Growth Factors, Cytokines, Cell Cycle Molecules

Matrix Metalloproteinase-12 Deficiency Worsens Relapsing-Remitting Experimental Autoimmune Encephalomyelitis in Association with Cytokine and Chemokine Dysregulation

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The elevation of several members of the matrix metalloproteinase (MMP) family promotes the pathophysiology of both multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). Nonetheless, given the multiple activities of MMPs, it remains possible that increased levels of a particular MMP may have beneficial functions during disease progression. We reported previously that *MMP-12*^{-/-} mice of the 129/SvEv strain had a poorer EAE outcome than wild-type controls. However, we did not determine further differences in disease profiles between these groups. Using the EAE model in 129/SvEv mice, we report that disease in both wild-type and *MMP-12*^{-/-} mice follows a relapsing-remitting course. Although both mouse groups had similar clinical onsets, subsequent relapses were more severe in *MMP-12*^{-/-} mice; their residual disability at remission was also higher compared with wild-type controls. The worsened relapses and remissions in *MMP-12*^{-/-} mice occurred despite a deficiency of the antigen recall capacity of lymph node-derived cells as well as a reduction in the proportion of macrophages in the spinal cord during the chronic phase of EAE. Significantly, large increases of levels of chemokines and cytokines were found in the spinal cords of *MMP-12*^{-/-} mice during chronic EAE. These results highlight MMP-12 as a beneficial enzyme in EAE and suggest that therapeutic interventions in multiple sclerosis should avoid targeting MMP-12. (*Am J Pathol* 2009, 174:898–909; DOI: 10.2353/ajpath.2009.080952)

system (CNS). There have been significant efforts in elucidating the mechanisms that regulate the entry of leukocytes into the CNS in MS, including in the expression of members of the matrix metalloproteinase (MMP) family. The production of MMPs by leukocytes helps degrade the basement membrane surrounding cerebral vessels,^{1,2} and, within the CNS, the expression of MMPs may produce demyelination and axonal injury, and promote neuroinflammation.^{3,4} Several members of the 25-MMP family are elevated in MS and experimental autoimmune encephalomyelitis (EAE)^{5,6} and, in support of their negative roles, pharmacological inhibitors of metalloproteinase activity reduce severity of EAE.⁷ Compared with wild-type animals, EAE is attenuated in mice deficient for MMP-8⁸ and -9⁹ and in MMP-2 and -9 double-null mice.¹

Although the majority of MMP members are thought to promote the EAE and MS disease process, it is possible that particular MMPs may have contrasting beneficial roles to help attenuate disease, given the wide range of substrates of MMPs.^{10,11} In particular, we have found that *MMP-12*^{-/-} mice on the 129/SvEv strain had worse EAE clinical disease compared with wild-type controls when daily disease scores were summed (sum of scores) throughout 73 days of evaluation.⁶ However, we were unable to differentiate the long-term disease course in the two groups given that the day of onset of initial clinical signs was highly variable among animals in that study.

The potential role of MMP-12 in alleviating disease requires further attention because MMP-12 is highly expressed in MS lesions.¹² Moreover, the use of nonselective MMP inhibitors to reduce inflammation may prove

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Multiple sclerosis (MS) is an inflammatory, demyelinating, and neurodegenerative disease of the central nervous

counterproductive if MMP-12 inhibition is not spared pharmacologically. Indeed, an inhibitor that targets MMP-12 has been contemplated for clinical trials in MS.¹³ Thus, with increasing experience in producing more consistent EAE disease onset in 129/SvEv mice, we have examined EAE disease activity in *MMP-12^{-/-}* mice and sought to understand its roles in secondary lymphoid organs and within the spinal cord. We report that EAE in 129/SvEv wild-type and *MMP-12^{-/-}* mice follows a relapsing-remitting course. Although *MMP-12^{-/-}* and wild-type mice have similar onset of disease, subsequent relapses were more severe in *MMP-12^{-/-}* mice and their residual disability at remission was also higher compared with wild-type controls. The worsened relapses and remission in *MMP-12^{-/-}* mice was accompanied by significant alterations within the spinal cord of CD4CD25⁺ regulatory T cells and macrophages in conjunction with an increase in the expression of pro- and anti-inflammatory cytokines during chronic EAE. These results provide new insights into the regulation of inflammation during relapsing-remitting EAE and MS and point to a role for MMP-12 in resolving disease.

Materials and Methods

EAE Induction in Female 129/SvEv Mice

MMP-12^{-/-} mice on the 129/SvEv strain were a kind gift from Dr. Steve Shapiro (Harvard Medical School, Boston, Massachusetts).^{6,14} Wild-type 129/SvEv mice were purchased from Taconics (Germantown, NY). These two lines were expanded in-house and used in the nonlittermate experiments. The lines were then crossed to produce heterozygote (*MMP-12^{+/-}*) breeders and the offspring ($\geq F_2$ generation) were used as littermate mice. Genotyping (Supplemental Figure S1, available at <http://ajp.amjpathol.org>) confirmed the identity of the *MMP-12^{+/+}*, *MMP-12^{+/-}*, and *MMP-12^{-/-}* mice.

EAE was induced in female mice when they were 8 to 9 weeks of age by injecting subcutaneously two injections (day 0 and day 7) of 100 μ g myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ in complete Freund's adjuvant (Fisher, Pittsburgh, PA) supplemented with 4 mg/ml of *Mycobacterium tuberculosis* (H37Ra) (Difco Laboratory, Detroit, MI). No pertussis toxin was used because the 129/SvEv strain did not require this reagent for development of EAE.

Animals were assessed daily using a 15-point disease score scale^{6,15,16} that replaced the more commonly used 5-point scale because the 15-point scale differentiates individual limb disability, rather than lumping both fore- or hindlimbs together, thus allowing for better characterization of disease progression. The 15-point scale ranges from 0 to 15 and is the sum of the disease state for the tail (0 to 2) and all four limbs (scored 0 to 3). Based on this scoring system a fully quadriplegic mouse would attain a score of 14 and mortality would be given a score of 15. The observer was blinded to genotype in all EAE experiments during behavioral and histological (see below) assessments. All animals were handled in accordance

with the policies outlined by the Canadian Council for Animal Care and the University of Calgary.

Genotyping of *MMP-12^{+/+}*, *MMP-12^{+/-}*, and *MMP-12^{-/-}* Mice

All mice were genotyped to determine their genetic background. Pups from heterozygous breeders were genotyped by isolating DNA from ear samples. A total of 5 ng of DNA in 2 μ l was added to 12 μ l of polymerase chain reaction (PCR) master mix (10 $\times$ PCR buffer, 10 mmol/L dNTP, 50 mmol/L MgCl₂, 20 μ mol/L oIMR0297 5'-CAC-GAGACTAGTGAGACGTG-3', 20 μ mol/L oIMR3207 5'-GCTAGAAGCAACTGGGCAAC-3', 20 μ mol/L oIMR3208 5'-5'-ACATCCTCACGCTTCATGTC-3', ddH₂O, and 1.25 U Taq polymerase). The PCR was run using the following conditions: 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 65°C for 1 minute, and 72°C for 1 minute, followed by 72°C for 2 minutes. The PCR products were resolved on a 1% agarose gel with the following molecular weights: *MMP-12^{+/+}*, 1064 bp fragments; *MMP-12^{+/-}*, 1064 and 1500 bp fragments; and *MMP-12^{-/-}*, 1500-bp fragments (Supplemental Figure S1, available at <http://ajp.amjpathol.org>).

Histology of Tissue from Control and EAE Mice

Animals were sacrificed via an overdose of ketamine/xylazine (Bimeda-MTC Animal Health Inc., Cambridge, Canada) and bled by aortic puncture for 5 minutes while the heart was pumping. Whole spinal cords were removed and fixed by submersion in 10% buffered formalin (Surgipath Canada Inc., Winnipeg, Canada) for 24 hours. Spinal cords were divided into thoracic and lumbar sacral portion and embedded in paraffin blocks. Thoracic cords were cut longitudinally on a microtome and four adjacent 6- μ m-thick sections were placed on glass slides (Fisher) for a total of four series of 48 sections from each animal. One series was then processed for hematoxylin, eosin, and Luxol fast blue (LFB). Our experience with these tissue-processing methods allowed us to adequately differentiate between cells trapped in blood vessels and those that are in the parenchyma, and perfusion before tissue collections did not affect our ability to differentiate between intravascular and parenchymal leukocytes (data not shown).

For histology, slides were warmed at 60°C for 1 hour, deparaffinized through a series of ethanol steps, and then placed in LFB (solvent 38; Sigma, St. Louis, MO) at 60°C for 3 hours. Slides were then placed in 95% ethanol, followed by water, and then 0.05% lithium carbonate before being placed in 70% ethanol. Once the appropriate myelin color differentiation was achieved slides were placed in hematoxylin (EMD, San Diego, CA) for 4 minutes. Slides were then sequentially dipped in 1% acid alcohol and ammonia water, with water washes in between, and then rinsed in water again, followed by 70% ethanol and 95% ethanol before being dipped in alcoholic eosin (EMD). Slides were then passed through a dehydrating step before being coverslipped using acry-

tol (Surgipath Canada Inc.). Images were captured using an Olympus BH-2 microscope (Olympus, Port Moody, Canada) and QCapture Pro software (version 5.1.1.14; Media Cybernetics Inc., Bethesda, MD).

For semiquantitative assessment of the extent of inflammation in the spinal cord, all stained longitudinal sections from the same coded mouse were first evaluated using the scoring system outlined in the Results (below). In this regard, the location (pial versus parenchymal) and number of inflammatory aggregates per section were documented, with parenchymal inflammation and larger and higher number of aggregates being ascribed greater inflammation scores. Finally, the number of inflammatory lesions/aggregates was counted per longitudinal section. Six different sections per animal were examined by a blinded observer and the average number of lesions per section was documented.

T-Cell Proliferation Assay Using MMP-12^{+/+}, MMP-12^{+/-}, and MMP-12^{-/-} Lymph Nodes (LNs)

Mice were sacrificed on day 10 after MOG immunization, just before clinical disease signs were expected, and LNs were removed. LNs were homogenized into a single cell suspension and washed in phosphate-buffered saline (PBS) two times at 1200 rpm. LN cells were plated in round-bottom plates at a density of 250,000 cells/100 μ l in RPMI containing 1% mouse serum (Invitrogen, Carlsbad, CA). The LN populations served to provide T cells for subsequent antigen-recall proliferation.

Cells for antigen-presenting cell (APC) function were harvested from spleens taken from nonimmunized animals. Spleens were homogenized and then layered onto Ficoll (GE Healthcare Bioscience, Uppsala, Sweden). The buffy layer containing cells was removed and washed two times in PBS before being suspended in RPMI containing 1% mouse serum. A tube containing the APC cells was then γ -irradiated at room temperature with a Gamma Cell 1000 (Nordion International Inc., Vancouver, Canada) using Cs-137 for 14 minutes at 300 rad, which was then followed by incubation of the APC cells with MOG₃₅₋₅₅ for 30 minutes at 4°C. The APCs (250,000 APCs/100 μ l) were then added to the LN cells described above for a total of 500,000 cells/well and incubated for 3 days at 37°C in 5% CO₂. During the last 18 hours of incubation cells were pulsed with 1 μ Ci of [³H] thymidine (Perkin Elmer, Waltham, MA) to determine the proliferative state of the cultures. After incubation, cells were harvested using a PHD cell harvester (Brandel Inc., Gaithersburg, MD) and [³H] thymidine incorporation was determined by using a Beckman LS3801 scintillation counter (Beckman Coulter, Mississauga, Canada). In one set of experiments T cells and APCs were plated together based on matching their genotype whereas in other experiments we plated T cells with nonmatching APCs.

Flow Cytometry of MMP-12^{+/+}, MMP-12^{+/-}, and MMP-12^{-/-} LN and CNS Samples Taken during Different Phases of EAE

Mice induced for EAE were sacrificed via an overdose of ketamine/xylazine and perfused with PBS. Draining LNs and lumbar sacral spinal cords were harvested at specific disease time points: Normal (no disease induction), presymptomatic (day 10 after MOG injection, just before signs of EAE symptoms), remission (2 days after the first signs of a decrease in peak EAE score), and chronic disease (day 35 after MOG injection) and placed in Hanks' balanced salt solution (Invitrogen). The tissue was pushed through a sieve with nylon mesh (pore size, 70 μ mol/L; BD Bioscience, Mississauga, Canada) and single cell suspensions were collected in RPMI containing 10% fetal bovine serum (BD Bioscience). The cells were washed twice with RPMI for 10 minutes at 1200 rpm. Cells were counted and plated at 0.5 to 1 $\times$ 10⁶/well before being stained for analysis. Cells were then washed in fluorescence-activated cell sorting (FACS) buffer [PBS, 2% fetal calf serum (v/v), 1% bovine serum albumin (w/v), 0.1% NaN₃ (v/v); BD Bioscience] and then blocked with mouse BD Fc block (BD Bioscience) for 20 minutes at 4°C. Cells were washed twice with FACS buffer and incubated with primary antibodies or isotype controls for 30 minutes at 4°C. Antibodies were CD3 PE-Cy5 (and isotype control: Armenian hamster IgG1 κ ; dilution range per 10⁶ cells/well, 0.2 μ g/ μ l), CD4 R-PE (rat IgG2a κ , 0.2 μ g/ μ l), CD8a FITC (rat IgG2a κ , 0.5 μ g/ μ l), CD45 PerCP (rat IgG2b κ , 0.2 μ g/ μ l), CD11b FITC (rat IgG2b κ , 0.5 μ g/ μ l), CD4 PerCP (rat IgG2a κ , 0.2 μ g/ μ l), and CD25 R-PE (rat IgG1 λ , 0.2 μ g/ μ l). These antibodies were from BD Bioscience. Cells were then washed twice with FACS buffer before being fixed with 1% formalin for 30 minutes; finally cells were suspended in 500 μ l of FACS buffer. For FoxP3 (FoxP3 FITC, rat IgG2a κ , 0.5 μ g/ μ l) staining cells were suspended in 1 $\times$ cytofix/cytoperm (BD Bioscience) for 20 minutes at 4°C, washed twice with permeabilization/wash buffer (BD Bioscience), and incubated with FoxP3 for 30 minutes at 4°C. Cells were then washed twice more and suspended in a final volume of 500 μ l of FACS buffer. All cells were captured and analyzed using a Becton Dickinson LSR 3 laser flow cytometer (BD Bioscience) and data analysis was done using Cell Quest Pro V4.0.2 software (BD Bioscience). Two separate experiments were analyzed and the results of the total percentage of gated events (n = 4 to 13 animals per genotype per time point) are then pooled. The percentage of gated events was evaluated using flow cytometry plots (Supplemental Figure S2, available at <http://ajp.amjpathol.org>) in which the region of interest was determined using side (x axis) and forward (y axis) scatter plots. Cells were then sorted based on their expression of specific markers and the dot plots show the region of interest used to determine the final gated events, which is shown as a percentage of gated events on the y axis of the summary line graph.

Examination of Inflammation in the CNS of $MMP-12^{+/+}$ and $MMP-12^{-/-}$ Mice during Different Phases of EAE Using Antibody Arrays

EAE was induced in $MMP-12^{+/+}$ and $MMP-12^{-/-}$ mice as outlined above and thoracic spinal cords from each genotype were removed during the following phases: normal (no disease induction), presymptomatic (day 10 after MOG injection, no signs of EAE symptoms), remission (2 days after the first signs of a decrease in peak EAE score), and chronic disease (day 35 after MOG injection) ($n = 3$ mice/genotype in each group). Spinal cords were homogenized in lysis buffer and supernatant used after centrifugation at 1300 rpm. The protein expression of 40 immune-related molecules was assessed using the mouse RayBio inflammatory antibody array from RayBiotech Inc. (Norcross, GA) (Supplemental Figure S3, available at <http://ajp.amjpathol.org>). All samples were processed together as follows; glass chip slides coated with the antibody arrays were first treated with blocking buffer before being incubated for 1 hour with various protein samples (100 μ g protein per slide) from the different genotypes during EAE. Slides were then washed and incubated sequentially with a biotin-conjugated secondary antibody solution, horseradish peroxidase-conjugated streptavidin, and horseradish peroxidase detection buffer. The signals were detected using chemiluminescence and densitometric analysis performed to determine the difference between the various samples. Slides were processed using a Gene Pix array 4000B scanner (Molecular Dynamics Inc., Sunnyvale, CA) and Gene Pix pro software (Molecular Dynamics Inc.). Positive control signals (a known amount of biotinylated antibody) were used to normalize the level of expression between samples being examined. For all arrays, each protein intensity value is divided by the average intensity value of the positive control on the same array; this value is then multiplied by the average intensity value of the positive control on the reference array giving the normalized level of expression for each protein examined. Normalization is essential for the identification and removal of the effects of systematic variation in the measured intensities, which are not attributable to differential expression.

Statistical Analysis

Statistical analysis was performed using Prism 5 (version 5.0) (GraphPad Software Inc., San Diego, CA). Statistical differences between groups with respect to disease onset, mean peak score, mean average disease score, mean cumulative disease, and histopathology were evaluated using a nonparametric analysis Mann-Whitney U -test. Statistical differences between groups with respect to daily clinical disease were evaluated using repeated measures two-way analysis of variance and Bonferroni post-hoc analyses. Statistical differences between groups with respect to T-cell cultures and antibody arrays were evaluated using one-way analysis of variance and

Figure 1. $MMP-12^{+/+}$ and $MMP-12^{-/-}$ nonlittermate animals have different EAE clinical profiles throughout 58 days of observation. **A:** Daily average EAE score of $MMP-12^{+/+}$ and $MMP-12^{-/-}$ animals throughout 58 days of observation. Values are mean $\pm$ SEM. Repeated measures two-way analysis of variance showed a significant difference ($P < 0.05$) between the two groups whereas posthoc Bonferroni tests indicated where the significance lies ($*P < 0.05$). **B:** Average cumulative clinical disease (the group average of the sum of daily disease scores from each animal throughout the observation period), showing that there is a significant difference between $MMP-12^{+/+}$ and $MMP-12^{-/-}$ animals in their overall disease burden. Values are mean $\pm$ SEM (nonparametric Mann-Whitney test, $*P < 0.05$). **C:** $MMP-12^{-/-}$ animals spent a significant percentage of their time with severe disability (disease score of 4 or greater) compared with $MMP-12^{+/+}$. Values are mean $\pm$ SEM. Nonparametric analyses of variance were significantly different ($P < 0.05$) and post-hoc analyses (Dunn's multiple comparison test) were significant when $*P < 0.05$ or $***P < 0.001$. In all panels, the number of mice in the $MMP-12^{+/+}$ group was 25, whereas that in $MMP-12^{-/-}$ was 27.

Tukey's multiple comparison test or two-way analysis of variance and Bonferroni post-hoc analyses.

Results

Relapsing-Remitting EAE Is Worsened in Mice without MMP-12

Nonlittermate $MMP-12^{+/+}$ and $MMP-12^{-/-}$ mice were induced for EAE using MOG₃₅₋₅₅ and animals were examined daily for their EAE progression for 58 days. There was no significant difference in onset of clinical signs between $MMP-12^{+/+}$ and $MMP-12^{-/-}$ mice, and both genotypes followed a relapsing-remitting course of disease (Figure 1A). Significantly, $MMP-12^{-/-}$ mice displayed a higher severity during a relapse, and they failed to remit to the low baseline disability of $MMP-12^{+/+}$ mice (Figure 1A). At the termination of the experiment on day 58, the residual deficit in $MMP-12^{-/-}$ mice was clearly higher than that of $MMP-12^{+/+}$ mice.

To further differentiate the severity of disease burden between individual $MMP-12^{+/+}$ and $MMP-12^{-/-}$ mice, the daily clinical disease for each mouse was summed throughout the 58 days of observation to provide the cumulative disease score per mouse. Figure 1B shows that $MMP-12^{-/-}$ mice had a significantly higher cumula-

tive score per animal (163.1 ± 16.3 versus 70.8 ± 7.9) in comparison with *MMP-12*^{+/+} mice, emphasizing the higher overall disease burden in the former. Moreover, when the percentage of time in which mice spent with mild (percent time at score 0 to 3) and severe disability (percent time at score ≥ 4) was documented, we found that *MMP-12*^{-/-} mice spent significantly less time with mild disability in comparison with *MMP-12*^{+/+} (Figure 1C). In this regard, the *MMP-12*^{-/-} mice spent a significant amount of time with tail involvement, hindlimb paralysis, and forelimb paresis whereas the *MMP-12*^{+/+} mice spent greater periods of time suffering only from tail limpness and mild hindlimb paresis. Overall, these results show that *MMP-12*^{-/-} nonlittermate mice succumb to a worse relapsing-remitting disease than their *MMP-12*^{+/+} counterparts.

We examined the thoracic spinal cords from *MMP-12*^{+/+} and *MMP-12*^{-/-} using LFB and H&E to determine whether differences in EAE severity translated to worsened neuropathology. Using a semiquantitative classification system (Figure 2), we were able to document general inflammation score. Normal spinal cords from both *MMP-12*^{+/+} and *MMP-12*^{-/-} mice were absent of demyelination and infiltrates, and we did not observe any gross anatomical or myelin structure abnormalities between the genotypes in nondisease condition. In EAE-afflicted mice, both *MMP-12*^{+/+} and *MMP-12*^{-/-} mice displayed inflammatory infiltrates and we found that *MMP-12*^{-/-} mice trended toward a higher inflammation score in comparison with *MMP-12*^{+/+} although statistical significance was not achieved (Figure 3A). We next counted the number of inflammatory lesions per longitudinal section of the thoracic cord, sampling six sections per mouse. Figure 3B shows that the average number of lesions per section was higher in *MMP-12*^{-/-} mice compared with that in *MMP-12*^{+/+} mice ($P = 0.03$). We did not observe any qualitative differences in the lesions between the genotypes in terms of the size, distribution, or location relative to blood vessels.

The above work consisted of the use of wild-type or *MMP-12*^{-/-} mice generated from separate breeding colonies. To preclude the possibility that EAE phenotype was confounded by epigenetic or environmental factors rather than the deletion of *MMP-12*, we sought to produce littermates for further analyses. *MMP-12*^{+/+} and *MMP-12*^{-/-} breeders were crossed to generate heterozygote breeders, which were further cross-bred to produce littermates of *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} mice, which were genotyped (Supplemental Figure S1, available at <http://ajp.amjpathol.org>). Examination of the genotype of >200 pups showed that the distribution of *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} mice was similar to what would be expected for Mendelian inheritance of this gene (Supplemental Figure S1, available at <http://ajp.amjpathol.org>). We deemed it important to use littermate mice (Figure 4) to confirm the data from nonlittermate experiments (Figure 1) because differential breeding, nursing, or housing properties, besides unknown genetic influences, may adversely affect outcomes.

Figure 2. Qualitative scoring of the degree of neuropathology in the spinal cord. Displayed are examples of histopathology in the spinal cord, cut longitudinally, and stained with LFB/H&E. In control normal tissue there are no leukocytes accumulated superficially or within the tissue. During EAE, there is an influx of leukocytes that usually is evident first in the pial lining and subsequently in the parenchyma. The number of lesions (some are indicated by **arrows**) present within a given spinal cord section analyzed is also indicative of the degree of inflammation. Thus, by the location and number (< or >5) of lesions on each section, a qualitative score can be documented in blinded analyses, with ascending scores representing greater severity. Areas of inflammation (hypercellularity) are usually correspondent with areas of demyelination where there is an interruption of LFB-positive profiles.

EAE was induced in littermate *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} using MOG₃₅₋₅₅ and animals were observed daily for 60 days. As with nonlittermates, there was no difference in disease onset between the genotypes and the course of disease in all groups was relapsing-remitting (Figure 4A). Significantly, *MMP-12*^{-/-} mice did not remit to the same level as *MMP-12*^{+/+}, and their relapse was more severe. Heterozygote *MMP-12*^{+/-} mice behaved in the same manner as wild-type controls, so the loss of both *MMP-12* alleles was necessary to confer a worsened EAE outcome. These results were

Figure 3. The degree of neuropathology in the spinal cord trended toward higher severity in the *MMP-12*^{-/-} animals. **A:** Inflammation score of the spinal cords of mice, assessed in sections stained with LFB/H&E, is similar between *MMP-12*^{+/+} and *MMP-12*^{-/-} animals, although there were eight null mice with an inflammation score of 5 or higher, compared with four in the wild-type group. Individual values are plotted and the bar represents the mean for the group. **B:** The lesion number, referring to the average number of inflammatory lesions that are encountered within each of six longitudinal spinal cord sections of a given mouse, was significantly higher in *MMP-12*^{-/-} animals compared with wild-type ($P = 0.03$). Nonparametric Mann-Whitney tests were significant when $*P < 0.05$ (*MMP-12*^{+/+}, $n = 10$; and *MMP-12*^{-/-}, $n = 12$).

confirmed by examination of average cumulative score throughout 60 days (Figure 4B) and the percentage of time mice spent with severe disability (Figure 4C), both of which were higher in the *MMP-12*^{-/-} mice. Overall, in littermate or nonlittermate experiments, the presence of MMP-12 plays a role in conferring a greater degree of remission from EAE and to prevent a severe relapse.

Figure 4. EAE profiles in *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} littermate animals throughout 60 days of observation. **A:** Daily average EAE score of *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} animals throughout 60 days of observation. Values are mean $\pm$ SEM. Repeated measures two-way analyses of variance were significant ($P < 0.05$) and Bonferroni posthoc analyses reveal periods of significance ($*P < 0.05$). **B:** Average cumulative score, displaying that there is a significant difference between *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} animals in their overall disease burden. Values are mean $\pm$ SEM. Nonparametric one-way ANOVAs were significant when $P < 0.05$. Posthoc analyses with Dunn's multiple comparison test were significant when $*P < 0.05$. **C:** *MMP-12*^{-/-} animals spend a significant percentage of their time with severe disability (disease score, >4). Values are mean $\pm$ SEM; post-hoc analysis with Dunn's multiple comparison test was significant when $*P < 0.05$ or $***P < 0.001$. There were 10 mice per genotype in all panels.

Figure 5. *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} littermate animals have different proliferation responses on challenge with MOG₃₅₋₅₅. **A:** *MMP-12*^{-/-} T cells have a depressed proliferative response on challenge with MOG₃₅₋₅₅ at varying concentrations (μ g/ml) compared with cells from *MMP-12*^{+/+} and *MMP-12*^{+/-} littermates. **B:** *MMP-12*^{-/-} T-cell-reduced proliferative responses are inherent properties of the T cells. T cells of all phenotypes proliferate to a similar extent when MOG₃₅₋₅₅ is presented by *MMP-12*^{-/-} APCs. In contrast, *MMP-12*^{-/-} T cells still have a depressed proliferative response when MOG₃₅₋₅₅ antigen is presented by *MMP-12*^{+/+} or *MMP-12*^{+/-} APCs. Values are mean $\pm$ SEM pooled from three separate experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with no MOG controls. (*MMP-12*^{+/+}, $n = 9$; *MMP-12*^{+/-}, $n = 6$; and *MMP-12*^{-/-}, $n = 9$).

MMP-12 Loss Confers Deficits in T-Cell Proliferation but Not in Antigen Presentation Capacity

To determine the mechanisms of the worsened clinical course of EAE in *MMP-12*^{-/-} mice, the ease of activation of leukocytes was first examined using a MOG₃₅₋₅₅ recall proliferation assay. Ten days after MOG immunization, and before clinical signs of disease were evident, LNs were removed and lymphocytes were incubated with irradiated antigen-presenting cells (APCs) from spleens of the same genotype, with or without MOG₃₅₋₅₅, for 3 days. Surprisingly, despite no difference in onset of clinical signs across genotypes, and a worse EAE outcome in *MMP-12*^{-/-} mice after the initial relapse (Figures 1A and 4A), *MMP-12*^{-/-} T cells had significantly decreased proliferative capacity in response to 20 and 100 μ g/ml MOG₃₅₋₅₅ when compared with T cells from wild-type or heterozygote littermates (Figure 5A). Thus, the subsequent worsening of EAE clinical scores in *MMP-12*^{-/-} mice after initial peak disease is not explained by a global

Figure 6. Leukocyte populations within the CNS during EAE in *MMP-12^{+/+}*, *MMP-12^{+/-}*, and *MMP-12^{-/-}* littermate animals. **A:** The percentage of CD3- and CD4-positive T cells increased during EAE in the CNS of *MMP-12^{+/+}*, *MMP-12^{+/-}*, and *MMP-12^{-/-}* littermates. *MMP-12^{-/-}* mice had a significant reduction in the percentage of CD3- and CD4-positive T cells during remission in comparison with the other genotypes. The percentage of CD3- and CD8-positive T cells increased in the CNS during EAE in a similar manner in all of the genotypes examined. **B:** The percentage of CD4- and CD25-positive T cells increased during EAE in the CNS of *MMP-12^{+/+}*, *MMP-12^{+/-}*, and *MMP-12^{-/-}* littermates. There was a significant elevation in the percentage of CD4- and CD25-positive T cells in the *MMP-12^{-/-}* mice during the remission phase. Values are mean $\pm$ SEM pooled from two experiments, $n = 4$ to 13 animals per genotype per time point. Two-way analysis of variance with Bonferroni post-test, $*P < 0.05$. The percentage of gated events was evaluated as described in the Materials and Methods.

increase of proliferative capacity of MOG-specific T cells; indeed, the contrary was found.

To determine the source of the diminished T-cell response to MOG_{35–55} we set out to examine whether *MMP-12^{-/-}* APCs might inefficiently present MOG_{35–55} to T cells (Figure 5B). T cells were presented MOG_{35–55} via APCs from nonmatching genotypes in the same manner as previous antigen recall experiments. The results show that *MMP-12^{-/-}* APCs were capable of presenting MOG_{35–55} with a similar degree of efficiency as *MMP-12^{+/+}* or *MMP-12^{+/-}* APCs. However, in the presence of either *MMP-12^{+/+}* or *MMP-12^{+/-}* APCs, *MMP-12^{-/-}* T cells had diminished proliferation. These results demonstrate that *MMP-12^{-/-}* T cells have an inherently diminished proliferative response to recall antigens.

Alterations of Inflammatory Cell Types in the Spinal Cord of *MMP-12^{-/-}* Mice during EAE

To better understand the phenotype of *MMP-12^{-/-}* mice, we investigated the cellular infiltrates in EAE at three phases of the disease: presymptomatic (day 10 after MOG injection), remission (2 days after the first signs of a decrease in peak EAE score), and chronic disease (day 35). Separation of clinical score was evident only in the chronic phase, in which *MMP-12^{-/-}* mice had higher severity than the other genotypes (Figures 1A and 4A). We focused on the CD4⁺ and CD8⁺ populations as well as CD4CD25⁺ T cells within draining LNs and the lumbar

sacral spinal cord. In the LN, CD3CD4⁺ T-cell numbers were fairly uniform across the different stages of disease per genotype. There was a trend toward increased numbers of CD3CD8⁺ T cells in all genotypes during remission (Supplemental Figure S2, available at <http://ajp.amjpathol.org>). Nonetheless, when comparing across genotypes, we did not observe any changes in the percentage of either CD3CD4⁺, CD3CD8⁺, or CD4CD25⁺ T cells within the LN at all disease stages analyzed (Supplemental Figure S2, available at <http://ajp.amjpathol.org>).

Investigations of the spinal cord found a marked increase of CD3CD4⁺ T cells within the CNS during the remission phase of EAE in all genotypes compared with normal conditions or presymptomatic or chronic EAE. Interestingly, *MMP-12^{-/-}* mice had significantly fewer CD3CD4⁺ T cells ($49.6 \pm 7.1\%$) during the remission phase compared with *MMP-12^{+/+}* mice ($62.3 \pm 11.1\%$) (Figure 6A). The number of CD3CD4⁺ T cells remained higher at the chronic phase of EAE as compared with normal animals but no significant differences were seen between the genotypes. The number of CD3CD8⁺ T cells within the CNS did not change significantly with EAE disease between the genotypes (Figure 6A).

With respect to CD4CD25⁺ cells within the CNS, we observed a marked increase during remission and chronic stages of EAE compared with normal or presymptomatic levels (Figure 6B). At remission, *MMP-12^{-/-}* mice had significantly more CD4CD25⁺ cells ($34.8 \pm 6.0\%$) than *MMP-12^{+/+}* ($26.2 \pm 5.9\%$) mice. A subset of

CD4CD25⁺ cells are regulatory T cells, which can be further identified by the expression of the transcription factor FoxP3. We did not detect any FoxP3 staining in any of our samples, which may be a function of the protocol used because cells were not restimulated *ex vivo* as has been reported in the literature.¹⁷

We next examined macrophages and microglia within the CNS because these populations are important in the presentation of antigen and play other inflammatory roles. Gating on CD11b⁺ and using the relative levels of CD45⁺ can allow the differentiation of macrophages (CD45^{high}) from microglia (CD45^{low}).^{18–21} There was a marked increase in the number of macrophages in the remission phase (Figure 7, A and B) compared with other time points; interestingly, *MMP-12*^{-/-} mice had significantly fewer macrophages (13.7 ± 2.8%) than that in *MMP-12*^{+/+} (19.5 ± 7.3%) and *MMP-12*^{+/-} (22.8 ± 6.5%) mice (Figure 7, A and B). By the chronic phase of disease, the number of macrophages decreased in all genotypes and there was no longer any significant difference between the genotypes.

The percentage of microglia within the CNS increased during the remission phase of disease and continued to remain high at the chronic phase of disease in all of the genotypes (Figure 7, A and C). The reduced percentage of macrophages during the remission phase of EAE in *MMP-12*^{-/-} in combination with the increasing percentage of microglia may reflect an imbalance in the inflammatory response of *MMP-12*^{-/-} mice contributing to the reduced ability to remit from EAE.

Levels of Inflammatory Molecules within the Spinal Cord of *MMP-12*^{-/-} Mice at Chronic Phase of EAE Are Substantially Altered in Comparison with Wild-Type Controls

To further our understanding of the worsening of EAE in *MMP-12*^{-/-} mice, we measured the levels of inflammatory molecules within the spinal cord using an antibody array that allowed us to chart 40 proteins (data not shown for BLC, TIMP-1, and TIMP-2). The thoracic spinal cords were taken from the *MMP-12*^{-/-} and *MMP-12*^{+/+} mice whose lumbar sacral cords were used for the flow cytometry analyses described earlier. We did not observe any significant difference between both genotypes for any of the proteins examined at the normal or presymptomatic phase of EAE (Supplemental Figures S4 and S5, available at <http://ajp.amjpathol.org>). Interestingly we found that 15 of the 40 proteins examined were significantly higher in *MMP-12*^{-/-} mice during the chronic phase of EAE. Of these 15 proteins, 4 were chemokines (MCP-1, fractalkine, I-TAC, and LIX) whereas the other 11 were cytokines or related molecules [interferon (IFN)- γ , interleukin (IL)-12p40/p70, IL-12p70, tumor necrosis factor (TNF)- α , IL-17, IL-4, IL-6, IL-3, IL-10, GM-CSF, and sTNF-R1] (Figure

Figure 7. Macrophage and microglia populations vary in the spinal cord during EAE in *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} littermate animals. **A:** The percentage of gated events was evaluated using flow cytometry plots in which the region of interest was determined using side (x axis) and forward (y axis) scatter plots. Cells were then sorted based on their expression of CD45-high CD11b (macrophages) or CD45-low CD11b (microglia) staining to determine the final gated events, which is shown as a percentage of gated events on the y axis of the summary line graph. **B:** The percentage of macrophages increased during EAE, with *MMP-12*^{-/-} mice having significantly fewer macrophages than their littermates. **C:** The percentage of microglia increased during EAE, peaking at chronic disease in all genotypes. Values are mean ± SEM pooled from two experiments, *n* = 4 to 13 animals per genotype per time point. Two-way analysis of variance, **P* < 0.05 with Bonferroni post-test. The percentage of gated events was evaluated as described in the Materials and Methods.

Figure 8. Changes in cytokine and chemokine expression between *MMP-12^{+/+}* and *MMP-12^{-/-}* during EAE. *MMP-12^{-/-}* mice had significantly altered levels of IL-3, IL-4, IL-6, IL-10, IL-12p40/p70, IL-12p70, and IL-17, as well as of fractalkine, I-TAC, LIX, MCP-1, G-CSF, GM-CSF, TNF- α , sTNF-RI, and IFN- γ during chronic EAE in comparison with wild-type littermates. Values are mean $\pm$ SEM of $n = 3$ animals per genotype per phase of disease. One-way analysis of variance with Tukey's multiple comparisons test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The y axis represents normalized level of expression (arbitrary units).

8). Not all of the proteins examined increased with chronic disease in the *MMP-12^{-/-}* mice; indeed, G-CSF had significantly decreased expression during the chronic phase of EAE while during the remission phase G-CSF was significantly higher in *MMP-12^{-/-}* than *MMP-12^{+/+}* mice (Figure 8).

Discussion

Several MMP members are elevated in MS and EAE in which they are thought to promote inflammation and pathology.^{3,4,22,23} However, it remains possible that particular MMP members at specific stages of disease may have benefits. Indeed, we reported that the highly up-regulated MMP-12 in EAE may be useful because *MMP-12^{-/-}* non-littermate mice have worse EAE clinical disease than wild-type mice throughout 73 days of evaluation.⁶ In that study, we found a higher Th1/Th2 profile in lymphocytes taken from the LN of *MMP-12^{-/-}* mice during the ascending phase of EAE when compared with lymphocytes harvested from LN from wild-type mice. Nonetheless, the spinal cord was not examined in our previous study for inflammatory profiles and the inconsistent onset and course of disease

among animals precluded an analysis of the nature of EAE in 129/SvEv mice.

In the present work, with increasing experience in handling EAE in 129/SvEv mice, we found that this strain of mice developed a relapsing-remitting course of EAE. Significantly, the lack of MMP-12 resulted in worsened EAE that manifested in an inability to remit completely from disease, a worse relapse and histology, and an exacerbation of proinflammatory molecules within the CNS during chronic disease.

We sought to provide explanations for the increased clinical score after MMP-12 loss. Because expansion of antigen-specific T cells plays an important role in EAE, our first goal was to determine the role of *MMP-12^{-/-}* T cells in antigen-specific recall by examining proliferative responses of LN-derived T cells to MOG₃₅₋₅₅. Expecting a positive correlation of increase in T-cell proliferation with worsened overall disease clinical score of *MMP-12^{-/-}* mice, we were surprised that *MMP-12^{-/-}* T cells proliferated significantly less than either *MMP-12^{+/+}* or *MMP-12^{+/-}* on restimulation with MOG₃₅₋₅₅. The lack of a correlation between T-cell proliferation and clinical score has been reported; older mice have been reported to have worse EAE despite having reduced proliferative T-cell responses.²⁴

Because T-cell proliferation provides evidence on the *ex vivo* response of cells to MOG, we also addressed the *in vivo* composition of infiltrating T cells in EAE-afflicted animals. We took CNS samples from *MMP-12*^{+/+}, *MMP-12*^{+/-}, and *MMP-12*^{-/-} mice at four stages. Our choice of time points was based on the periods during EAE when there was no difference in clinical disease between genotypes (normal and presymptomatic), when mice started separating in clinical disease (remission), and finally when *MMP-12*^{-/-} mice showed a clear divergence in disease from wild-type (chronic). We found that *MMP-12*^{-/-} mice had significantly fewer CD3CD4⁺ T cells during remission. Despite that, *MMP-12*^{-/-} mice did not remit as efficiently as *MMP-12*^{+/+} or *MMP-12*^{+/-} mice. One possibility is that a CD4⁺ subset with anti-inflammatory or regulatory roles is selectively lost in *MMP-12*^{-/-} mice. Particularly, it has become apparent that CD4⁺ T cells comprise a subclass of regulatory T cells (Tregs, CD4CD25⁺), which can suppress the function of encephalitogenic CD4⁺ T cells²⁵ and confer protection against EAE.²⁶⁻²⁸ We thus investigated whether *MMP-12*^{-/-} mice had reduced CD4CD25⁺ T cells within the CNS during remission but the converse was found (Figure 6B). It remains to be determined whether these CD4CD25⁺ cells, although more numerous, have functionally altered suppressive capabilities in *MMP-12*^{-/-} spinal cord.

We examined changes in the macrophage and microglia populations during EAE because these cells interact with T cells and play important roles in EAE pathology by producing cytokine, proteases, and free radicals.²⁹⁻³¹ Although T cells can initiate EAE disease, the effector mechanisms leading to inflammation and demyelination within the CNS are aided by cells types such as infiltrating macrophages and endogenous microglia. Early work by Brosnan and colleagues³² demonstrated that macrophage depletion by treatment with silica dust delayed the onset and reduced the severity of EAE in Lewis rats. The elimination of macrophages by the use of liposomes containing dichloromethylene diphosphonate prevented the clinical manifestations of disease.^{33,34} Mice without *MAC-1*^{-/-} (CD11b), an important molecule for the trafficking of monocytes/macrophages to sites of inflammation and subsequent phagocytosis, had reduced EAE severity suggestive of a role in damage.³⁵ The ablation of microglia after ganciclovir treatment of CD11b-HSVTK transgenic mice also repressed the development of EAE.³⁶ In the current study, we used flow cytometry, CD11b, and relative amounts of CD45 to differentiate between macrophage (CD45^{high}) and microglia (CD45^{low}).^{18,19,37} We found that *MMP-12*^{-/-} mice had significantly decreased numbers of macrophages and normal microglia levels in the CNS during remission. The reduced number of infiltrating macrophages in *MMP-12*^{-/-} compared with wild-type mice, concomitant with the reduced ability to remit, runs counter to the majority of data suggesting a detrimental role for macrophages.³³⁻³⁵ Nonetheless, the exact role of macrophage/microglia in EAE and MS is still unclear and debatable, and there are beneficial roles ascribed to these cells as well.^{38,39} The absence of macrophages within the CNS during remission in *MMP-12*^{-/-} could result in increased myelin debris and subsequent

epitope spreading and axonal damage. This possibility of beneficial macrophages as suggested by the *MMP-12*^{-/-} mice at remission deserves further clarification. It is important to recognize the importance of polymorphonuclear leukocytes in EAE,⁴⁰ and because we did not directly examine these cells, we cannot exclude their roles in our studies.

Because there were cellular differences between the genotypes studied herein (Figures 6 and 7) and given that these cellular studies did not reveal the pro- or anti-inflammatory nature of the cells, we next determined whether there were specific molecular alterations in 40 proteins known to be involved in inflammation. We found that the majority of the proteins examined (fractalkine, I-TAC, LIX, MCP-1, GM-CSF, sTNF-RI, IL-3, IL-4, IL-6, IL-10, IFN- γ , IL-12p40/p70, IL-12p70, IL-17, and TNF- α) were significantly increased in *MMP-12*^{-/-} mice during the chronic phase. Only G-CSF was significantly higher in *MMP-12*^{-/-} mice during remission then switching to a significantly lower expression during chronic disease. The reversal of EAE using G-CSF has been described by two groups via the administration of recombinant G-CSF to EAE-afflicted mice.^{41,42} The observation that *MMP-12*^{-/-} mice showed significantly reduced expression of G-CSF during chronic EAE suggests that this molecule may be important in their worsened disease. How MMP-12 and G-CSF interact mechanistically will be the subject of future study.

The majority of the molecules found to be increased in the *MMP-12*^{-/-} mice during the chronic phase are known mediators of EAE particularly with regards to cellular recruitment (fractalkine, I-TAC, MCP-1, and GM-CSF) and Th1/17 pro-inflammatory T cell profiles (IL-3, IL-6, IFN- γ , IL-12p40/p70, IL-12p70, IL-17, sTNF-RI, and TNF- α). Of note, Th2 or regulatory cytokines (IL-4 and IL-10) traditionally associated with anti-inflammatory roles, were also altered in the chronic phase of EAE in *MMP-12*^{-/-} mice.

We do not know the precise mechanisms by which MMP-12 leads to the altered levels of inflammatory molecules during the chronic phase of EAE. As a protease, the functions of MMP-12 rely on the spectrum of substrates that it cleaves. Many cytokines and chemokines can be posttranslationally modified by the actions of MMPs,⁴³⁻⁴⁵ so these interactions are a potential step toward gross changes in inflammatory molecules in the spinal cord after MMP-12 loss. Such possibilities remain to be addressed in future studies, and our results have highlighted some molecules (eg, G-CSF) for scrutiny. It could be particularly interesting to evaluate whether the apparent protective effects of G-CSF in EAE is a consequence of particular aspects of MMP-12 activity during the chronic phase of disease.

In summary, we report that MMP-12 is an important molecule in EAE. Its absence results in the reduced ability to remit effectively from a relapse, and the clinical severity of subsequent relapses is worsened. These results highlight that the expression of MMP-12 at the active edge of MS lesions¹² could be a protective phenomenon. Therapeutic interventions in MS that are aimed at reducing MMP activity should avoid MMP-12 as a target. Alter-

nately, strategies to elevate MMP-12 levels at the chronic phases of disease may lead to improvements in clinical disability although this requires further dissection in future studies.

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