Tim-3 Regulation of Central Nervous System Autoimmune Disease

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Abstract

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Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) mediated by self-reactive, myelin-specific T cells. Both CD4+ and CD8+ T cells play important roles in the pathogenesis of MS. MS is studied using experimental autoimmune encephalomyelitis (EAE), an animal model mediated by myelin-specific T cells. Tim-3 is a cell-surface receptor expressed on CD4+ IFN-γ-secreting Th1 cells, and triggering Tim-3 signaling ameliorated EAE by inducing death in pathogenic Th1 cells in vivo. This suggested that enhancing Tim-3 signaling might be beneficial in patients with MS. However, Tim-3 is also

expressed on activated CD8+ T cells, microglia, and dendritic cells (DCs), and the combined effect of manipulating Tim-3 signaling on these cell types during CNS autoimmunity is unknown. Furthermore, CD4+ IL-17-secreting Th17 cells also play a role in MS but do not express high levels of Tim-3. We investigated Tim-3 signaling in EAE models that include myelin-specific Th17, Th1 and CD8+ T cells. We found that preventing Tim-3 signaling in CD4+ T cells altered the inflammatory pattern in the CNS due to differential effects on Th1 versus Th17 cells. In contrast, preventing Tim-3 signaling during CD8+ T cell-mediated EAE exacerbated disease. We also analyzed the importance of Tim-3 signaling in EAE in innate immune cells. Tim-3 signaling in DCs and microglia did not affect the manifestation of EAE in these models. These results indicate that the therapeutic efficacy of targeting Tim-3 in EAE is dependent on the nature of the effector T cells contributing to the disease.

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List of Abbreviations

Ab	antibody
Ag	antigen
APC	antigen presenting cell
Bat3	human leukocytes antigen B-associated transcript 3
BBB	blood-brain barrier
BCR	B cell receptor
BMC	bone marrow chimera
CFA	complete Freund's adjuvant
CPM	counts per minute
CSF	blood-cerebrospinal fluid
CNS	central nervous system
EAE	experimental allergic encephalomyelitis
DC	dendritic cell
FACS	fluorescence-activated cell sorter
GM-CSF	granulocyte-macrophage colony-stimulating factor
golli	gene of oligodendrocyte lineage
GWAS	genome-wide association studies
HLA	human leukocyte antigen
ICC	intracellular cytokine staining
IFN-γ	interferon gamma
IL-17	interleukin-17
i.p.	intraperitoneal
iTreg	inducible regulatory T cell
i.v.	intravenous
LN	lymph node
LPS	lipopolysaccharide
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PLP	proteolipid protein
PP	primary progressive
PRR	pattern-recognition receptor
Ptx	pertussis toxin
Rag	recombinase activating gene
RR	relapsing–remitting
SP	secondary progressive
TCR	T cell receptor
Tg	transgenic
Th	T helper
Tip	TNF- and iNOS-producing
TLR	Toll-like receptor

Tim-3	T-cell immunoglobulin and mucin domain-3
WT	wild-type

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DEDICATION

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Chapter 1: Introduction

The Immune system

The immune system is comprised of a variety of cells and molecules that form a versatile defense network to protect the host from invading pathogens while maintaining tolerance to self. An immune response can be divided into two functions: recognition and response. The immune system is capable of specifically recognizing an immense diversity of pathogens and is able to discriminate between non-self- and self-proteins. When an invading pathogen has been recognized, the immune system recruits the appropriate immune cells and molecules to elicit an effector response to clear the infection. If the same pathogen has been previously exposed to the host, a memory response would be induced and is characterized by a more rapid recognition and stronger immune reaction that serve as a rationale for vaccination.

Innate Immunity

The mammalian immune system can be divided into two components: innate immunity and adaptive immunity. The innate immune system is the first line of defense immediately after infection, before the development of an adaptive immune response. The innate immune response utilizes a set of pathogen-resistance mechanisms that are not specific to a particular microbe. The innate immune system is comprised of physical and anatomic barriers such as skin and mucus, phagocytic cells such as macrophages, dendritic cells and neutrophils, and antimicrobial and inflammatory compounds. The innate immune cells utilize a set of disease-resistance mechanisms that are not specific to a particular pathogen; instead, it contains cellular and

molecular components such as pattern-recognition receptors (PRRs) that recognize highly conserved structures specific to frequently encountered pathogens. For example, Toll-like receptors (TLRs) are a type of PRRs usually expressed in macrophages and dendritic cells that recognize foreign molecules including lipopolysaccharide (LPS), double-stranded RNA of viruses, and flagellin [1]. Upon recognition of infectious agents, phagocytes ingest pathogens and release bactericidal and reactive oxygen species to eliminate invading organisms. Macrophages can elicit addition help from other immune cells to the site of infection by releasing soluble mediators such as chemokines and cytokines. In addition, activated dendritic cells play an essential role in linking the innate and adaptive immune system by priming naïve T cells in secondary lymphoid organs.

Adaptive Immunity

While the more primitive innate immune system is found in certain invertebrate organisms, the adaptive immune system has evolved to work together intricately with the innate immune system to mount a more specific immune response to ensure high degree of protection in vertebrates. The adaptive arm of the immune system is also responsible for the generation and maintenance of memory responses to previous infection. The specificity and ability of the adaptive immune system to strengthen over the course of a response is possible because the receptors expressed on adaptive immune cells such as T and B lymphocytes are generated via sophisticated genetic mechanisms. T cell receptor (TCR) and B cell receptor (BCR) gene segments undergo somatic recombination during development and maturation, which allow them

to randomly generate a vast number of antigen receptors for specific recognition of most evolving pathogens. Because adaptive immune responses require five to seven days to generate, the innate immune response is essential during the initial stage of host defense. Together, the innate and adaptive immune systems work in a precisely coordinated manner to provide acute and long-lived immunity against variety of pathogens.

CD4+ T helper cell activation and differentiation

CD4+ T helper (Th) cells play an important role in the immune system. They are capable of influencing other immune cells, including activation and maintenance of CD8+ T cell and B cell responses, optimization of macrophage and dendritic cell functions, and regulation of peripheral tolerance. Two stimulatory signals are generally required for the activation of naïve T cells. Signal 1 is the initial signal generated by the binding of TCR on T cells to processed antigenic peptide bound to major histocompatability complex (MHC) class I or II molecule on the surface of antigen presenting cells (APCs); signal 2 describes a subsequent antigen-nonspecific co-stimulatory signal provided primarily by the interaction between CD28 receptor on the T cell and members of the B7 family (CD80 and CD86) expressed on activated APCs [2]. DCs are considered highly efficient APCs in activating naive T lymphocytes, although B cells and macrophages can also present antigens to T cells [3-9]. Upon full activation of CD4+ T cells, they undergo significant proliferation and differentiate into distinct Th cell subsets of specific phenotypes. Depending on the strength and the duration of TCR engagement and signaling as well as the cytokine milieu at the site of T cell activation [10-12], naïve CD4+ T cells may differential into at least four distinct Th cell subsets: Th1, Th2, Th17, and inducible regulatory T cells (iTregs) [13]. Each subset expresses unique transcription factors and is distinguished by their specific patterns of cytokine production and function that is summarized in **Table 1**.

Immune responses in the central nervous system

The central nervous system has been considered an immune privilege site. Anatomical and physiological barriers are in place to isolate the CNS from the immune system under normal conditions. The blood-brain barrier (BBB) is comprised of specialized endothelial cell that form tight junctions surrounding the majority of CNS capillaries. Tight junctions strictly regulate the movement of ions, molecules, and cells between the blood and the CNS [14]. Cerebrospinal fluid (CSF) produced in the choroid plexus of the brain occupies the subarachnoid space, lining the ventricles in the brain and spinal cord [15]. Another element that hinders the immune response in the CNS is the absence of a formal lymphatic drainage seen in other peripheral tissues. Lack of a lymphatic drainage system decreases CNS antigens from entering nearby lymph nodes, thus limiting the presentation of CNS antigens by APCs in the periphery [16]. Furthermore, the low expression of MHC class II molecules in CNS resident cells restricts antigen presentation and T cell reactivation under healthy conditions [17]. Together, these anatomical and structural properties of the CNS minimize immune surveillance by T cells from the periphery under healthy condition.

Microglia

In order to main optimal functioning of the CNS, resident microglia play important roles in the survival and regeneration of neuronal cells and immune surveillance [18]. Microglia are considered the resident macrophages of the CNS as they have the ability to phagocytose and clear debris and dead cells, as well as provide the first line of defense against infectious agents.

In the healthy CNS parenchyma, microglia are confined in an immunosuppressive environment and express little to no MCH class II molecules [19-21]. However, microglia can upregulate MHC class II, CD80, CD86, and CD40 upon pathogen encounter to facilitate T cell activation [22, 23]. While microglia share many functional similarities with macrophages, they are distinct from the monocyte or macrophage lineage as they originate from a yolk sac progenitor [24], and they are maintained in the CNS throughout life without reconstitution from the bone marrow [25, 26].

CNS antigen trafficking

It is suggested that the adaptive immune responses against CNS antigens are initiated in the periphery and subsequently propagated to the CNS [27]. Since the CNS lacks a conventional lymphatic system, extracellular fluids containing CNS antigens can drain through CSF to the cervical lymph nodes (LNs) [28]. Additional means for transporting CNS antigens is provided by macrophages and DCs, which are located in CNS compartments including choroid plexus, meningeal, and perivascular space outside the CNS parenchyma [29-31]. Perivascular macrophages and DCs phagocytose and process CNS antigens and migrate to the cervical LNs [32-34], where they can stimulate antigen-specific responses in naïve and memory T cells in the periphery [14]. Activated and memory T cells express a specific combination of adhesion molecules, chemokine receptors and integrins that allow them to cross the BBB and enter the CNS [14]. Finally, resident APCs presenting CNS antigens in the brain and spinal cord can re-stimulate these T cells and propagate a competent immune response resulting in neuroinflammation.

Autoimmunity

Autoimmunity is an inappropriate response of the immune system against its own cells and tissues mediated by self-reactive lymphocytes. During T cell and B cell development in the thymus and bone marrow, immature lymphocytes are subjected to negative selection in which immature T and B lymphocytes that have high affinity for self-antigens are eliminated, resulting in self-tolerance. Negative selection is not perfect, as self-reactive lymphocytes can still be found in the periphery of healthy individuals, however, they do not typically initiate autoimmune

responses. There are mechanisms in place to maintain peripheral tolerance including clonal deletion, anergy, and immune suppression by regulatory immune cells. Clonal deletion is a process in which mature lymphocytes that bind to self-antigens with high affinity for the first time in the periphery undergo apoptosis. Anergy is a state of nonresponsiveness to antigen, whereas T and B cells do not respond to engagement of their specific antigens. When T and B cells are under chronic exposure to the same antigen, they become inactivated or anergic. Furthermore, in the presence of inhibitory signals such as CTLA-4, or absence of inflammatory cytokines and the expression of co-stimulatory molecules on the APCs, recognition of a peptide-MHC complex by naïve T and B cells induce tolerogenic signal from the antigen receptor, resulting in anergy. Regulatory T cells (Tregs) play an important role in the maintenance of peripheral tolerance. They are characterized by expression of the transcription factor *Foxp3* and cell surface markers CD4 and CD25 [35]. Tregs express a diverse TCR repertoire for antigen recognition that is distinctly different from that of CD4+ conventional T cells [36-38]. Two lineages of Tregs have been identified: natural Tregs (nTregs) and inducible Tregs (iTregs). nTregs originate from the thymus in a process similar to normal T cell development [39, 40], whereas iTregs are differentiated from naïve CD4+ conventional T cells in the periphery in the presence of IL-2 and TGF-β [41, 42]. Both nautral and inducible Tregs can suppress self-reactive T cells and modulate ongoing immune response. Failure in maintaining peripheral tolerance by clonal deletion, anergy, and Tregs leads to activation of self-reactive T or B cells against self-antigens that can cause severe damage to cells and organs, resulting in development of autoimmune diseases such as type-I diabetes, lupus, rheumatoid arthritis, and multiple sclerosis.

	Th1	Th2	Th17	iTreg
Signature cytokines	IFN-γ	IL-4 IL-5 IL-13	IL-17A IL-17F IL-22	IL-10 TGF-β
Lineage-specific transcription factor	*T-bet*	*GATA-3*	*RORγt*	*Foxp3*
Specialized functions	Cell-mediated immunity; autoimmunity	Extracellular immunity; allergy & asthma	Bacterial & fungal infection; autoimmunity	Suppression of immune response

Table I. T helper cell subsets

Upon activation, naive CD4+ T cells differentiate into distinct Th cell subsets. Th1, Th2, Th17, and iTreg are defined based on the cytokines produced. Each cell subset expresses unique lineage-specific transcription factors and they exhibit specialized functions that contribute to the clearance of specific types of pathogens and are involved in certain inflammatory and autoimmune diseases. This table is adapted from [43].

Multiple sclerosis

Overview

Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system affecting approximately 350,000 people in the United States and 2.5 million worldwide. It is most commonly diagnosed during early adulthood and affects more females than males [44]. MS is characterized by focal inflammatory infiltrates, demyelinating plaques, and axonal damage, resulting in a wide spectrum of neurological defects including sensory motor disturbances, ataxia, fatigue, and cognitive impairment [45]. Although the exact cause of the disease is not known, it is believed to be of autoimmune origin in which self-reactive immune cells recognize myelin antigens and infiltrate into the CNS [46-48]. MS susceptibility is associated with a complex interplay of genetic [49] and environmental factors [50]. A long-standing hypothesis is that environmental factors trigger MS in genetically susceptible individuals by promoting the activation of myelin-specific T cells that normally circulate in the periphery in a tolerant state. Once activated, these T cells can enter the CNS and initiate an autoimmune response. A major role for CD4+ T cells in this process is strongly supported by the observation that the strongest association of genetic susceptibility to MS is with MHC class II alleles that present antigen to CD4+ T cells. However, a role for MHC class I alleles that present antigen to CD8+ T cells was also confirmed by the genome-wide association studies (GWAS) [51], suggesting a complex pathogenesis.

Myelin antigens in MS

The pathology of MS is generally believed to reflect autoimmune attack upon myelin auto-antigens. There are four myelin proteins thought to be involved in MS disease progression, including myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin-associated glycoprotein (MAG) [52-54]. MBP is a major component of the myelin sheath of oligodendrocytes and Schwann cells in the central and peripheral nervous system. It is the second most abundant protein in myelin after PLP in the CNS, and it plays a central role in maintaining the structural integrity of the myelin sheath [55]. MBP family actually refers to a family of isoforms that arise by differential splicing of a single mRNA transcript initiated from one of three transcription start-sites within the gene complex called gene of oligodendrocyte lineage (golli) [56]. Although golli proteins are generated from the first transcription start-site and they share some common MBP epitopes, proteins in this family differ from the classic MBPs in which they contain a unique 133-amino-acid sequence. In contrast to the classic MBPs that are predominantly expressed in the CNS, golli-MBP proteins are expressed more ubiquitously in the immune system [55], including bone marrow [57], spleen [58], macrophages [59], and thymocytes [60]. The functions of golli proteins are largely unknown, however, they have been suggested to serve as additional sources of MBP epitopes in autoimmunity [61-63] as well as regulate T cell activation and differentiation [58, 64]. In addition, our laboratory has shown that the expression of MBP epitopes within golli proteins in the thymus could induce central tolerance of MBP_{79-87}-specific CD8+ thymocytes [65]. On the other hand, the

classic MBPs that are incorporated into myelin are produced from the second and third transcription start-sites of the gene, but these protein share the first three exons that are also utilized in transcripts encoding golli-MBP proteins. T cells specific for MBP are found in both healthy individuals and patients with MS [66], however, T cell clones isolated from patients with MS produce significantly more Th1 cytokines than those generated from healthy individuals when activated with MBP peptides in vitro [67].

MOG is a transmembrane glycoprotein expressed on the surface of myelinating oligodendrocytes and on the external lamellae of myelin sheaths in the CNS. MOG is a quantitatively minor component of total myelin protein relative to PLP and MBP [68]. Although the primary function of MOG is not well understood, it is speculated to function as adhesion molecule on the myelin sheath to maintain structural integrity and facilitate interactions between myelin and the immune system [69]. More importantly, MOG is identified as an important autoantigen in MS [70]. PLP is a hydrophobic integral membrane protein and produced predominantly by mature myelinating cells such as oligodendrocytes [71]. The major functions of PLP in the CNS include compaction and stabilization of myelin sheaths, maintenance and survival of axons, and maturation of oligodendrocytes [72]. MAG is a type I transmembrane glycoprotein localized in periaxonal Schwann cells and glial membranes of myelin sheaths present in both central and peripheral nervous system [73]. The functions of MAG include formation and maintenance of oligodendrocytes and myelinated axons [74, 75]. Preferential MAG

loss associated with oligodendrocyte dystrophy at the edge of some acute lesions is detected in some patients with MS [76, 77].

Clinical courses

MS is a heterogeneous disease manifested with diverse clinical course and pathological features. Four common clinical courses of MS have been recognized by the National Multiple Sclerosis Society in 1996 based on the frequency and severity of neurological signs, the accumulative damage in the CNS, and the capability of recovery. Relapsing–remitting form of MS (RR-MS) is diagnosed in approximately 85% of patients with MS and can persist for many years, during which patients experience episodes of neurological disability with full recovery of function between relapses [78, 79]. Approximately 50% of those with an initial RR-MS eventually develop secondary progressive MS (SP-MS), when the period of remission after each acute attack diminishes [80]. The reasons for this shift to increasing clinical disability and lack of return to baseline are not known. A less common form of MS is primary progressive (PP-MS), which comprises approximately 10-20% of all MS cases. Patients with PP-MS lack the acute exacerbations seen in patients with RR-MS; instead, they develop steadily worsening clinical disability from the time of their initial diagnosis [81]. Patients with PP-MS respond poorly to most commonly used immune-modulating MS therapeutics [82], suggesting that a different pathogenic pathway may be at play in these patients compared to those with RR-MS. The prognosis of patients with MS depends on the form of the disease and varies among individuals but is usually not life shortening.

Lesion patterns

The locations of lesions within the CNS are variable among patients, and they are the key determinant of clinical symptoms. The majority of lesions are found in the brain, particularly in the periventricular white matter, cerebellum, brainstem, and optic nerves. Many patients exhibit lesions in the spinal cord as well as the brain, while only 2-10% of patients exhibit inflammation in the spinal cord and optic nerves without extensive involvement of the brain (referred to as opticospinal MS) [83, 84]. The mechanisms promoting differences in localization patterns of lesions in the CNS during MS are not well understood.

Pathology of MS lesions

The pathology of the MS lesions is highly variable, exhibiting heterogeneous immune cell composition. The CNS pathology in MS is characterized by inflammatory lesions, demyelination, remyelination, neurodegeneration, and glial scar formation [85]. Criteria have been developed that allow the lesions seen in patients with MS to be divided into four categories (Patterns I-IV). The criteria are based on the distribution of myelin loss, plaque geography and extension, pattern of oligodendrocyte injury, and immunopathological evidence of immunoglobulin and activated complement deposits [76]. Patterns I and II lesions are dominated by T cells and macrophages and are centered around blood vessels, with preservation of oligodendrocytes. The main difference between pattern I and pattern II lesions is the presence of antibody and complement deposition in pattern II lesions. In contrast, patterns III and IV are

associated with oligodendrocyte cell death. Pattern III lesions involve apoptosis of oligodendrocytes and they have more diffuse inflammation not centered on blood vessels. The feature of pattern IV lesions is non-apoptotic oligodendrocyte degeneration [86]. Studies of MS lesions revel that multiple active plaques analyzed from an individual belong to the same pattern, indicating a lack of intra-individual heterogeneity. This suggests that the pathological patterns may reflect distinct pathogenic pathways occurring in different individuals rather than the evolution of lesions over time within an individual [76].

Genetic susceptibility of MS

The idea that genetic factors may be associated with MS started to unfold in the 1890s with the identification of familial aggregation [87]. Familial based studies have shown that first, second, and third-degree relatives of people with MS are more likely to have the disease than the general population [88]. Specifically, genome linkage studies identified a strong correlation of HLA class II genes and MS susceptibility, whereas HLA class I genes that increase and decrease the genetic susceptibility to MS were identified [89]. However, the relatively low concordance rates of twins (25.4% in identical twins and 5.4% in fraternal twins) support the importance of non-genetic factors such as environmental factors in the overall MS susceptibility and disease outcome [90].

Environmental factors in susceptibility of MS

Both non-infectious and infectious agents have been proposed to contribute to pathogenesis of MS [91]. Geographically, there is a higher prevalence of MS in people who live farther from the north or south of the equator, which inversely correlates with duration and intensity of sunlight. These data have attracted the interest of studying the role of UV and vitamin D in influencing MS development [50]. However, the large range of incidence and prevalence seen in different regions in some countries does not necessarily follow latitudinal gradient. For example, MS is common in regions populated by Northern Europeans but is rare in the Asian race [92]. Immigration studies correlate the risk of MS with place of residence in childhood, in which if an individual migrated from a region of high frequency of MS to a region of low frequency before age 15, the low risk was acquired, whereas migration after 15 did not change the risk [93].

Animal models of MS

Introduction

Developing therapies to treat or prevent MS requires an in-depth understanding of the pathogenesis of the disease. Mechanistic studies in MS are challenging because central nervous system tissue is difficult to access and immune responses within this tissue cannot be easily monitored. Therefore, animal models are essential in defining the mechanisms underlying MS. These models are important not only to discover new therapeutic targets, but also to test new therapies prior to translation to patients. One of the major challenges in developing animal models of MS is that patients with MS vary widely both in disease presentation and in response to therapeutics. Nevertheless, developing animal models that recapitulate different aspects of the disease seen in patients with MS is important because it could provide a fundamental framework for understanding the complex pathogenesis of MS.

Experimental autoimmune encephalomyelitis

EAE is the most widely used animal model of T cell-mediated CNS autoimmune disease that shares many clinical and histological features with MS. EAE is induced either by immunization with myelin antigens (active induction) or by adoptive transfer of T cells isolated from animals immunized with myelin antigens that are reactivated in vitro prior to transfer into naïve animals (passive induction) [94, 95]. EAE has been studied in a variety of animal species, including mouse, rat, rabbit, guinea pigs, and non-human primates. The manifestation of EAE

can vary greatly depending on the strain or genotype of rodent and sometimes the mode of induction (active versus passive). Since no single model replicates the full spectrum of inflammatory mechanisms and neurodegeneration seen in MS, studies using different EAE models are necessary to represent the complexity and heterogeneity of CNS autoimmune disease.

CD4+ T cell-mediated EAE models

The strongest known genetic risk-factor of MS is the HLA classes II genes, implicating HLA class II molecules and CD4+ T cells in MS pathogenesis [89]. The pathogenicity of CD4+ T cells was also demonstrated in various EAE models. Most immunization protocol of EAE using pertussis toxin and myelin antigens emulsified in complete Freund's adjuvant (CFA) results CD4+ T cell activation, and adoptive transfer of CD4+ T cells from immunized mice was sufficient to induce EAE [96]. In addition, it has been shown that mice expressing transgenic TCRs specific for MHC class II-restricted myelin epitopes developed spontaneous EAE mediated by myelin-specific CD4+ T cells [74,95-97]. Together, these adoptive transfer and spontaneous disease studies identify CD4+ T cells as a major effector cell type in EAE.

Both Th1 and Th17 cells are found in the CNS and CSF of patients with MS [97-100]. Although IFN-γ-producing Th1 cells were originally suggested to be the effector CD4+ T cell subset that induced inflammation in EAE [86], IL-23-dependent Th17 cells was later identified as another T cell subset that can also induce EAE [101]. To further investigate the role of Th1 and Th17 cells in EAE, disease was induced in animal models that are genetically deficient in IFN-γ or IL-17. Surprisingly, both IFN-γ and IL-17 were not required for EAE induction [102-104]. When

additional cytokines were evaluated for their role in EAE pathogenesis, GM-CSF was identified as a critical pathogenic cytokine in EAE models as GM-CSF$^{-/-}$ mice are resistant to EAE [105]. Unlike IFN-γ and IL-17, GM-CSF-producing T cells are required for EAE induction [106], and that T cells producing GM-CSF can induce EAE in the absence of both IFN-γ and IL-17 [107, 108]. Recent studies showed that GM-CSF can be produced by both Th1 and Th17 cells. This finding helps explain how adoptive transfer of either Th1 or Th17 cells induces EAE independently of IFN-γ or IL-17, respectively.

"Classic" CD4+ T cell-mediated EAE model

EAE is typically induced by immunization with myelin antigens emulsified in complete Freund's adjuvant (CFA) accompanied by injections of pertussis toxin. This immunization protocol activates mostly MHC class II-restricted CD4+ T cells as myelin antigens are introduced exogenously; subsequent studies demonstrated that adoptive transfer of CD4+ T cells from mice immunized with myelin antigens was also sufficient to induce EAE [96]. However, the distribution of lesions in the CNS differs between MS and most murine EAE models. While the majority of patients with MS have parenchymal lesions in the brain and the spinal cord [84], most EAE models induce inflammation targeting the spinal cord and optic nerves without extensive involvement of the brain. This spinal cord-dominant inflammatory pattern generated by most EAE models is referred to as "classic" EAE.

“Atypical” CD4+ T cell-mediated EAE model

There are a few EAE models, however, in which mice develop parenchymal inflammation in the brain (with or without accompanying spinal cord inflammation), referred to as “atypical EAE”. Both IFN-γ and IL-17 appear to play important roles in differentially regulating inflammatory responses in the brain and spinal cord [109]. Th17 cells in particular have been implicated as important promoters of brain inflammation. Our studies in C3HeB/Fej mice showed that the relative abundance of myelin-specific Th1 compared to Th17 cells infiltrating the CNS determines whether parenchymal inflammation occurs in the brain [110]. When Th1 cells predominate in the infiltrate, inflammation is restricted primarily to the spinal cord, resulting in classic EAE. A predominance of Th17 cells; however, results in an inflammatory pattern more similar to that seen in MS with lesions occurring in both brain and spinal cord. The increase in parenchymal brain lesions causes distinct clinical signs that include leaning, rolling, and ataxia. Our study showed that parenchymal lesions in the brain occurred only at Th17:Th1 ratios >1, while inflammation in the spinal cord occurred at a wide range of Th17:Th1 ratios [110]. Thus, Th1 and Th17 cells appear to be critical determinants of inflammatory infiltrates as well as the inflammatory pattern.

The role of CD8+ T cells in MS

Increasing evidence from patients with MS indicates that CD8+ T cells play an important role in the pathogenesis of MS [27, 111, 112]. CD8+ T cells outnumber CD4+ T cells in acute and chronic CNS lesions in MS [47, 113], and axonal damage correlates with the number of CD8+ T cells and macrophages, but not CD4+ T cells [128,129]. In addition, CD8+ and not CD4+ T cell clonal expansion are detected in the blood and CNS of patients with MS [114-118], and the frequency of neuroantigen-specific CD8+ T cells is higher in the CNS of patients with MS than healthy individuals [126,127]. Most importantly, anti-CD4 depleting antibody treatment in patients with MS did not show significant clinical improvement in patients whereas therapies that deplete all lymphocyte populations reduced relapse rate and new lesion formation. [119-121]. These data support a pathogenic role of CD8+ T cells in MS.

CD8+ T cell-mediated EAE models

Most animal studies of myelin-specific CD8+ T cells demonstrated a pathogenic role for CD8+ T cells in EAE [122-126]. Similar to passive EAE induction by myelin-specific CD4+ T cell, adoptive transfer of -CD8+ T cells can induce inflammation in the CNS. However, animals receiving MBP_{79-87}-specific CD8+ T cell displayed some pathological features similar to a subset of patients with MS that are not typically seen in CD4+ T cell-mediated models [122]. Whereas CD4+ T cell-mediated EAE models mostly induce inflammation localized in the spinal cord, lesions in recipients of MBP_{79-87}-specific CD8+ T cells were observed only in the brain. Lesions were most commonly located within the white matter of the cerebellum, although widely

scattered, focal involvement of gray and white matter in the midbrain, cerebral cortex, and brain stem was sometimes observed. The lesions pattern seen in this CD8-mediated EAE model exhibited significant oligodendrocyte cell death, resembling some characteristics of Patterns III and IV seen in patients with MS [76].

Our laboratory developed two TCR transgenic mouse models based on two different H-$2K^k$-restricted, MBP_{79-87}-specific CD8+ T cell clones: Vα8 and Vβ6 (designated 8.6) and Vα8 and Vβ8 (designated 8.8) [65]. These models differ in terms of tolerance of the T cells; naïve 8.6 T cells are subjected to central and peripheral tolerance that prevent them from contributing to autoimmunity. On the other hand, naïve 8.8 T cells show no evidence of tolerance induction and 8.8 T cells populated the periphery in a seemingly naïve state [65]. However, both 8.6 and 8.8 T cells induce EAE when activated in vitro before adoptive transfer into mice (passive induction). In addition, tolerance in 8.8 T cells can be broken to induce EAE by infecting the transgenic mice with viruses encoding MBP, as well as WT vaccinia or adenovirus virus that did not express MBP (active induction) [126] . To better understand how EAE can be induced in these transgenic mice infected by WT virus not encoding MBP, we found that a small subset of 8.8 T cells express dual TCRs, one specific for the virus and one for MBP_{79-87}. Viral infection activates these dual TCR-expressing transgenic CD8+ T cells by binding to the TCR specific for the virus. Consequently, the same cell can propagates inflammation in the CNS and induce EAE using the second TCR specific for MBP_{79-87}. This model provides an intriguing explanation for how a common viral infection could potentially trigger autoimmunity by demonstrating that activation

of dual TCR-expressing CD8+ T cells are capable of recognizing both MBP and viral antigens [126].

Tim-3

Introduction

T-cell immunoglobulin and mucin domain-3 (Tim-3) is expressed by both innate and adaptive immune cells, including DCs, microglia, IFN-γ-secreting CD4+ T cells (Th1), and activated CD8+ T cells [127, 128]. It was later found to be expressed on Th17 cells at lower levels than on Th1 cells [129-131]. Tim-3 is a type-I membrane glycoprotein containing common structural motifs that include an IgV domain embedded in the cell membrane via a mucin stalk. The Tim-3 gene is located on mouse chromosome 11 and encoded in a genetic locus that has been linked to disease susceptibility in several different autoimmune disease models such as EAE and systemic lupus erythematosis [132-135]. Tim-3 has been shown to transmit an inhibitory signal to activated Th1 cells via interaction with its ligand, galectin-9. Galectin-9 is a β-galactose binding protein that can induce calcium flux, cell aggregation, and cell death in activated Th1 cells (in vitro), and selective loss of IFN-γ producing T cells and ameliorates EAE (in vivo) [136]. Furthermore, the effect of Tim-3 signaling in negatively regulating IFN-γ producing CD8+ T cells has also been demonstrated in studies of murine graft-versus-host disease [137] and herpes simplex virus infection [138]. These data demonstrate a crucial role of Tim-3 in negatively regulating CD4+ Th1 and CD8+ T cell responses.

Recently, Tim-3 has been shown to be expressed on exhausted T cells in other disease settings including cancer [139-142] and chronic viral infection [143-145]. These Tim-3-expressing, exhausted T cells are characterized by their inability to proliferate and exert effector function

upon antigen activation [146], and blocking Tim-3 signaling in these situations restores CD8+ T cell proliferation and enhances cytokine production [139, 145, 147]. These data suggest the effects of Tim-3 signaling on T cell activity during an immune response will likely depend upon the nature of the effector cells mediating a particular immune response.

Tim-3 signaling

As described above, surface expression of Tim-3 plays a dual role as an inhibitory receptor of Th1 and effector CD8+ T cells [136-138], as well as a T cell exhaustion marker in chronic viral infections [143-145] and cancer [139-142]. Although the functions of Tim-3 have been widely studied in different physiological settings in the past decade, the molecular mechanisms modulating Tim-3 function were not well understood until recently [148]. It has been shown that human leukocytes antigen B-associated transcript 3 (Bat3) is an intracellular binding partner for Tim-3 under normal physiological condition, binding to residues 252-270 of Tim-3 intracellular tail. However, interaction of Tim-3 and its ligand galectin-9 can abrogate the Bat3-Tim-3 binding activity, allowing Tim-3 to transduce inhibitory signaling that limit Th1 effector functions. Specifically, binding of galectin-9 to Tim-3 induces phosphorylation of Tyr256 and Tyr263, resulting in the release of Bat3 from Tim-3 in the intracellular tail. Bat3 is primarily expressed in Tim-3^+ Th1 cells, and its expression is reduced in exhausted Tim-3^+ T cells isolated from mouse tumors and untreated HIV-1-infected individuals. While Bat3 is able to enhance Th1 effector responses by binding to and inhibiting Tim-3, promoting Bat3 and Tim-3 interaction in

exhausted T cells could reverse their dysfunction. These data suggest that Bat3 can potentially regulate both T cell death and exhaustion.

The role of Tim-3 in MS and EAE

The function of Tim-3 was first studied in an EAE model mediated primarily by CD4+ T cells [127, 136]. Tim-3 has been shown to play a crucial role in negatively regulating IFN-γ secreting Th1 cells [136]. In EAE induced in C57BL/6 mice, administration of Tim-3 ligand, galectin-9, inhibited Th1 responses by reducing the number of IFN-γ-producing CD4+ T cells and ameliorated disease [136]. On the other hand, inhibition of Tim-3 signaling exacerbated EAE. These findings instigated analysis of CD4+ T cell clones isolated from cerebrospinal fluid of patients with MS. Studies revealed that CD4+ T cell clones from patients with MS expressed lower levels of Tim-3 and produced more IFN-γ compared to CD4+ T cells from healthy controls. Furthermore, treatment with glatiramer acetate or IFN-β appeared to restore normal levels of Tim-3 expression to CD4+ T cells from patients with MS, as well as their responsiveness to Tim-3 blockade [149]. These data suggest that the level of Tim-3 expression on T cells could influence the pathogenic immune response in MS [149, 150]. Specifically, reduced Tim-3 expression may contribute to enhanced activity of pathogenic T cells in MS [150], although the function of Tim-3 in CD8+ T cell-mediated autoimmunity is not well understood.

Questions to address

This dissertation examines the function of Tim-3 expressed on particular innate and adaptive immune cells in regulating autoimmune responses in the central nervous system. In particular, we address whether Tim-3 signaling in CD4+ T cells influences inflammatory patterns in the CNS during CD4+ T cell-mediated EAE. Furthermore, we investigate the function of Tim-3 in pathogenic myelin-specific CD8+ T cells in CD8+ T cell-mediated EAE, and the role of Tim-3 in DCs and microglial cells in both CD4+ and CD8+ T cell-mediated EAE models. Together, this dissertation studies the combined effects of manipulating Tim-3 signaling in both innate and adaptive immune cells on disease pathogenesis during CNS autoimmunity.

Chapter 2: Tim-3 signaling in Th1 cells affects localization of inflammation in the CNS

Brief Introduction

The kinetics of Tim-3 expression are different between innate and adaptive immune cells. While Tim-3 is constitutively expressed on DCs and microglia under healthy condition, expression of Tim-3 is induced only on activated Th1 and CD8+ T cells during an immune response [127, 128, 151]. The complex pattern of Tim-3 expression indicates the need to understand how Tim-3 signaling affects multiple innate and adaptive immune cell types in vivo. To better understand the role of Tim-3 in CNS autoimmunity, the use of multiple animal models of MS is essential because not all characteristics of the human disease that could be influenced by Tim-3 signaling are reproduced well in a single EAE model. For example, most EAE models induce classic EAE, in which inflammation predominantly targets only the spinal cord, while most patients with MS exhibit lesions in both the brain and the spinal cord [27].

Our laboratory recently developed an EAE model in C3HeB/Fej mice in which mice develop parenchymal inflammation in the brain and the spinal cord, referred to as "atypical EAE" [110]. We demonstrated that EAE was strongly affected by the relative frequency of myelin-specific Th1 and Th17 cells within the T cell population infiltrating the CNS. While inflammation in the spinal cord was induced by transferring T cells with a wide range of Th17:Th1 ratios; inflammation in the brain was induced only when the Th17:Th1 ratio was equal to or greater than 1 [110]. The increase in parenchymal brain lesions results in atypical EAE in

which distinct clinical symptoms and pathology indicating inflammation occurs in the cerebellum and brain stem in addition to the paralysis associated with spinal cord inflammation. These findings strongly suggest that IL-17 signaling promotes inflammation in the brain. However, Tim-3 is expressed only at a low level on activated IL-17-secreting Th17 cells [129, 130]. Thus, the ability of Tim-3 to influence CNS autoimmunity may be limited to responses that engage only certain subsets of effector T cells. Importantly, the therapeutic effect of triggering Tim-3 signaling with galectin-9 in vivo was demonstrated only in a classic EAE model in C57BL/6 strain [127, 136]. This raises the question of whether manipulating Tim-3 signaling in vivo would have a different disease outcome in an atypical EAE model in which myelin-specific Th17 cells play a more influential role in the pathogenesis but express low levels of Tim-3 compared to Th1 cells.

Results

Tim-3 signaling affects the inflammatory pattern but not incidence or severity in CD4+ T cell-induced EAE

We investigated the effect of eliminating Tim-3 signaling in an EAE model in which a substantial component of the inflammatory infiltrate is comprised of Th17 cells [110]. C3HeB/FeJ mice respond to two distinct epitopes of rMOG, MOG_{97-114} and MOG_{79-90}. MOG_{97-114}-specific T cells differ from MOG_{79-90}-specific T cells in that they exhibit a Th17:Th1 ratio >1 upon activation and typically trigger extensive brain inflammation and atypical EAE [110]. Since Tim-3 is expressed at a high level on Th1 cells compared to Th17 cells, we hypothesized that preventing Tim-3 signaling in this atypical EAE model may increase the number of Th1 cells without affecting the number of Th17 cells because Th1 cells would no longer be preferentially susceptible to Tim-3-induced T cell death. Eliminating Tim-3-mediated Th1 cell death without affecting Th17 cells would decrease the Th17:Th1 ratio and potentially inhibit brain inflammation. To test this hypothesis, we first confirmed that Tim-3 was differentially expressed on Th1 compared to Th17 cells in our animal model. After C3HeB/FeJ mice were immunized with rMOG, splenocytes were harvested seven days later, restimulated with MOG peptide and stained for CD4, Tim-3, IFN-γ and IL-17 (**Fig. 1.1A**). Consistent with previous findings, Tim-3 expression was strongly increased on CD4+ IFN-γ^+ cells, while only a slight increase in Tim-3 expression was detected on CD4+IL-17^+ T cells (**Fig. 1.1B**). We then analyzed the number of Th1 and Th17 cells in the spleens of both WT and Tim-$3^{-/-}$ mice immunized with rMOG by

ELISPOT. Although the total numbers of CD4+ T cells in the spleens of immunized WT and Tim-3$^{-/-}$ mice were comparable (**Fig. 1.2A**), significantly more Th1 cells were found in the spleens of Tim-3$^{-/-}$ mice (**Fig. 1.2B**). In a few experiments (2 out of 5), we also observed a decrease in Th17 cells in the spleens of Tim-3$^{-/-}$ mice, which may reflect competition for antigen or growth factors between Th17 and Th1 cells during priming, and/or production of soluble factors by Th1 cells that inhibit Th17 expansion. Importantly, Tim-3$^{-/-}$ mice primed with rMOG consistently exhibited a lower Th17:Th1 ratio in the spleen compared to WT mice (**Fig. 1.2C**), suggesting that Tim-3-induced death of Th1 cells is a major determinant of the Th17:Th1 ratio during an ongoing immune response.

We next analyzed EAE induced in WT and Tim-3$^{-/-}$ mice by rMOG/CFA immunization and pertussis toxin. In contrast to the increased mortality and clinical score observed in C57BL/6 mice when Tim-3 signaling was blocked during EAE induction [127], no differences in disease incidence, onset or severity were observed between the WT and Tim-3$^{-/-}$ mice on the C3HeB/FeJ background (**Table II**). Instead, the manifestation of EAE differed between WT and Tim-3$^{-/-}$ mice. A significantly higher incidence of classic EAE was observed in Tim-3$^{-/-}$ mice compared to WT mice (**Fig. 2.1A**). Consistent with the increase in classic EAE observed in Tim-3$^{-/-}$ mice, the Th17:Th1 ratio of T cells isolated from the brains of Tim-3$^{-/-}$ mice with EAE was significantly lower compared to that seen in WT mice (**Fig. 2.1B**).

To determine whether the altered inflammatory pattern observed in Tim-3$^{-/-}$ mice with EAE was due to Tim-3 deficiency only on T cells, activated WT and Tim-3$^{-/-}$ MOG_{97-114}-specific

CD4+ T cells were adoptively transferred into WT recipients to induce EAE. Similar disease onset, severity and progression were observed in recipients of WT and Tim-3$^{-/-}$ T cells (**Fig. 2.2, A and B**). However, the WT recipients of Tim-3$^{-/-}$ T cells exhibited a significantly higher incidence of classic EAE compared to the WT recipients of WT T cells (**Fig. 2.2C**). These data indicate that Tim-3 signaling in CD4+ T cells influences the localization of inflammation in the brain and spinal cord via alterations in the Th17:Th1 ratio without affecting disease onset and severity.

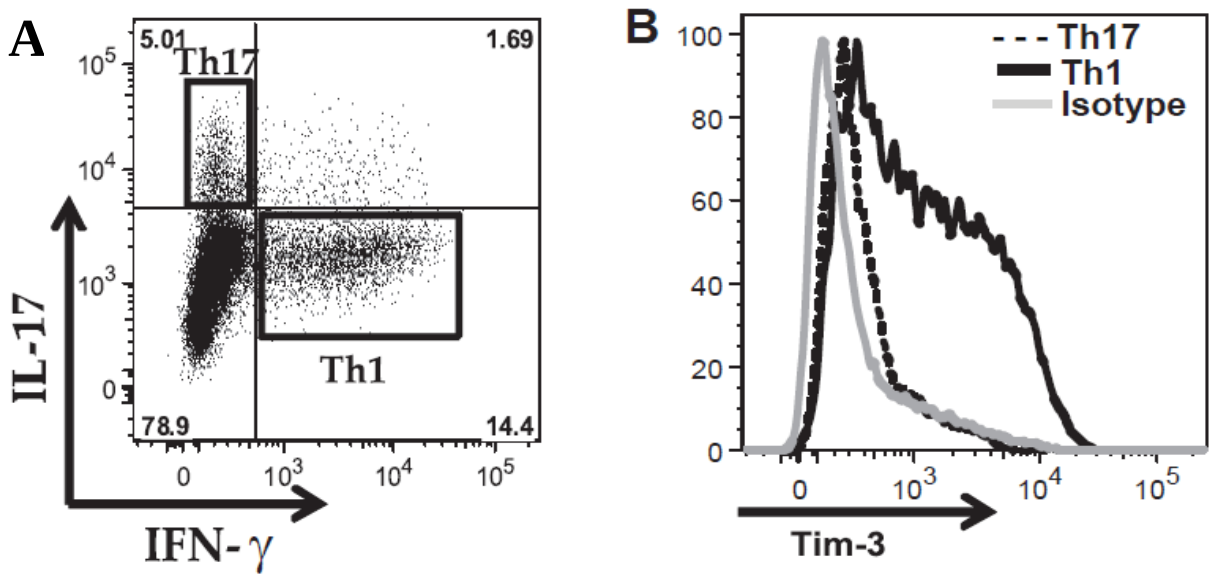

Figure 1.1. Th1 but not Th17 cells express a high level of Tim-3.

(A) Flow cytometric analyses of splenocytes that were isolated from WT mice previously immunized with rMOG, cultured for 7 hrs with MOG_{97-114} peptide and Golgi plug, and stained with mAbs specific for CD4, Tim-3, and intracellularly for IL-17 and IFN-γ. Data are gated on CD4+ T cells. (B) The expression of Tim-3 is shown for CD4+IFN-γ^+-gated and CD4+IL-17^+-gated cells. Data are representative of two independent experiments with a total of 5 mice.

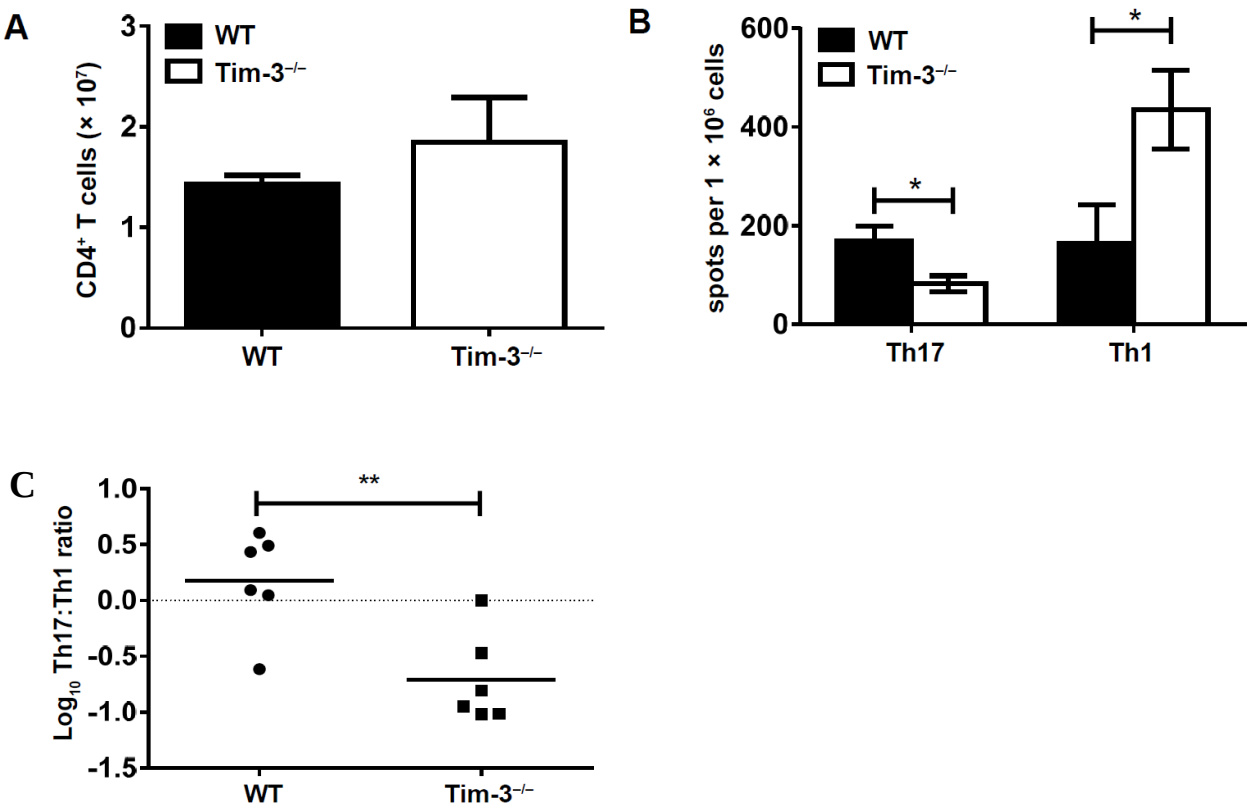

Figure 1.2. Tim-3 signaling determines the Th17:Th1 ratio among responding CD4+ T cells in vivo.

(A) The number of total CD4+ T cells in the spleens of WT and Tim-3$^{-/-}$ mice 7 days post-immunization with rMOG is shown. Splenocytes from immunized mice were counted, stained for CD4 and analyzed by flow cytometry. CD4+ T cell number was calculated as the total splenocyte number × % CD4+ cells. Error bars represent s.e.m. (n = 6). (B) The number of IFN-γ - (Th1) and IL-17-secreting (Th17) cells in the spleens of WT and Tim-3$^{-/-}$ mice 7 days post-rMOG immunization is shown. The numbers of Th1 and Th17 cells were determined by ELISPOT using MOG_{97-114} to stimulate the T cells. Error bars represent s.e.m. (n = 6), *p <0.05. (C) The Th17:Th1 ratio observed in the spleens of Tim-3$^{-/-}$ mice compared to WT mice after

rMOG immunization is shown. The ratios were calculated from the data in (B); each dot represents an individual mouse. **p <0.01. Data are representative of three independent experiments with 6 mice/group in each experiment.

Table II. Disease incidence, onset, and severity were comparable between the WT and Tim-3$^{-/-}$ mice after active induction of CD4+ T cell-mediated EAE

	Incidence	Mortality	Onset[1]	Maximal score[2]
WT	30 of 30	6 of 30 (20%)	18.8 ± 2.86	6.19 ± 0.40
Tim-3$^{-/-}$	30 of 30	4 of 30 (13%)	22.2 ± 4.39	6.12 ± 0.34

[1] Onset indicates the day that clinical signs are first detected (mean ± s.d.).

[2] Maximal score is the maximum disease score achieved according to the grading scale described in *Materials and Methods* (mean ± s.d.).

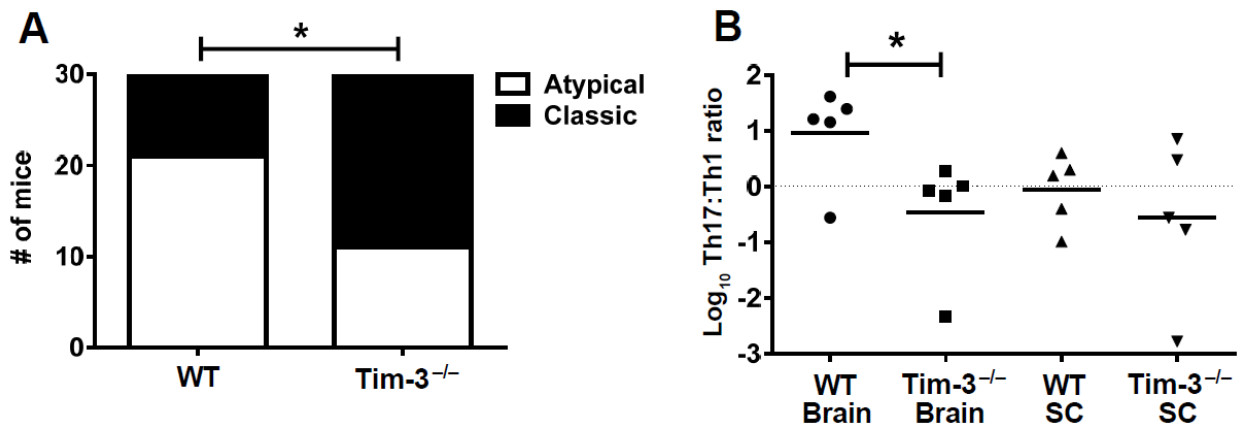

Figure 2.1. Deficiency in Tim-3 signaling increases the incidence of classic EAE.

(A) The numbers of WT and Tim-3$^{-/-}$ mice exhibiting atypical or classic EAE following rMOG immunization are shown. Data are pooled from three independent experiments with a total of 30 mice/group. *p <0.05. (B) The Th17:Th1 ratios of MOG_{97-114}-specific T cells isolated from the brains and spinal cords (SC) of WT and Tim-3$^{-/-}$ mice are shown. The numbers of Th1 and Th17 cells were determined by ELISPOT within 1 d of disease onset. Each dot represents an individual mouse. *p <0.05. Data are representative of three independent experiments using 5 mice/group in each experiment.

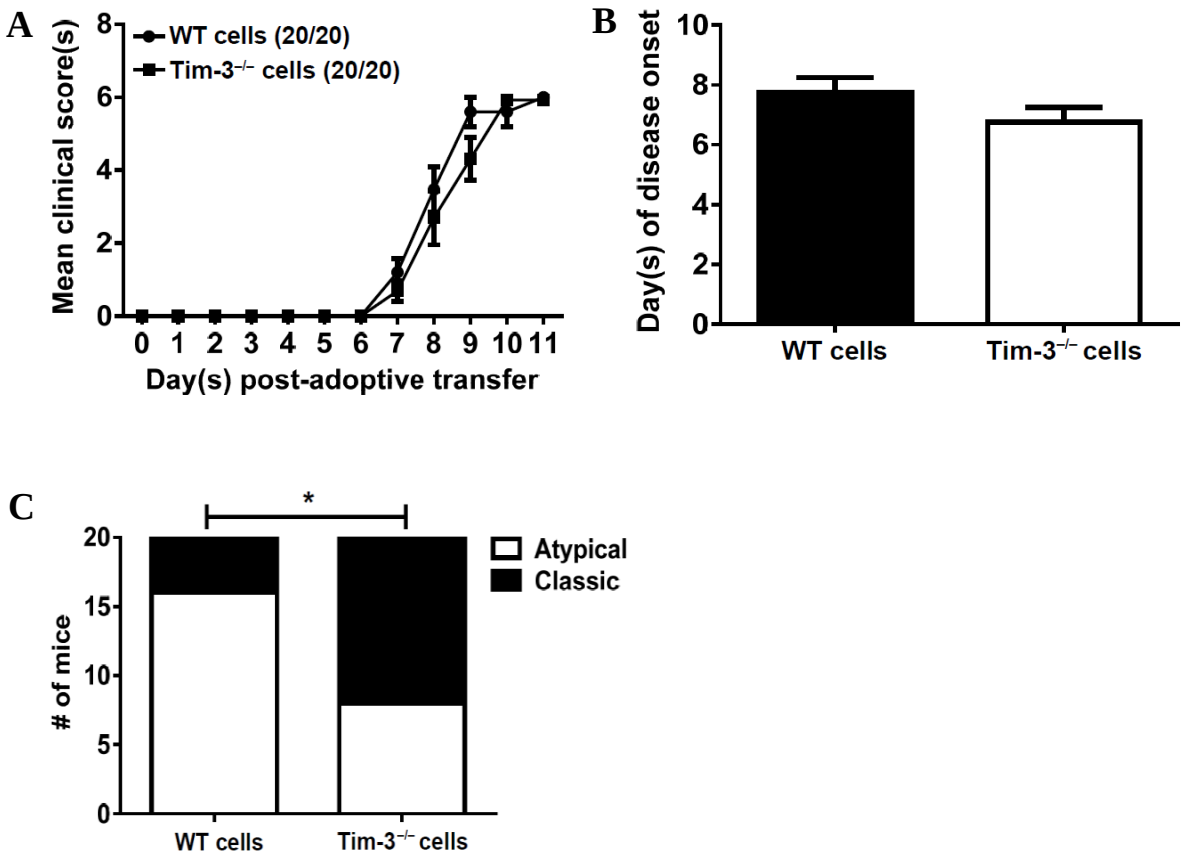

Figure 2.2. Lack of Tim-3 signaling in T cells alters the clinical signs of EAE without affecting disease severity or onset.

(A) Disease course in WT recipients after adoptive transfer of WT or Tim-3$^{-/-}$ CD4+ MOG_{97-114}-specific T cells. Disease incidence was 100% in both groups (20/20). (B) Average day of onset of EAE is shown for mice receiving WT or Tim-3$^{-/-}$ cells. Error bars represent s.e.m. (n = 20). (C) Numbers of WT mice exhibiting atypical or classic EAE after adoptive transfer of CD4+ MOG_{97-114}-specific WT or Tim-3$^{-/-}$ cells (n = 20). *p <0.05. Data are pooled from three independent experiments.

Chapter 3: Tim-3 signaling inhibits the activity of myelin-specific CD8+ T cells during EAE

Brief Introduction

CD8+ T cells have been suggested to play an important role in the pathogenesis of MS [27, 111, 112, 152], and Tim-3 is expressed on activated CD8+ T cells. However, the role of Tim-3 in myelin-specific CD8+ T cells during CNS autoimmunity has not been investigated. Previous studies in other animal disease models showed that Tim-3 signaling induces cell death in activated IFN-γ-secreting CD8+ T cells during murine acute graft-versus-host disease [137] and herpes simplex virus infection [138]. On the other hand, Tim-3 is expressed on exhausted CD8+ T cells in patients with tumors [140, 141] and chronic viral infections, such as HIV [144] and HCV [143]. Blocking Tim-3 signaling in these situations restores CD8+ T cell proliferation and enhances cytokine production [139, 145, 147]. We are interested in investigating the effect of manipulating Tim-3 signaling in myelin-specific CD8+ T cells during CNS autoimmunity.

Results

Tim-3 signaling inhibits the activity of myelin-specific CD8+ T cells during EAE

We analyzed the role of Tim-3 signaling in CD8+ T cells during CNS autoimmunity using a transgenic mouse model developed in our laboratory in which mice express a transgenic TCR specific for MBP_{79-87} presented by H-2Kk [65]. The transgenic mice are referred to as "8.8" mice because the transgenic TCR is comprised of Vα8 and Vβ8. CD8+ T cell-mediated EAE can be induced either by adoptive transfer of in vitro activated 8.8 T cells into WT recipients, or by viral infection with WT vaccinia virus [126]. We first confirmed that Tim-3 expression was induced in vitro on activated 8.8 T cells and that galectin-9 treatment triggered cell death. Tim-3 expression was detected on 8.8 T cells three days after stimulation with MBP_{79-87}, and the level of Tim-3 expression increased after a second in vitro stimulation (**Fig. 3.1A**). Exposure of activated 8.8 T cells to increasing concentrations of galectin-9 increased the percentage of Annexin V^+/7-AAD^+ 8.8 T cells and decreased the number of viable cells in a dose-dependent manner (**Fig. 3.1B and C**). In contrast, the number of viable, naïve 8.8 T cells that lack Tim-3 expression was unaffected by addition of galectin-9 (**Fig. 3.1C**). These results indicate that Tim-3 signaling induces cell death in activated MBP-specific CD8+ T cells via Tim-3/galectin-9 interaction in vitro.

To evaluate the influence of Tim-3 signaling during CD8+ T cell-mediated EAE, activated 8.8 T cells were adoptively transferred into WT recipients that were treated with either Tim-3 blocking Abs [153] or control Abs every other day. EAE mediated by 8.8 T cells can result

in both classic and atypical clinical signs; however, weight-loss is the most quantitative measure of disease progression in this model [126]. Recipients of 8.8 T cells that were treated with Tim-3 blocking Abs exhibited more severe weight loss compared to the control treatment group (**Fig. 3.2A**), indicating that inhibition of Tim-3 signaling exacerbates EAE induced by $MBP_{79\text{-}87}$-specific CD8+ T cells. We also investigated the role of Tim-3 signaling in actively induced CD8+ T cell-mediated EAE by infecting Tim-$3^{+/+}$ and Tim-$3^{-/-}$ 8.8 mice with vaccinia virus. Although similar neurological symptoms and disease progression were observed in both groups of mice, Tim-$3^{-/-}$ 8.8 mice consistently exhibited earlier disease onset compared to Tim-$3^{+/+}$ 8.8 mice (**Fig. 3.2B**). We hypothesized that the earlier disease onset in Tim-$3^{-/-}$ 8.8 mice might be due to greater accumulation of CD8+ effector T cells in the absence of Tim-3-mediated CD8+ T cell death in vivo. Consistent with this notion, the number of CD8+ T cells in the CNS was greater in Tim-$3^{-/-}$ compared to Tim-$3^{+/+}$ 8.8 mice seven days post-vaccinia infection (**Fig. 3.2C**). Tim-$3^{-/-}$ 8.8 T cells did not produce more IFN-γ than Tim-$3^{+/+}$ 8.8 T cells as the average mean fluorescence intensity (MFI) for IFN-γ staining was similar for CD8+ T cells isolated from the CNS of Tim-$3^{+/+}$ and Tim-$3^{-/-}$ 8.8 mice (**Fig. 3.2D**). Furthermore, intracellular granzyme B levels between Tim-$3^{+/+}$ and Tim-$3^{-/-}$ 8.8 T cells were also comparable (data not shown). These data indicate that the earlier disease onset observed in Tim-$3^{-/-}$ 8.8 mice is likely due to the increased accumulation of MBP-specific CD8+ T cells in the CNS rather than enhanced effector function exerted by Tim-$3^{-/-}$ CD8+ T cells. Together these results indicate that Tim-3 signaling inhibits the activity of myelin-specific CD8+ T cells during CNS autoimmunity in addition to limiting CD4+ Th1 cell activity.

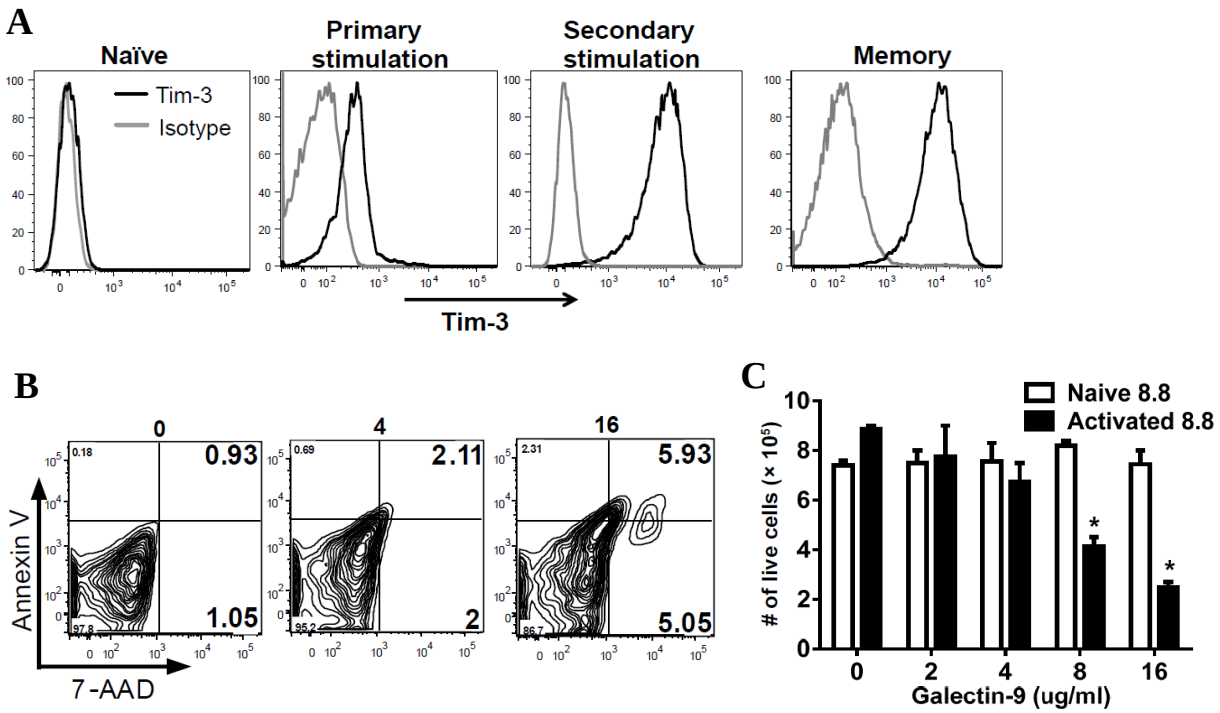

Figure 3.1. Tim-3 signaling induces death in activated CD8+ T cells.

(A) Flow cytometric analysis of Tim-3 expression on naïve 8.8 T cells and 8.8 T cells stimulated once or twice with MBP_{79-87} peptide in vitro. Data are gated on CD8+ T cells and are representative of three experiments. (B) Flow cytometric analysis of Annexin V and 7-AAD expression on 8.8 T cells that were stimulated with MBP_{79-87} peptide for 7 d and then cultured overnight with increasing concentrations of galectin-9. The numbers above the plots indicate the concentration of galectin-9 (μg/ml) added to the culture. Data are gated on CD8+ T cells and are representative of three independent experiments. (C) Naïve and previously activated 8.8 T cells were incubated with increasing concentrations of galectin-9 overnight and the number of live cells was determined in each culture by counting cells stained with trypan blue ($n = 5$). *$p < 0.05$. Data are representative of three independent experiments.

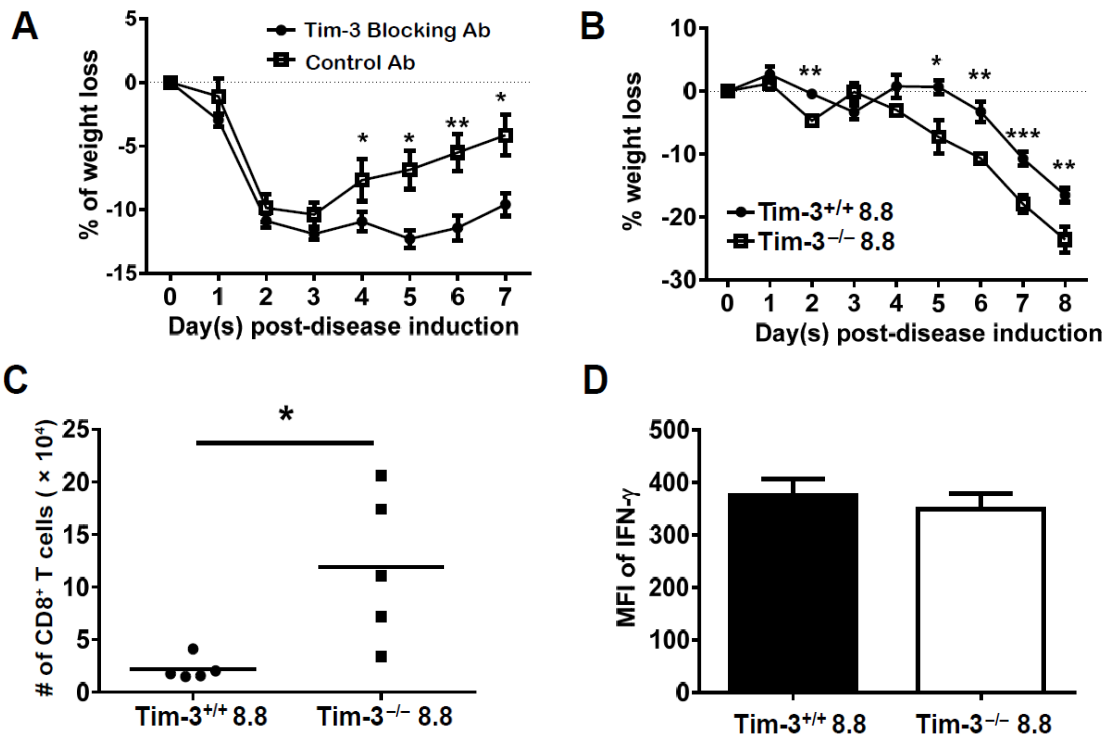

Figure 3.2. Severity and onset of CD8+ T cell-mediated EAE is influenced by Tim-3 signaling.

(A) Disease course of CD8+ T cell-mediated EAE induced by adoptive transfer of activated CD8+ 8.8 T cells into recipients that were treated every other day with either 100 μg Tim-3 blocking Ab or control Ab (rat IgG1, κ). The percent weight loss relative to d 0 is shown (n = 5). *p <0.05, **p <0.01. Data are representative of three experiments with 5 mice/group in each experiment. (B) Disease course of CD8+ T cell-mediated EAE induced in Tim-$3^{+/+}$ and Tim-$3^{-/-}$ 8.8 mice by vaccinia virus infection. Error bars represent s.e.m (n = 5). *p <0.05, **p <0.01, ***p <0.001. Data are representative of three experiments with 5 mice/group in each experiment. (C) The numbers of CD8+ T cells in the CNS of Tim-$3^{+/+}$ and Tim-$3^{-/-}$ 8.8 mice 7 days post-vaccinia virus infection are shown. The numbers were determined by staining CNS mononuclear

cells with Abs specific for CD8 and Vβ8. Each dot represents an individual mouse. *p <0.05. (D) The mean fluorescence intensity (MFI) for IFN-γ intracellular staining is shown for CD8+ T cells isolated from the CNS of Tim-$3^{+/+}$ and Tim-$3^{-/-}$ 8.8 mice 7 days post-vaccinia infection. Error bars represent s.e.m. (n = 5). Data are representative of 6 mice from three independent experiments.

Chapter 4: Tim-3 signaling in DCs and microglia do not play a significant role in EAE

Brief Introduction

Tim-3 is constitutively expressed on DCs and microglia under healthy conditions; however, the effects of Tim-3 signaling on DCs and microglial cells in CNS autoimmunity has not been studied. Microglia are considered the resident APCs in the CNS, and antigen presentation by inflammatory DCs plays an essential role in initiating and propagating EAE [154, 155]. Tim-3 signaling in DCs was initially reported to be pro-inflammatory as exposure of splenic DCs to both galectin-9 and LPS in vitro resulted in enhanced TNF-α secretion compared to LPS treatment alone [128]. However, a recent study suggested that Tim-3 is a regulator of pro- and anti-inflammatory responses by innate immune cells as blocking Tim-3 signaling led to an increase in TLR-mediated IL-12 and IL-10 production in human peripheral blood monocytes [156]. We want to determine whether Tim-3 signaling on innate immune cells has opposing or synergistic effects on the inflammatory response in EAE.

Results

Expression of Tim-3 on DCs does not influence T cell priming in vitro

In contrast to its function in Th1 and activated CD8+ T cells, the role of Tim-3 signaling in innate immune cells including DCs and microglia has not been defined. Pro-inflammatory effects were reported in studies that used galectin-9 to trigger Tim-3 expressed on murine DCs upon LPS stimulation in vitro; however, suppressing Tim-3 signaling on human monocytes via Tim-3 antibody blockade or expression of Tim-3-specific siRNA revealed both pro- and anti-inflammatory regulation of Tim-3 signaling in these cells [128, 156]. If Tim-3 signaling influences the phenotype of the DCs that present antigen following immunization with CFA, then lack of Tim-3 expression by DCs could affect T cell priming and contribute to the changes in the manifestation of EAE observed in Tim-3$^{-/-}$ mice. To investigate whether Tim-3 signaling could alter the phenotype of DCs exposed to microbial antigens in adjuvant, we compared the levels of pro-inflammatory cytokine production by splenic DCs from WT and Tim-3$^{-/-}$ mice in response to LPS stimulation in vitro. DCs were sorted from the spleens of WT and Tim-3$^{-/-}$ mice and cultured with and without LPS for 24 hours. Cytokines in the supernatants from these cultures were analyzed by Bioplex assay. While exposure to LPS enhanced overall cytokine production by splenic DCs, the only significant difference observed between LPS-treated Tim-3$^{-/-}$ and WT DCs was a slightly higher level of IL-6 production by the Tim-3$^{-/-}$ DCs (**Fig. 4.1**). Significant differences were not observed for production of IL-1β, TNF-α, IL-12(p40), IL-12(p70), IL-1α or IFN-γ. WT and Tim-3$^{-/-}$ splenic DCs also expressed comparable levels of activation markers

after LPS stimulation, including MHC class II, CD40, CD80, and CD86 **(Fig. 4.2**), suggesting that the expression of Tim-3 may not significantly alter the response of DCs to inflammatory stimuli.

Although we did not detect differences in cytokine production by Tim-$3^{-/-}$ DCs, we investigated whether a lack of Tim-3 expression on DCs influenced their ability to prime myelin-specific CD4+ and CD8+ T cells in vitro. Splenic DCs from WT and Tim-$3^{-/-}$ mice were used as APCs with and without LPS stimulation to present MOG_{97-114} peptide to naïve MOG_{97-114}-specific TCR transgenic CD4+ T cells recently developed in our laboratory (Castelli and Goverman, unpublished data). No differences were detected in IFN-γ, IL-17, and GM-CSF production by CD4+ T cells activated by either WT or Tim-$3^{-/-}$ splenic DCs, even when the DCs were stimulated with LPS (**Fig. 4.3A**). Similarly, no differences in IFN-γ production (**Fig. 4.3B**) or in proliferation (**Fig. 4.3C**) by CD8+ 8.8 T cells were observed when the 8.8 T cells were activated by WT or Tim-$3^{-/-}$ DCs presenting MBP_{79-87}. Thus, the small difference in IL-6 production observed in vitro for Tim-$3^{-/-}$ DCs stimulated with LPS did not influence the priming of naive CD4+ or CD8+ T cells. These data suggest that the impact of Tim-3 deficiency observed in CD4+ and CD8+ T cell-mediated EAE models is unlikely to reflect differences in T cell priming by Tim-$3^{-/-}$ DCs.

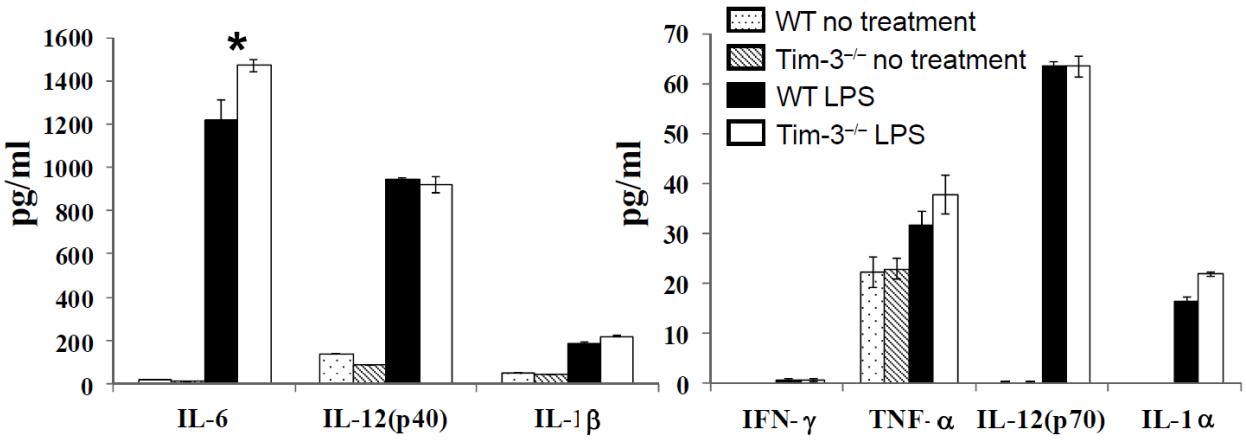

Figure. 4.1. WT and Tim-3$^{-/-}$ DCs produce similar amounts of inflammatory cytokines.

Splenic DCs sorted from WT and Tim-3$^{-/-}$ mice were cultured with or without LPS for 24 hr. Supernatants from the cultures were analyzed for the indicated cytokines using a BioPlex system (Bio-Rad). Error bars represent s.d. (n = 3). *p <0.05. Data are representative of three independent experiments with 3 mice/group in each experiment.

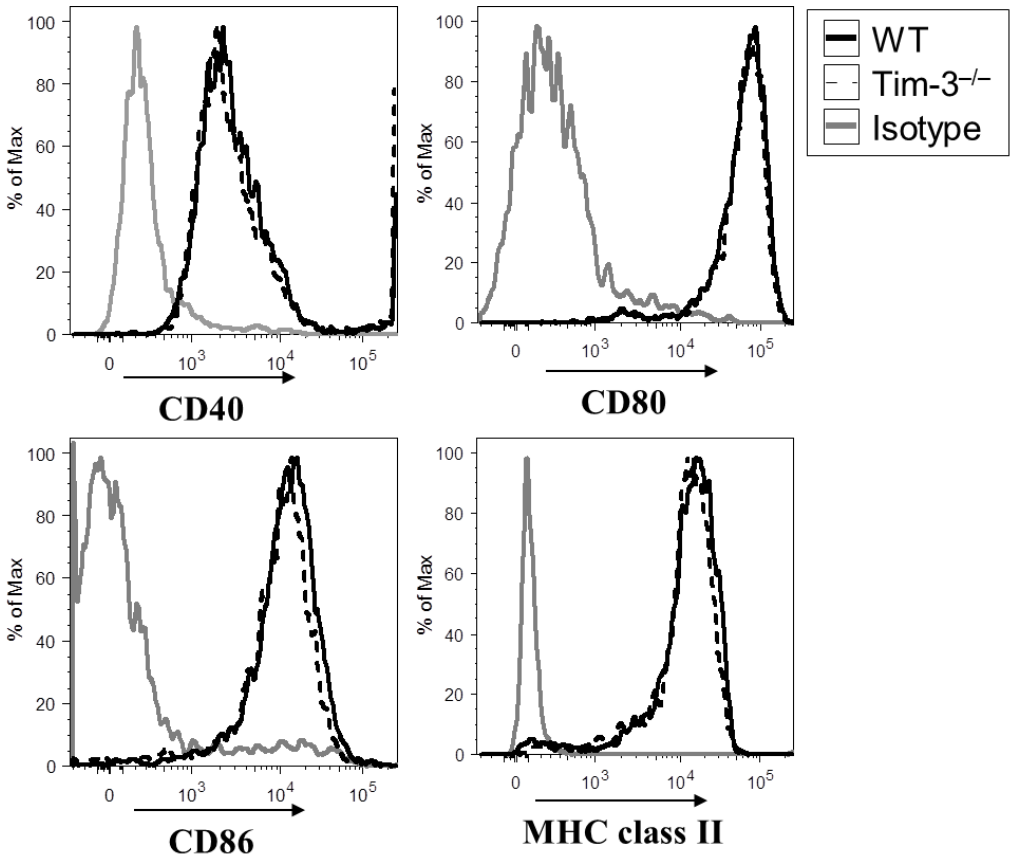

Figure 4.2. WT and Tim-3$^{-/-}$ DCs express comparable levels of cell surface activation markers.

Splenic DCs sorted from WT and Tim-3$^{-/-}$ mice were cultured with LPS for 24 hrs. DCs were stained with antibodies specific for CD11c, CD40, CD80, CD86, and MHC class II for flow cytometric analysis. Data are gated on CD11c^{+} cells and are representative of three independent experiments.

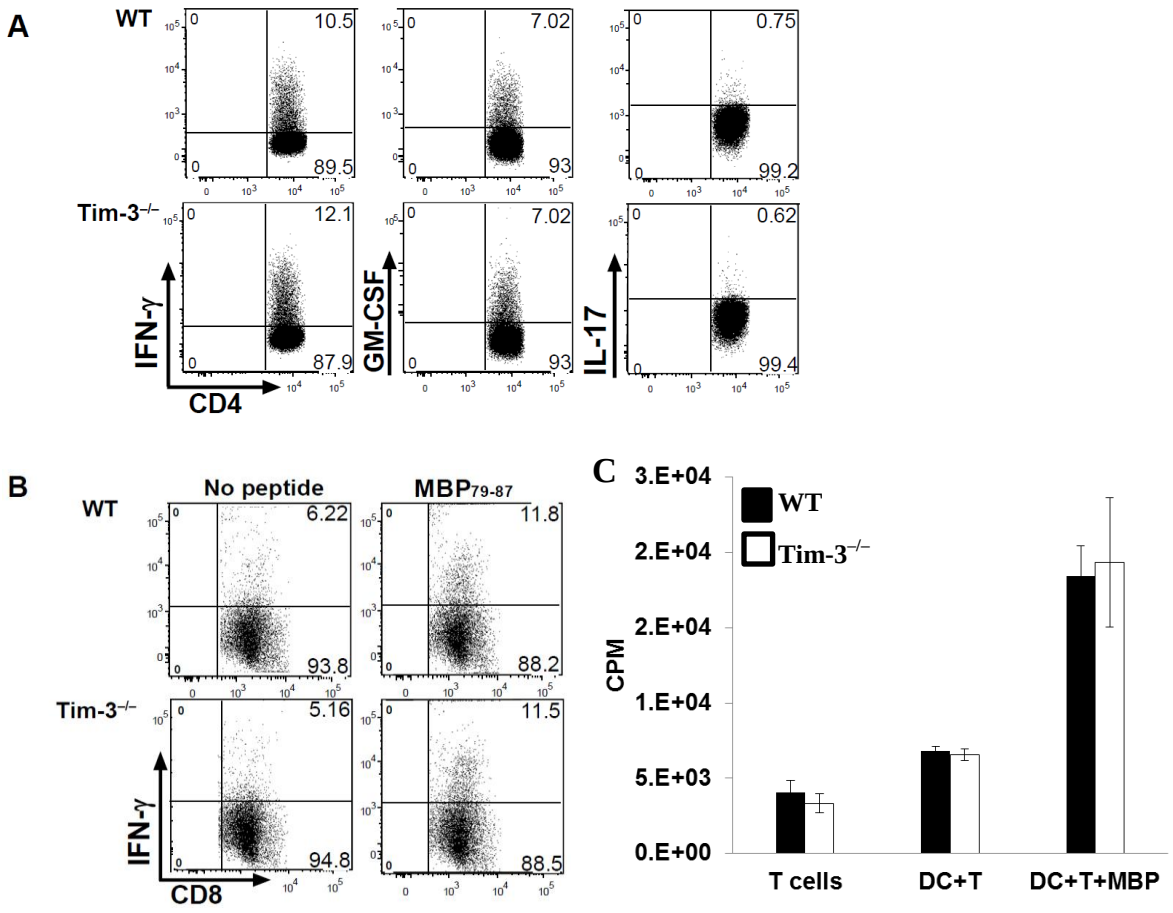

Figure 4.3. The effector function of myelin-specific CD4+ and CD8+ T cells primed in vitro is not affected by expression of Tim-3 on DCs.

(A) Flow cytometric analyses of CD4+ MOG_{97-114}-specific TCR transgenic T cells that were incubated with sorted WT (*top*) or Tim-$3^{-/-}$ (*bottom*) splenic DCs, MOG_{97-114} peptide and LPS for 7 d. Data are gated on CD4+ T cells. (B) Flow cytometric analysis of MBP-specific CD8+ 8.8 T cells that were incubated with either sorted WT (*top*) or Tim-$3^{-/-}$ (*bottom*) splenic DCs in the absence (*left*) or presence (*right*) of MBP_{79-87} peptide for 7 d. Data are gated on CD8+ T cells. Data are representative of 6 mice analyzed from three independent experiments for (A) and (B). (C) WT or Tim-$3^{-/-}$ splenic DCs were co-cultured with naïve MBP-specific CD8+ T cells

with or without MBP_{79-87} peptide. T cell proliferation was measured 48 hrs later with ^{3}H-thymidine incorporation assay. Error bars represent s.d. (n = 5). Data are representative of three independent experiments.

Tim-3 signaling in microglia does not influence EAE pathogenesis

The function of Tim-3 signaling in microglia is not known. Tim-3 is expressed constitutively at a high level on microglial cells in healthy mice, and expression of Tim-3 is maintained on microglia during EAE (**Fig. 5.1, A and B**). To determine if Tim-3 signaling in microglial cells influences either CD4+ or CD8+ T cell-mediated EAE, we constructed bone marrow chimeras (BMCs) in which cells from WT bone marrow were transferred into lethally irradiated Tim-$3^{-/-}$ or WT recipients. Microglia in Tim-$3^{-/-}$ mice that received WT bone marrow cells expressed a very low level of Tim-3, indicating that there was minimal repopulation of microglia by cells derived from the WT bone marrow (**Fig. 5.1C**). EAE was induced by adoptive transfer of MOG-specific CD4+ T cells into the WT→Tim-$3^{-/-}$ and WT→WT BMCs. No differences in disease onset or severity were observed in the different types of BMCs (Data not shown). In contrast to the shift from atypical to classic EAE observed when Tim-$3^{-/-}$ CD4+ T cells were transferred into WT recipients, the incidence of classic and atypical disease did not change in these different BMCs (**Fig. 5.2A**). Activated CD8+ 8.8 T cells were also adoptively transferred into both types of BMCs and no differences in the manifestation of EAE, disease onset or severity were found between the two groups (**Fig. 5.2B**). Together, our results indicate that Tim-3 expression on microglial cells does not significantly influence manifestation of CD4+ and CD8+ T cell-mediated EAE.

The data described above suggest that Tim-3 signaling in either DCs or microglia does not significantly influence the manifestation of EAE. However, we could not generate animals

that lack Tim-3 only on DCs in vivo. Therefore, we asked whether Tim-3 signaling in the innate immune cell compartment as a whole (DCs and microglial cells together) influenced CNS autoimmunity by transferring activated WT T cells into Tim-3$^{-/-}$ recipients. In these experiments, the transferred T cells expressed Tim-3 but both the DCs and microglial cells in the recipients lacked Tim-3 expression. Disease onset, severity and incidence of classic versus atypical disease were comparable between WT and Tim-3$^{-/-}$ recipients that received WT MOG-specific CD4+ T cells (**Fig. 5.2C**). In addition, disease progression was similar when activated CD8+ 8.8 T cells were transferred into WT or Tim-3$^{-/-}$ recipients (**Fig. 5.2D**). These results support the notion that Tim-3 signaling in DCs and microglial cells does not play an influential role in EAE pathogenesis in our EAE models. Together, these data indicate that Tim-3 signaling in activated myelin-specific CD8+ T and CD4+ Th1 cells is primarily responsible for the differences exhibited by WT and Tim-3$^{-/-}$ animals during CD4+ and CD8+ T cell-mediated EAE.

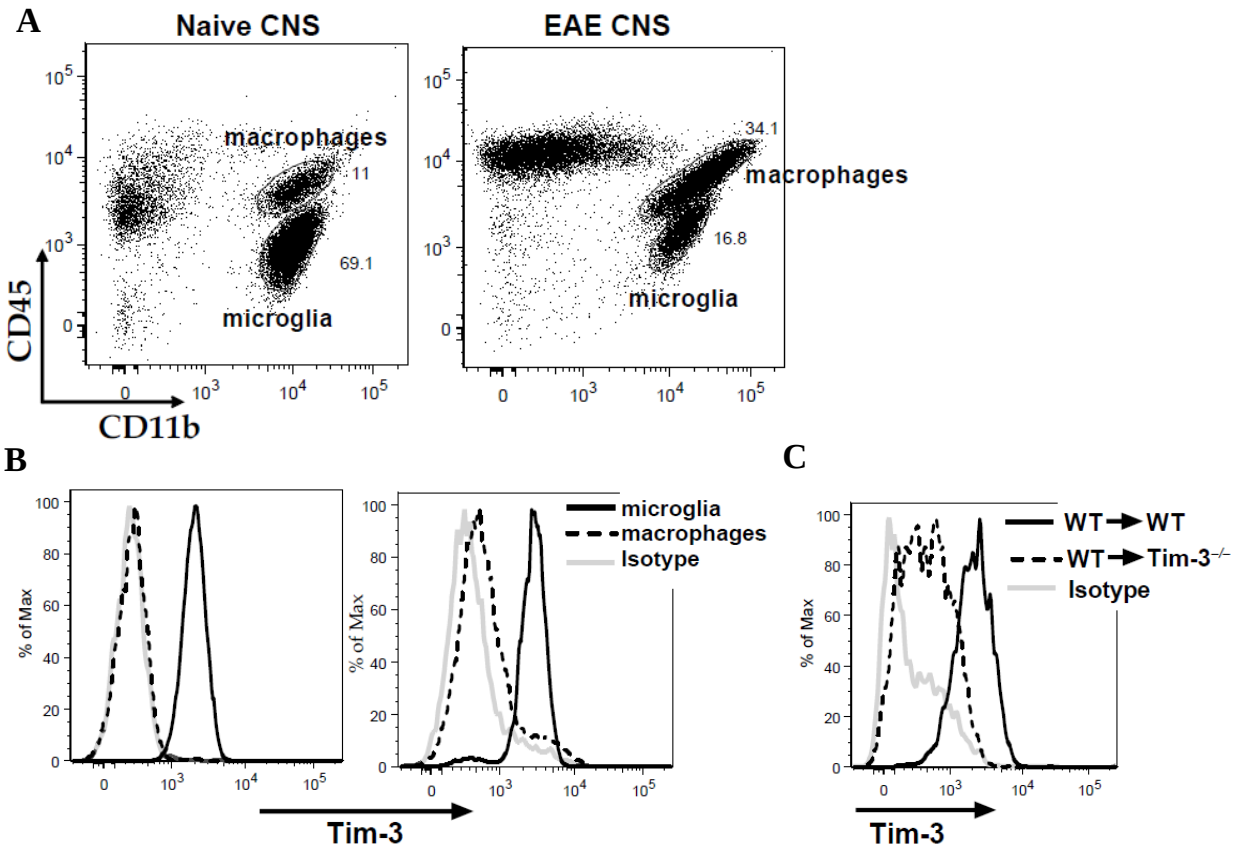

Figure. 5.1. Microglia in healthy mice and mice with EAE express Tim-3.

(A) Flow cytometric analysis of CNS mononuclear cells showing the gates defined by CD45 and CD11b expression used to distinguish macrophages from microglial cells. Mononuclear cells isolated from the CNS of naïve (left) and EAE (right) WT mice were stained with Abs specific for CD11b, CD45, and Tim-3. Microglial cells are identified as $CD11b^{+}CD45^{low}$ cells and macrophages as $CD11b^{+}CD45^{high}$ cells. (B) Expression of Tim-3 is shown on microglia- and macrophage-gated cells from mice as in (A), data are representative of 5 mice. (C) Tim-3 expression on microglia remains low in WT→Tim-$3^{-/-}$ BMCs. Data are representative of 5 BMCs.

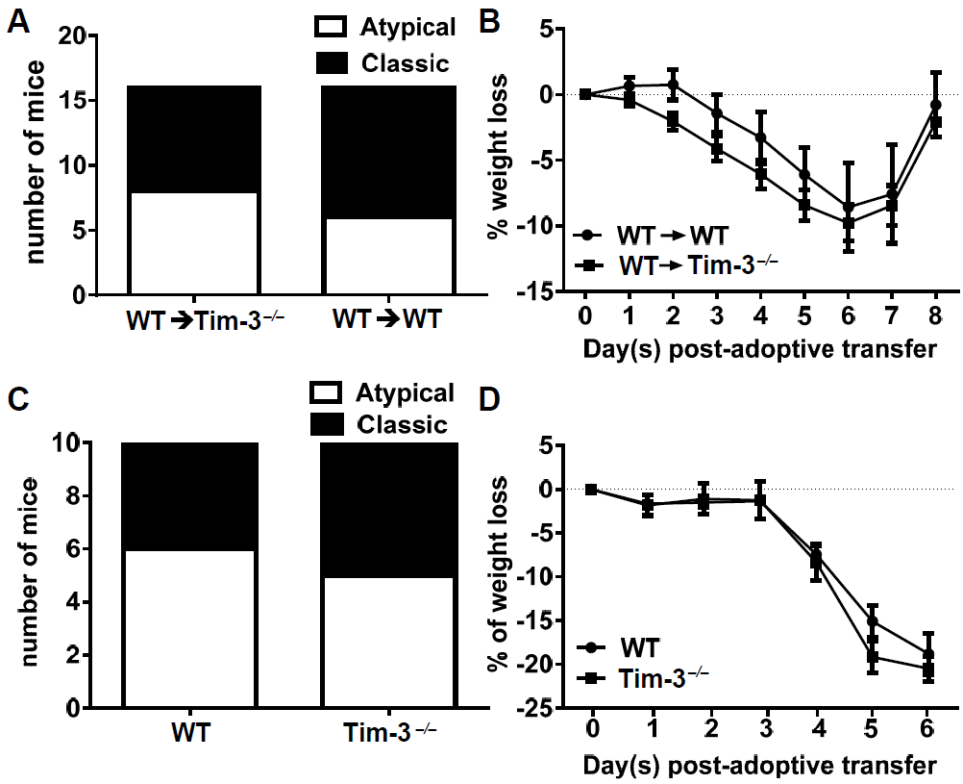

Figure. 5.2. Lack of Tim-3 expression on innate immune cells does not influence the manifestation of EAE.

(A) The number of indicated BMC recipients exhibiting classic or atypical EAE after adoptive transfer of activated WT MOG_{97-114}-specific CD4+ T cells is shown (n = 16). (B) Disease course of EAE in the indicated BMC recipients following adoptive transfer of activated CD8+ 8.8 T cells. The percent weight loss relative to day 0 is shown; differences in weights between the two groups of mice were not statistically significant. Error bars represent s.e.m. (n = 5). Data are representative of three independent experiments with 5 mice/group in each experiment. (C) The number of mice with classic or atypical EAE is shown for WT or Tim-$3^{-/-}$ recipients of activated WT MOG_{97-114}-specific CD4+ T cells (n = 10). (D) Disease course of EAE in WT or Tim-$3^{-/-}$

recipients of activated CD8+ 8.8 T cells. Error bars represent s.e.m. (n = 4). Data are representative of three experiments with 4 mice/group in each experiment.

Chapter 5: Discussion and Future Questions

Tim-3 was originally identified as a cell surface marker specifically expressed on Th1 cells in which Tim-3 signaling inhibited pro-inflammatory Th1 responses [136]. Since its first discovery, extensive studies were carried out to investigate the function of Tim-3 in different innate and adaptive immune cell types, as well as its functional relevance to human diseases. Tim-3 has been implicated in playing an immunoregulatory role in autoimmune diseases [151], transplant tolerance [157], chronic viral infections [143-145], and anti-tumor immunity [139-142, 148]. Understanding how Tim-3 influences the immune response in each of these diseases has been difficult because the function of Tim-3 is different between innate and adaptive immune cells, and the effect of Tim-3 signaling is variable between different diseases even within the same cell type. For example, Tim-3 functions as a T cell exhaustion marker during chronic viral infections and tumor immunity and its expression seems to be required to maintain T cells in a state of non-responsiveness. Blocking Tim-3 interactions in these situations allows exhausted T cells to recover their effector function and is beneficial in these chronic diseases [139, 145, 147]. On the other hand, Tim-3 expression on Th1 cells during autoimmunity allows these cells to be susceptible to cell death such that promoting Tim-3 signaling is beneficial in autoimmune disease settings where Th1 cells play a major role. This notion is demonstrated in EAE studies in which enhancing Tim-3 signaling in vivo ameliorated Th1-mediated EAE [127, 136], suggesting that Tim-3 may be a potential therapeutic target in MS.

Many questions need to be addressed before we can consider Tim-3 as a potential therapeutic target in MS as the role of Tim-3 signaling in CNS autoimmunity is not well understood. MS is a heterogeneous disease in both clinical course and lesion pathology [158]. The variation of inflammatory cells found in MS lesions suggests that the distinct effector cell types contributing to disease pathogenesis may be different between patients with MS. Furthermore, although Tim-3 is expressed on Th1 cells, activated CD8+ T cells, DCs, and microglia and these cell types all have been shown to play important roles in MS. The effect of Tim-3 signaling in Th17 cells, CD8+ T cells, DCs and microglia on the pathogenesis of CNS autoimmunity is not defined. Therefore, determining the efficacy of Tim-3 as a therapeutic target in MS requires understanding the effects of manipulating Tim-3 signaling on all of these diverse cell types. This dissertation examines the function of Tim-3 expressed by both innate and adaptive immune cells in EAE. We used a unique model of CD8+ T cell-mediated CNS autoimmunity as well as a CD4+ T cell-mediated EAE model in which both Th17 and Th1 cells are prominent effector T cells. We found that eliminating Tim-3 signaling resulted in a shift in inflammatory pattern from atypical to classic EAE without affecting disease severity or incidence in CD4+ T cell-mediated EAE. Furthermore, blocking or eliminating Tim-3 signaling during CD8+ T cell-mediated EAE exacerbated disease. In contrast, we found that Tim-3 expression by DCs and microglia did not play a significant role in determining the manifestation of both CD4+ and CD8+ T cell-mediated EAE.

Although earlier studies in C57BL/6 mice reported exacerbation of EAE upon blocking Tim-3 signaling, we observed a change in the clinical manifestation rather than increased disease

severity when Tim-3 signaling was eliminated in our CD4+ T cell-mediated EAE models in C3HeB/FeJ mice. This discrepancy is possibly due to the distinct effector T cells that induce EAE between these two strains. Th1 cells are the predominant effector subset inducing EAE in C57BL/6 mice such that animals exhibit classic EAE where inflammation primarily targets the spinal cord. Enhancing Tim-3 signaling in vivo in this model causes a loss of pathogenic IFN-γ-secreting T cells and results in ameliorated EAE in C57BL/6 mice [136]. On the other hand, C3HeB/FeJ mice exhibit atypical EAE in which extensive parenchymal lesions are observed in the brain as well as the spinal cord. This inflammatory pattern resembles more closely to those seen in many patients with MS. In contrast to Th1-mediated EAE in C57BL/6, both Th1 and Th17 cells contribute to EAE induced in C3HeB/FeJ mice. We have previously demonstrated that the increase in parenchymal brain inflammation results from a higher Th17:Th1 ratio among T cells infiltrating the CNS in C3HeB/FeJ mice [110]. Consistent with results reported by other groups, Tim-3 is expressed at a high level on Th1 but not Th17 cells in our CD4+ T cell-mediated EAE model. We found that eliminating Tim-3 signaling during EAE resulted in an increase in accumulation of Th1 cells due to a lack of Tim-3-induced cell death in this subset, while the survival of Th17 cell was not affected by manipulation of Tim-3 signaling. The increase in the Th1 population caused a decrease in the ratio of Th17:Th1 cells infiltrating the CNS, resulting in decreased inflammation in the brain but not the spinal cord. Thus, while the incidence of classic EAE increased in Tim-3$^{-/-}$ mice, the overall disease onset and severity were unchanged. The differential survival of Th1 versus Th17 cells in Tim-3$^{-/-}$ mice during EAE demonstrates an important role for Tim-3 in determining lesion localization, and suggests that

the effect of targeting Tim-3 in patients with MS will depend on the relative abundance of pathogenic Th1 and Th17 cells that are active in their disease.

Although Tim-3 is expressed on activated CD8+ T cells and CD8+ T cells have been suggested to play an important role in the pathogenesis of MS [27,118,119,136], the function of Tim-3 signaling in myelin-specific CD8+ T cells has not been studied in CNS autoimmunity[27, 111, 112, 152]. We found that Tim-3 was expressed on activated but not naïve MBP-specific CD8+ T cells, and galectin-9 treatment induced cell death in Tim-3 expressing, MBP-specific CD8+ T cells in vitro. Preventing Tim-3 signaling in vivo using Tim-3 blocking antibodies and in Tim-$3^{-/-}$ 8.8 mice caused increased severity in clinical signs and an earlier disease onset during CD8+ T cell-mediated EAE. The exacerbated disease pathogenesis observed in Tim-$3^{-/-}$ 8.8 mice is correlated with increased accumulation of MBP-specific CD8+ T cells in the CNS of Tim-$3^{-/-}$ 8.8 mice compared to Tim-$3^{+/+}$ 8.8 mice. These data indicate that myelin-specific CD8+ T cells are similar to Th1 cells in their susceptibility to Tim-3-induced cell death, and that patients with MS may benefit from strategies that enhance Tim-3 signaling when CD8+ T activity is prominent in their disease.

DCs have been shown to play a critical role in reactivating T cells that infiltrate the CNS and microglia may play a role in the development of EAE [155, 159]. Both cell types express high levels of Tim-3 under healthy conditions. However, the role of Tim-3 signaling in DCs and microglia in the context of CNS autoimmunity is not well understood. Studying Tim-3 function in DCs and microglia is challenging as both cell types are more difficult to purify and culture ex vivo compared to CD4+ and CD8+ T cells. We first investigated the effect of Tim-3 signaling in

DCs using bone marrow-derived cell culture in vitro. We found that bone marrow-derived DCs from Tim-3$^{-/-}$ mice consistently produced more inflammatory cytokines than WT bone-marrow-derived DCs in response to LPS (data not shown). However, the physiological relevance of bone marrow-derived DCs was unclear as WT bone-marrow-derived DCs express a much lower level of Tim-3 relative to splenic DCs ex vivo. Furthermore, most of the differences between WT and Tim-3$^{-/-}$ DCs were not significant when splenic DCs were analyzed. Although splenic DCs from Tim-3$^{-/-}$ mice produced slightly more IL-6 in vitro in response to LPS, myelin-specific CD4+ and CD8+ T cells produced comparable amounts of cytokines and exhibited a similar activated phenotype when activated by either WT or Tim-3$^{-/-}$ DCs in vitro. While it is not possible to isolate Tim-3 deficiency only to DCs in vivo using the genetic tool of Cre-Lox recombination in our C3HeB/Fej mice, these results suggest that lack of Tim-3 signaling on DCs would not greatly influence T cell differentiation into effector cells or their activity in the development of EAE. Similarly, no effect on EAE pathogenesis was observed when Tim-3 deficiency was isolated to microglia in vivo using bone-marrow chimeric mice. Interestingly, we found that Tim-3 is developmentally regulated as it is not expressed on microglia derived from neonatal CNS cultures (unpublished observations). However, the number of microglia in the CNS of healthy and Tim-3$^{-/-}$ mice was comparable, indicating that Tim-3 signaling is not required for microglia survival under steady state conditions.

In conclusion, our results suggest that Tim-3 could be a potential therapeutic target in MS treatment. Specifically, enhancing Tim-3 signaling may be beneficial in patients with MS if Th1 cells and/or CD8+ T cells are the predominant effector cells in their disease (**Fig. 6**). However,

targeting Tim-3 might not be useful in patients with MS if Th17 cells are the major cell type contributing to their disease pathogenesis. As the Th17 cell subset does not appear to be affected by Tim-3 signaling, the reduction in Th1 cells will increase the Th17:Th1 ratio in the pathogenic T cell population, potentially resulting in a shift in inflammatory pattern rather than amelioration of disease. In the future, identification of an inhibitory cell-surface marker expressed on Th17 cells that exhibits similar activity as Tim-3 for Th1 and activated CD8+ T cells could be therapeutically beneficial in MS when used in combination therapy with Tim-3 treatment.

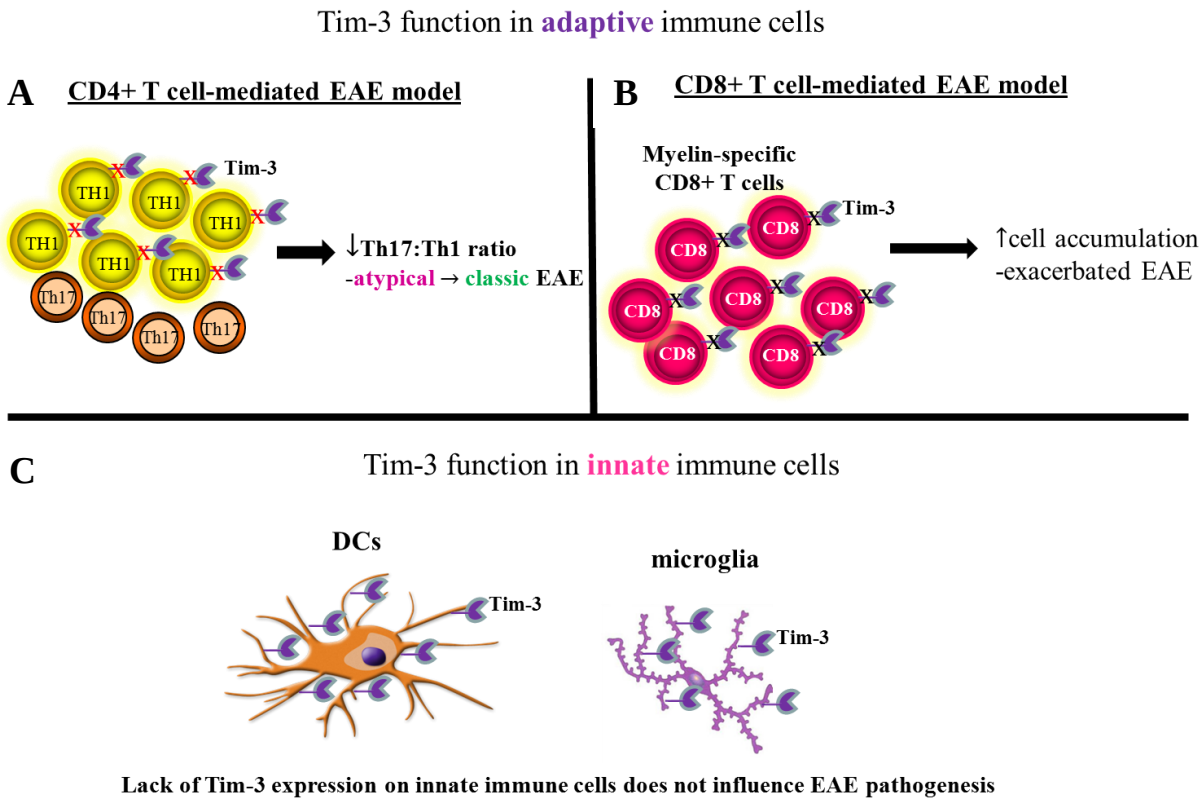

Figure 6. Working model of Tim-3 regulation in CNS autoimmune disease.

(A) Preventing Tim-3 signaling in CD4+ T cells altered the inflammatory pattern in the CNS due to differential effects on Th1 versus Th17 cells. (B) Preventing Tim-3 signaling during CD8+ T cell-mediated EAE increased accumulation of myelin-specific CD8+ T cells, resulting in exacerbated disease. (C) Tim-3 signaling in DCs and microglia did not affect the manifestation of EAE in these models.

Chapter 6: Materials and Methods

Mice

C3HeB/FeJ mice were purchased from The Jackson Laboratory and bred in our colony. BALB/c Tim-3$^{-/-}$ mice were kindly provided by Dr. V. Kuchroo (Harvard Medical School, Boston, MA) and were backcrossed for ten generations onto the C3HeB/FeJ background. C3HeB/FeJ mice expressing a transgenic TCR comprised of Vα8 and Vβ8 specific for residues 79-87 of myelin basic protein (MBP_{79-87}) have been previously described (referred to as 8.8 mice) [65]. 8.8 mice lacking Tim-3 expression were generated by breeding 8.8 mice to C3HeB/FeJ Tim-3$^{-/-}$ mice. Mice expressing a transgenic TCR specific for residues 97-114 of myelin oligodendrocyte glycoprotein (MOG) were generated directly in C3HeB/FeJ mice (Castelli and Goverman, manuscript in preparation). Thy-1.1 C3HeB/FeJ mice were generated by backcrossing of the allele encoding Thy-1.1 from C57BL/6 onto the C3HeB/FeJ background for 12 generations. All mice were bred and maintained in a specific pathogen-free facility at the University of Washington (Seattle, Washington). Mice used for EAE induction were between 8–12 wks of age. All procedures have been approved by the Institutional Animal Care and Use Committee at the University of Washington.

Reagents and peptides

We produced recombinant MOG (rMOG, residues 1-125 from rat) protein in Escherichia coli as described [160]. Recombinant galectin-9 was provided by Dr. V. Kuchroo (Harvard Medical

School, Boston, MA). $MOG_{97–114}$ (rat sequence TCFFRDHSYQEEAAVELK)) and MBP_{79-87} (mouse sequence DENPVVHFF) peptides were purchased from GenScript.

CD4+ T cell-mediated EAE induction

Active CD4+ T cell-mediated EAE was induced by immunizing mice with 100 μg of rMOG emulsified in complete Freunds adjuvant (CFA) containing 1 mg/ml mycobacteria (Sigma) accompanied by two injections of 200 ng pertussis toxin (List Biological Laboratories) as described [95]. Passive CD4+ T cell-mediated EAE was induced by culturing splenocytes (1×10^7 cells per ml) from rMOG-immunized mice for 3 days with 10 μM $MOG_{97–114}$ peptide and 10 ng/ml of rIL-23 (eBioscience). Viable cells were isolated from a Lympholyte gradient (Cedarlane) and intraperitoneally injected (2×10^7 cells per mouse) into sublethally irradiated (250 rads) mice. We scored the severity of EAE as follows (a grade was assigned when any one of its associated signs was observed): grade 1, paralyzed tail, hindlimb clasping, hyperactivity; grade 2, head tilt, hindlimb weakness; grade 3, one paralyzed leg, mild body leaning; grade 4, two paralyzed legs, moderate body leaning; grade 5, forelimb weakness, severe body leaning; grade 6, hunched, breathing difficulty, body rolling; grade 7, moribund. Atypical EAE was determined by the presence of one or more of the following symptom(s): hyperactivity, head tilt, body leaning and rolling.

CD8+ T cell-mediated EAE induction

Passive CD8+ T cell-mediated EAE was induced by adoptive transfer of in vitro-activated 8.8 T cells. Splenocytes (2.5×10^6 cells per ml) from naïve 8.8 mice were incubated with 1 μM

MBP_{79-87} peptide and 15 IU/ml IL-2 for 7 days. The cells were collected and re-stimulated with 0.5 μM MBP_{79-87} peptide and 15 IU/ml IL-2 for 2 days. Mice were injected i.v. with the activated cells (8-10 × 10^6) and 100 IU IL-2 was administered i.p. daily until disease onset. CD8+ T cell-mediated EAE was actively induced by i.p. injection of vaccinia virus infection (1 × 10^6 PFU of New York City Board of Health vaccinia virus) as previously described [126]. Disease course was monitored by weight loss; mice were sacrificed when they lost more than 20% of their original body weight. Neurological symptoms, such as knuckling, hypersensitivity or difficulty in walking usually appeared 7 days post-disease induction.

In vivo Tim-3-blocking Ab treatment

100 μg Tim-3-blocking mAb (clone 8B.2C12, eBioscience) or rat IgG1 isotype control mAb (eBioscience) was administered i.p. on day 0, 2, 4, and 6 post-transfer of 8.8 T cells during passive CD8+ T cell-mediated EAE induction. Mice were weighed daily to monitor disease progression.

In vitro galectin-9 treatment of 8.8 T cells

Naïve and activated 8.8 T cells (1 × 10^6 per well) were cultured overnight with the indicated concentrations of galectin-9 prior to staining for apoptotic cells. Activated 8.8 T cells were generated by culturing naïve 8.8 splenocytes with 0.5 μM MBP_{79-87} in RPMI 1640 complete medium containing 20 IU/ml IL-2. The 8.8 T cells were either used 3 days post-stimulation or split on day 3 with complete media containing 20 IU/ml IL-2 and used 7-20 days after stimulation.

Isolation of CNS cells

Mononuclear cells were isolated from the CNS of perfused EAE mice as previously described [161]. Briefly, brain and spinal cord were dissociated with a 5-ml syringe plunger through a sterile stainless steel mesh and centrifuged with brake for 10 min at 3000 rpm. Cell pellets were resuspended in 30% percoll, overlaid onto 70% percoll, and centrifuged without brake for 20 min at 2800 rpm. Cells were collected from the 30%-70% percoll interface.

Isolation and activation of splenic DCs

Spleens from WT and Tim-3$^{-/-}$ mice were digested while shaking for 30 min at 37°C with 1 mg/ml Collagenase D (Roche) and 50 μg/ml DNase I (Roche) in HBSS medium. EDTA (10 mM) was added to detach adherent cells while samples were stirred vigorously for 10 min at 25°C. DCs were enriched using a Dynabeads mouse DC enrichment kit (Invitrogen), stained for CD11c and sorted on a FACS Aria cell sorter (BD biosciences). Purity was >98%.

Splenic DCs and myelin-specific CD4+ and CD8+ T cell co-culture and proliferation assay

DCs from WT and Tim-3$^{-/-}$ spleens were isolated as described above. Myelin-specific CD4+ and CD8+ T cells were purified from MOG_{97-114}-specific and 8.8 TCR transgenic mice, respectively, using CD4+ and CD8+ T cell isolation kits and an AutoMACS separator (Miltenyi Biotec). DCs purified from WT or Tim-3$^{-/-}$ mice (2×10^5) were cultured with 1×10^5 myelin-specific CD4+ or CD8+ T cells in duplicate with 1 μg/ml LPS, and MOG_{97-114} peptide for CD4+ T cell culture or MBP_{79-87} peptide for CD8+ T cell culture for 7 days at 37°C prior to intracellular cytokine

staining. T cell proliferation was measured 48 hours later with ^{3}H-Thymidine incorporation assay.

Flow Cytometry

Cells were incubated with Fc block (clone 2.4G2; eBioscience) in 5% normal mouse serum for 15 min at room temperature, washed and stained with mAbs for 30 min at 4°C. mAbs from eBioscience were: anti-Tim-3 (clone RMT3-23), rat IgG2a isotype control, anti-CD8 (clone 53-6.7), anti-CD4 (clone GK1.5), anti-CD11c (clone N418), anti-Vβ8 (clone F23.1), anti-CD45 (clone 30-F11), anti-CD11b (M1/70), hamster IgG isotype control, anti-CD40 (clone 1C10), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), anti-MHC class II (clone NIMR-4). mAbs from BD Biosciences were: anti-IFN-γ (clone XMG1.2), anti-GM-CSF (clone MPI-22E9), anti-IL-17 (clone TC11-18H10), and rat IgG1 isotype antibody (R3–34). The annexin V/7-AAD apoptosis detection kit was from BD Biosciences. Cells were analyzed with a FACS Canto cytometer (BD Biosciences) and FlowJo software version 8.8.7 (Tree Star).

Intracellular staining

CNS mononuclear cells (1×10^{6}) from vaccinia-infected mice were incubated for 2 hrs at 37°C with splenocytes from Thy-1.1 C3HeB/FeJ mice that were either naïve or pulsed with 5 μM $MBP_{79\text{-}87}$. Golgi Plug was then added and the cells were stained for CD8 and Thy-1.2 after an additional 5 hrs incubation. The cells were fixed and intracellularly stained with anti- IFN-γ or rat IgG1 isotype mAb according to manufacturer's directions (BD Biosciences). Cytokine production by either MOG-specific CD4+ T cells or 8.8 CD8+ T cells that had been cultured

with DCs was assessed by harvesting the cells after 7 days of culture and incubating them with naive splenocytes pulsed with either 1 μg/ml MOG_{97-114} for CD4+ T cell cultures or 1 μg/ml MBP_{79-87} for CD8+ T cell cultures for 2 hrs at 37°C followed by an additional 5 hrs incubation with Golgi Plug. Cells were then stained for CD4 or CD8 and intracellularly stained for IFN-γ, IL-17, GM-CSF or with rat IgG1 isotype control mAb. Samples were analyzed on a FACS Canto cytometer (BD Biosciences) as above.

Enzyme-linked immunosorbent spot assays (ELISPOT)

Splenocytes (1×10^6 cells per well) or total mononuclear cells isolated separately from the brains and spinal cords of perfused mice (typically $1–10 \times 10^5$ cells per well) were plated in duplicate wells of 96-well Enzyme-linked immunosorbent spot (ELISPOT) plates (Millipore) and ELISPOT assays were carried out according to BD Biosciences protocols and analyzed on an ImmunoSpot Analyzer (CTL). IFN-γ–specific mAb pairs (Cat.# 551083), IL-17–specific (TC11-18H10) and biotinylated IL-17–specific (TC11-8H4.1) mAbs were from BD Biosciences. Background spots obtained by plating T cells in the absence of exogenous antigen (<10 spots per well) were subtracted from the total number of spots with antigen.

Generation of bone marrow chimeras

Cells (1×10^7) isolated from femurs of WT mice were transferred i.v. on day 0 into lethally irradiated WT or Tim-$3^{-/-}$ mice (1,000 rads on day -1). Recipients were provided neomycin sulfate (2 mg/ml; Sigma) in their drinking water from day -2 to day 21, and mice were used for EAE induction 6-8 weeks after transfer of cells.

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