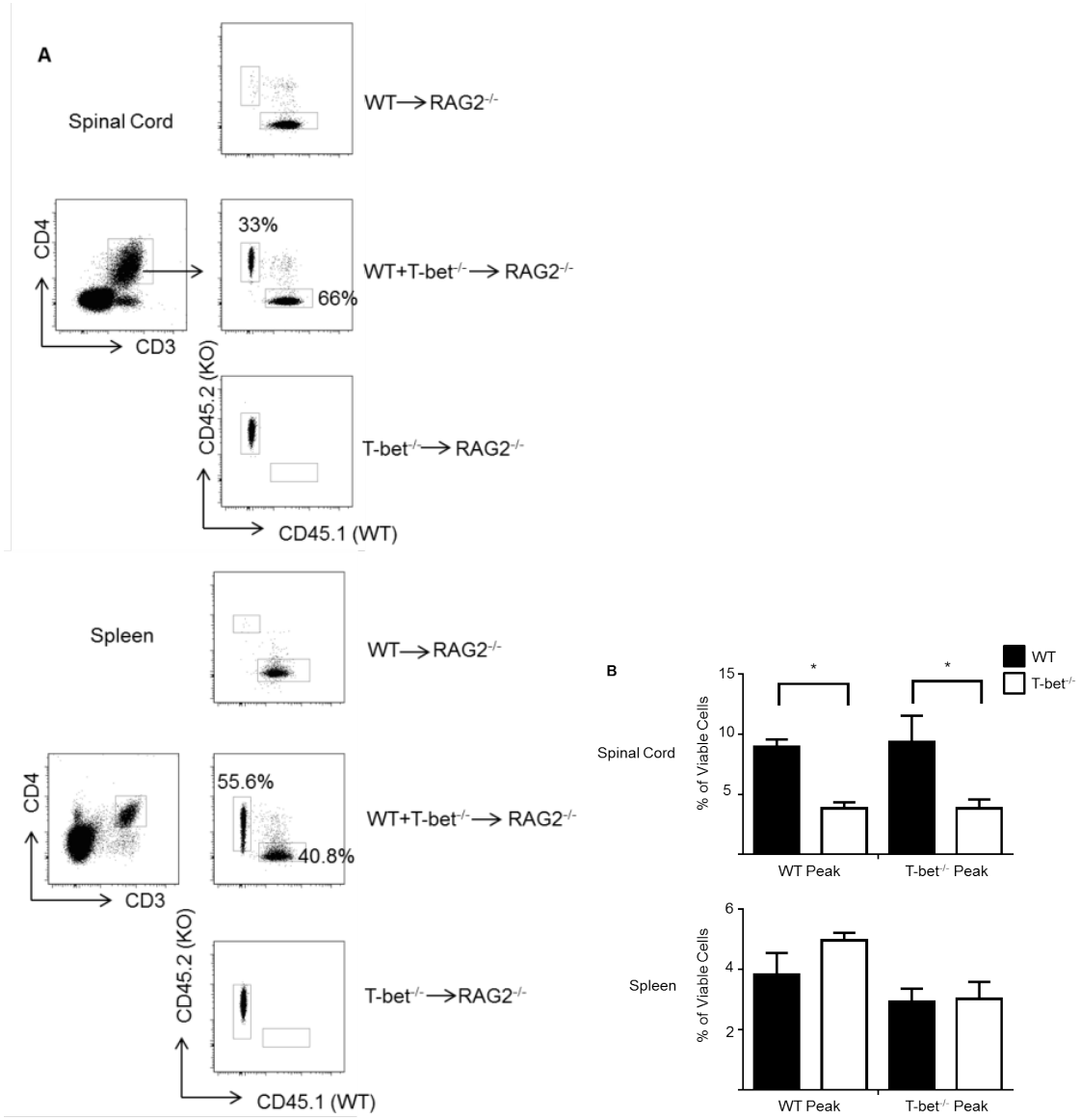

Figure 4.2. T-bet$^{-/-}$ CD4+ cells accumulate less in the CNS, but not the periphery, relative to wild-type CD4+ cells when placed in the same host. Following disease onset in RAG2$^{-/-}$ recipients of wild-type and/or T-bet$^{-/-}$ CD4+ T cells, spinal cord mononuclear cells and spleens were recovered and analyzed for frequency of wild-type or T-bet$^{-/-}$ CD4+ cells. A) Representative dot plots indicating gating scheme and typical results from spinal cord (top) and spleen. B) Bar graph of combined data. WT Peak indicates tissue harvested at early time point, T-bet$^{-/-}$ Peak indicates tissue harvested at later time point.

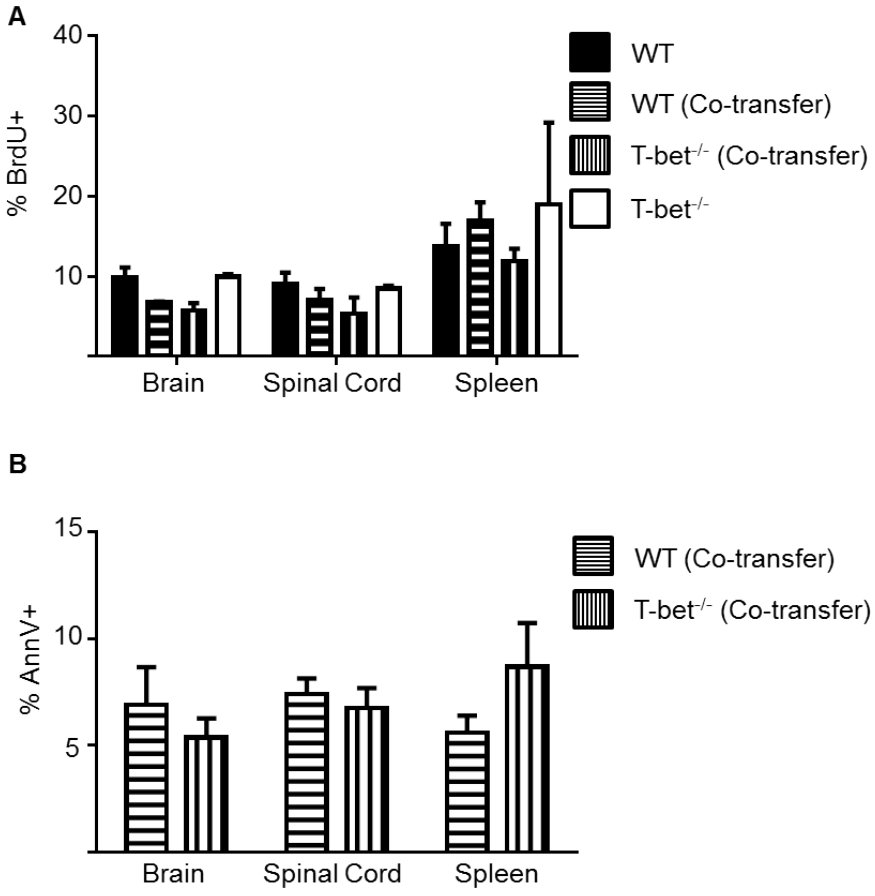

Figure 4.3. T-bet$^{-/-}$ Th17 cells have no defect in proliferation or survival. RAG2$^{-/-}$ recipients of wild-type, T-bet$^{-/-,}$ or wild-type and T-bet$^{-/-}$ (Co-transfer) Th17 cells were harvested at peak disease. Cells were isolated from brains, spinal cords, and spleens. A) BrdU incorporation was measured to assess proliferation across tissues. Data shown is graphed on wild-type or T-bet$^{-/-}$ donor CD4+ T cells. B) AnnexinV staining was measured to assess apoptosis across tissues. Data shown is graphed on wild-type or T-bet$^{-/-}$ donor CD4+ T cells.

In the absence of differences in survival or proliferation, we measured trafficking cytokine differences. As previously mentioned, several studies have described altered homing patterns in T-bet-deficient CD4+ T cells. Typically, these studies focused on IL-12-polarized Th1 cells to elucidate the mechanisms in which T-bet conferred Th1 function [8, 10, 11]. However, more recent data, including our own observations, have described the role of T-bet following IL-23 polarization, thus its role of controlling homing and trafficking in Th17 cells is worth revisiting [3, 7-9]. Furthermore, since T-bet-deficient Th17 cells are considered to be highly polarized Th17 cells as they produce high, sustained levels of IL-17, it is possible homing molecules associated with Th17 cells are highly expressed on T-bet-/- Th17 cells. Several integrins, chemokines, and their receptors have been reported to be preferentially upregulated during either Th1 or Th17 responses. If the acquisition of Th1-like properties via Th17 cell plasticity involves the upregulation of Th1-associated trafficking molecules, perhaps the stability associated with T-bet-deficient Th17 cells also inhibits that upregulation. To investigate this, we measured the expression of chemokine receptors, integrins, and other molecules associated with trafficking of cells to various sites of inflammation within our co-transfer model. In the spinal cord at peak disease, we found that transferred T-bet$^{-/-}$ CD4+ T cells expressed significantly higher levels of CCR6, $\alpha 4\beta 7$, $\alpha 4\beta 1$, and CD127 (IL-7Rα). Data was similar in the spleen, though T-bet-/- cells in the spleen expressed significantly less CXCR3. CCR6 has been shown to be critical for Th17 cell homing to the CNS and its ligands

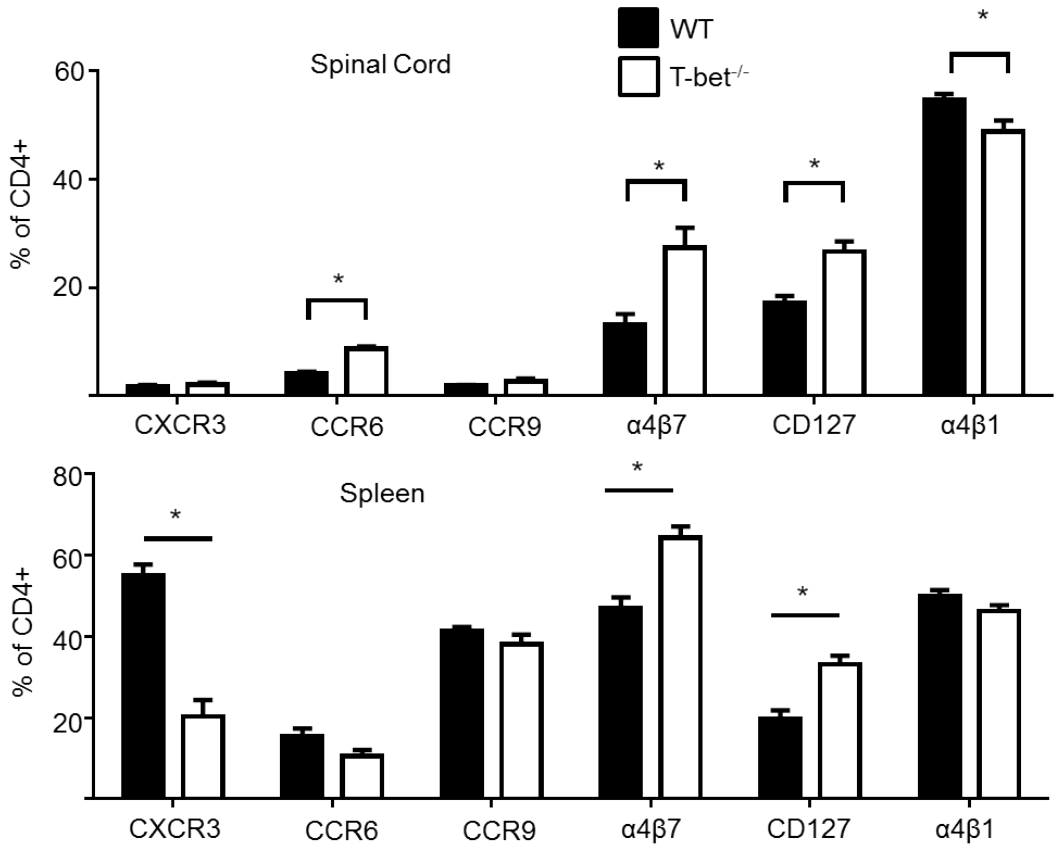

Figure 4.4. T-bet$^{-/-}$ Th17 cells have altered homing molecule expression in the setting of inflammation. Wild-type and T-bet-/- Th17 cells were co-transferred into RAG2-/- hosts. At peak disease, cells from spinal cord (A) and spleen (B) were isolated and analyzed for homing molecules using FACS

CCL19 and CCL20 are expressed in the CNS during EAE [16-18], The integrin $\alpha4\beta7$ binds the gut-associated ligand MadCAM-1. $\alpha4\beta1$, alternatively, was demonstrated to promote Th1 cell entry into the spinal cord during EAE and Th17 cells are thought to traffic to the CNS independently of the $\alpha4$ integrin [19, 20]. IL-7Rα binds IL-7 and is associated with susceptibility to multiple sclerosis and has been shown to be a survival factor for Th17 cells [21]. We saw no difference in expression of the gut-homing chemokine receptor CCR9 between wild-type and T-bet$^{-/-}$ Th17 cells, nor did we see differences in S1P1 receptor expression or p-selectin binding (data not shown). These data suggest that T-bet may play a role in Th17 cell homing, similar or related to its published role in Th17 cell homing, and in the absence of T-bet, Th17 cells have altered trafficking patterns to sites of inflammation.

While we observed differences in chemokine receptor and adhesion molecule expression between transferred wild-type and T-bet$^{-/-}$ Th17 cells, we investigated other mechanisms that could lead to the reduced potency of T-bet$^{-/-}$ cells. We considered the possibility that T-bet$^{-/-}$ Th17 cells acquired a regulatory or suppressive phenotype or promoted the expansion of host cells which could suppress autoimmunity. As previously mentioned, mice which received both wild-type and T-bet$^{-/-}$ T cells developed a similar disease course to that seen in mice which received wild-type cells alone. This suggests that T-bet$^{-/-}$ cells could not suppress the encephalitogenicity of wild-type cells and did not acquire a regulatory phenotype. We also did not observe FoxP3 expression by T-bet$^{-/-}$

cells. Furthermore, as previously mentioned in chapter III, RAG2$^{-/-}$ mice that received T-bet$^{-/-}$ T cells developed splenomegaly relative to RAG2$^{-/-}$ mice that received wild-type cells. This was a result of a significant Ly6c+Ly6g+ myeloid cell accumulation. These myeloid cells could be the recently described myeloid-derived suppressor cells, which are typically studied in the setting of tumor immunity [22]. However, Ly6c+Ly6g+ myeloid cell numbers in spleens of co-transfer mice were similar to those that received wild-type cells alone (figure 4.5). Wild-type cells were dominant in promoting disease and were not controlled by those myeloid cells. In the absence of those cells in the co-transfer model, we observed the described paucity of T-bet$^{-/-}$ cells in the CNS, which is the most likely explanation for the reduced disease severity seen in RAG2$^{-/-}$ recipients of T-bet$^{-/-}$ cells. This observation did not eliminate the possibility that T-bet$^{-/-}$ CD4+ T cells did elicit myeloid-derived suppressor cells when transferred into RAG2$^{-/-}$ mice, and future studies will be to investigate the role of myeloid cell accumulation in this model.

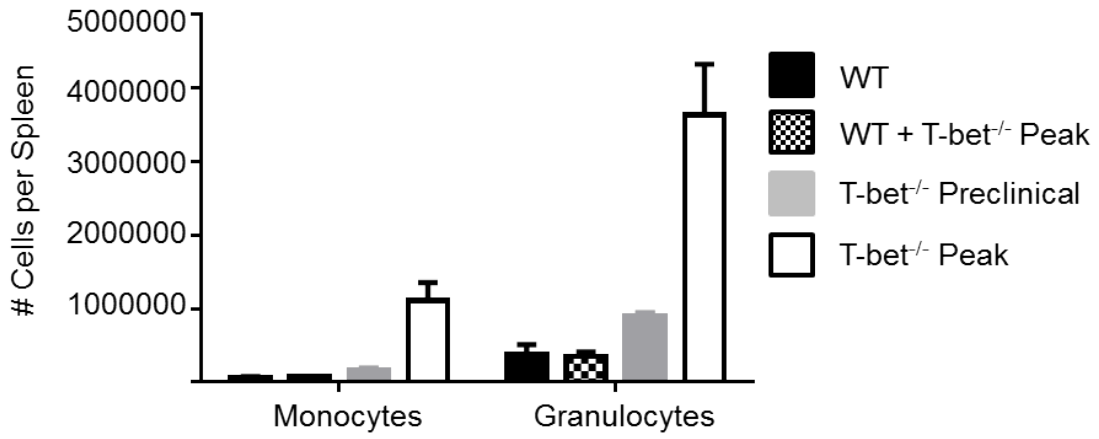

Figure 4.5. Recipients of both wild-type and T-bet$^{-/-}$ Th17 cells did not develop myeloid cell accumulation in the spleens of RAG2$^{-/-}$ hosts. Spleen cells were recovered from RAG2$^{-/-}$ recipients of wild-type, wild-type and T-bet$^{-/-}$ cells, or T-bet$^{-/-}$ alone at an early and late time point. Monocytes were gated on CD11b^{+}Ly6c^{+}Ly6g^{-} cells while granulocytes are gated on CD11b^{+}Ly6c^{+}Ly6g^{+} cells. (*p<.05)

Discussion

While several groups have determined a critical role for T-bet in Th1-associated function and trafficking, newer data have described the importance for T-bet in Th17 plasticity and IL-23 polarization. We previously published that T-bet deficient Th17 cells induce EAE in RAG2$^{-/-}$ and wild-type hosts, however with reduced severity and delayed onset. We determined using co-transfer models of disease that in T-bet$^{-/-}$ Th17 cells accumulate less in the CNS but to similar levels in the periphery. We determined this was not a result of deficits in survival or proliferation, nor the promotion of an anti-inflammatory or suppressive environment, and was likely a defect in CD4+ T cell trafficking to the CNS.

Though our results were inconclusive as to which particular receptors were critical to the defects in T-bet$^{-/-}$ homing, we could determine that the defect was intrinsic to T-bet deficient Th17 cells rather than differences in host responses between wild-type and T-bet$^{-/-}$ Th17-mediated adoptive transfer models of EAE. The advantage of using a co-transfer model was that wild-type and T-bet-deficient CD4+ T cells could be analyzed within the same environment of inflammation following EAE onset, eliminating the possibility that host proinflammatory cells and molecules were not properly activated in the presence of T-bet$^{-/-}$ CD4+ T cells.

In future experiments, we hope to better clarify the particular homing receptors that are regulated by T-bet to promote proper Th17 cell trafficking. We will gain a better understanding of the significance of the upregulation of certain gut-homing molecules in T-bet$^{-/-}$ Th17 cells by measuring transferred T-bet$^{-/-}$ accumulation in the gut. Furthermore, we appreciate the possibility that pleiotropic functions of T-bet as a transcription factor may lead to several dysregulated transcriptional networks in its absence, leading to a large number of possible solutions to the diminished potency of T-bet$^{-/-}$ cells in this model. Using this co-transfer adoptive transfer model will promote understanding of the effect of T-bet deficiency in Th17 cells within the same setting of inflammation as functional wild-type cells. Lastly, these data have reinforced the understanding that T-bet, though critical for Th1 differentiation, is certainly involved in IL-23-driven Th17 cell immunology.

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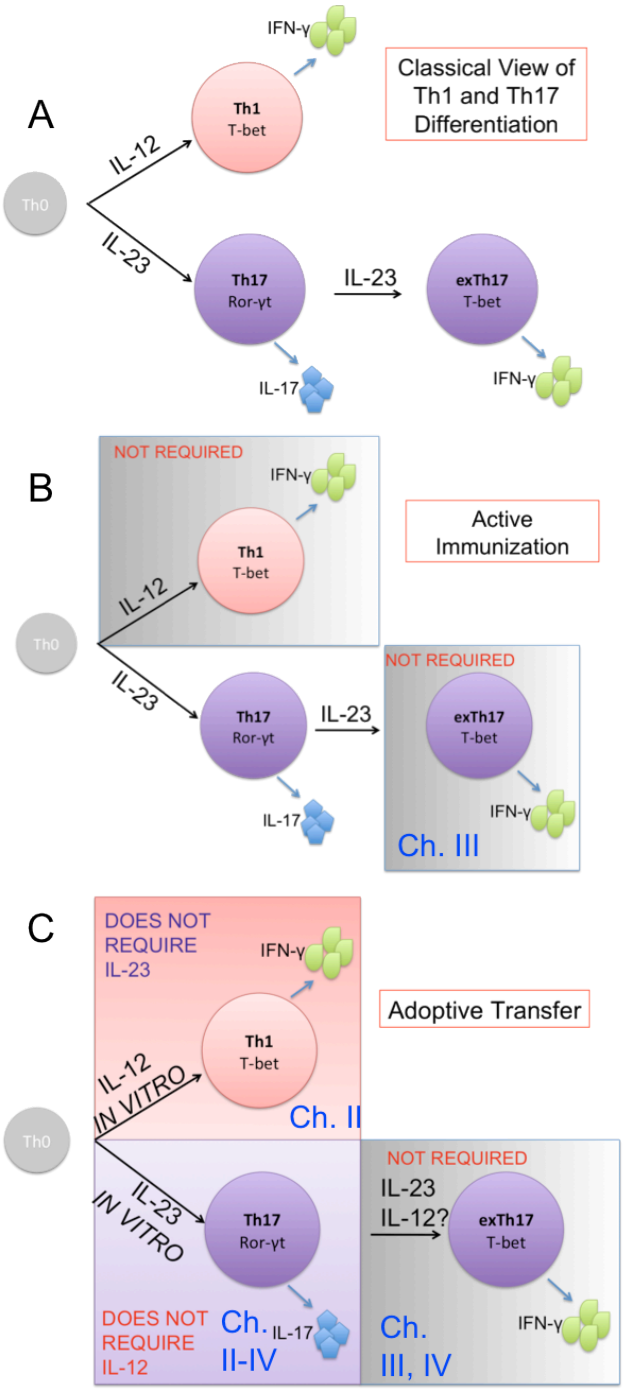
CHAPTER V

DISCUSSION

Based on previously published literature, the contributions of Th1 cells, stable Th17 cells, and exTh17 cells to disease development in the adoptive transfer model of EAE were unclear. The research described in this dissertation attempted to answer the following questions:

- *Is IL-23 required for Th1 CD4+ T cell encephalitogenicity?*
- *Is plasticity a critical feature of Th17 cell encephalitogenicity?*
- *Do stable IL-23-polarized cells have fundamentally different properties than plastic Th17 cells?*

The answers to these questions, described below, augment the current model of adoptive transfer EAE. This updated model, its analogous active immunization model of EAE, and chapters in which specific features have been discussed, as well as a classical model for T cell polarization and remaining questions, are illustrated in figure 5.1. Table 5.1 demonstrates the notable features of disease driven by each lineage.



Remaining Questions

- Why is IL-12 insufficient to induce encephalitogenicity in IL-23-deficient mice?
- Is the requirement for IL-23 rooted in specific homing or activation properties required for encephalitogenicity?

Remaining Questions

- What are the properties of exTh17 and Th1 cells that contribute to pathogenicity?
- What are the factors which promote exTh17 cells to efficiently traffic to the CNS?

Figure 5.1. IL-12 and IL-23 in the development of encephalitogenic CD4+ T cell lineages during EAE. A) The role of IL-12 and IL-23 in classical pathways of Th1, Th17, and exTh17 development and the critical and dispensable elements in B) the active immunization model of EAE and C) the adoptive transfer model of EAE. Several conclusions illustrated here were drawn from data published in this dissertation.

	Properties	Encephalitogenic Potential
Th1	*Enriched monocyte recruitment, accumulation* *Does not require IL-23*	++
Th17 (stable)	*Enriched neutrophil recruitment, accumulation* *Does not require IL-12* *Does not require T-bet* *Less efficient at inducing EAE following adoptive transfer than Th1 or exTh17 cells*	+
exTh17	*More efficient than stable Th17 cells in homing to the CNS* *More efficient than stable Th17 cells in inducing EAE* *IL-12 signaling by host promotes exTh17 development*	++

Table 5.1. Properties of EAE induced by Th1 cells, stable Th17 cells, and exTh17 cells.

IL-12 polarized Th1 cells do not require IL-23 for encephalitogenicity

In Chapter II, we showed that IL-12-polarized Th1 cells induce EAE independently of IL-23. This is a novel finding that contradicts the current dogma that IL-23 exposure is universally critical for CD4+ T cell encephalitogenicity. Furthermore, IL-23-independent Th1 cells induced disease more efficiently than IL-12-independent Th17 cells. Each lineage was highly polarized as defined by IFN-γ and IL-17 cytokine expression patterns prior to transfer and maintained those cytokine profiles following disease onset. IL-23-independent Th1 transfers elicited a monocyte-rich pattern of infiltration compared to IL-12-independent Th17 transfers, and a distinct global profile of cytokine and chemokine production. Using serum collected from MS patients, we used relative IL-12 and IL-23 levels to define MS patients groups. IL-12-skewed, IL-23-skewed, and unskewed patients formed three distinct groups in ordination space based on the expression levels of several cytokine and chemokines.

These data suggest that IL-12 and IL-23 drive distinct disease mechanisms in EAE and that adoptive transfer models can be used to determine features of those mechanisms. Furthermore, relative IL-12 and IL-23 expression, as opposed to IFN-γ and IL-17 expression, may be a better way to define Th1- or Th17-biased MS patient groups since the expression of those so-called signature cytokines is more mutable than the expression of innate polarizing cytokines.

Future studies using IL-12-independent and IL-23-independent EAE models will measure properties of highly polarized, encephalitogenic Th1 and Th17 cells following transfer, including survival, proliferation, and trafficking. According to the literature, IL-23 signaling drives both the plasticity and encephalitogenicity of Th17 cells. Hence, it was surprising to find that donor IL-23-polarized Th17 cells fail to convert to ex-Th17 cells in IL-12 deficient hosts. Furthermore, Th17 polarized myelin-specific T cells were less potent in IL-12 deficient than WT hosts. Preliminary data have demonstrated that, following transfer and EAE onset, IL-12Rβ2$^{-/-}$ Th17 cells sometimes "disappear" and cannot be detected in the CNS or periphery. This, along with data in this dissertation that demonstrate that transferred IL-12Rβ2$^{-/-}$ Th17 cells are stable, may suggest that IL-12 signaling promotes the survival of *bona fide* Th17 cells during the effector phase of EAE. Elucidating the mechanisms by which IL-12R promotes Th17 cell survival and plasticity would lead to novel findings that would add new perspectives on the distinction between Th1 and Th17 cells. Other future studies include examining the relevance of various effector cytokines and cell populations in IL-12 *versus* IL-23-driven disease. We also hope to determine the significance of the enrichment of monocytes and possible reduction in neutrophils in the CNS of mice with Th1-driven EAE *versus* Th17-driven EAE. While transfer of IL-23-independent Th1 cells into CCR2$^{-/-}$ hosts, which have impaired monocyte trafficking, resulted in reduced disease relative to wild-type hosts, we have not demonstrated that phenomenon is specific for highly polarized Th1-mediated disease but not Th17-mediated disease. Similarly, it is unclear if IL-12-

independent Th17-mediated disease, but not IL-23-independent Th1-mediated disease, is dependent on neutrophil recruitment to the CNS.

Currently, data and patient samples are available that were not used in the analyses published here, including MRI activity and clinical disease severity. Adding more parameters and patients to the human datasets may better refine our hypothesis that relative IL-12 and IL-23 expression defines distinct patient groups. Furthermore, if other published open-access MS patient studies that are available include patient IL-12 or IL-23 expression in any context, we can consider those data to determine if our conclusions hold across institutions and populations.

Data generated from MS patient serum averaged over 12 blood draws per patient at various stages of disease (and a very small number of patients) could be considered a poor comparison to data from brain and spinal cord homogenates from mice at the peak of EAE. Furthermore, the adoptive transfer model of EAE involves a great deal of manipulation *in vitro*. Active immunization eliminates the variables associated with *ex vivo* culture conditions. Studies with genetically engineered C57BL/6 mice indicate that EAE induced by active immunization requires IL-23-polarized Th17 cells and not Th1 cells for clinical manifestation. It is unclear why IL-12 is insufficient to promote disease in actively immunized IL-23-deficient mice. The adjuvant in CFA used for active immunization, *Mycobacterium tuberculosis,* is a potent inducer of IL-12. One can

speculate that in C57BL/6 mice, myelin antigen-specific CD4+ T cells must traffic through a region where a high level of IL-23 is produced prior to activation or initiation of effector function in the CNS. For example, high levels of IL-23 are produced in the gut, and Th17 cells are found at a high frequency in gut-associated lymphoid tissue (GALT). It is possible that in IL-23-deficient mice, myelin antigen-specific CD4+ T cells traffic through the GALT but are not secondarily activated or licensed by IL-23 to become fully encephalitogenic. Nevertheless, the adoptive transfer data shown here demonstrate the ability of IL-12 to circumvent the requirement for IL-23 for the acquisition of encephalitogenicity in vitro. The results of our human, as well as murine, studies support the hypotheses that IL-12 and IL-23 drive distinct CNS-targeted autoimmune diseases and suggest that these pathways could be differentially targeted.

Th17 cell plasticity is not required to induce EAE

In Chapter III, we demonstrated that IL-23-polarized T-bet$^{-/-}$ Th17 cells have characteristics of stable, highly polarized Th17 cells. Following adoptive transfer, T-bet$^{-/-}$ Th17 cells induced EAE in wild-type and RAG2$^{-/-}$ hosts. Furthermore, following transfer, T-bet$^{-/-}$ Th17 cell populations remained high IL-17-producers and produced little IFN-γ, while wild-type Th17 cell populations were predominantly IFN-γ-producers and produced little IL-17. Therefore, these data reinforce previous findings that T-bet is required for Th17 cell plasticity, and also

demonstrate the novel finding that the feature of plasticity is not absolutely required for IL-23-polarized cells to become encephalitogenic.

Stable Th17 cells exhibit unique homing properties

While T-bet$^{-/-}$ Th17 cells could induce EAE, disease was mild and delayed relative to wild-type controls. Chapter IV pursued those data reported in Chapter III and investigated the mechanisms of the reduced potency of T-bet$^{-/-}$ Th17 cells. Using co-transfer models where IL-23 polarized wild-type and T-bet$^{-/-}$ CD4+ T were injected into the same RAG2$^{-/-}$ host, we determined that T-bet$^{-/-}$ cells accumulate less in the CNS, but not in the periphery, relative to wild-type cells. This did not appear to be a result of defective proliferation or elevated apoptosis in T-bet$^{-/-}$ cells. However, the transferred T-bet$^{-/-}$ CD4+ cells expressed different homing molecules than WT cells. Specifically, T-bet$^{-/-}$ cells expressed CCR6, and $\alpha 4\beta 7$ while WT cells expressed CXCR3.

Like Chapter II, Chapters III and IV have broad implications in Th17 cell biology and the properties that promote Th17 cell encephalitogenicity. The data demonstrate that Th17 cell plasticity is dispensable for Th17 cell function in EAE, but it may promote Th17 cell potency. Previous reports have demonstrated that though T-bet is considered to be a Th1-associated factor, it is upregulated in Th17 cells following IL-23 exposure and during EAE, even in the absence of IFN-γ and IL-12 [1]. Our data suggest that T-bet is not absolutely required for

autoimmune Th17 cell function but does influence T cell trafficking and, consequently, the efficiency with which effector cells infiltrate their target organ.

Future studies will examine the trafficking properties of T-bet$^{-/-}$ Th17 cells in detail. Based on the integrins and receptors that were upregulated on Th17 cells, it is possible that these cells preferentially home to non-CNS tissues, including gut-associated lymphoid tissue. The ligand for $\alpha4\beta7$, MadCAM-1, is expressed highly in gut associated tissues. We hypothesize that administration of $\alpha4\beta7$- and MadCAM-1 blocking reagents to the adoptive transfer recipients of T-bet$^{-/-}$ Th17 cells will result in a redistribution of donor cells such that less accumulate in the GALT and more in the CNS. If that is the case those blocking agents will paradoxically cause disease exacerbation in recipients of T-bet$^{-/-}$ Th17 cells.

Questions also remain regarding the relevance of the Ly6G^{+} myeloid cells that expand in the spleens of RAG2$^{-/-}$ hosts following transfer of wild-type, but not T-bet$^{-/-}$ Th17 cells. It is possible those Ly6G^{+} cells are suppressive and contribute to the reduced severity of EAE seen in recipients of T-bet$^{-/-}$ Th17 cells. In tumor models, Ly6g+ myeloid cells, or myeloid-derived suppressor cells (MDSC) have a suppressive effect on T cell responses to promote tumor survival [2]. MDSC have been previously described in EAE, though their identification and veracity as CD4+ T cell suppressors *in vivo* is unclear [3, 4]. Comparing these cells to previously described myeloid-derived suppressor cells by measuring cell-surface

marker and cytokine expression, as well as functional T cell suppression assays, will help determine whether they are contributing to reduced disease.

Several groups have published on the role of T-bet in efficient T cell trafficking, though most of those studies have been done in the context of IL-12-polarized Th1 cells or other cell lineages. T-bet controls CXCR3 expression in CD8+ cells, Th1 cells and regulatory T cells [5]. T-bet deficient cells also express less sphingosine-1-phosphate receptors 1 and 5 and P-selectin ligand [6, 7]. However, the role of T-bet in Th17 cell trafficking is largely unexamined. The data published here suggest that IL-23-polarized T-bet deficient T cells are reminiscent of highly polarized Th17 cells. Therefore it would be reasonable to predict that Th17-associated trafficking molecules, including $\alpha 4\beta 7$, CCR6, and other gut-homing receptors are enriched on T-bet deficient IL-23-polarized cells. Furthermore, as T-bet is a pleiotropic transcription factor that regulates not just Th1 development but several other pathways, including the conversion of Th17 cells to exTh17 cells, it would seem that the acquisition of the exTh17 profile *via* T-bet would include the altered expression of Th1-associated trafficking molecules. This would confirm the assumption that the conversion to an exTh17 cell is not simply the upregulation of IFN-γ and downregulation of IL-17, but a shift in the entire expression profile of the Th17 cell. It would be interesting if this shift resulted in a “hybrid” of Th1 and Th17 cell trafficking profiles, contributing to the unique properties of exTh17 cells.

The role of Th17 cells, exTh17 cells, and Th1 cells in autoimmune disease

Collectively, this dissertation describes the factors that are dispensable or required for the encephalitogenicity of Th1, Th17, or exTh17 cells, as well as distinct and overlapping properties of EAE induced by each of these subsets (Table 5.1). While these data clarify the contributions of exTh17 cells and IL-12 to EAE (and possibly MS), further questions remain and are listed in figure 5.1. Furthermore, the aforementioned questions regarding the relevance of EAE to MS and the artificial nature of the adoptive transfer model raises the possibility that our results are an artifact of culture conditions or specific for mouse models of neuroinflammation. Nonetheless, there is evidence that Th1, Th17, or exTh17 play similar roles in other rodent models of autoimmunity and disease. Muranski et al. determined that tumor-specific Th17 cells could efficiently eliminate tumors following adoptive transfer. Not only did this absolutely require the ability for Th17 cells to acquire exTh17 cell properties (including the expression of IFN-γ and T-bet), but transferred Th1 cells were unable to eradicate tumors, suggesting a functional distinction between Th1 cells and exTh17 cells [8]. In fact, unlike data published in this dissertation, T-bet was absolutely required for Th17 cells to clear tumors, suggesting exTh17 cells are also functionally distinct from Th17 cells in this model. This also suggests the presence and function of exTh17 cells in models outside of autoimmunity. In another example, adoptive transfer models of diabetes demonstrate that disease can be induced with Th1 cells, and to a lesser extent Th17 cells, but the upregulation of IFN-γ by those Th17 cells is

critical to their pathogenicity [9]. These data suggest that even in other mouse models, exTh17 cell development promotes or is critical to Th17 cell function, and exTh17 cell function is distinct from that of Th1 cells.

T-bet-deficient Th17 cells and IL-12 signaling-deficient Th17 cells fail to upregulate IFN-γ following disease onset and induce disease less efficiently than either wild-type Th17 cells or IL-23-deficient Th1 cells. IL-12R expression is controlled by T-bet in a positive feedback loop following IFN-γ exposure, but T-bet is also upregulated by T cells following IL-23 exposure. Furthermore, it has been demonstrated that T-bet is upregulated in CD4+ cells during EAE in even in mice deficient in IL-12- and IFN-γ signaling pathways [1]. T-bet should then be sufficiently upregulated by IL-23-polarized IL-12R-deficient Th17 cells transferred into wild-type hosts. However if that were the case, why are IL-12R-deficient Th17 cells stable? It is also curious that although actively immunized mice deficient in IL-12 signaling are susceptible to EAE, the IL-12p40-deficient transfers of IL-23-polarized cells were a great deal less efficient at inducing disease. Therefore, there are paradoxes that seem to link IL-12 to exTh17 development and Th17 cell encephalitogenicity *in vivo*.

The description of the exTh17 lineage has been fascinating because it blurs the lines between two functionally distinct CD4+ T cell subsets, whose function in several other disease models and human diseases depends on the signature cytokines they produce. IFN-γ production by CD4+ cells is critical for mice to

survive *Listeria monocytogenes, Toxoplasma gondii* infection, and *Salmonella enterica* [10]. Incidentally, *Listeria* infection is cleared in the absence of IL-23R signaling. IFN-γ is thought to mainly activate macrophages and promote MHCII expression, as well as its aforementioned role in the expression of CXCR3 and its ligands. IL-17A on the other hand, is critical for clearance of extracellular bacterial and fungal infections, including *Klebsiella pneumoniae* and *Candida albicans*, and is thought to promote activation and recruitment of neutrophils [11]. However, during EAE as well as collagen-induced arthritis, a rodent rheumatoid arthritis disease, IFN-γ expression not required for disease [12-15]. Not only does this observation promote the assumption that Th17 cells and not Th1 cells are the single critical mediators of EAE, it undermines the relevance of IFN-γ-producing exTh17 cells. Indeed, in mice lacking IL-23R, Th17 cells develop, but are not encephalitogenic, and do not convert to exTh17s [10, 16]. However, it is important to consider that our lab has demonstrated that IL-23-polarized myelin-specific T cells require IFN-γ and not IL-17 to promote EAE. Also, the shift in a transcriptional program induces a network of change, and while it is convenient to examine two informative cytokines, alterations of other critical factors could be overlooked. Further description and comparison of these three subsets will define those factors. Unfortunately, until those definitions arise, the relevance of *bona fide* exTh17 cells in human disease will be difficult to measure. Nonetheless, cells expressing both Rorc and T-bet, as well as IFN-γ and IL-17, have been recovered from patients with Crohn's Disease; Th17 cells that are able to convert into Th1 and other CD4+ cell lineages *ex vivo* have been recovered from patients

with ulcerative colitis, graft versus host disease, and colon cancer [17, 18]. Furthermore, as mentioned previously, there have been reports on the presence of IFN-γ- and IL-17-producing T cells in the periphery and CNS of patients with active MS, and more recent reports have described IFN-γ-producing Th17 cells recovered from the periphery of patients with MS are able to cross a model of the blood brain barrier [19, 20]. These data suggest the existence of a population of Th17 cells with Th1-associated properties in human disease, and whether their relevance is similar to that seen in mouse models is yet to be determined.

As previously discussed, MS is a highly heterogeneous disease and patients have variable responses to available disease modifying therapies. Overall, the data presented here suggest distinct molecular mechanisms for CNS-targeted autoimmune diseases in mouse and human. Though IL-12 and IL-23 expression and exTh17 development has been measured and controlled in these rodent studies to determine their pathogenicity independently of each other, it is certainly possible that those lineages have a common downstream mechanism of pathogenesis. Indeed, our research using the EAE model determined those both IL-12- and IL-23-driven diseases have similar clinical phenotypes, so the possibility that the two pathways converge to form a similar outcome would not be surprising. If this were the case, treatment of MS could be uniform and relatively straightforward across patients. However, the differences in inflammatory cytokine production, T cell trafficking, myeloid cell recruitment to the CNS and in the periphery, patterns of infiltration, and overall potency of the Th

lineages suggest EAE--and likely MS--has several non-overlapping mechanisms that result in autoimmune disease. MS risk is determined by genetic influences, infection, and environmental factors that could each promote distinct innate cytokine profiles. Thus, any of those factors could promote the induction of one pathway over another to induce disease. If a multi-pathway theory were true, treatment for MS would require the identification of dominating mechanisms within each patient using biomarkers, then targeting treatment toward those mechanisms. For example, it has been reported that accepted treatments for MS, including IFN-β, fingolimod, and dimethyl fumarate can suppress Th17 cell development and function [21-24]. Other groups have attempted to divide patients into responders and non-responders and determine IL-17 expression profiles, though with limited success [25]. Ultimately, stratifying patients based on distinct molecules or features is the appropriate strategy of determining whether those features are relevant for MS.

In the adoptive transfer model of EAE, IL-12 and IL-23 can be mutually exclusive and exTh17 cell function is separate from Th1 and Th17 cell development. Using the models described, further clarification of each of those populations could lead to better understanding their roles in other mouse models, as well as in autoimmune disease in human populations.

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