

Collagenase-2 Deficiency or Inhibition Impairs Experimental Autoimmune Encephalomyelitis in Mice*

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Alicia R. Folgueras[‡], Antonio Fueyo[§], Olivia García-Suárez[¶], Jennifer Cox^{||}, Aurora Astudillo[¶], Paolo Tortorella^{**}, Cristina Campestre^{††}, Ana Gutiérrez-Fernández[‡], Miriam Fanjul-Fernández[‡], Caroline J. Pennington^{§§}, Dylan R. Edwards^{§§}, Christopher M. Overall^{||}, and Carlos López-Otín^{†1}

From the [‡]Departamento de Bioquímica y Biología Molecular, and [§]Biología Funcional, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, and [¶]Servicio de Anatomía Patológica, Hospital Central de Asturias, Oviedo 33006, Spain, the ^{||}Departments of Oral Biological and Medical Sciences, and Biochemistry and Molecular Biology, Centre for Blood Research, University of British Columbia, Vancouver, V6T 1Z3 Canada, the ^{**}Dipartimento Farmaco-Chimico, Università degli Studi di Bari, Bari 70125, Italy, the ^{††}Dipartimento di Scienze del Farmaco, Università G. d'Annunzio, Chieti 66013, Italy, and the ^{§§}School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ United Kingdom

Matrix metalloproteinases (MMPs) have been implicated in a variety of human diseases, including neuroimmunological disorders such as multiple sclerosis. However, the recent finding that some MMPs play paradoxical protective roles in these diseases has made necessary the detailed study of the specific function of each family member in their pathogenesis. To determine the relevance of collagenase-2 (MMP-8) in experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, we have performed two different analyses involving genetic and biochemical approaches. First, we have analyzed the development of EAE in mutant mouse deficient in MMP-8, with the finding that the absence of this proteolytic enzyme is associated with a marked reduction in the clinical symptoms of EAE. We have also found that *MMP-8*^{-/-} mice exhibit a marked reduction in central nervous system-infiltrating cells and demyelinating lesions. As a second approach, we have carried out a pharmacological inhibition of MMP-8 with a selective inhibitor against this protease (IC₅₀ = 0.4 nM). These studies have revealed that the administration of the MMP-8 selective inhibitor to mice with EAE also reduces the severity of the disease. Based on these findings, we conclude that MMP-8 plays an important role in EAE development and propose that this enzyme may be a novel therapeutic target in human neuro-inflammatory diseases such as multiple sclerosis.

Multiple sclerosis (MS)² is an inflammatory disease of the central nervous system (CNS) characterized by autoreactive

T-cell infiltration that causes myelin sheath destruction and axonal loss (1, 2). Although the origin of MS remains unclear, CD4⁺ Th1 cells are believed to be the main mediators of the autoimmune reaction (3, 4). According to this, the injection of myelin peptides into susceptible mice generates experimental autoimmune encephalomyelitis (EAE), a murine model of human multiple sclerosis, and leads to myelin-specific recruitment of T cells that subsequently differentiate into Th1-type effector cells (5). The extravasation of reactive leukocytes into the CNS parenchyma across the blood-brain barrier correlates with the appearance of clinical symptoms (6).

Matrix metalloproteinases (MMPs) have been largely implicated in MS and EAE progression due to their ability to degrade the main extracellular matrix components that maintain the integrity of the blood-brain barrier (7, 8). Consistent with this putative role of MMPs in the development or progression of MS, several members of this metalloproteinase family have been shown to be up-regulated in serum, cerebrospinal fluid, and brain samples of MS patients (9, 10) as well as in murine models of demyelinating lesions (11, 12). Likewise, expression of MMPs has been detected in diverse cell types involved in the pathogenesis of the disease (13, 14). Interestingly, the administration of MMP inhibitors has reduced the severity of the disease in different EAE murine models (15–18). Furthermore, young MMP-9-deficient mice and MMP-2/MMP-9 double knock-out mice are more resistant to the development of the disease than wild-type animals (19, 20). However, and somewhat unexpectedly, *MMP-2*^{-/-} and *MMP-12*^{-/-} mice are more susceptible to EAE induction than their wild-type counterparts suggesting that these metalloproteinases could play protective roles in the course of MS (21, 22). These findings, together with the variety of processes in which MMPs are involved (23, 24), suggest that the contribution of these enzymes to the progression of neuro-inflammatory diseases is much more complex than originally anticipated. Thus, and besides the degradative action of MMPs during blood-brain barrier disruption, these enzymes may also contribute to the progression of MS through their ability to degrade myelin components releasing encephalitogenic peptides (25, 26). Likewise, MMPs may play important roles in the regulation of the inflammatory stimuli responsible for autoreactive T cell recruitment

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¹ To whom correspondence should be addressed. Tel.: 34-985-104-201; Fax: 34-985-103-564; E-mail: clo@uniovi.es.

² The abbreviations used are: MS, multiple sclerosis; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MMP, Matrix metalloproteinase; MOG, myelin oligodendrocyte glycoprotein; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; WT, wild type.

Impaired Autoimmune Encephalomyelitis in $MMP-8^{-/-}$ Mice

and infiltration (27). In fact, MMPs have been demonstrated to modulate inflammation through the proteolytic cleavage of a number of cytokines and chemokines that have been implicated in the pathobiology of MS (27, 28). This functional diversity of MMPs makes necessary the detailed analysis of the specific role that each individual member of this complex family of metalloproteinases might play in the pathogenesis of MS.

Our study in this regard has focused on the analysis of the putative implication of collagenase-2 (MMP-8) in the development and progression of neuro-inflammatory diseases such as MS. MMP-8 is a potent collagenolytic enzyme frequently associated with inflammatory conditions, including asthma, hepatitis, ulcerative colitis, atherosclerosis, periodontitis, and rhinosinusitis (29–34). Interestingly, several works have also evidenced the up-regulation of MMP-8 in EAE, and its expression has been correlated with disease severity (12, 14). To further explore the possibility that this metalloproteinase could play a role in MS pathogenesis, we have used mutant mice deficient in MMP-8 and analyzed their susceptibility to EAE. In this work, we report that $MMP-8^{-/-}$ mice are more resistant to EAE than their wild-type counterparts, and show a marked reduction in CNS-infiltrating cells and demyelinating lesions. On this basis, we propose that MMP-8 plays an important role in EAE development and can be a therapeutic target in human neuro-inflammatory diseases such as MS.

EXPERIMENTAL PROCEDURES

EAE Induction and Clinical Evaluation—Wild-type ($MMP-8^{+/+}$) and MMP-8-null mice ($MMP-8^{-/-}$) were generated in a C57BL6/129Sv background, as previously described (35). Control and mutant mice used in all experiments were littermates derived from interbreeding of $MMP-8^{+/-}$ heterozygotes. For EAE induction, 8- to 10-week-old female mice were injected subcutaneously in the flank on days 0 and 7 with 300 μ g of myelin oligodendrocyte glycoprotein (MOG_{35–55}) peptide. The peptide was thoroughly emulsified in 100 μ l of complete Freund's adjuvant containing 500 μ g of heat-inactivated *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). Mice were also injected intraperitoneally on days 0 and 2 with 200 μ l of PBS containing 500 ng of Pertussis toxin (List Biologicals Laboratories). After immunization with MOG, mice were observed daily, and the disease severity was scored on a scale of 0–5 with graduations of 0.5 for intermediate clinical signs. The score was defined as follows: 0, no detectable clinical signs; 1, weakness of the tail; 2, hind limb weakness or abnormal gait; 3, complete paralysis of the hind limbs; 4, complete hind limb paralysis with forelimb weakness or paralysis; 5, moribund or death. Paralyzed mice were given easy access to food and water. Mouse experimentation was done according to the guidelines of the Universidad de Oviedo, Oviedo-Spain.

In Vivo MMP-8 Inhibition Studies—The cyclohexylamine salt of (*R*)-1-(3'-methylbiphenyl-4-sulfonylamino)methylpropyl phosphonic acid, a new phosphonate inhibitor with potent ($IC_{50} = 0.4$ nM) and selective action against MMP-8 (36), was dissolved in PBS with 5% Me₂SO at 2.5 mg/ml. Treated mice were injected intraperitoneally daily with a dose of 25 mg/kg body weight of the inhibitor, starting at the time of MOG

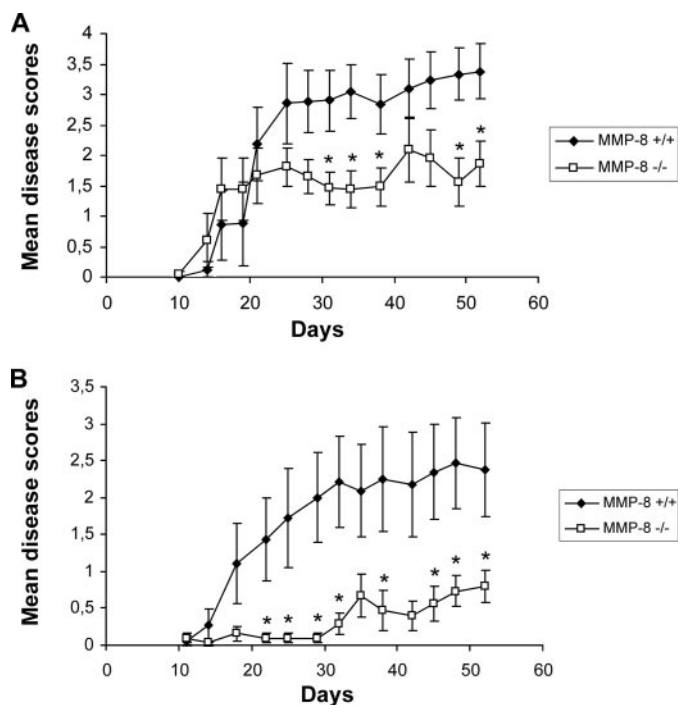


FIGURE 1. Clinical severity of EAE is reduced in $MMP-8^{-/-}$ mice. Wild-type and $MMP-8^{-/-}$ female mice were immunized with the encephalitogenic peptide MOG_{35–55} and scored daily according to the severity of their symptoms. The experiment was performed with 8- to 10-week-old $MMP-8^{+/+}$ and $MMP-8^{-/-}$ mice ($n = 6$ mice per group) (A) and 4-week-old $MMP-8^{+/+}$ ($n = 7$) and $MMP-8^{-/-}$ ($n = 8$) mice (B). Results are expressed as mean disease score \pm S.E. *, $p \leq 0.05$.

immunization. All mice were monitored daily until the time of sacrifice.

Analysis of Expression of MMPs, TIMPs, ADAMs, and ADAMTSs—Spinal cords from immunized wild-type and $MMP-8^{-/-}$ mice were isolated during the chronic phase of the disease. Tissues were homogenized and total RNA was extracted by using a commercial kit (RNeasy MiniKit, Qiagen). One microgram of RNA was reverse transcribed to make cDNA. TaqMan PCR was used to profile mRNA levels of all members of the MMP family and the four TIMPs and several members of the ADAM and ADAMTS families, as previously described (14). The 18 S rRNA gene was used as an endogenous internal control.

Isolation of Splenocytes and Cytokine Assays—Spleens from immunized wild-type and $MMP-8^{-/-}$ mice were isolated and dispersed into a single cell suspension. After lysis of red blood cells, the splenocytes were washed and resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, 2-mercaptoethanol, and streptomycin/penicillin. Cells were plated at 4×10^6 cells/well in 24-well plates containing 1 ml of culture medium with 0, 5, or 50 μ g/ml MOG_{35–55}. Supernatants were collected at 48 h for cytokine analysis. Quantitative enzyme-linked immunosorbent assay was performed for TNF- α , IFN- γ (R&D), IL-4, IL-10 (BD Biosciences), and TGF- β (Promega) according to the protocol supplied by the manufacturer.

Splenocyte Proliferation Assay—Spleens from wild-type and $MMP-8^{-/-}$ mice were removed 21 days after immunization and processed as described above. Cells were plated in tripli-

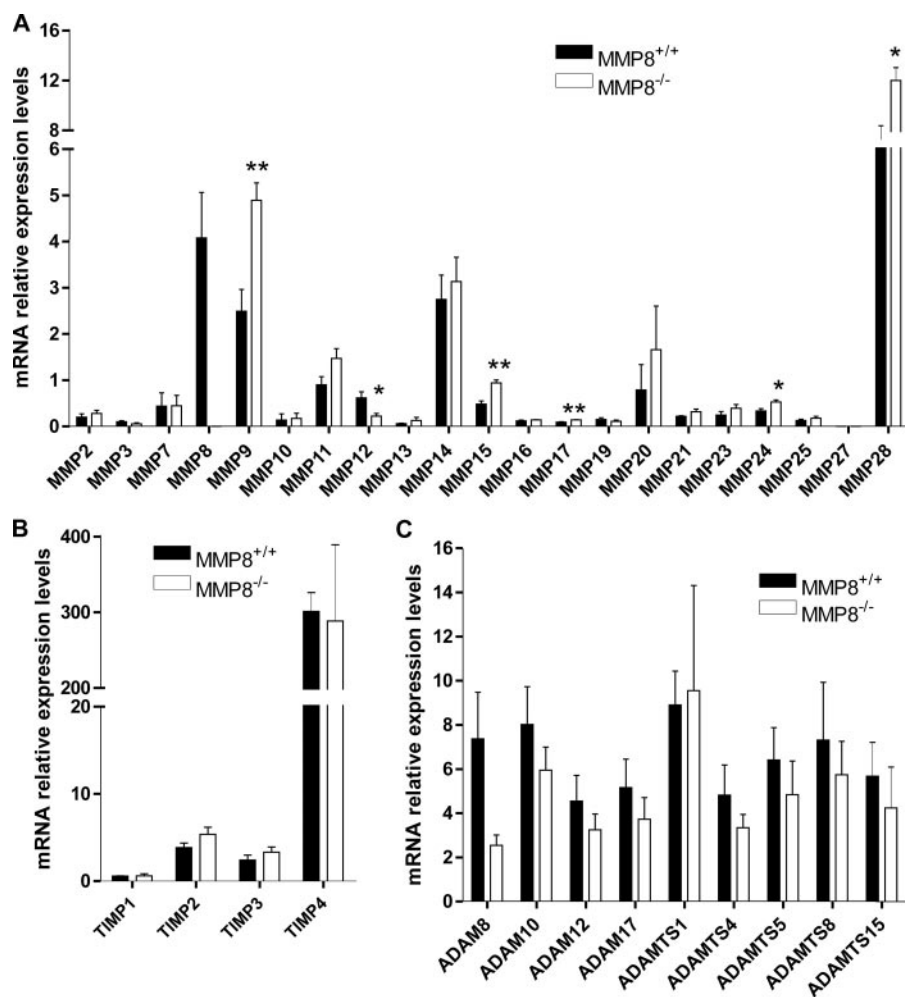


FIGURE 2. Analysis of MMP, ADAM, ADAMTS, and TIMP expression levels in 8-week-old wild-type and $MMP-8^{-/-}$ immunized mice. TaqMan real-time PCR analysis of MMPs (A), TIMPs (B), and ADAMs and ADAMTSs (C) expressed in spinal cord samples from wild-type (black bar) and $MMP-8^{-/-}$ immunized mice (white bar). mRNA levels on the y-axis are expressed relative to 18 S rRNA levels. Both forms of murine $MMP-1$ (43) were undetectable. Values are means \pm S.E. *, $p \leq 0.05$; **, $p \leq 0.01$. $n = 4$ mice per group.

icates at 7×10^5 cells/well in 96-well microtiter plates containing 200 μ l of culture medium with 0, 5, or 50 μ g/ml MOG_{35–55}. They were cultured for 48 h and then pulsed with 1 μ Ci/well [*methyl*-³H]thymidine for the last 16 h. Cells were collected and precipitated with 5% trichloroacetic acid for 4 h at 4 °C. The precipitated DNA was filtered using G/C glass fiber filters and radioactivity was determined in a scintillation counter.

Histological Analysis and Immunofluorescence—Wild-type and $MMP-8^{-/-}$ immunized mice with representative clinical scores were selected from each group. Brains and spinal cords were isolated, fixed in 4% paraformaldehyde, and embedded in paraffin. Each sample was serially sectioned 5- μ m thick at 100- μ m intervals and stained with hematoxylin and eosin. To evaluate the degree of inflammation, the “depth” of inflammatory infiltrates was measured in serial sections of spinal cord samples and quantified using Image Tool HUCA software. To assess the degree of demyelination, an immunohistochemical analysis was performed using a primary antibody against myelin basic protein. To perform immunohistochemistry, deparaffined, and rehydrated sections were rinsed in PBS (pH 7.5). Endogenous peroxidase activity and nonspecific binding were

blocked with peroxidase block buffer (DakoCytomation) and 1% bovine serum albumin, respectively. Sections were incubated overnight at 4 °C with a monoclonal antibody anti-myelin basic protein (a gift from Dr. Sternberger), diluted 1:1500. Then, sections were incubated with an anti-mouse EnVision system-labeled polymer (DakoCytomation) for 30 min, washed in buffer solution, and visualized with diaminobenzidine. Sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted in Entellan®. Inflammatory and demyelinating lesions were evaluated by a neuropathologist. To quantify the different cellular profiles, sampling was systematically randomized, and two sections of the spinal cord from wild-type and $MMP-8^{-/-}$ immunized mice, with representative clinical scores, were selected to perform the immunohistochemical analysis. To detect T lymphocytes, deparaffined and rehydrated sections were heated in 10 mM citrate buffer solution (pH 6.5) in a pressure cooker for 7 min. Antibody nonspecific binding was blocked using 1% bovine serum albumin in PBS. Samples were incubated overnight at 4 °C with a rabbit anti-human CD3 antibody (Aton Pharma Inc.), diluted 1:100. Then, slides were incubated with an anti-

rabbit EnVision system-labeled polymer for 30 min, washed in buffer solution, and visualized with diaminobenzidine. To perform macrophage and polymorphonuclear (PHN) immunohistochemistry, sections were incubated for 2 h at 37 °C and overnight at 4 °C with a rat anti-mouse neutrophils (Serotec) or a rat anti-mouse F4/80 (Serotec), diluted 1:50. After that, slides were incubated with a goat anti-rat secondary antibody diluted 1:50. Sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted in Entellan®. The total number of each cellular profile was referred to the area of white matter analyzed. This area was calculated in each section using Image Tool HUCA software. For double immunofluorescence, spinal cords were isolated, fixed in 4% paraformaldehyde, and embedded in OCT. Cryosections were blocked with 20% serum in PBS and 0.2% Triton X-100 for 30 min. Then, slides were incubated overnight at 4 °C with a rat monoclonal antibody to mouse Ly6G (BD Pharmingen) diluted 1:25. After washing with PBS, samples were incubated for 1 h at room temperature with Alexa Fluor 488-goat anti-rat IgG secondary antibody diluted 1:100. A primary antibody against MMP-8 (35) was added to the slides diluted 1:3000 in blocking buffer and incubated for 1 h. Finally,

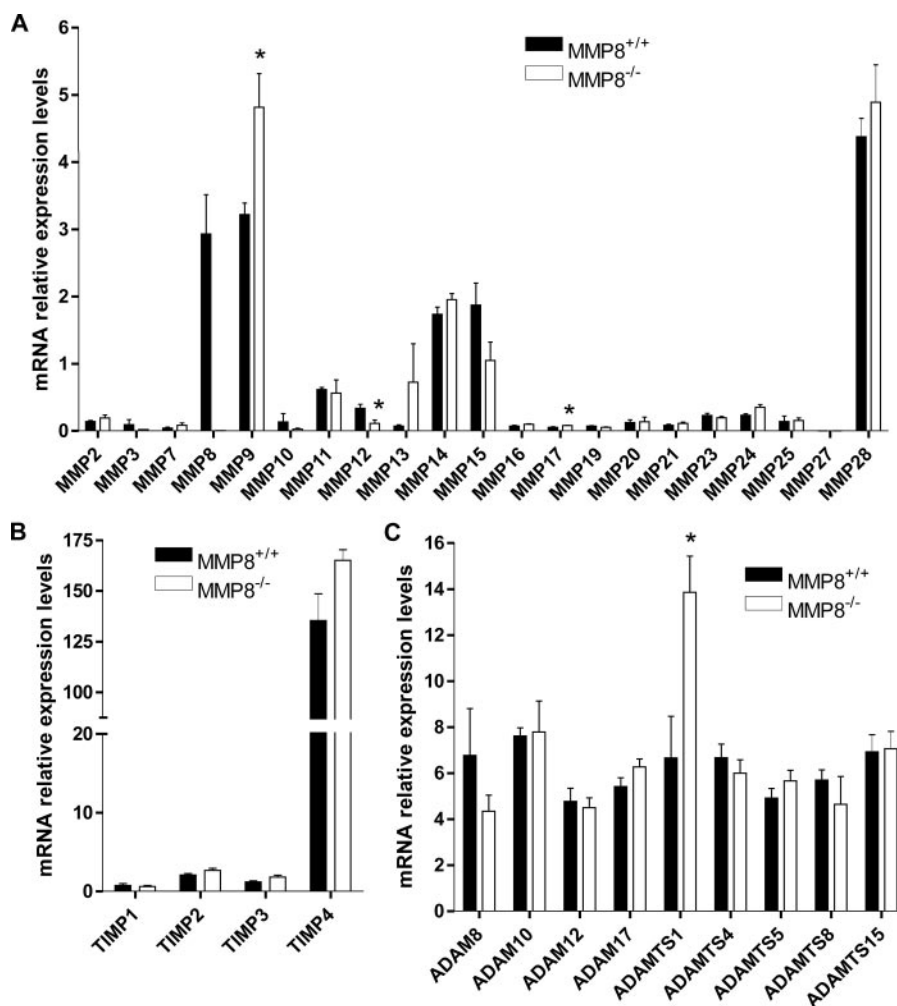


FIGURE 3. Analysis of MMP, ADAM, ADAMTS, and TIMP expression levels in 4-week-old wild-type and *MMP-8^{-/-}* immunized mice. TaqMan real time PCR analysis of MMPs (A), TIMPs (B), and ADAMs and ADAMTSs (C) expressed in spinal cord samples from wild-type (black bar) and *MMP-8^{-/-}* immunized mice (white bar). mRNA levels on the y-axis are expressed relative to 18 S rRNA levels. Both forms of murine *MMP-1* were undetectable. Values are means \pm S.E. *, $p \leq 0.05$; **, $p \leq 0.01$. $n = 4$ mice per group.

samples were incubated for 1 h at room temperature with Alexa Fluor 594-goat anti-rabbit IgG secondary antibody diluted 1:2000. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Sections were examined using a Confocal-Ultra Spectral Leica TCS-SP2-AOBS microscope.

MMP and ADAM Inhibition Assays—MMP inhibition assays have been previously described (36). Recombinant ADAM-10 and ADAM-17 were purchased from R&D Systems. For assaying ADAMs, the inhibitor (*R*)-1-(3'-methylbiphenyl-4-sulfonylamino)methylpropylphosphonic acid stock solution (100 mM) was further diluted at six different concentrations (0.01 nM to 10 μ M) in the fluorometric assay buffer (25 mM Tris, pH 8.0, 25 μ M ZnCl₂, 0.005% Brij-35). The enzyme (final concentration of 19 nM for ADAM-10 and 1.4 nM for ADAM-17) and inhibitor solutions were incubated in the assay buffer for 4 h at 25 °C. After addition of 5 μ M (final concentration) of the fluorogenic substrate Mca-Pro-Leu-Ala-Gln-Ala-Val-Dap (Dpn)-Arg-Ser-Ser-Ser-Arg-NH₂ (Bachem), the hydrolysis was monitored recording the increase of fluorescence ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 405$ nm) using a LS55 spectrofluorometer from PerkinElmer

Life Sciences. The assays were performed in duplicate in a total volume of 100 μ l per well in 96-well microtiter plates (Nunc). The percentage of inhibition was calculated from control reactions without the inhibitor. IC₅₀ was determined using the formula: $V_i/V_0 = 1/(1 + [I]/IC_{50})$, where V_i is the initial velocity of substrate cleavage in the presence of the inhibitor at concentration $[I]$ and V_0 is the initial velocity in the absence of the inhibitor. Results were analyzed using GraphPad Software.

Statistical Analysis—Values shown are mean \pm S.E. Comparison of clinical scores, cytokine production levels, and cellular profiles between the various treatment groups were analyzed by using two-tailed Student's *t* test. A value of $p \leq 0.05$ was considered significant. Statistically significant differences are shown with asterisks.

RESULTS

MMP-8^{-/-} Mice Are More Resistant to EAE—To investigate the possible contribution of MMP-8 to the initiation and progression of EAE, wild-type and *MMP-8*-deficient mice were immunized with the encephalitogenic peptide MOG_{35–55} and scored according to the severity of their symptoms. A total of 66 female mice (8–10 weeks old, *MMP-8^{+/+}*, $n = 32$, *MMP-*

8^{-/-}, $n = 34$) were immunized in three independent experiments, and the results of a representative experiment are shown in Fig. 1A. Although the disease onset is similar in both groups, after day 20 from the immunization, the clinical scores observed in *MMP-8^{-/-}* mice were significantly reduced during the chronic phase of the disease. Also, the maximal disease score was diminished in *MMP-8^{-/-}* mice compared with wild type (wild type = 3.4 versus knock-out = 2.1, $p = 0.09$). Considering that the lack of MMP-8 could be compensated in the adult life by other members of the MMP family or by alternative proteolytic pathways, we also induced EAE in 4-week-old wild-type ($n = 7$) and *MMP-8^{-/-}* mice ($n = 8$). As shown in Fig. 1B, the severity and the onset of the disease were significantly reduced in young *MMP-8^{-/-}* compared with young wild-type mice. As expected, the maximal disease severity score of young *MMP-8^{-/-}* mice (wild type = 2.5 versus knock-out = 0.8, $p \leq 0.05$) was also diminished in comparison to that obtained with *MMP-8^{+/+}* young mice. The greatest differences between wild-type and *MMP-8^{-/-}* mice EAE susceptibility obtained in this experiment suggest the existence of a compensatory mech-

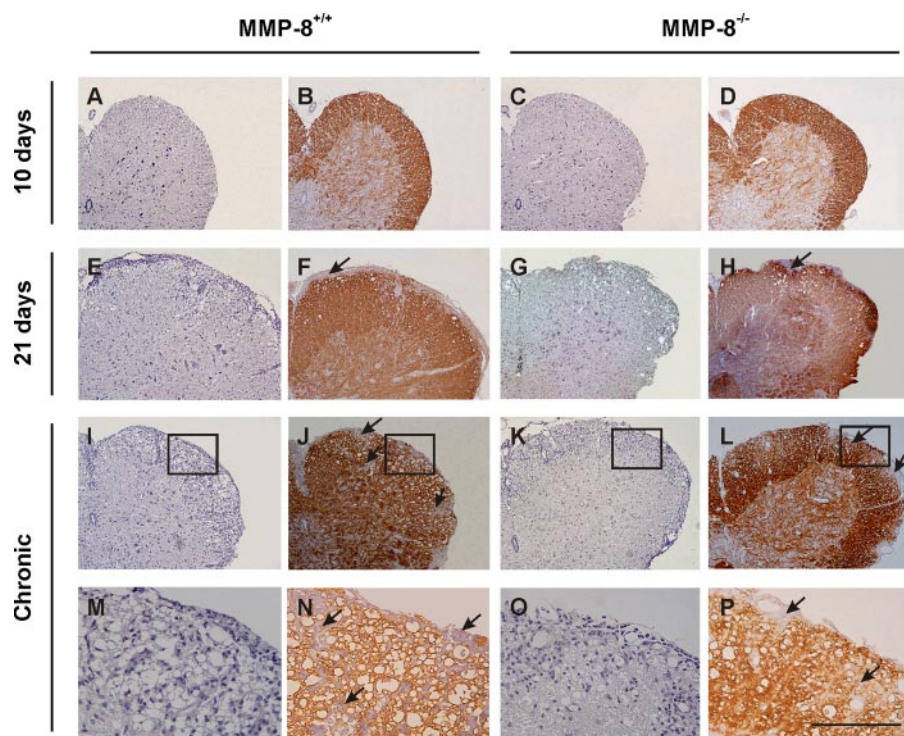


FIGURE 4. Diminished CNS inflammation during the chronic phase of EAE in $MMP-8^{-/-}$ mice immunized with MOG₃₅₋₅₅. Hematoxylin and eosin staining (A, C, E, G, I, K, M, and O) and myelin basic protein immunohistochemistry (B, D, F, H, J, L, N, and P) of spinal cords from 8-week-old $MMP-8^{+/+}$ and $MMP-8^{-/-}$ mice immunized with MOG₃₅₋₅₅. There is no inflammation in CNS samples from $MMP-8^{+/+}$ and $MMP-8^{-/-}$ mice sacrificed on day 10 post-immunization (top panel). Typical multifocal inflammation in the leptomeninges, around blood vessels, and incipient demyelinating lesions (indicated by arrows) are observed in $MMP-8^{+/+}$ and $MMP-8^{-/-}$ mice sacrificed on day 21 post-immunization. Representative tissue sections from $MMP-8^{+/+}$ and $MMP-8^{-/-}$ mice sacrificed during the chronic phase of the disease (I–L) show vacuolization in the white matter, parenchymal inflammation, and severe demyelination in $MMP-8^{+/+}$ mice, whereas inflammatory lesions are reduced in $MMP-8^{-/-}$ mice. Magnification, 10 \times . Scale bar, 100 μ m. A higher magnification image of the tissue lesion, indicated by a rectangle, is shown (M–P). Magnification, 40 \times . Scale bar, 100 μ m.

anism in the adult life. To evaluate this possibility, we analyzed the expression levels of all murine MMPs and their endogenous inhibitors (TIMPs), as well as selected members of the structurally related families of ADAMs and ADAMTSs, in the spinal cords of 8-week-old ($n = 4$ mice per group) and 4-week-old ($n = 4$ mice per group) wild-type and $MMP-8^{-/-}$ immunized mice, sacrificed during the chronic phase of the disease. Interestingly, the relative expression level of $MMP-8$ was one of the highest in the wild-type diseased mice (Figs. 2A and 3A). This analysis revealed that there are six genes, $MMP-9$, $MMP-12$, $MMP-15$, $MMP-17$, $MMP-24$, and $MMP-28$, whose expression levels were significantly different between $MMP-8^{+/+}$ and $MMP-8^{-/-}$ 8-week-old mice. However, three of them, $MMP-9$, $MMP-12$, and $MMP-17$, also showed different expression patterns in young mice (Figs. 2A and 3A). These results suggest that the occurrence of compensatory events, elicited by the lack of $MMP-8$, may have appeared during earlier stages of knock-out mice development. By contrast, there are certain genes whose expression pattern is markedly different between 8- and 4-week-old $MMP-8^{-/-}$ mice. Thus, $MMP-15$, $MMP-24$, and $MMP-28$ were significantly up-regulated in adult $MMP-8^{-/-}$ mice compared with wild type, whereas there were not significant differences between their expression levels in $MMP-8^{+/+}$ and $MMP-8^{-/-}$ 4-week-old mice (Figs. 2A and 3A). In an opposite way, $ADAMTS1$ expression levels were significantly up-

regulated in $MMP-8^{-/-}$ 4-week-old mice compared with wild-type, whereas no difference was observed between $MMP-8^{+/+}$ and $MMP-8^{-/-}$ adult mice. The relative expression levels of the four TIMPs and the analyzed ADAMs remained unaltered in both groups (Figs. 2B, 2C, 3B, and 3C). Altogether, these findings confirm the existence of different expression patterns between 8- and 4-week-old $MMP-8^{-/-}$ mice that may contribute to explain the differences observed between the EAE susceptibility of adult and young $MMP-8^{-/-}$ mice.

Reduced Inflammation and Demyelination in $MMP-8^{-/-}$ EAE Mice—To evaluate whether $MMP-8$ may contribute to activated cell recruitment and CNS inflammation, histopathological examination of spinal cord and brain samples from 8-week-old wild-type and $MMP-8^{-/-}$ immunized mice was performed at different times in the course of the disease. We first analyzed samples extracted 10 days after the initiation of the experiment, before any clinical symptoms were observed. As expected, no detectable lesions were present at this time point in both wild-type and $MMP-8^{-/-}$ mice (Fig. 4, A–D). In contrast, samples extracted during the acute phase of the disease (21 days) showed a marked increase in the number of infiltrating cells, although the demyelinating lesions were still very limited. Despite the fact that this time marks the point of divergence in EAE progression between wild-type and $MMP-8^{-/-}$ mice, as the severity of EAE symptoms continues to increase in wild-type animals while is maintained in $MMP-8^{-/-}$ mice, no histological differences were observed between both groups (Fig. 4, E–H).

Finally, we analyzed CNS tissues from wild-type and $MMP-8^{-/-}$ mice sacrificed at the chronic phase of the disease. Interestingly, although animals were chosen with similar clinical symptoms in both genotypes, the extent of the inflammatory infiltrates were significantly reduced in knock-out tissues compared with wild-type counterparts (wild type = $334.1 \pm 57.2 \mu$ m, $n = 3$, versus knock-out = $125.0 \pm 39.5 \mu$ m, $n = 3$, $p \leq 0.05$) (Fig. 4, I, K, M, and O). In addition, myelin basic protein immunohistochemistry revealed extensive demyelinating lesions in the wild-type tissues, while demyelination was less severe in $MMP-8^{-/-}$ mice (Fig. 4, J, L, N, and P). A detailed analysis of the cellular types present in the inflammatory lesions revealed that, despite the fact that T lymphocytes were the predominant subpopulation in the parenchyma infiltrates, a great number of macrophages and neutrophils were also detected in

Impaired Autoimmune Encephalomyelitis in *MMP-8*^{-/-} Mice

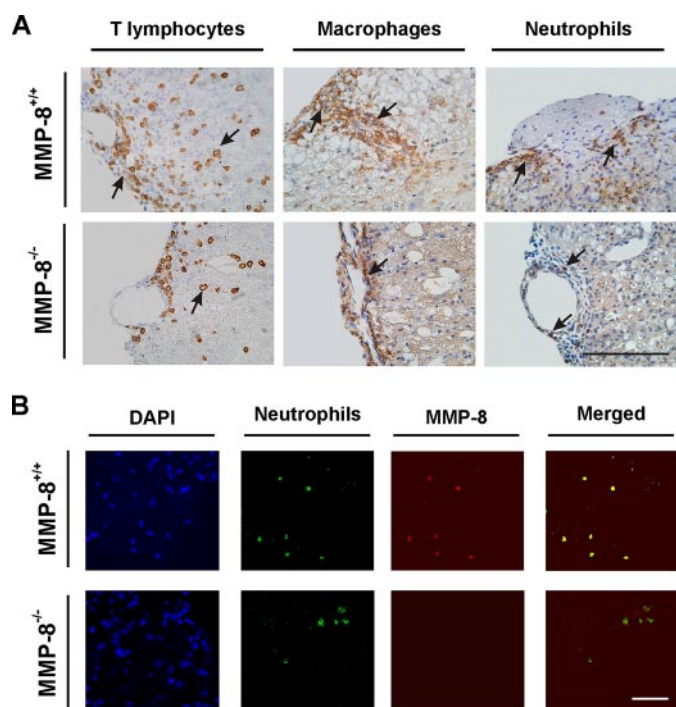


FIGURE 5. Cellular profiles and specific localization of MMP-8 in the inflammatory infiltrates of mice subjected to EAE. *A*, representative immunostaining showing the presence of T lymphocytes (*left panels*), macrophages (*middle panels*), and neutrophils (*right panels*) in spinal cords from 8-week-old *MMP-8*^{+/+} and *MMP-8*^{-/-} mice immunized with MOG_{35–55} and sacrificed during the chronic phase of the disease. Magnification, 40 \times . Scale bar, 100 μ m. *B*, immunofluorescence reveals Ly6G-positive neutrophils that colocalize with specific MMP-8 immunostaining in spinal cords from EAE-induced *MMP-8*^{+/+} and *MMP-8*^{-/-} mice sacrificed during the chronic phase of the disease. Magnification, 63 \times . Scale bar, 30 μ m.

TABLE 1

Cellular profiles in spinal cords of 8-week-old *MMP-8*^{+/+} and *MMP-8*^{-/-} mice subjected to EAE

Data represent the number of each cell type per mm² of white matter. Values are means \pm S.E. (*n* = 4 mice per group).

	T lymphocytes	Macrophages	Neutrophils
<i>MMP-8</i> ^{+/+}	410.2 \pm 67.5	102.1 \pm 50.6	28.7 \pm 11.8
<i>MMP-8</i> ^{-/-}	265.6 \pm 84.9	29.9 \pm 9.1	16.9 \pm 7.3

wild-type mice. The same cellular profiles were observed in *MMP-8*^{-/-} mice, although the number of inflammatory cells was markedly reduced (Fig. 5*A* and Table 1). To determine the cellular source of MMP-8, we performed immunofluorescence analysis of spinal cord samples obtained from immunized mice. These experiments revealed that neutrophils, present at the sites of inflammation, were the main source of MMP-8 (Fig. 5*B*), whereas no co-localization was observed with lymphocytes or macrophages (data not shown).

Splenocyte Activation in *MMP-8*^{-/-} Mice during EAE—Because *MMP-8*^{-/-} mice exhibit both marked reduction in the extent of inflammatory/demyelinating EAE lesions and less severe clinical symptoms, we further investigated if this phenotype was associated with alterations in the antigen-specific immune response. To this purpose, splenocytes from 8-week-old wild-type (*n* = 8) and *MMP-8*^{-/-} (*n* = 8) immunized mice were isolated at day 21 after immunization and cultured in the presence or absence of different concentrations of MOG_{35–55} peptide. Then, the secretion of TNF- α and IFN- γ , two pro-

inflammatory Th1 cytokines involved in EAE pathogenesis, was evaluated. As shown in Fig. 6 (*A* and *B*), there was no defect in the ability of splenocytes derived from *MMP-8*^{-/-} mice to secrete TNF- α or IFN- γ when compared with those from wild-type mice. In addition, we analyzed the production of Th2 cytokines such as IL-10 or IL-4 (Fig. 6, *C* and *D*) and TGF- β (*n* = 4) (Fig. 6*E*). However, no significant differences were seen in the secretion of these cytokines. In addition, we determined if the proliferative response was altered in *MMP-8*^{-/-} mice, but the culture of stimulated splenocytes with [*methyl*-³H]thymidine did not reveal significant differences between both groups (Fig. 6*F*). We next evaluated the possibility that this pattern of secretion could change during the chronic phase of the disease. Thus, we isolated splenocytes from 8-week-old wild-type (*n* = 4) and *MMP-8*^{-/-} (*n* = 3) immunized mice at day 50, and cells were stimulated as described above. As can be seen in Fig. 7, no significant differences in cytokine production were observed between wild-type and knock-out mice. However, as shown Fig. 7*F*, when we analyzed the relative increase in cytokine production during the chronic phase *versus* the average levels obtained in each group during the acute phase of the disease, we observed certain differences in the pattern of cytokine secretion between wild-type and knock-out splenocytes. Thus, IL-4 and IL-10 levels were markedly increased in splenocytes derived from *MMP-8*^{-/-} mice during the chronic phase compared with the increased production observed in the cultures derived from wild-type mice. Considering that the relative secretion of TNF- α and IFN- γ was maintained, these results suggest a greater ability of *MMP-8*^{-/-} splenic cells to increase the Th2-type response during the chronic phase of the disease.

MMP-8 Inhibition Reduces the EAE Symptoms in the Acute Phase of the Disease—To investigate whether the administration of a selective MMP-8 inhibitor (Table 2) would reduce the clinical symptoms of EAE disease, we designed an experimental procedure based on previously published results obtained with broad spectrum MMP inhibitors. To this purpose, wild-type female mice (8–10 weeks old) were subjected to EAE induction as described above. The control group was composed of 9 mice that were compared with 11 mice injected with the MMP-8 inhibitor. Treated mice were injected intraperitoneally daily with a dose of 25 mg/kg body weight of the inhibitor, starting at the time of MOG immunization. Wild-type mice daily injected with the vehicle reached similar disease scores as untreated mice (data not shown), thus ruling out any significant influence of the experimental procedure on the observed results. As shown in Fig. 8*A*, which is representative of two independent experiments, MMP-8 inhibitor treatment markedly reduces the disease severity during the early stages of EAE. This was apparent by day 21 post induction, where the mean clinical disease score of the control group was 2.6 \pm 0.6 *versus* 0.9 \pm 0.4 of the treated mice (*p* \leq 0.05). However, during the chronic phase of the disease, the severity of EAE progressively increased in the treated mice showing that MMP-8 inhibition was not effective at later time points. At this time point, mice were sacrificed and CNS tissues were histopathologically evaluated. No differences were observed in

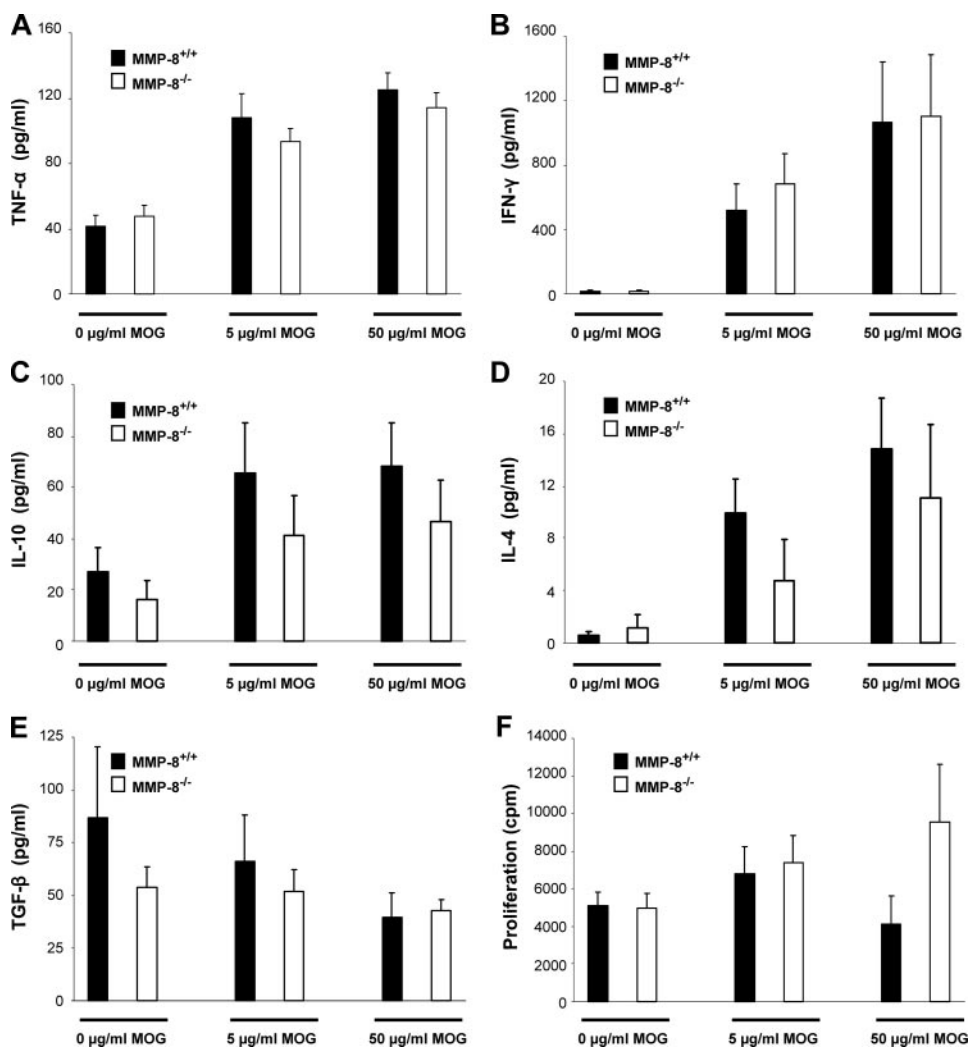


FIGURE 6. Antigen-specific cytokine production and proliferative responses of splenic cells from 8-week-old *MMP-8*^{+/+} and *MMP-8*^{-/-} mice. Splenocytes were isolated on day 21 post-immunization and stimulated *in vitro* with 0, 5, and 50 µg/ml MOG₃₅₋₅₅. Supernatants were collected at 48 h and TNF-α (A), IFN-γ (B), IL-10 (C), IL-4 (D), and TGF-β (E) concentrations were determined by enzyme-linked immunosorbent assay. To determine the proliferative responses, [³H]methylthymidine was added after 72 h of antigen stimulation, and its incorporation was measured 16 h later (F). Values are means ± S.E. *n* = 8 mice per group.

the severity of the inflammatory and demyelinating lesions (Fig. 8B).

DISCUSSION

The pathogenesis of multiple sclerosis is characterized by the massive entry of inflammatory auto-reactive T cells in the parenchyma of the CNS. Several reports have directly related the degradative actions of MMPs with the disruption of the blood-brain barrier (20, 37, 38). Thus, it has been demonstrated that the administration of MMP inhibitors reduces the transmigration of leukocytes in EAE. However, despite this pathogenic role classically attributed to the MMP family of proteases, the availability of murine models deficient in specific MMPs has demonstrated that certain members of this group of enzymes may play protective roles in neuro-inflammatory diseases (21, 22). This fact is likely a consequence of the great variety of bioactive substrates that the MMPs can proteolytically process, including cytokines, chemokines, or adhesion

molecules that modulate the recruitment of inflammatory infiltrating cells into the CNS parenchyma (27, 28). These previous findings make of special interest the analysis of the putative role of MMP-8 in MS. Interestingly, MMP-8 combines a potent collagenolytic activity with a reported ability to cleave several chemokines involved in inflammatory processes (39, 40), thereby having the potential to modulate the evolution of the disease in different ways.

To determine the beneficial or detrimental functions that MMP-8 may play in the progression of MS, we have analyzed the susceptibility of MMP-8-deficient mice to EAE. Our results have demonstrated that these mutant mice have attenuated clinical symptoms during the chronic phase of the disease when compared with their wild-type counterparts. Thus, and despite the fact that *MMP-8*^{-/-} and wild-type mice show similar disease scores during the onset and the acute phase of the disease, we have observed that *MMP-8*^{-/-} mice manifest a clear recovery at later time points. Moreover, considering the possibility that a compensatory mechanism may act in the adult life as a consequence of the absence of a certain MMP (19, 21), we performed the same study with younger mice. Interestingly, we found several differences in the expression levels of certain MMPs

and ADAMTSs between wild-type and *MMP-8*^{-/-} mice, suggesting the existence of alternative proteolytic pathways that can restore some functions of this collagenolytic MMP. One of the main candidates to perform these activities was *MMP-9*, which was markedly up-regulated in 8-week-old *MMP-8*^{-/-} mice compared with wild types, and its relevance in the pathogenesis of EAE has been largely demonstrated (15, 19, 20). However, we observed that its expression level was also increased in 4-week-old *MMP-8*^{-/-} mice, indicating that there are compensatory mechanisms but they may have appeared earlier during *MMP-8*^{-/-} mice development. These results suggest that the differences observed in EAE susceptibility between adult and young *MMP-8*^{-/-} mice derive from the differences found in the expression pattern of other genes, such as *MMP-15*, *MMP-24*, *MMP-28*, or *ADAMTS1*; however, to date, the particular contribution of these genes to the development of this disease remains unclear.

Impaired Autoimmune Encephalomyelitis in $MMP-8^{-/-}$ Mice

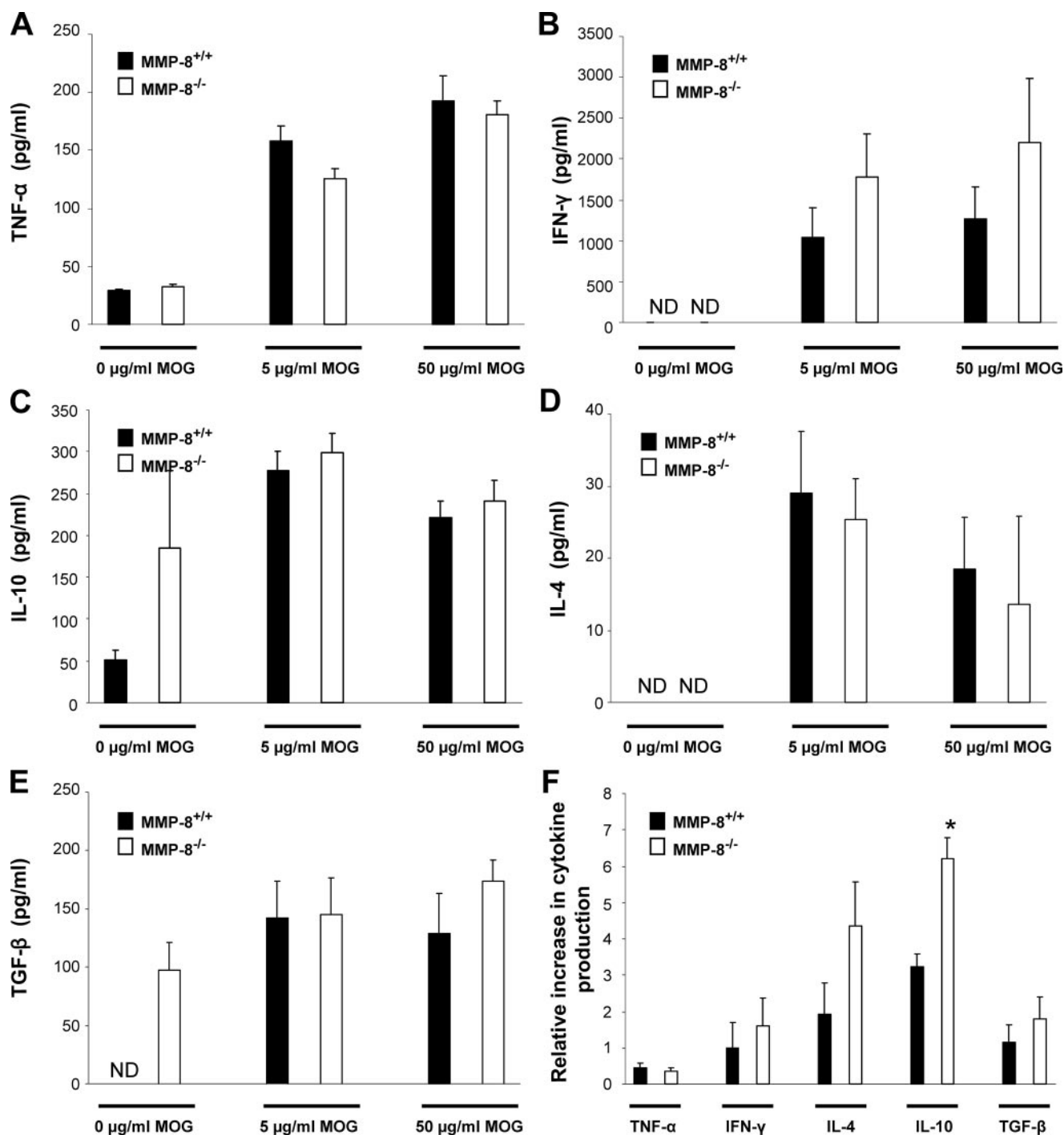


FIGURE 7. Increased Th2-type cytokine production in splenocytes from $MMP-8^{-/-}$ mice during the chronic phase of EAE. Splensins from 8-week-old $MMP-8^{+/+}$ ($n = 4$) and $MMP-8^{-/-}$ ($n = 3$) mice with representative disease scores were isolated during the chronic phase of the disease. Splenic cells were cultured and stimulated *in vitro* with 0, 5, and 50 $\mu\text{g/ml}$ MOG₃₅₋₅₅. Supernatants were collected at 48 h and the production of TNF- α (A), IFN- γ (B), IL-10 (C), IL-4 (D), and TGF- β (E) was determined by enzyme-linked immunosorbent assay. ND, not detectable. The relative increase in cytokine production during the chronic phase was calculated by comparison with the average of cytokine production obtained in each group during the acute phase of the disease. These results correspond to the values obtained with cultured splenocytes stimulated with 5 $\mu\text{g/ml}$ MOG₃₅₋₅₅ (F). Values are means \pm S.E. *, $p \leq 0.05$.

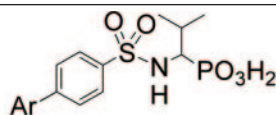
The histopathological analysis of the spinal cord and brain parenchyma revealed a marked correlation between the observed clinical symptoms and the degree of inflammation. Thus, samples taken during the acute phase of the disease showed similar meningeal inflammatory foci in both wild-type and $MMP-8^{-/-}$ mice. However, during the chronic phase of the disease, the severity of the infiltrating and demyelinating


lesions was markedly reduced in $MMP-8^{-/-}$ mice. Considering that a large number of neutrophils was present at the sites of inflammation and that it has been reported that these cells play a key role in the recruitment of inflammatory cells during the effector phase of the disease (41), our results suggest that MMP-8 may contribute to this regulatory function of the immune response once the inflammation has been initiated.

TABLE 2

Inhibitory activity of (*R*)-1-(3'-methylbiphenyl-4-sulfonylamino)methylpropylphosphonic acid against MMPs and ADAMs

IC₅₀ (nM); errors are in the range of 5–10% of the reported value.



No.	Ar	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-13	MMP-14	ADAM-10	ADAM-17
(<i>R</i>)-14i		320	24	230	1100	0.4	64	15	26	>10000	>10000

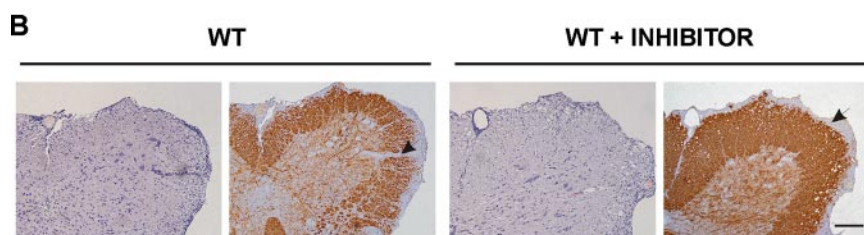
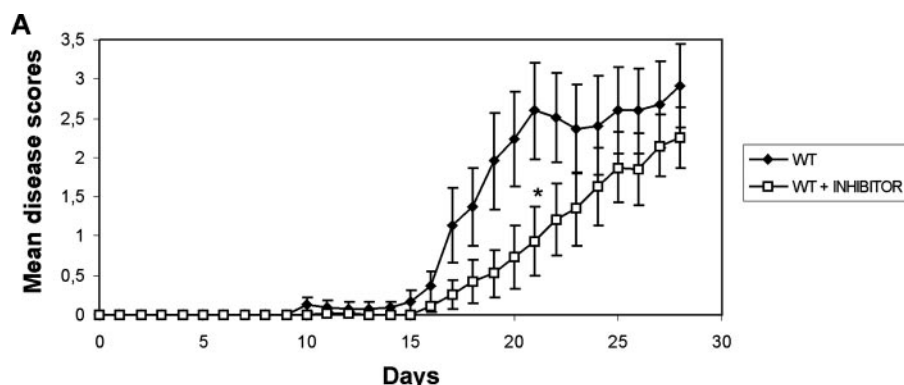


FIGURE 8. Pharmacological inhibition of MMP-8 attenuates clinical severity of EAE during the acute phase of the disease. Eight-week-old wild-type mice were immunized with MOG_{35–55} and injected intraperitoneally daily with a dose of 25 mg/kg body weight of an MMP-8 inhibitor, starting at the time of MOG immunization (*n* = 9). This group was compared with a control group of wild-type mice immunized with MOG_{35–55} (*n* = 11). Mice were monitored daily and scored for clinical symptoms of EAE. Results are expressed as mean disease score ± S.E. *, *p* ≤ 0.05. *A*, representative hematoxylin and eosin staining (*first and third panels*) and myelin basic protein immunohistochemistry (*second and fourth panels*) of spinal cords from control and treated mice sacrificed during the chronic phase of the disease (*B*). The data shown are representative of two independent experiments. Magnification, 10×. Scale bar, 100 μm.

Consistent with this possibility, the analysis of the antigen-specific responses of splenocytes isolated from wild-type and *MMP-8^{-/-}* mice revealed a greater increase in certain immunomodulatory cytokines in the *MMP-8^{-/-}* cultures during the chronic phase of the disease, compared with the response observed in wild-type samples. Thus, levels of IL-10, which is critical in the regulation of EAE progression (42), are significantly increased in splenocytes from *MMP-8^{-/-}* mice at this chronic phase, suggesting that the absence of MMP-8 may affect Th1/Th2 polarization responses in *MMP-8^{-/-}* splenic cells during the chronic phase of EAE.

The results described herein with *MMP-8^{-/-}* mice and the EAE model should be consistent with the idea that therapeutic inhibition of MMP-8 may reduce the clinical symptoms of the treated mice. In agreement with this proposal, we have demonstrated in this work that the administration of an MMP-8-selective inhibitor markedly reduces the severity of EAE. Interestingly, the protective effect of the inhibitor was more

pronounced in the early stages of the disease, whereas data from mutant mice indicate a critical role for the protease at later stages during the disease development. A possible explanation for these results derives from the temporal window in which the proteolytic function of MMP-8 is targeted. Thus, in *MMP-8^{-/-}* mice, the absence of the protease encoded by the targeted gene can induce compensatory or adaptive mechanisms during the embryonic development until adult life in an attempt to replace the functions provided by this enzyme. However, this response is limited when the inhibition is performed in a particular moment during the adult life, as is the case of pharmacological interventions like that based on the administration of the MMP-8-selective inhibitor used in this study. We have also to consider that this inhibitor, although it mainly targets MMP-8, can also block the activity of other related MMPs. Accordingly, additional studies with MMP-

8-specific inhibitors currently unavailable as well as with animal models deficient in other MMPs will be necessary to define the precise relevance of MMP-8 in the pathogenesis of MS.

In conclusion, our results provide the first causal evidence that MMP-8 plays an important role in EAE development and open a novel therapeutic possibility to reduce the clinical symptoms of human neuro-inflammatory diseases by specific targeting of this member of the large and complex family of matrix metalloproteinases.

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Impaired Autoimmune Encephalomyelitis in MMP-8^{-/-} Mice

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