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J Immunol 2013; 190:4991-4999; Prepublished online 5 April 2013;
doi: 10.4049/jimmunol.1300083
http://www.jimmunol.org/content/190/10/4991

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/04/05/jimmunol.1300083.DC1

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The Influence of T Cell Ig Mucin-3 Signaling on Central Nervous System Autoimmune Disease Is Determined by the Effector Function of the Pathogenic T Cells

Sarah Y. Lee and Joan M. Goverman

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the 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. T cell Ig mucin-3 (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, 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 dendritic cells 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. The Journal of Immunology, 2013, 190: 4991–4999.

T cell Ig mucin-3 (Tim-3) is a type I transmembrane glycoprotein expressed by both innate and adaptive immune cells, including dendritic cells (DCs), microglia, IFN-γ-secreting CD4+ T cells (Th1), and activated CD8+ T cells (1). The kinetics of Tim-3 expression differ between innate and adaptive immune cells. Whereas Tim-3 is constitutively expressed on DCs and microglia, its expression is induced on CD4+ Th1 and CD8+ T cells only upon activation (1–3). Galectin-9 has been identified as a ligand for Tim-3 (4). Binding of galectin-9 to Tim-3 expressed on Th1 cells in vitro induces calcium flux and cell aggregation, followed by death, suggesting that Tim-3 may play a role in attenuating immune responses by eliminating activated T cells (4). However, Tim-3 is expressed only at a low level on activated IL-17–secreting Th17 cells (5, 6) and is not detectable on Th2 cells that secrete IL-4 (2). Thus, the ability of Tim-3 to influence an immune response may be limited to responses that engage only certain subsets of effector T cells.

The function of Tim-3 was first studied in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) (2, 4). MS is an inflammatory, demyelinating disease of the CNS that is believed to result from the activity of autoreactive CD4+ and CD8+ T cells (7). MS is a genetically complex disease; >50 loci have been associated with susceptibility to MS by genome-wide association studies (8). The vast majority of these loci correspond to genes with immune function, supporting an autoimmune etiology for MS. EAE is induced by immunization with myelin Ags, which typically activates myelin-specific CD4+ rather than CD8+ T cells (7). In EAE induced in C57BL/6 mice, administration of galectin-9 reduced the number of IFN-γ-secreting, myelin-specific CD4+ T cells and ameliorated disease (4), and inhibition of Tim-3 signaling exacerbated EAE (2). These findings prompted studies of CD4+ T cell clones isolated from cerebrospinal fluid of patients with MS. Compared with CD4+ T cells from healthy controls, CD4+ T cell clones from patients with MS expressed lower levels of Tim-3 and produced more IFN-γ (9). Furthermore, treatment with glatiramer acetate or IFN-β restored normal levels of Tim-3 expression to CD4+ T cells from patients with MS, as well as their responsiveness to Tim-3 blockade (10). Together, these data suggested that the level of Tim-3 expression on CD4+ T cells influences their pathogenic activity in MS and EAE.

The results described above implicated Tim-3 as a potential therapeutic target in MS. However, the complex pattern of Tim-3 expression indicates the need to understand how Tim-3 signaling affects multiple cell types in vivo. This requires the use of multiple animal models of MS 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, the distribution of lesions in the CNS differs between MS and most murine EAE models. The majority of patients with MS have parenchymal lesions in the brain, and the spinal cord is frequently involved as well. Only a
small subset of patients exhibits opticospinal MS in which inflammation targets the spinal cord and optic nerves without extensive involvement of the brain (11). In contrast, inflammation predominantly targets only the spinal cord in most EAE models, whereas the brain is relatively spared of parenchymal lesions (7). This spinal cord–dominant inflammatory pattern results in ascending flaccid paralysis, which is referred to as classic EAE. However, we recently developed a new EAE model in C3HeB/FeJ mice in which extensive parenchymal lesions occur in both the brain and spinal cord. We found that the relative abundance of myelin-specific Th1 compared with Th17 cells infiltrating the CNS determines whether parenchymal inflammation occurs in the brain (12). 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 atypical EAE in which distinct clinical signs reflecting inflammation in the cerebellum and brain stem occur in addition to the paralysis associated with spinal cord inflammation. Parenchymal lesions in the brain occurred only at Th17/Th1 ratios ≥1, whereas inflammation in the spinal cord occurred at a wide range of Th17/Th1 ratios (12). Importantly, the therapeutic effect of triggering Tim-3 signaling with galectin-9 in vivo was demonstrated only in a classic EAE model (2, 4). This raises the question of whether manipulating Tim-3 signaling in vivo would have a different outcome in an atypical EAE model in which myelin-specific Th17 cells play a greater role in the pathogenesis, but express low levels of Tim-3 compared with Th1 cells.

Another limitation of the models used to study Tim-3 signaling in EAE is that the disease in these models is mediated exclusively by CD4+ T cells because the immunization protocol used to induce disease predominantly activates CD4+ T cells (7). However, CD8+ T cells appear to play an important role in the pathogenesis of MS (7, 13–15). CD8+ T cells outnumber CD4+ T cells in MS lesions (16, 17), and axonal damage correlates with the number of macrophages and CD8+, but not CD4+, T cells (18, 19). CD8+ and not CD4+ T cell clones also persist over time in both the blood and CNS of patients with MS (17, 20–22). As Tim-3 signaling induces TNF-α secretion of CD8+ T cells in vitro activated 8.8 T cells. Splenocytes (2.5 × 10^6) were transferred into sublethally irradiated (250 rad) mice. We scored the severity of EAE by monitoring body weight loss; mice were sacrificed when they lost >20% of their body weight.

In this study, we used both CD4+ and CD8+ T cell–mediated EAE models to analyze the role of Tim-3 expressed on adaptive and innate immune cells in regulating autoimmune responses in the CNS. We show that preventing Tim-3 signaling in CD4+ T cells did not increase disease incidence and severity in C3HeB/FeJ mice. Instead, preventing Tim-3 signaling altered the pattern of inflammation in the brain and spinal cord due to differential effects on Th1 versus Th17 cells. In contrast, preventing Tim-3 signaling during CD8+ T cell–mediated EAE exacerbated disease severity due to increased myelin-specific CD8+ T cell accumulation in the CNS. We also show that preventing Tim-3 signaling in innate immune cells did not affect the manifestation of EAE in these models.

Materials and Methods

Mice

C3HeB/FeJ mice were purchased from The Jackson Laboratory and bred in our colony. BALB/c Tim-3– mice were provided by V. Kuchroo (Harvard Medical School, Boston, MA) and were backcrossed for 10 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) (35). The 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 97–141 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, WA). Mice used for EAE induction were between 8 and 12 wk 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 (36). Recombinant galectin-9 was provided by V. Kuchroo. MOG_97-114 (rat sequence TCFRFDHSYQEEAVELK) and MBP_79-87 (mouse sequence DENVPVHFF) 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 CFA containing 1 mg/ml mycobacteria (Sigma-Aldrich) accompanied by two injections of 200 ng pertussis toxin (List Biological Laboratories), as described (37). Passive CD4+ T cell–mediated EAE was induced by culturing splenocytes (1 × 10^7 cells/ml) from rMOG-immunized mice for 3 d with 10 μM MOG_97–114 peptide and 10 ng/ml rIL-2 (eBioscience). Viable cells were isolated from a lymphocyte gradient (Cedarlane) and i.p. injected (2 × 10^7 cells per mouse) into sublethally irradiated (250 rad) 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 clasp, 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 symptoms: 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 CD4+ T cells in vitro activated 8.8 T cells. Splenocytes (2.5 × 10^7 cells/ml) from naive 8.8 mice were incubated with 1 μM MBP_79-87 peptide and 15 IU/ml IL-2 for 7 d. The cells were collected and restimulated with 0.5 μM MBP_79-87 peptide and 15 IU/ml IL-2 for 2 d. Mice were injected i.v. with the activated cells (8–10 × 10^7), 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 vacccinia virus infection (1 × 10^7 PFU New York City Board of Health vaccinia virus), as previously described (38). Disease course was monitored by weight loss; mice were sacrificed when they lost >20% of
their original body weight. Neurological symptoms, such as knackling, hypersensitivity, or difficulty in walking, usually appeared 7 d postinfection.

In vivo Tim-3–blocking Ab treatment
A total of 100 μg Tim-3–blocking mAb (clone 8B.2C12; eBioscience) or rat IgGl isotype control mAb (eBioscience) was administered i.p. on days 0, 2, 4, and 6 after 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 × 10^9 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 MBP79–87 in RPMI 1640 complete medium containing 20 IU/ml IL-2. The 8.8 T cells were either used 3 d after stimulation or split on day 3 with complete media containing 20 IU/ml IL-2 and used 7–20 d after stimulation.

Isolation of CNS cells
Mononuclear cells were isolated from the CNS of perfused EAE mice, as previously described (39). Briefly, brain and spinal cord were dissociated with a 2 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
Splenic 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 coculture
DCs from WT and Tim-3−/− spleens were isolated, as described above. Myelin-specific CD4+ and CD8+ T cells were purified from MOG<sub>97–114</sub>– 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<sub>97–114</sub> peptide for CD4+ T cell culture or MBP<sub>79–87</sub> peptide for CD8+ T cell culture for 7 d at 37°C prior to intracellular cytokine staining.

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 as follows: anti–Tim-3 (clone RMT3-23), rat IgG2a isotype control, anti–CD11c (clone Gl1.5), anti–CD11b (clone M1/70), and hamster IgG isotype control mAbs from BD Biosciences as follows; anti–IFN-γ (clone XMG1.2), anti–GM-CSF (clone MPI-22E9), anti–IL-17 (clone TC11-18H10), and rat IgG1 isotype Ab (R3-34). The annexin V77–aquoaminocoumarin D (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<sup>6</sup>) from vaccinia-infected mice were incubated for 2 h at 37°C with splenocytes from Thy-1.1 C3HeB/FeJ mice that were either naive or pulsed with 5 μM MBP<sub>79–87</sub>. Golgi Plug was then added, and the cells were stained for CD8 and Thy-1.2 after an additional 5 h incubation. The cells were fixed and intracellularly stained with anti–IFN-γ or rat IgGl 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 d of culture and incubating them with naïve splenocytes pulsed with either 1 μg/ml MOG<sub>97–114</sub> for CD4+ T cell cultures or 1 μg/ml MBP<sub>79–87</sub> for CD8+ T cell cultures for 2 h at 37°C, followed by an additional 5 h 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 IgGl isotype control mAb. Samples were analyzed on a FACS Canto cytometer (BD Biosciences), as above.

ELISPOT assays
Splenic cells (1 × 10<sup>6</sup> cells/well) or total mononuclear cells isolated separately from the brains and spinal cords of perfused mice (typically 1–10 × 10<sup>6</sup> cells/well) were plated in duplicate wells of 96-well 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 (catalogue 551083) and 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 Ag (<10 spots per well) were subtracted from the total number of spots with Ag.

Generation of bone marrow chimeras
Cells (1 × 10<sup>5</sup>) isolated from femurs of WT mice were transferred i.v. on day 0 into lethally irradiated WT or Tim-3−/− mice (1000 rad on day −1). Recipients were provided neomycin sulfate (2 mg/ml; Sigma-Aldrich) in their drinking water from day −2 to day 21, and mice were used for EAE induction 6–8 wk after transfer of cells.

Statistical analysis
Statistical analyses were performed with Prism version 5.0 (GraphPad Software), using an unpaired two-tailed Student <i>t</i> test, unless indicated otherwise. A <i>p</i> value <0.05 was considered significantly different.

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 (12). C3HeB/FeJ mice respond to two distinct epitopes of rMOG, MOG<sub>97–114</sub>– and MOG<sub>79–90</sub>–MOG<sub>97–114</sub>–specific T cells differ from MOG<sub>79–90</sub>–specific T cells in that they exhibit a Th17/Th1 ratio ≥1 and typically trigger extensive brain inflammation and atypical EAE (12). We hypothesized that preventing Tim-3 signaling in this 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 death of Th1 cells 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 with Th17 cells in this strain. C3HeB/FeJ mice were immunized with rMOG, and splenocytes were harvested 7 d later and stained for CD4, Tim-3, IFN-γ, and IL-17 (Supplemental Fig. 1A). Tim-3 expression was strongly increased on CD4+IFN-γ+ cells; however, only a slight increase in Tim-3 expression was seen on CD4+IL-17+ T cells (Supplemental Fig. 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. 1A), significantly more Th1 cells were found in the spleens of Tim-3−/− mice (Fig. 1B). In a few experiments (two of five), we also observed a decrease in Th17 cells in the spleens of Tim-3−/− mice, which may reflect competition for Ag 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 with WT mice (Fig. 1C), suggesting that Tim-3–induced death of Th1 cells during an ongoing immune response is a major determinant of the Th17/Th1 ratio.
We next analyzed EAE induced in WT and Tim-3−/− mice by rMOG/CFA immunization. In contrast to the increased mortality and clinical score observed in C57BL/6 mice when Tim-3 signaling was blocked during EAE induction (2), no differences in disease incidence, onset, or severity were observed between the WT and Tim-3−/− mice on the C3HeB/FeJ background (Table I). 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 with WT mice (Fig. 2A). A 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 with that seen in WT mice (Fig. 2B).

We investigated whether the altered inflammatory pattern observed in Tim-3−/− mice with EAE was due to Tim-3 deficiency only on effector T cells by inducing EAE via adoptive transfer of activated WT and Tim-3−/− MOG97–114-specific CD4+ T cells into WT recipients. Similar disease onset, severity, and progression were observed in recipients of WT and Tim-3−/− T cells (Fig. 3A, 3B). However, the WT recipients of Tim-3−/− T cells exhibited a significantly higher incidence of classic EAE compared with the WT recipients of WT T cells (Fig. 3A, 3C), similar to our results in actively induced EAE in Tim-3−/− mice. Importantly, this effect on the manifestation of EAE resulted from the lack of expression of Tim-3 only on the adoptively transferred effector T cells as the recipients in these experiments were all WT mice. This excludes the possibility that potential differences in effector function between Tim-3+ and Tim-3−/− Foxp3+ T regulatory cells (40) could be contributing to the change in manifestation of EAE in recipients of Tim-3−/− T cells. 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.

### Table I. Disease incidence, onset, and severity were comparable between the WT and Tim-3−/− mice after active induction of CD4+ T cell-mediated EAE

<table>
<thead>
<tr>
<th>Incidence</th>
<th>Mortality</th>
<th>Onset</th>
<th>Maximal Score</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>30 of 30</td>
<td>6 of 30 (20%)</td>
<td>18.8 ± 2.86</td>
</tr>
<tr>
<td>Tim-3−/−</td>
<td>30 of 40</td>
<td>4 of 30 (13%)</td>
<td>22.2 ± 4.39</td>
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*aOnset indicates the day that clinical signs are first detected (mean ± SD).
*bMaximal score is the maximum disease score achieved according to the grading scale described in Materials and Methods (mean ± SD).

*Fig. 1.* 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 d after 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 × percentage of CD4+ cells. Error bars represent SEM (n = 6). (B) The number of IFN-γ (Th1)– and IL-17 (Th17)–secreting cells in the spleens of WT and Tim-3−/− mice 7 d after rMOG immunization is shown. The numbers of Th1 and Th17 cells were determined by ELISPOT using MOG97–114 to stimulate the T cells. Error bars represent SEM (n = 6). *p < 0.05. (C) The Th17/Th1 ratio observed in the spleens of Tim-3−/− mice compared with WT mice after rMOG immunization is shown. The ratios were calculated from the data in (B); each dot represents an individual mouse. Data are representative of three independent experiments with six mice per group in each experiment. **p < 0.01.

Tim-3 signaling facilitates the elimination of myelin-specific CD8+ T cells during EAE

We analyzed the role of Tim-3 signaling in CNS autoimmunity mediated by CD8+ T cells using a mouse model developed in our laboratory in which mice express a transgenic TCR specific for MBP79–87 presented by H-2Kb (35). 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 transferring activated 8.8 T cells into WT recipients, or by infecting 8.8 mice with WT vaccinia virus (38). We first confirmed that Tim-3 expression was induced in vitro on activated 8.8 T cells and that exposure to galectin-9 triggered their death. Tim-3 was detected on 8.8 T cells 3 d after stimulation with MBP79–87, and the level of Tim-3 expression increased after a second in vitro stimulation (Fig. 4A). 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 fashion (Fig. 4B, 4C). In contrast, the number of viable, naive 8.8 T cells that lack Tim-3 expression was unchanged by addition of galectin-9 cells (Fig. 4C). 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 assess the influence of Tim-3 signaling during CD8+ T cell–mediated EAE, activated 8.8 T cells were transferred into WT...
recipients that were treated with either Tim-3–blocking Ab (41) or control Ab 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 (38). Recipients of 8.8 T cells that were treated with Tim-3–blocking Ab exhibited more severe weight loss compared with the control treatment group (Fig. 5A), indicating that inhibition of Tim-3 signaling exacerbates EAE induced by adoptive transfer of activated 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 neurologic symptoms and disease progression were observed in both groups of mice, Tim-3−/− 8.8 mice consistently exhibited earlier disease onset compared with Tim-3+/+ 8.8 mice (Fig. 5B). We hypothesized that the earlier disease onset in Tim-3−/− 8.8 mice might be due to greater expansion 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 with Tim-3+/+ 8.8 mice 7 d after vaccinia infection (Fig. 5C). 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 for IFN-γ staining was similar for CD8+ T cells isolated from the CNS of Tim-3+/+ and Tim-3−/− 8.8 mice (Fig. 5D). These data indicate that the earlier disease onset observed in Tim-3−/− 8.8 mice is most 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 serves to limit the activity of myelin-specific CD8+ T cells during CNS autoimmunity in addition to limiting CD4+ Th1 cell activity.

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

In contrast to its function in activated T cells, the role of Tim-3 signaling in innate immune cells has not been defined. Proinflammatory effects were reported in studies that used galectin-9 to

![FIGURE 3.](http://www.jimmunol.org/) 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+ MOG97–114–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 SEM (n = 20). (C) Numbers of WT mice exhibiting atypical or classic EAE after adoptive transfer of CD4+ MOG97–114–specific WT or Tim-3−/− cells (n = 20). Data are pooled from three independent experiments. *p < 0.05.

![FIGURE 4.](http://www.jimmunol.org/) Tim-3 signaling induces death in activated CD8+ T cells. (A) Flow cytometric analysis of Tim-3 expression on naive 8.8 T cells and 8.8 T cells stimulated once or twice with MBP79–87 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 MBP79–87 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) Naive 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). Data are representative of three independent experiments. *p < 0.05.
trigger Tim-3 expressed on LPS-stimulated murine DCs in vivo; however, suppressing Tim-3 signaling on human monocytes via Tim-3 Ab blockade or expression of Tim-3–specific small interfering RNA revealed anti-inflammatory effects of Tim-3 signaling in these cells (1, 34). If Tim-3 signaling influences the phenotype of the DCs that present Ag 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 Ags in adjuvant, we compared the responses of splenic DCs from WT and Tim-3−/− mice to LPS in vitro. DCs were sorted from the spleens of WT and Tim-3−/− mice and cultured with and without LPS for 24 h. Cytokines in the supernatants from these cultures were analyzed by Bioplex assay. Whereas 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 (Supplemental Fig. 2). Significant differences were not observed for production of IL-1β, TNF-α, IL-12(p40), IL-12(p70), IL-10, 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 (data not shown), 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 MOGp7–114 peptide to naive MOGp7–114–specific TCR-transgenic CD4+ T cells recently established in our laboratory (L. Castelli and J. Goverman, unpublished observations). 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. 6A). Similarly, no differences in proliferation (data not shown) or IFN-γ (Fig. 6B) production by CD8+ 8.8 T cells were observed when the 8.8 T cells were activated by WT or Tim-3−/− DCs presenting MBPp9–87. Thus, the small difference in IL-6 production observed in vitro for Tim-3−/− DCs stimulated with LPS did not influence the priming of naïve 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.

**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 (Supplemental Fig. 3A, 3B). To determine whether Tim-3 signaling in microglial cells influences either CD4+ or CD8+ T cell–mediated EAE, we constructed bone marrow chimera (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 (Supplemental Fig. 3C). 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. 6C). 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. 6D). 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 ablation of Tim-3 on 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 (data not shown), and incidence of classic versus atypical disease (Fig. 6E) were comparable between WT and Tim-3−/− recipients that received WT MOG-specific CD4+ T cells. In addition, disease progression was similar when activated CD8+ 8.8 T cells were transferred into WT or Tim-3−/− recipients (Fig. 6F). 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 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.

**Discussion**

Since its first description as an inhibitor of proinflammatory Th1 responses, Tim-3 has been implicated in regulating immune responses in autoimmune diseases (3), transplant tolerance (42), chronic viral infections (27–29), and antitumor immunity (25, 26, 31, 43, 44). Understanding how Tim-3 influences the immune response in each of these diseases has been challenging for several reasons. First, multiple cell types express Tim-3, and Tim-3–mediated signaling may have different effects in each cell type. Second, the outcome of Tim-3 signaling can be different even within the same cell type. For example, Tim-3 expression on exhausted T cells in chronic viral infections and in tumor immunity appears to be required to maintain T cells in the peripheral repertoire in a state of nonresponsiveness. Blocking Tim-3 interactions allows exhausted T cells to regain their effector function and is beneficial in these diseases (29–31). In contrast, Tim-3 expression on Th1 cells during autoimmunity confers susceptibility to cell death such that enhancing Tim-3 signaling is beneficial in autoimmune diseases in which Th1 cells play a major role. Indeed, studies in EAE demonstrated that promoting Tim-3 signaling in vivo ameliorates Th1-mediated EAE (2, 4). This finding led to the notion that Tim-3 may be an effective therapeutic target in MS. However, many questions remained about the role of Tim-3 signaling in CNS autoimmunity. MS is a heterogeneous disease in both clinical signs and lesion structure (45). The variation in the composition of inflammatory cells found in lesions suggests that...
the relative activity of distinct effector cell types may differ among individual patients. Furthermore, whereas T cells, DCs, and microglia all express Tim-3 and all play important roles in the pathogenesis of CNS autoimmune disease, the effect of Tim-3 signaling is not understood in DCs and microglia. Even the role of Tim-3 signaling in other T cell subsets that contribute to the pathogenesis of MS, such as Th17 cells and CD8\(^+\) T cells, has not been defined. Determining the efficacy of Tim-3 as a therapeutic target in CNS autoimmunity requires understanding the effects of manipulating Tim-3 signaling on all of these diverse cell types. In this study, we used both a unique model of CD8\(^+\) T cell–initiated CNS autoimmunity as well as a CD4\(^+\) T cell–mediated EAE model in which both Th17 and Th1 cells are prominent effector T cells to analyze the importance of Tim-3 expression by both innate and adaptive immune cells. We found that Tim-3 expression by DCs and microglia did not play a large role in determining the manifestation of EAE. In contrast, we found that eliminating Tim-3 signaling exacerbated EAE mediated by CD8\(^+\) T cells and resulted in a shift from atypical to classic EAE without increasing disease severity or incidence in CD4\(^+\) T cell–mediated EAE.

Although earlier reports in C57BL/6 mice described exacerbation of EAE upon blocking Tim-3 signaling, we observed a change in the clinical manifestation rather than the severity of disease when Tim-3 signaling was prevented in CD4\(^+\) T cell–mediated EAE in C3HeB/FeJ mice. This difference is most likely due to the nature of the effector T cells that induce EAE in these two strains. C57BL/6 mice exhibit classic EAE in which Th1 cells are the predominant effector subset and inflammation primarily targets the spinal cord. Triggering Tim-3 signaling in vivo in this model causes a loss of pathogenic IFN-γ–secreting T cells with a decrease in severity of EAE (4). The atypical EAE seen in C3HeB/FeJ mice results from extensive parenchymal lesions in the brain as well as the spinal cord, and is more similar to the inflammatory pattern seen in many patients with MS. The increase in parenchymal brain inflammation results from a higher Th17/Th1 ratio among T cells infiltrating the CNS in C3HeB/FeJ mice (12). Consistent with the difference in Tim-3 expression on activated Th17 and Th1 cells, we found that eliminating Tim-3 signaling during EAE in this model caused an increase in survival of Th1 cells due to a lack of Tim-3–induced cell death in this subset, whereas the survival of Th17 cell was unchanged. 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, whereas the incidence of classic EAE relative to atypical EAE was increased in Tim-3\(^{-/-}\) mice, the overall severity of disease and the incidence 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 Th1 versus Th17 cells that are active in their disease.

The role of Tim-3 signaling on myelin-specific CD8\(^+\) T cells has not been previously examined in EAE, although increasing evidence suggests that CD8\(^+\) T cells play an important role in the pathogenesis of MS (7, 13–15). We found that Tim-3 signaling induces cell death in MBP-specific CD8\(^+\) T cells, and that preventing Tim-3 signaling in vivo using Tim-3–blocking Ab and in Tim-3\(^{-/-}\) 8.8 mice exacerbated CD8\(^+\) T cell–mediated EAE. Increased severity in clinical signs and an earlier disease onset correlated with increased accumulation of MBP-specific CD8\(^+\) T cells in the CNS of Tim-3\(^{-/-}\) 8.8 mice compared with Tim-3\(^{+/+}\) 8.8 mice. Thus, our data indicate that self-reactive CD8\(^+\) T cells are similar to Th1 cells in their sensitivity to cell death mediated by Tim-3, and that patients with MS for whom CD8\(^+\) T cell activity is prominent may benefit from strategies that enhance Tim-3 signaling.

Little is known about the role of Tim-3 signaling in DCs and microglia in the context of CNS autoimmunity, although 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 (33, 46). Studying Tim-3 function in DCs and microglia is challenging, as both cell types are more difficult to purify and culture ex vivo compared with CD4\(^+\) and CD8\(^+\) T cells. DCs are typically studied in vitro using cells derived from cultures of bone marrow cells. 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 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 activated in vitro by either WT or Tim-3\(^{-/-}\) DCs produced comparable amounts of cytokines and exhibited a similar activated phenotype. Thus, although it is not possible to isolate Tim-3 deficiency only to DCs in vivo, these results suggest that lack of Tim-3 signaling on DCs would not influence T cell differentiation into effector cells or their activity in the development of EAE. Similarly, no effect on EAE 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 in neonatal mice (our 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 sum, our findings suggest that strategies designed to enhance Tim-3 signaling may be beneficial in patients with MS in which Th1 cells and/or CD8\(^+\) T cells are the primary effector cells in the disease. Caution should be used with this potential treatment, however, as the number of effector Th17 cells does not appear to be affected by Tim-3 signaling. A reduction in Th1 cells will alter the Th17/Th1 ratio in the pathogenic T cell population, and, if there are substantial numbers of Th17 cells active in the disease process, this may result in a shift in inflammatory pattern rather than amelioration of disease. Identification of a cell surface marker expressed on Th17 cells that exhibits similar inhibitory activity as Tim-3 for Th1 and activated CD8\(^+\) T cells could be therapeutically beneficial in combination therapies that would target all potential effector T cell subsets in MS.

Acknowledgments
We thank N. Maurillo for animal husbandry and technical assistance and Emily Pierson and Sarah Simmons for critical reading of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

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