Persistent Macrophage/Microglial Activation and Myelin Disruption after Experimental Autoimmune Encephalomyelitis in Tissue Inhibitor of Metalloproteinase-1-Deficient Mice

Stephen J. Crocker,* Jason K. Whitmire,*
Ricardo F. Frausto,* Parntip Chertboonmuang,*
Paul D. Soloway,[†] J. Lindsay Whitton,* and
Iain L. Campbell*[‡]

From the Molecular and Integrative Neurosciences Department,*
The Scripps Research Institute, La Jolla, California; the Division
of Nutritional Sciences,[†] College of Agriculture and Life Sciences,
Cornell University, Ithaca, New York; and the School of
Molecular and Microbial Biosciences,[‡] University of Sydney,
Sydney, Australia

Increased leukocyte trafficking into the parenchyma during inflammatory responses in the central nervous system (CNS) is facilitated by the extracellular proteolytic activities of matrix metalloproteinases that are regulated, in part, by the endogenous tissue inhibitors of metalloproteinases (TIMPs). In experimental autoimmune encephalomyelitis (EAE), TIMP-1 gene expression is induced in astrocytes surrounding inflammatory lesions in the CNS. The physiological importance of this temporal and spatial relationship is not clear. Herein, we have addressed the functional role of TIMP-1 in a myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅)-induced model of EAE using TIMP-1-deficient (TIMP-1^{-/-}) C57BL/6 mice. Although CD4⁺ T-cell immune responses to myelin in wild-type (WT) and TIMP-1^{-/-} mice were similar, analysis of CNS tissues from TIMP-1^{-/-} mice after EAE revealed more severe myelin pathology than that of WT mice. This disruption of myelin was associated with both increased lymphocyte infiltration and microglial/macrophage accumulation in the brain parenchyma. These findings suggest that induction of TIMP-1 by astrocytes during EAE in WT mice represents an inherent cytoprotective response that mitigates CNS myelin injury through the regulation of both immune cell infiltration and microglial activation. (*Am J Pathol* 2006; 169:2104–2116; DOI: 10.2353/ajpath.2006.060626)

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS). The etiology of MS is not known but inflammation and autoimmunity are central components of this disease. Many of the key features of MS can be modeled in rodents or nonhuman primates by immunization with myelin or myelin peptides that produce a condition called experimental autoimmune encephalomyelitis (EAE). In EAE, the development of myelin-specific T-cell responses and the infiltration of activated lymphocytes into the CNS parenchyma result in inflammation-mediated myelin injury. The infiltration of activated leukocytes into the CNS is required for immune-mediated myelin injury in both MS and EAE.¹ Extravasation of immune cells into the CNS is facilitated by matrix metalloproteinases (MMPs). This large and diverse family of extracellular proteases are important regulators of tissue homeostasis and also are associated with cellular injury and pathology in a wide variety of CNS diseases, including MS.^{2–4} In EAE, increased expression and activity of MMPs has been reported.⁵ MMPs are produced predominantly by activated immune cells,^{6,7} and MMPs facilitate the transmigration of these cells across the blood brain barrier by the proteolytic cleavage of substrates within the extracellular matrix.⁸ Deletion of MMP genes and pharmacological inhibition of MMP proteins lead to reduced immune cell trafficking into the CNS and attenuated demyelination during EAE.^{2,9} These findings indicate that elevated MMP expression and activity reported in the cerebrospinal fluid of MS patients likely reflects inadequate endogenous regulation

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Address reprint requests to Stephen J. Crocker, Ph.D., The Scripps Research Institute, SP30-2110, 10550 North Torrey Pines Rd., La Jolla, CA 92037. E-mail: crocker@scripps.edu.

of MMPs as a feature of this disease that contributes to CNS demyelination.

The activity of MMPs are regulated, in part, by the production and actions of an endogenous family of proteins called the tissue inhibitors of metalloproteinases (TIMPs).¹⁰ The proteolytic balance between MMPs and TIMPs is thought to modulate MMP activities in a tissue-specific manner, with an imbalance associated with human diseases, including neurodegeneration and neuroinflammation.¹¹ Previous studies have shown that expression of TIMP-1 is very low in the adult CNS, but it is robustly induced during EAE coincident with clinical disability.^{7,12,13} Astrocytes that express TIMP-1 were localized to areas of the immune cell infiltrates and associated demyelinating lesions in the spinal cord of mice with active EAE.¹² This temporal and spatial relationship of TIMP-1 with both the phase and sites of demyelinating injury has led to the proposal that expression of the *TIMP-1* gene represents an endogenous response to restrict the spread of activated immune cells within the brain parenchyma and minimize MMP-mediated myelin injury during inflammation in the CNS.¹² However, the physiological role of TIMP-1 in autoimmunity and demyelination is not well understood. Previous studies have indicated that increased expression of TIMP-1 by delivery of a recombinant virus had little effect on CNS myelin injury, whereas collagen-induced autoimmune-mediated tissue injury was reportedly exacerbated by enhanced TIMP-1 expression.^{14,15} In this study, we sought to fill this gap in our current understanding of TIMP-1 function in the CNS by determining the impact of TIMP-1 deficiency in a mouse model of EAE.

Materials and Methods

Experimental Autoimmune Encephalomyelitis (EAE)

TIMP-1-deficient (*TIMP-1*^{-/-}) mice were generated previously.¹⁶ These mice were backcrossed onto the C57BL/6 background for 10 successive generations and then bred to homozygosity. Wild-type (WT) C57BL/6 mice were used as controls for these experiments. To induce EAE, *TIMP-1*^{-/-} ($n = 22$) or WT C57BL/6 mice ($n = 21$), all between 8 to 10 weeks of age, were immunized with myelin oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅, 3 mg/ml; The Scripps Research Institute Peptide Synthesis Core Facility, La Jolla, CA). MOG₃₅₋₅₅ peptide was emulsified with complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO) containing *Mycobacterium tuberculosis* (200 ng/ml; Difco, Detroit, MI), and 100 μ l of emulsion was deposited subcutaneously in the region of the thigh of each hind leg (these mice are hereafter referred to as EAE groups). Control animals of each genotype were immunized with an equivalent volume of CFA emulsion that did not contain MOG peptide (CFA groups). Each animal was administered pertussis toxin (islet activating protein, 500 ng, i.p.; List Biological Laboratories, Inc., Campbell, CA) at the time of immunization and again 2 days later. Mice were evaluated on a daily

basis for changes in gross body weight, overt signs of illness, and clinical signs of EAE using the following scoring system: 0, no physical signs; 0.5, distal tail limpness; 1, full tail limpness; 2, mild or unilateral hind limb paresis; 3, full bilateral hind limb paralysis; 4, moribund; and 5, death attributable to EAE.

RNA Isolation and Multiprobe RNase Protection Assay

Total RNA was isolated from CNS tissues using Tri-Reagent (Sigma-Aldrich) as per the manufacturer's instructions, and RNase protection assays were performed as described previously.^{5,17}

Histology, Immunohistochemistry, and Image Analysis

Sagittal sections (6- to 8- μ m thick) were prepared from spinal cord and brain tissues that were collected into formalin (10%) fixative and were then paraffin embedded. Tissues were processed for hematoxylin and eosin or Luxol Fast Blue (LFB) staining for myelin using standard protocols. Immunohistochemistry was performed using the following antibodies: CD3 (pan leukocyte marker; Biocare Medical, Walnut Creek, CA), Iba-1 (microglial marker; Wako, Osaka, Japan), and myelin basic protein (MBP; Chemicon, Temecula, CA). Immunostaining was visualized using either biotin-conjugated secondary antisera (all purchased from Jackson ImmunoResearch, West Grove, PA) or Alexa-fluorophore-conjugated secondary antisera. Immunopositive staining for biotinylated antisera was developed using Vectastain DAB kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions and then counterstained with Accustain hematoxylin solution (Sigma-Aldrich). Immunofluorescent histochemistry was used to comparatively measure the intensity (and therefore the quantity) of MBP staining. Digital images were acquired using Axio-Vision software (Carl Zeiss Microimaging, Thornwood, NY) on an Axiovert 200 inverted microscope (Zeiss). All sections used for analysis were processed in parallel for immunodetection of MBP using the same reagents, concentrations, and incubation times. Images were collected at the same time using identical settings with respect to image exposure time and image compensation settings. Images were then imported into Image J software (NIH Imaging; <http://rsb.info.nih.gov/ij/>) and quantification of the relative MBP fluorescent staining was performed by obtaining the threshold range of real over background signal and then using the average real threshold range to measure the percent coverage in the region of interest.¹⁸ The region of interest for all samples covered 93,796 μ m² of white matter in the posterior column of the thoracic spinal cord. Analysis was performed on multiple sections per experimental group and genotype: $n = 3$ to 4 per treatment group per genotype.

Fluorescence-Activated Cell Sorting

Brain and spinal cord leukocytes were isolated from CFA- and MOG₃₅₋₅₅-immunized WT and TIMP-1^{-/-} mice either during the acute (day 18) or chronic (day 52) phase of EAE-associated illness. To analyze the cellular infiltrate within the CNS parenchyma, mice were perfused intracardially with 10 ml of saline, and brain and spinal cord tissues were collected separately into Dulbecco's modified Eagle's medium. The tissues were minced manually with a Dounce homogenizer, and a cell suspension was prepared in 44% Percoll, floated onto a 56% Percoll cushion, and separated by centrifugation. Leukocytes were identified as the primary cellular constituent at the interface between the two Percoll layers. Cells were then stained directly *ex vivo* with anti-CD4, anti-CD8, anti-CD44, Mac1, or B220 antibodies, analyzed using a BD Biosciences FACSCalibur, and then analyzed by FlowJo software (Tree Star, Ashland, OR).

Intracellular Cytokine Staining

The number and magnitude of antigen-specific CD4⁺ T cells was determined by stimulating isolated lymphocytes *ex vivo* with MOG₃₅₋₅₅ peptide (1 μ mol/L) and measuring interferon (IFN)- γ production using an anti-IFN- γ antibody (e-Biosciences), as described previously.¹⁹ In brief, 2×10^6 cells/well were stained for 1 hour (4°C) in primary antisera (either α CD4 or α CD8), washed, and then fixed in 2% formaldehyde for 5 minutes. Cells were then permeabilized [0.1% saponin, 0.1% sodium azide, and 1% fetal bovine serum in phosphate-buffered saline (PBS)] and stained using 0.5 μ g/ml rat anti-mouse IFN- γ for 30 minutes (4°C). Cells were then rinsed with permeabilizing solution followed with a wash in 5% fetal bovine serum (in PBS). Cellular labeling was acquired on a FAC-Scan flow cytometer and data analyzed using FlowJo software.

Statistical Analyses

EAE scores were tabulated as treatment group averages for each day starting with the day of immunization of MOG₃₅₋₅₅. Animals that died during the course of this study (ie, score of 5) were excluded from group average scores beyond that day. Repeated measures analysis of variance was performed followed by Bonferroni posthoc tests for comparisons to identify significance ($P < 0.05$) within and between individual treatment groups and experimental time points.

Results

EAE Progression and Clinical Severity in TIMP-1^{-/-} Mice

To determine the role of TIMP-1 production during EAE, TIMP-1^{-/-} ($n = 22$) or WT ($n = 21$) mice were immunized with MOG₃₅₋₅₅ emulsified in CFA and assessed on a daily

basis for health and development of EAE signs (Figure 1). Additional groups of mice were immunized with CFA alone as controls ($n = 8$ per genotype). Analysis of group mean EAE scores revealed that WT C57BL/6 or TIMP-1^{-/-} mice began developing EAE signs beginning 14 days after MOG₃₅₋₅₅ immunization. Signs of EAE persisted in both WT and TIMP-1^{-/-} mice throughout the 52-day study endpoint and differed significantly from CFA-treated mice that did not develop signs of EAE (analysis of variance, $P < 0.0001$). Among MOG₃₅₋₅₅-injected animals there was a slight, yet not significant, delay in the time to peak EAE in TIMP-1^{-/-} compared with EAE-WT mice. Likewise, the distribution of individual peak EAE scores within groups of MOG₃₅₋₅₅ WT or TIMP-1^{-/-} mice did not differ markedly (Figure 1B).

We assessed gross body weight as a measure of overall animal health. Weight loss in MOG-immunized mice coincided with the onset and duration of EAE signs (Figure 1C). The average weights of EAE mice differed significantly from CFA-treated mice coincident with the time phase of peak clinical signs ($P < 0.001$, Figure 1C). Significant weight loss among TIMP-1^{-/-} mice lasted from days 20 to 30, whereas this range was longer in WT mice ranging from day 17 through day 35. Mice of both genotypes immunized with MOG₃₅₋₅₅ exhibited a high prevalence of EAE: 80.95% of WT and 86.36% of TIMP-1^{-/-} developed an EAE score of at least one (Figure 1D, hatched bar). In addition, mortality rates (EAE score of 5) among EAE-WT and EAE-TIMP-1^{-/-} mice were similar (9.52 and 9.05%, respectively; Figure 1D, black bar), as were the numbers of TIMP-1^{-/-} or WT mice (1 of 22 and 2 of 21, respectively) that failed to develop EAE (score of ≥ 1) in response to MOG₃₅₋₅₅ immunization (Figure 1D, white bar). Thus, by several clinical criteria, the course of EAE in mice lacking TIMP-1 appeared similar to that observed in WT mice.

TIMP Gene Expression in MOG₃₅₋₅₅-Immunized TIMP-1^{-/-} Mice

In some instances, deletion of genes can result in the compensatory expression of other physiologically related genes that can potentially impact the interpretation of experimental findings.²⁰ To address the possibility that expression of the other matrix metalloproteinase inhibitor genes (TIMPs-2, -3, -4) were differentially expressed in MOG₃₅₋₅₅-immunized TIMP-1^{-/-} mice, we examined the expression of these genes by RNase protection assay in the spinal cords from WT and knockout mice treated with CFA or during the acute and chronic phases of EAE. As shown in Figure 2, basal levels of expression of the four TIMP family genes differed in control mice (CFA-treated) with only the TIMP-1 gene exhibiting a very low level of basal expression. During the acute phase of EAE, however, the level of TIMP-1 mRNA was markedly increased, whereas the levels of the other TIMP mRNAs were unaltered throughout the clinical course of MOG₃₅₋₅₅-induced EAE. Importantly, expression of the TIMP-2, -3, and -4 genes was comparable in both WT and TIMP-1^{-/-} mice,

Figure 1. EAE severity in TIMP-1^{-/-} and WT mice. **A:** Daily evaluation of EAE scores in MOG₃₅₋₅₅-immunized (EAE) or control-treated (CFA) mice revealed comparable progression of EAE in TIMP-1^{-/-} mice ($n = 22$) when compared with WT mice ($n = 22$). Mice treated with CFA ($n = 8$ per genotype) did not exhibit any signs of EAE. **B:** Analysis of peak EAE scores among MOG-immunized TIMP-1^{-/-} or WT mice indicated a similar overall severity of EAE within each group as indicated by the numbers of animals developing EAE at each clinical grade. **C:** Daily analysis of weight revealed weight loss coincident with inflammation among all MOG-immunized mice during the acute phase and recovery of this loss during the chronic phase of EAE. **D:** Comparison of the proportions of mortality, incidence-developed EAE (grade, >1), and animals that did not develop EAE (grade, <1) among all MOG₃₅₋₅₅-immunized mice of either WT or TIMP-1^{-/-} genotype. Data in **A** and **B** are presented as mean + SEM for each treatment group for each time point with statistical comparisons made using two-way analysis of variance (95% confidence), followed by Bonferroni posttests for intergroup determinations of significance; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

indicating that these genes do not undergo compensatory changes in the absence of TIMP-1.

EAE-Induced Demyelination in the Spinal Cord

LFB myelin staining of spinal cord transverse sections from CFA-treated WT or TIMP-1^{-/-} mice revealed no notable differences in myelination (Figure 3, A and B). As expected, immunization with MOG₃₅₋₅₅ peptide induced significant myelin injury in both WT and TIMP-1^{-/-} mice, as evidenced by reduced LFB staining of spinal cord sections (Figure 3, C and D). In addition, two differences between WT and TIMP-1^{-/-} mice were observed. First, increased numbers of infiltrating immune cells were observed during the acute phase of EAE in TIMP-1^{-/-} mice (Figure 3D). Second, during the chronic phase of EAE, myelination of the spinal cord appeared to improve in WT mice (Figure 3E) but much less so in TIMP-1^{-/-} mice (Figure 3F).

To corroborate this observation we performed immunostaining for MBP in adjacent spinal cord sections from WT (Figure 3, G, I, and K) and TIMP-1^{-/-} (Figure 3, H, J, and L) mice and quantified the degree of myelination. Myelin staining of spinal cord sections with antisera against MBP determined that there was less MBP expression in TIMP-1^{-/-} mice compared with CFA-treated WT mice ($P < 0.01$) (Figure 3, G versus H, and M). During the acute phase of EAE the degree of demyelination, as indicated by the loss of MBP staining, was proportionally equivalent in TIMP-1^{-/-} and WT mice with reductions of 80.3 and 80.9%, respectively (Figure 3, I, J, and M). During the chronic phase of EAE, WT mice exhibited an increase in the density and quality of the myelin in their spinal cords, as reflected by an increase in MBP immunostaining ($P < 0.01$ versus acute, Figure 3K) although the degree of myelin loss remained significantly less than unlesioned animals ($P < 0.01$). MBP staining in the spinal cords of the TIMP-1^{-/-} mice during the chronic phase of

Figure 2. Analysis of TIMP family mRNA levels during EAE. RNase protection assay was used to examine the expression of TIMP genes in samples of spinal cord from CFA-treated WT (black bars) or TIMP-1^{-/-} mice and during acute or chronic phases of MOG-induced EAE. The level of individual TIMP mRNAs was corrected for loading relative to the ribosomal gene L32, and the result presented as arbitrary units. Data are presented as mean + SEM of *n* = 3 per genotype per treatment for each gene. Statistical comparisons made using two-way analysis of variance (95% confidence), followed by Bonferroni posttests for significance (***) *P* < 0.001 versus CFA, chronic, and all TIMP-1^{-/-} time points).

EAE did not exhibit a similar improvement in myelination relative to the acute phase of EAE (*P* > 0.05; Figure 3, L and M). Thus, the disorganized appearance of the LFB

and MBP staining of myelin in the TIMP-1^{-/-} mice verified that there was persistent disruption of myelination in TIMP-1^{-/-} mice after EAE. Further, nuclear counterstain-

Figure 3. Evaluation of spinal cord myelination and myelin integrity during EAE. Spinal cord samples were analyzed by histochemical staining for LFB and immunohistochemistry for MBP in spinal cord cross sections of WT (A, C, E, G, I, K) or TIMP-1^{-/-} mice (B, D, F, H, J, L) that had been either CFA-treated or MOG₃₅₋₅₅-immunized (as indicated). **A–F:** Representative LFB staining for myelin in spinal cord sections from CFA or EAE-treated, WT, or TIMP-1^{-/-} mice revealed significant disruption of myelin in the anterior column area of the spinal cord during the acute phase of EAE. **G–L:** Immunohistochemistry for MBP validated the observed changes in spinal cord myelination evaluated by LFB staining and determined that during the chronic phase of EAE myelin in the spinal cords of TIMP-1^{-/-} mice was reduced (K, L) relative to WT mice. Nuclei, stained with 4,6-diamidino-2-phenylindole, also demonstrate in the TIMP-1^{-/-} mice an increase in the cellularity of the spinal cord during EAE (J, L). **M:** Quantification of MBP staining densities (as described in Materials and Methods) in WT and TIMP-1^{-/-} mice and during the acute and chronic phases of EAE. Statistical analyses made using analysis of variance, using evaluation of *n* = 3 per group CFA and acute EAE; *n* = 6 per group chronic EAE, followed by Bonferroni posttests for significance: **P* < 0.05, CFA WT versus CFA TIMP-1^{-/-}; ***P* < 0.01; ****P* < 0.001 versus CFA treatment of same genotype.

Figure 4. Analysis of MOG₃₅₋₅₅-specific T-cell responses during EAE. Scatterplot analysis of cellular infiltrates isolated from the spinal cords of CFA or MOG₃₅₋₅₅-immunized WT and TIMP-1^{-/-} mice. *Ex vivo* stimulation of pooled isolated cells with MOG₃₅₋₅₅ peptide identified antigen-specific CD4⁺ T-cell (x axis) responses by the evoked production of interferon-γ (y axis) as measured by flow cytometry. Data are representative of *n* = 3 to 4 per group.

ing with 4,6-diamidino-2-phenylindole in these histological sections confirmed that this pathology was associated with a hypercellularity, suggesting that differential or increased cellular infiltration during EAE may account for the observed differences in myelin status.

CD4⁺ T-Cell Responses during EAE

We next sought to determine the phenotype(s) of the infiltrating cells in the CNS of TIMP-1^{-/-} mice and to ascertain whether TIMP-1 deficiency may have resulted in an enhanced myelin-specific immune response that contributed to the observed differences in neuropathology. The first cell type we examined was the CD4⁺ T cell, which is responsible for mediating EAE.^{21,22} Since previous work had shown that TIMP-1 can be expressed by CD4⁺ T cells,²³ we considered that the absence of TIMP-1 may have altered the development or magnitude of a myelin-specific CD4⁺ T-cell response in MOG₃₅₋₅₅-immunized mice. To test this, we isolated and pooled the cellular infiltrates from spinal cords of WT and TIMP-1^{-/-} mice, either acute phase (18 days) or chronic phase (52 days), after immunization with MOG₃₅₋₅₅ and used flow cytometry to identify and quantify the antigen-specific T-cell response, using peptide-stimulated IFN-γ production as a marker of activation. As shown in Figure 4, very few CD4⁺ T cells and no antigen-specific (MOG₃₅₋₅₅-specific) T cells were detected in CFA-treated (control) animals. During the acute phase of EAE there was a dramatic increase in the number of CD4⁺ T cells in both WT (19.4% of lymphocytes isolated from spinal cord) and TIMP-1^{-/-} mice (19.7%). MOG₃₅₋₅₅-specific CD4⁺ T cells from spinal cords were similarly increased during the acute phase of EAE, and there was a modest increase in the proportion of these antigen-specific cells detected in the TIMP-1^{-/-} mice (4.1 versus 5.5%, respectively). During the chronic phase of EAE, the magnitude of the MOG₃₅₋₅₅-responsive CD4⁺ T cells was reduced to ~2% in both TIMP-1^{-/-} and WT mice. Thus, although notable changes in the myelin-specific CD4⁺

T-cell response were measured in the spinal cords of MOG₃₅₋₅₅-immunized mice, there were no significant differences in the development, duration, or magnitude of the MOG₃₅₋₅₅-specific CD4⁺ T-cell response in TIMP-1^{-/-} mice versus WT mice.

Demyelination and T-Cell Infiltration in the Cerebellum

MOG₃₅₋₅₅-induced EAE in C57BL/6 mice is a disease of ascending paralysis that is correlated with the infiltration of immune cells into the spinal cord.³ Because the absence of TIMP-1 exacerbated myelin-related pathology in the spinal cord (Figure 3), we next determined whether there were any differences in immune cell infiltration and myelination in the brains of MOG₃₅₋₅₅-immunized WT and TIMP-1^{-/-} mice (Figure 5). LFB staining revealed immune cell infiltration into the brain with occasional perivascular cuffing observed in MOG₃₅₋₅₅-immunized WT mice during the acute phase of EAE (data not shown). Cellular infiltration and myelin damage became less pronounced during the chronic phase of EAE (Figure 5C). During the acute phase of EAE in TIMP-1^{-/-} mice, myelin disruption accompanied by widespread immune cell infiltrates into the CNS parenchyma was observed (data not shown). In contrast to WT mice, however, these changes in neuroinflammatory pathology in the TIMP-1^{-/-} mice also persisted during the chronic phase of EAE and were associated with patches of myelin loss (Figure 5D). No differences between WT or TIMP-1^{-/-} mice were observed after CFA treatment alone (Figure 5, A and B). Immunohistochemistry for immune (CD3⁺) cells revealed few detectable cells within the brains of CFA-treated WT or TIMP-1^{-/-} mice (Figure 5, E and F). In MOG₃₅₋₅₅-immunized WT mice, the majority of CD3⁺ cells were localized to perivascular areas (Figure 5G). Consistent with the observed differences of myelination in TIMP-1^{-/-} mice, CD3⁺ cells were more prevalent in the perivascular infiltrates but also found scattered within the brain parenchyma surrounding the extensive perivascular cuffs (Figure 5H, arrows).

To determine whether these differences in CD3-positive T-cell histological staining reflected disparity in the amount of CD4⁺ T cells within the brains of WT and TIMP-1^{-/-} mice, we also performed a flow cytometry analysis of cellular infiltrates isolated from the brains of CFA-treated and MOG₃₅₋₅₅-immunized mice during the acute and chronic phases of EAE. As shown in Figure 5I, analysis of pooled samples isolated from brain indicated that there was a considerable influx of CD4-positive cells into the brains of MOG-immunized mice during the acute phase of EAE that was greater in the brains TIMP-1^{-/-} mice (24.3 versus 16.0% in WT mice). A small proportion of these lymphocytes were found to respond to *ex vivo* stimulation with MOG₃₅₋₅₅ peptide (as measured by IFN-γ production) in either WT or TIMP-1^{-/-} mice during the acute phase of EAE (Figure 5I). Although this antigen-specific CD4-positive T-cell response in brain was small in magnitude in both groups, the proportion of

Figure 5. Enhanced dissemination of cellular infiltrates into brains of MOG₃₅₋₅₅-immunized TIMP-1 $^{-/-}$ mice. LFB staining of myelin in the cerebellum of WT and TIMP-1 $^{-/-}$ mice that were either treated with CFA or EAE. Although WT mice exhibited little myelin disruption within the brain during EAE (C), disorder of myelin in the brain of TIMP-1 $^{-/-}$ mice was apparent and remained throughout the 52-day experimental endpoint (ie, chronic phase) (D). A and B: No apparent differences were noted in the myelination of adult brains from CFA-treated WT or TIMP-1 $^{-/-}$ mice. Immunohistochemical detection of infiltrating T cells (α CD3 $^{+}$) in CFA-treated mice (E, F) revealed sparse cellular staining, whereas in WT mice, perivascular CD3 $^{+}$ cells were occasionally observed (G). In contrast, there were many CD3 $^{+}$ cells within the CNS parenchyma of TIMP-1 $^{-/-}$ mice 52 days after induction of EAE (H). I: Contour plots of flow cytometric analysis of antigen (MOG₃₅₋₅₅)-specific immune responses of cells isolated from the brains of mice. Data represent $n = 3$ per group per time point and demonstrate increased proportions of CD4 $^{+}$ T cells (x axis) during the acute phase of EAE in TIMP-1 $^{-/-}$ mice. Red text denotes percentage of lymphocytes isolated that were CD4 $^{+}$ cells. *Ex vivo* stimulation with MOG₃₅₋₅₅ peptide reveal a small proportion of antigen-specific T cells via production of IFN- γ (y axis) that was increased in TIMP-1 $^{-/-}$ mice.

MOG₃₅₋₅₅-specific T cells was severalfold higher in TIMP-1 $^{-/-}$ mice compared with WT mice.

Microgliosis and Macrophage Infiltration during EAE

Immune-mediated myelin injury requires the involvement of microglia and macrophages as well as autoreactive T cells.²⁴ In this context, macrophages, as well as resident microglia, have been shown to be important mediators of myelin injury through direct cellular injury (phagocytosis)

and through promoting autoreactive T-cell responses by functioning as antigen presenting cells.²⁸⁻³⁰ To address the possibility that enhanced microgliosis/macrophage activity contributed to the EAE-related pathology in TIMP-1 $^{-/-}$ mice, we examined whether the spinal cords from MOG₃₅₋₅₅-immunized WT and TIMP-1 $^{-/-}$ mice exhibited any differences in the infiltration of peripheral macrophages and/or activation of microglia.

Immunohistochemistry for ionized calcium-binding adaptor molecule-1 (Iba-1), a marker for macrophages and microglia, revealed sparsely labeled cells within the

Figure 6. Enhanced and persistent macrophage/microgliosis in spinal cords of TIMP-1^{-/-} mice after EAE. **A:** Immunohistochemical detection of the macrophage/microglial marker Iba-1 in the spinal cords of WT C57BL/6 mice or TIMP-1^{-/-} mice treated with CFA or during the acute or chronic phases of EAE after MOG₃₅₋₅₅ immunization, as indicated. During the acute phase of EAE, the boundary of infiltrating cell types is demarcated by the dotted black line relative to region of predominant macrophage/microglial reactivity. **B:** Flow cytfluorometric analysis of mononuclear leukocytes isolated from spinal cords of WT and TIMP-1^{-/-} mice reveal the dramatic expansion/infiltration of CD11b⁺/MHCII⁺ macrophage/microglial cells into the spinal cord during the acute and chronic phases of EAE. Note the enhanced proportion of CD11b⁺ cells remaining (top left quadrant, chronic phase) in the TIMP-1^{-/-} mice relative to EAE-WT mice.

white and gray matter of spinal cords from CFA-treated WT or TIMP-1^{-/-} mice (Figure 6A). In dramatic contrast, numerous Iba-1⁺ cells were present in the white matter of the spinal cord during the acute phase of EAE (Figure 6A). In TIMP-1^{-/-} mice, the density of the Iba-1⁺ cells

was greater than in WT mice, reflected in a deeper boundary of the cellular infiltration into the spinal cords of these mice (Figure 6A, acute, dashed line). During the chronic phase of EAE, the pattern of macrophage/microglial staining in WT mice was greatly reduced, with an

occasional cluster of Iba1⁺ cells observed (black arrow). In contrast, many more intensely stained clusters of cells (white arrows) along with evidence of myelitis (dashed line) were found to persist in the spinal cords of TIMP-1^{-/-} mice at this same time point.

We had demonstrated a phenotypic change in macrophages/microglia in TIMP-1^{-/-} mice during EAE, so we next used flow cytometry to determine the proportion of MHC class II-positive/CD11b-positive cells among the mononuclear cells isolated from spinal cords of CFA-treated and MOG₃₅₋₅₅-immunized mice (Figure 6B). A dramatic and equivalent increase in the proportion of activated macrophages/microglia (MHCII⁺/CD11b⁺) was identified during the acute phase of EAE in both WT and TIMP-1^{-/-} mice. This incursion subsided during the chronic phase of EAE but did not completely abate, because a significant population of macrophage/microglia cells remained in the spinal cords during this phase of EAE when compared with CFA-treated mice (Figure 5B). During the chronic phase of EAE in TIMP-1^{-/-} mice, a larger residual population of macrophage/microglia cells was also observed (Figure 6B).

Because changes in macrophages and microglia were identified within the spinal cords of EAE-treated mice, we next examined whether similar changes in macrophage/microglial activation occurred in the brain. Immunohistochemistry for the macrophage/microglial marker Iba-1 revealed labeling of resting microglia in CFA-treated WT and TIMP-1^{-/-} mice (Figure 7, A and B). During the chronic phase of EAE in WT mice, strongly labeled microglia and other Iba1-positive perivascular macrophages were also observed in these mice (Figure 7C). Iba-1-positive immunostaining of brain tissues from TIMP-1^{-/-} mice displayed a more robust and widespread microgliosis with abundant infiltrating macrophage cells (Figure 7D). We also examined whether similar differences in astrogliosis existed. Immunohistochemistry for glial acidic fibrillary protein (GFAP), a phenotype marker for astrocytes, revealed a notable increase in astrocyte labeling in the cerebella of WT mice (Figure 7, G and K), whereas GFAP⁺ immunostaining in cerebella of MOG₃₅₋₅₅-immunized TIMP-1^{-/-} mice was not as enhanced but instead resembled GFAP staining in CFA-treated mice (Figure 7, E, F, H, I, J, and L).

Mononuclear leukocytes were isolated from the brains of WT and TIMP-1^{-/-} mice and were pooled (on account of their small numbers, $n = 3$ to 4 per group per time point); flow cytometric analyses revealed that, during the acute phase of EAE, there was a significant increase in the number of the macrophage/microglia cells in both WT and TIMP-1^{-/-} mice (Figure 7M). Similar to the pathology and changes observed in the spinal cords of TIMP-1^{-/-} mice, during the chronic phase of EAE, a larger residual population of MHCII/CD11b⁺ cells was identified in the TIMP-1^{-/-} mice (67.7 versus 47.1% in WT mice). These flow cytometric data are consistent with the increased Iba1⁺ immunostaining observed in the TIMP-1^{-/-} mice and indicate that these mice exhibited a lasting microglial activation and infiltration of macrophage-like cells during the chronic phase of EAE.

Discussion

Previous studies have identified important roles for MMPs in the pathogenesis of CNS demyelination. However, less is known about the physiological role(s) the endogenous MMP inhibitors, TIMPs, play during CNS inflammation. Among the four known TIMP genes, TIMP-1 has been reported to be induced in many models of EAE, including immunization with MBP,¹² spinal cord homogenate,³¹ MOG₃₅₋₅₅ (this study), or after adoptive transfer of MBP-reactive T cells.⁷ TIMP-1 is consistently among genes with the highest up-regulation during inflammatory demyelination during EAE.^{13,7,32} To our knowledge, this study is the first to directly examine the *in vivo* function of TIMP-1 during inflammatory demyelination. Herein, we have shown that EAE in TIMP-1-deficient mice resulted in enhanced dissemination of immune cells into the CNS parenchyma, which was associated with persistent microgliosis/macrophage activity and prolonged myelin injury.

The primary pathological change in the TIMP-1^{-/-} mice was not altered in lymphocyte responses during EAE but rather a persistent disruption of myelination. Measurement of MBP staining in the spinal cords of TIMP-1^{-/-} mice indicated that there is a slight reduction in amount of myelination in these mice. This phenotype could be attributed to either a worsened injury attributable to increased mononuclear cell activity within the CNS of TIMP-1^{-/-} mice or to an inherent difference in the process of remyelination in these mice. Because the proportion of demyelination during EAE (relative to basal conditions) was equivalent between WT and TIMP-1^{-/-} mice, we hypothesize that the impairment of myelin recovery during the chronic phase of EAE may indicate an inherent defect in myelin production or myelin organization in the TIMP-1^{-/-} mice. TIMP-1^{-/-} mice appear phenotypically normal and have no known defect in myelination. However, the physiology and kinetics of myelination in these mice has not been carefully explored. These studies are currently underway in our laboratory.

One surprising aspect of our study was that the clinical signs of EAE in TIMP-1^{-/-} mice did not differ from those observed in WT mice in terms of the onset, severity, or duration (Figure 1). This appears incongruous with the disrupted myelination observed during the chronic phase of EAE in the TIMP-1^{-/-} mice. We have two plausible explanations for this apparent contradiction. First, the classic clinical criteria for evaluating EAE accurately reflect spinal inflammation but may not provide a true assessment of the overall status of CNS myelination. Indeed, previous work has also shown that dysmyelination in mice with null mutations for PLP-DM20 exhibit a subtle behavioral phenotype that worsened with age.³³ Moreover, immunization of rats with anti-MOG antibodies can evoke demyelination without gross neurological deficit,³⁴ and transfer of MOG-specific T cells can initiate neuroinflammation but not cause any clinical disease.³⁵ Hence, more sensitive measures of behavioral function may be required to resolve any differences in motor performance in these circumstances.³⁶ A second possible explanation for the dichotomy between the clinical and histological evaluations is that the axons of TIMP-1^{-/-} mice may be

Figure 7. Enhanced macrophage/microglial reactivity in brains of TIMP-1^{-/-} mice. Immunohistochemistry for the macrophage/microglial marker Iba-1 revealed scattered resting microglia in the cerebella of CFA-treated WT (A) or TIMP-1^{-/-} mice (B). During the chronic phase of EAE (52 days after MOG₃₅₋₅₅ immunization), clusters of Iba-1⁺ cells were noted in WT animals (C), whereas widespread intense Iba-1⁺ staining was observed in TIMP-1^{-/-} mice (D). Astrocyte labeling with GFAP revealed a band of strong GFAP⁺ cells surrounding the perimeters of the cerebellar commissure (dotted black line). During the chronic phase of EAE, widespread GFAP⁺ staining was observed in the cerebellar commissure of WT mice (G) but not TIMP-1^{-/-} mice (H). I-L: High-magnification images of GFAP staining in regions marked by red boxes in plates E-H. M: Cell sorting (FACS) analysis of CD11b⁺/MHCII⁺ macrophage/microglia isolated from the brains of CFA-treated or MOG₃₅₋₅₅-immunized mice during the acute or chronic phases of EAE, as indicated. Note the increased proportion of CD11b⁺ cells (top left quadrant, chronic phase) in TIMP-1^{-/-} during chronic EAE compared with WT mice at this same phase of disease. Analysis was performed on pooled samples, *n* = 3 to 4 per group per time point.

more resistant to injury during chronic myelin disruption. This possibility would help to explain the reduced astrogliosis in TIMP-1^{-/-} mice after EAE and would also be supported by recent work by Rivera and colleagues³⁷ demonstrating that TIMP-1^{-/-} mice exhibit a significant

reduction in kainic acid-induced neuronal injury. Together with our data, this would suggest that although EAE evoked a persistent oligodendrocyte injury, the typical neuronal impact resulting from this injury, and its behavioral sequelae, were prevented by the absence of

TIMP-1. The reduced astrogliosis observed in TIMP-1^{-/-} mice during the chronic phase of EAE may reflect this difference. Further study will be necessary to elucidate the mechanism(s) through which TIMP-1 affects neural plasticity in response to inflammatory stress and demyelination-associated injury.

In this study of immune-mediated damage of the CNS in an EAE model of demyelination, TIMP-1^{-/-} mice were distinguished from the WT mice by the protracted injury to oligodendrocytes. The persistent myelin disruption in TIMP-1^{-/-} mice during the chronic phase of EAE suggested that differences in the immune response may underlie the observed differences in myelin pathology. Because these CD4⁺ T cells are important mediators of pathology in MOG₃₅₋₅₅-induced EAE in C57B/6 mice, we initially tested the possibility that there was a differential accentuation of the myelin-specific T-cell response in TIMP-1^{-/-} mice. We did observe an increase in CD3⁺ T cells in the brains of TIMP-1^{-/-} mice and a somewhat increased CD4⁺ T-cell response was measured in the brains, but not the spinal cords, of knockout mice during the acute phase of EAE. However, during the chronic phase of EAE, the CD4⁺ T-cell response resolved in both WT and TIMP-1^{-/-} mice. Hence, we also assessed the possibility that differential activation of macrophages/microglia in TIMP-1^{-/-} mice may be associated with the persistent myelin disruption during the chronic phase of EAE in these mice. Indeed, we determined that there were measurable increases in the presence and activation of macrophages/microglia in both the spinal cords and brains of TIMP-1^{-/-} mice. Thus, further study will be required to resolve whether differences in either acute phase T-cell responses and/or the protracted activation of macrophages/microglia are important contributors to the persistent disruption of CNS myelination during MOG₃₅₋₅₅-induced EAE in TIMP-1^{-/-} mice.

The role of microglia/macrophages in neuropathology and their contribution to immune-mediated myelin injury or repair is the subject of considerable debate. Microglia are endogenous brain macrophages and have been associated with the promotion of T-cell responses and directly contributing to myelin injury. Indeed, neutralization of microglial activation/macrophage recruitment has been reported to suppress EAE in rodents.^{27,38} From these reports it could be deduced that macrophages/microglia are deleterious during neuroinflammation. However, in a recent study by Butovsky and colleagues³⁹ the potential beneficial effects of macrophages/microglia on oligodendrocyte progenitor cell differentiation, and thus myelin repair, were found to be influenced by exposure to IFN- γ ; high concentrations of IFN- γ promoted tumor necrosis factor- α production from macrophages/microglia and was detrimental to oligodendrocyte progenitor cell differentiation. In their study, administration of IL-4 suppressed the negative effects of IFN- γ , and IL-4-stimulated microglia suppressed MOG₃₅₋₅₅-induced EAE in C57BL/6 mice.³⁹ The findings presented in our study support the former notion that, in the absence of TIMP-1, enhanced or sustained microglial activation and increased macrophage recruitment correlated with the attenuated myelin recovery in the TIMP-1^{-/-} mice. Be-

cause microglia and/or macrophages release factors that directly contribute to oligodendrocyte injury,⁴⁰ we propose that TIMP-1 may be an endogenous regulator of macrophage/microglial activation either through inhibition of a metalloproteinase, or perhaps even through a direct MMP-independent process.⁴¹ Thus, an important physiological action of TIMP-1 may be to delimit macrophage/microglial activities and therein permit effective remyelination after injury, and, consequently, TIMP-1^{-/-} mice exhibit more persistent oligodendrocyte disruption.

These findings are consistent with the prevailing hypothesis that endogenous expression of TIMP-1 during CNS inflammation is a primary factor responsible for the regulation of MMP-dependent activities of immune cell migration into the CNS parenchyma and myelin injury.^{2,42,43} However, our data also indicate that TIMP-1 likely serves dual functions during immune-mediated myelin injury: the control of cellular extravasation and spread of these immune cell infiltrates into the CNS parenchyma but also the regulation of glial responses to inflammatory myelin injury.

Several studies throughout the past years have examined the levels of MMPs and TIMP-1 in MS, as measured by protein levels in cerebrospinal fluid or serum of human cases, or analysis based on postmortem pathology. In MS, TIMP-1 expression is normal or reduced compared with healthy control patients.⁴⁴⁻⁴⁶ In contrast, and in parallel to our findings in TIMP-1^{-/-} mice, increased expression of MMPs by macrophages has been reported in the active lesions from human cases of MS.⁴⁷ Many studies therefore have relied on the ratio of TIMP-1 levels relative to MMP expression as an indicator of net proteolytic activity.^{45,48,49} Although this approach may reflect the overall function of MMPs, it reveals little of the contribution of TIMP-1 to this disease. Given the general lack of increased TIMP-1 expression in human cases of MS, the dramatic increase in TIMP-1 during EAE models of demyelination represent a primary difference between MS in humans and autoimmune-induced myelin damage in WT animals. Our findings on the EAE neuropathology in the TIMP-1^{-/-} mice suggest that the paucity of TIMP-1 in MS may contribute to the pathology of this disease. Future studies addressing the role and regulation of TIMP-1 in myelin physiology and in MS may provide relevant insights and potential therapeutic avenues to address the chronic pathology of MS.

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