The FASEB Journal express article 10.1096/fj.04-2030fje. Published online August 4, 2005.

An elevated matrix metalloproteinase (MMP) in an animal model of multiple sclerosis is protective by affecting Th1/Th2 polarization

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ABSTRACT

Inflammation in multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), is manifested by changes in matrix metalloproteinase (MMP) expression and in the ratio of T helper (Th) 1 and 2 effector cytokines. Here, we provide a comprehensive documentation of MMPs in EAE and report that of all the MMPs that could be measured at peak disease in spinal cord tissue, MMP-12 was the most highly up-regulated. In contrast to previously published findings of MMPs in EAE, this increase in MMP-12 expression was associated with protection, as MMP-12 null mice had significantly worse maximum severity and EAE disease burden compared with wild-type (WT) controls. When spleen and lymph node cells were removed from EAE-afflicted WT and MMP-12 null mice at the same disease score before divergence of disease and restimulated in vitro, the MMP-12 null cells had significantly higher Th1 to Th2 cytokine ratio. Measurements of the transcriptional regulators of T cell polarization revealed that MMP-12 null cells had increased T-bet and reduced GATA-3 expression, a condition that favors a Th1 bias. These results emphasize that specific MMPs can have beneficial roles in inflammation, and they implicate MMPs in T effector polarization for the first time.

Key words: autoimmunity • EAE/MS • cytokines • proteases • neuroimmunology

Multiple sclerosis (MS) and one of its animal models, experimental autoimmune encephalomyelitis (EAE), are diseases of the central nervous system (CNS) that are influenced by the particular pattern of cytokine expression characteristic of T helper (Th) 1 effector subsets. In addition, recent evidence has implicated the matrix metalloproteinases (MMPs) in the pathogenesis of MS and EAE (reviewed in refs 1, 2).

T cell polarization has been the subject of intense research since the initial characterization of CD4+ Th1 and Th2 effector cell populations by Mossman et al. (3). These subsets have

differential roles (4); the Th1 effector subset, traditionally responsible for the production of proinflammatory cytokines such as interferon-gamma (IFN-γ), has been implicated in the pathogenesis of autoimmunity. Conversely, the Th2 cytokines, including interleukin (IL)-5, are important in parasitic infections as well as the development of asthma. In addition, T helper lymphocyte polarization can result in repression of the opposite effector cytokine expression, such that Th2 cells have the ability to balance the Th1 cytokines and alleviate the severity of autoimmune diseases.

MMPs are a family of 23 zinc-bound proteases that, collectively, have the capability to degrade all protein components of the extracellular matrix (ECM). For this reason, the initial roles attributed to MMPs have been in processes that require remodeling of the ECM, such as developmental events, wound healing, and tumor metastasis (5). However, recent evidence has implicated MMPs in more complex roles, including apoptosis, cell signaling, and immune regulation (5, 6).

MMPs are implicated in MS and EAE pathology (1, 2). Increased expression of various MMP members (MMP-2, -3, -7, -9, and -14) has been found in the serum, cerebrospinal fluid, and autopsied brains of patients with MS, compared with controls (7–9). In EAE, MMP–3, -7, -8, -9, -12, and -14 are up-regulated and expression of some has been correlated with disease severity (10–13). Young MMP-9 null mice are less susceptible to EAE induction than WT mice (14). In support of a pathogenic role of MMPs in EAE, synthetic inhibitors of metalloproteinases attenuate the EAE disease course (12, 13, 15, 16). In MS, one mechanism attributed to the efficacy of interferon-β is the inhibition of MMP production by T lymphocytes (17). Overall, it is thought that MMPs contribute to MS and EAE pathology through a variety of mechanisms, including leukocyte trafficking, blood-brain barrier disruption, and cleavage of myelin proteins (1, 2).

Currently, most research on MMPs in EAE has focused on the detrimental roles of a very narrow spectrum of MMP family members. Whether other MMPs are involved and whether MMPs have beneficial functions in the disease are unknown. In addition, no research has addressed a link between MMP activity and the polarization of the Th1 and Th2 effector T cell subsets. In this manuscript, by using the myelin oligodendrocyte glycoprotein (MOG)-induced model of relapsing-remitting EAE in 129/SvEv mice, we have analyzed the expression of 22 of 23 murine MMP family members during peak EAE severity. We have found that MMP-12 (metalloelastase) is highly up-regulated at peak disease course. Unexpectedly, this protease plays an important protective role in attenuating EAE, since mice lacking MMP-12 have a significantly worse disease outcome than wild-type (WT) mice. Finally, we demonstrate that splenocytes and lymph node cells from MMP-12-deficient animals during disease remained highly Th1 polarized, whereas WT cells at the same disease score had a higher Th2 to Th1 cytokine ratio. These data indicate for the first time that an up-regulated MMP member is a protective molecule in EAE and that MMP-12 functions in part by modulating the Th1/Th2 effector cytokine balance. M

MATERIALS AND METHODS

Induction of relapsing-remitting MOG EAE

Female 129/SvEv mice, aged 8-10 wk, were induced for EAE using MOG_{35-55} in complete Freunds adjuvant (CFA); in this strain of mice, this protocol produces a relapsing-remitting

model of disease. Animals were injected subcutaneously in their flanks, at day 0, and again at day 7, each with 100 μg of MOG_{35-55} in 100 μl of CFA, supplemented with an additional 4 mg/ml of *Mycobacterium tuberculosis* (H37Ra) as described previously (12). No pertussis toxin was used.

Daily assessments of disease in mice

Animals were evaluated daily using a scoring system that makes the assessment of disease in 129/SvEv mice more responsive to change. This new scale ranges from 0 to 15 and is the sum of the state of the tail and all of the four limbs. For the tail, a score of 0 reflects no signs, 1 represents a half paralyzed tail, while a score of 2 is given to a mouse with a fully paralyzed tail. For each of the hind- or forelimbs, each assessed separately, 0 signifies no signs, a score of 1 is a weak or altered gait, 2 represents paresis, while a score of 3 denotes a fully paralyzed limb. Thus, a fully paralyzed quadriplegic animal would attain a score of 14. Mortality equals a score of 15. Typically, a mouse undergoing an attack would first have loss of function in the tail followed by one of the hindlimbs; therefore, a low score of 3 or 4 indicates tail involvement and a weak hindlimb, akin to Grade 2 on the commonly used 5 point scale (12). The ability of our scoring method to differentiate between animals is highlighted by an example of two animals where one remains at a score of 12 (both hindlimbs and tail are fully paralyzed and forelimbs are partially paralyzed) and another that has remitted from 12 to a score of 7 (where the animal has recovered function in both hindlimbs and forelimbs but not completely). Both of these states would be given a score of 4 by the old scoring method (12), because the forelimbs were involved even though clinically these animals behaved very differently. For this reason, the new scoring method was preferred. For studies at peak disease, animals were killed at a point when disease signs were no longer worsening and animals were just beginning to show signs of remission.

RNA analyses

Immediately after death, the lumbar/sacral spinal cord was removed into 1 ml of Trizol® and the total RNA was then extracted. An RNase protection assay (RPA) was first used to compare the regulation of MMPs in control and EAE animals (129/SvEv WT mice) at peak disease. A probe set with nine MMPs, containing the ribosomal protein RPL32-4A (or L32) as an internal loading control, was employed as described previously (12).

TaqMan polymerase chain reaction (PCR) was also used to profile mRNA levels when animals reached peak disease, since this allowed MMPs not included in the RPA to be analyzed. One microgram of RNA was reverse transcribed to make cDNA. Only WT samples were used for these analyses, with four normal nonimmunized animals, and six animals at peak EAE disease. All primers and methodology have been described previously (18, 19). The 18S rRNA gene was used as an endogenous internal control. The cycle number was recorded as the fluorescence crossed a threshold, determined by the linear phase of replication, and relative amounts were determined using a standard curve and twofold serial dilutions covering the range of 20-0.625 ng of RNA (for 18S analyses, the range was from 4 to 0.125 ng). Ranges were determined using a precalculated standard curve developed from the measurement of individual molecules of each RNA species.

Localization of the source of MMP-12 in EAE

Mice at peak EAE were killed, and spinal cord was removed for in situ hybridization for MMP-12 transcripts as described previously (19). The cellular source of the MMP-12 signal was achieved by immunohistochemistry using antibodies to cell type specific markers before the in situ hybridization process (19).

Comparison of MMP-12 null mice and WT in relapsing-remitting EAE

MMP-12 null mice on a 129/SvEv background were used for MOG EAE to analyze phenotypic characteristics in comparison to WT mice on the same background. The MMP-12 null mice have been previously detailed (20) and are on the 129/SvEv background. MMP-12 null mice and WT 129/SvEv animals were genotyped using PCR of tail DNA and by casein zymography for protein expression (data not shown). Histological examinations of the spinal cords were performed as described previously for indications of inflammation and neural changes (12). The spinal cords from five mice of each group were analyzed at their respective peak disease.

Isolation of splenocytes and lymph node cells

To examine whether the lack of MMP-12 would affect the polarization and cytokine expression in T cell subsets of mice induced with EAE, both WT and MMP-12 null mice were induced with EAE as described above. At a clinical score of 4, akin to Grade 2 on the commonly used EAE scale (12), the spleen and draining lymph nodes (LN) from four animals were removed and ground together into a suspension in sterile PBS. The splenocyte/LN cell (LNC) population was isolated using a Ficoll gradient (21). Isolated splenocytes were plated in triplicate at 5×10^6 cells/ml in enriched RPMI media in 100 μl wells of a 96-well round bottom plate (21). This clinical score of 4 was chosen, as mice of both the WT and MMP-12 null mice were still similar in signs, but this would be the point of eventual divergence of disease: WT mice would begin to remit after this while MMP-12 null mice would progressively worsen.

Analysis of cytokine expression in supernatants of splenocytes and LNCs

Isolated splenocytes/LNCs were either left untreated as controls, treated with MOG_{35-55} to observe antigen induced cytokine expression, or treated with concanavalin A (ConA; Sigma-Aldrich) at 5 μg/ml as a positive control (each in triplicate). Cytokine levels in total supernatants of cell cultures were assessed by ELISA for IFN-γ after 48 or 72 h of treatment and for IL-5 after 120 h, as per instructions provided with each kit (Biosource International).

Analysis of cytokine mRNA expression

Splenocytes and LNCs were treated as above and cultured in a 24-well plate at 5×10^6 cells in 1 ml of enriched RPMI media for 48 h. Cultured cells were resuspended, removed of supernatant and lysed in Trizol®. RNA was reverse transcribed to make cDNA and analyzed for cytokine and transcription factor expression using SYBR®-green real-time PCR analyses. The primers (forward and reverse) were as follows: IFN-γ (ACTGGCAAAAGGATGGTGAC and TGAGCTCATTGAATGCTTGG), IL-5 (GAAGTGTGGCGAGGAGAGA and GCACAGTTTTGTGGGGTTTT), T-bet (CAACAACCCCTTTGCCAAAG and TCCCCCAAGCAGTTGACAGT), and GATA-3 (AGAACCGGCCCCTTATCAA and AGTTCGCGCAGGATGTCC).

RESULTS

Complete spectrum of MMP alterations at peak EAE disease demonstrates a dramatic up-regulation of MMP-12

Immunization of female mice with MOG_{35-55} on the 129/SvEv strain produces a relapsing remitting disease course where initial disease signs are less predictable in onset (ranging from days 12-22; average of 14 days) than other mouse strains (data not shown). Animals primarily lose function of tail and limbs in a caudal to rostral progression, with a peak in disease averaging around day 18 and they then remit and regain most or complete function in a rostral to caudal manner. Remission may last for several days up to 2 wk before clinical signs reappear. Animals will typically repeat the disease course for two or three cycles before the disease becomes a slow steady progression.

An initial RNase protection assay (RPA) was used to survey the regulation of 9 MMPs at peak disease (Fig. 1). Although many MMPs were up-regulated, including MMP-2, -3, -9, -12, and -14, MMP-12 elevation was the most prominent. Real-time PCR (18, 19) for 22 MMPs was then performed, and quantitative expression of each MMP as a fraction of 18S rRNA demonstrated the involvement of many MMPs in EAE at peak disease (Fig. 2). We found no discrepancy between the RPA and TaqMan data. Whereas MMP-7 and MMP-10 were below the level of detection by RPA, TaqMan PCR demonstrated that these were unaltered and increased, respectively, in EAE. Some of the most prominent increases in gene expression, besides MMP-12, included MMP-3, -8, -10, -19, and -25. Interestingly, some MMPs were down-regulated in EAE compared with normals and these included MMP-15, -16, -17, -21, and -24. We also profiled the endogenous tissue inhibitors of metalloproteinases (TIMPs); TIMP-1 was elevated in EAE, TIMP-4 was unaltered, while TIMP-2 and -3 were reduced. A display of the fold increase of all MMPs and TIMPs from unaffected normal spinal cord is presented in Fig. 3.

The real-time PCR data confirmed, most notably, the massive up-regulation of MMP-12 detected through RPA, in that there was almost 1000-fold increase in the transcript encoding this MMP member in the spinal cord of EAE afflicted animals compared with normals (Fig. 3).

We determined the source of MMP-12 at peak EAE using in situ hybridization and found strong message emanating from the subpial surfaces of the spinal cord (Fig. 4). Cell type-specific immunohistochemistry demonstrates that the majority of cells expressing MMP-12 were Iba1-positive, representing cells of macrophage/microglia lineage (19, 22; Fig. 4).

MMP-12 null mice have significantly worse disease outcomes than their WT counterparts

Since MMP-12 transcript levels were notably increased during peak disease in EAE, we addressed further its involvement in EAE. We immunized MMP-12 null or WT mice with MOG and compared the resultant disease severity. Animals were followed over 73 days, during which the daily scoring was used to assess disease progress. Surprisingly, the results of the clinical scores demonstrated that MMP-12 null mice did significantly worse than their WT counterparts. While the maximum disease severity score for WT animals was an average of 5.2 ± 2.3 (means±SD; akin to Grade 2 on the commonly used 5 point EAE scale, representing hindlimb involvement), that for MMP-12 animals was nearly doubled at 9.7 ± 2.6 (Fig. 5*A*; akin to Grade 4 on the commonly used EAE scale, with forelimb and hindlimb involvement).

Because the onset of signs in 129/SvEv tended to be variable between animals, we could not accurately display the average daily disease score of groups of WT or null mice. However, each animal's disease burden over the entire experiment could be computed by adding their daily disease score to obtain the "sum of scores" for that mouse. Thus, we demonstrate that MMP-12 null mice had a significantly worse sum of scores, representing a more extensive disease burden (Fig. 5*B*). In this regard, the WT mice typically reached their peak in disease and then remitted to the point where the animals recovered full function of all four limbs and their tail. In contrast, MMP-12 null mice achieved a higher peak disease score and for longer periods of time. When disease remitted in MMP-12 null mice, most animals never fully recovered function and were left with lasting impairment and weakness in tail and hindlimbs (data not shown).

Although the onset of signs was variable amongst mice, group means indicate that both the WT and MMP-12 null mice had similar average day of onset of disease (Fig. 5*C*). Thus, the worse disease outcome in MMP-12 null was due to the course of disease after onset, rather than the time taken to succumb to disease.

The spinal cord histology was examined from mice at peak disease to determine the basis for the worse disease score in MMP-12 null mice. Figure 6 demonstrates that there was qualitatively more cellular infiltration and demyelination in MMP-12 null mice compared with WT animals. Thus, a worse disease score in animals without MMP-12 is correlated with increased inflammation and neuropathology in the CNS.

Overall, the data implicate MMP-12 as a protective enzyme in the pathology and course of EAE.

MMP-12 is involved in T effector cell polarization during MOG induced EAE

To determine the mechanisms of MMP-12 in EAE, we first addressed whether MOG immunization generates a differential antigenic response in WT and MMP-12 null mice before the onset of signs of disease. Ten days after MOG treatment, and before the appearance of symptoms, splenocytes and LNCs were isolated from mice and retreated with MOG in vitro. Measurement of the content of IFN-γ in the conditioned medium 3 days after shows that there was no difference between WT and MMP-12 null cells, either with respect to the basal non-MOG stimulated amount, or in response to MOG incubation (Fig. 7*A*). Similarly, [^{3}H]thymidine incorporation assays show that the increase in the ratio of antigen- vs. basal-proliferation of WT cells was 2.6 ± 0.6 (*n* of 4 mice, 8 wells per condition per mice), while that for MMP-12 null cells was essentially similar (2.5±0.5; $P>0.05$). These results, together with the similar time of onset of clinical signs in both genotypes, suggest that MMP-12 did not play a role in the generation of encephalogenic T cells but rather that its effect occurred after clinical signs of disease had begun.

To evaluate further the mechanisms of MMP-12 in EAE, we made note of the results that while WT mice were achieving a maximum disease score of 4-6 before the disease remitted, MMP-12 null mice at this juncture continued to become more diseased, subsequently achieving a peak score of between 8 or 10. Thus, we killed animals at a similar disease score of 4, a point of divergence between WT and MMP-12 null mice, to evaluate possible differences in cytokine expression by lymphoid cells in both groups. Specifically, we tested the hypothesis that because Th1 and Th2 effector cells are crucial to EAE disease development and resolution, the difference between the two groups could be attributed to mechanisms in the control of the Th1/Th2

polarization. WT and MMP-12 null mice were immunized with MOG and killed when they reached an EAE score of 4. Splenocytes and LNCs were removed, cultured in enriched RPMI media, and were either left untreated or were restimulated with MOG for analysis of cytokine expression. A proliferation assay first revealed that there was no significant difference between splenocytes and LNCs from MMP-12 null and WT mice after stimulation with MOG (data not shown), indicating that the precursor frequency of MOG antigen-specific cells was similar between both groups. When supernatants from cultured cells were assayed by ELISA for the Th1 cytokine, IFN-γ, we found that both the 10 and 20 μg/ml MOG-induced IFN-γ levels were significantly greater ($P<0.05$, two tailed unpaired *t* test) in the MMP-12 null samples than in the WT cells (Fig. 7*B*). IL-5 was below the level of ELISA detection.

To evaluate the Th cell polarization further, RNA was extracted from splenocytes and LNCs of mice at a disease score of 4 and examined for changes in IFN-γ and IL-5 using real-time PCR. MMP-12 null cells upon MOG antigen restimulation in vitro displayed a 14-fold increase in IFN-γ levels relative to the unstimulated cells (Fig. 8*A*; $P<0.001$, two tailed unpaired *t* test), while IL-5 was decreased ($P<0.01$, two-tailed unpaired *t* test, compared with unstimulated cells; Fig. 8*B*). We then used the ratio of IFN-γ to IL-5 for each individual mouse, which was further averaged across each group. Such analyses highlight that MMP-12 null splenocytes were significantly skewed toward a Th1 phenotype ($P<0.001$, two tailed unpaired *t* test; Fig. 8*C*). In contrast, WT splenocytes were biased toward a Th2 polarizing condition (Fig. 8*D*), and this ratio was significantly different from 1 ($P<0.05$, two tailed unpaired *t* test, compared with unstimulated WT cells). The modest ratio of Th2 to Th1 of 2 for the WT cells presumably reflects a disease stage in which the WT mice were just beginning to remit and where Th2 cytokines were just beginning to increase over those of Th1. In studies by others, EAE is highly Th1 polarized in the initial stages of disease, while Th2 cytokines predominate during the remission phases (23, 24).

Collectively, these data indicate that the mechanism by which MMP-12 is protective in EAE is accounted for, in part, by altering a Th1 to a Th2 milieu.

Transcriptional regulators of Th1/Th2 polarization are altered in MMP-12 null mice

We addressed possible mechanisms that could underlie the differences in the T effector cell polarization between WT and MMP-12 null mice. Specifically, by using splenocytes and LNCs harvested from mice at a disease score of 4, as described above, we examined the expression of the transcriptional regulators for Th1 and Th2 promotion, which are T-bet and GATA-3, respectively (25, 26). Real-time PCR analyses show that the level of T-bet transcripts was significantly increased in MMP-12 null cells after MOG stimulation ($P<0.001$, two tailed unpaired *t* test, compared with unstimulated state; Fig. 8*E*). In contrast, there was a significant decrease in GATA-3 expression after MOG stimulation in null cells ($P<0.01$ compared with unstimulated state).

Overall, the combined data from cytokine and transcription factor analyses indicate that at a disease score of 4, WT T cells are beginning to show a slight Th2 bias and mice subsequently remit. In contrast, MMP-12 null animals at this same score have increased T-bet and reduced GATA-3 expression, favoring a T effector phenotype that is highly polarized toward a Th1 type; in concordance, animals continue to worsen clinically. Therefore, the data indicate that despite similar clinical deficits at this point in the disease, WT and MMP-12 null mice have profoundly

divergent T effector cell responses driving disease pathology, thus linking an MMP to T cell polarization for the first time.

DISCUSSION

EAE is a prototypical animal model of a Th1-mediated autoimmune disease (27, 28) that shares many clinical and pathological features of MS. The differentiation of T helper cells into Th1 or Th2 effector cells is believed to be crucial to the severity and outcome of the inflammation in both diseases. Other families of inflammatory molecules, including chemokines, adhesion molecules, and MMPs, are altered in MS and EAE, and these are also thought to contribute to the disease process. A pathogenic role for MMPs in MS and EAE is attributed to the observations that inhibitors of metalloproteinase activity alleviate disease severity in EAE (12, 13, 15, 16) and to the discovery that young MMP-9 null mice are less susceptible to the induction of EAE (14). Clinically, a mechanism by which interferon-β is thought to alleviate the MS disease process is through the inhibition of MMP-9 production by T cells, thus attenuating their ability to transmigrate across barriers into the CNS (17).

A limited number of MMPs have thus far been investigated in EAE and elevations of MMP-3, -7, -8, -9, -12, and -14 have been found in the disease (10–13, 27). Whether other MMPs are likewise altered, or which MMP is a crucial regulator of the disease, is unclear. Recently, by using a comprehensive TaqMan screen, Toft-Hansen et al. (29) reported the elevation of MMP-8, -10, and -12, as well as a related member, ADAM-12, in EAE induced by the adoptive transfer of T cells. In this study, we have systematically evaluated the expression of 22 of 23 MMP members during peak EAE in MOG-immunized 129/SvEv mice. Our data reveal that many MMP members are elevated in peak EAE, while some MMP members are down-regulated during the disease process. In correspondence between the TaqMan and RPA data, MMP-12 transcripts are most abundantly up-regulated in peak EAE.

The source of MMP-12 is Iba1-positive cells (Fig. 4), representing microglia and infiltrating macrophages in the CNS (19, 22); it is currently not possible to delineate reliably between microglia and macrophages in tissue sections. The description of MMP-12 in microglia/macrophages supports that of Toft-Hansen et al. (29), who used PCR to detect MMP-12 transcripts in distinct populations of cells isolated by flow cytometry from the spinal cord of adoptive transfer EAE mice. In active demyelinating lesions of MS, Vos et al. (30) found that MMP-12 detected by immunohistochemistry was localized to phagocytic macrophages.

We note that the results of this manuscript have focused on the expression of transcripts, which may not always equate with protein expression (31). Although it is desirable to determine protein expression as well, the reagents (e.g., antibodies) to detect most nongelatinase MMPs, including MMP-12, are rudimentary and the specificity suspect. Future studies will have to define better MMP protein expression and secretion to extend the current data. It must also be kept in mind that the changes in MMP expression in EAE may reflect the profile of MMPs expressed by different leukocyte subsets, as these infiltrate into the CNS in EAE. Nonetheless, the current results do suggest MMP members, based on their high expression, for further detailed studies.

We sought to elucidate the function of MMP-12 in EAE. By using MMP-12 null mice to evaluate the significance of MMP-12 in EAE, however, we discovered that MMP-12 had a protective role in the disease, since the null mutants had a worse disease phenotype than WT

controls (Fig. 5). These data emphasize for the first time that an up-regulated MMP in EAE has a beneficial role.

Recently, there have been other reports of MMP-12 being significantly up-regulated in EAE (28, 32). Unlike the current manuscript, these authors did not further evaluate the beneficial or detrimental function of MMP-12 in EAE.

Since EAE and MS are diseases dominated by Th1 cytokines and to evaluate the disparity in disease severity between WT and MMP-12 null mice, we examined cytokine expression in splenocytes and LNCs isolated at the same disease score, before the subsequent divergence of disease scores. Individual WT animals demonstrated an attenuated Th1 response and a trend toward Th2 cytokine polarization. In contrast, MMP-12 deficient animals remained highly Th1 polarized. The basis of this Th1 polarization in MMP-12 null mice is attributed to the altered expression of the transcriptional regulators of Th1/Th2 bias: increased T-bet and decreased GATA-3. These data indicate for the first time that an MMP may function through modulating the Th1/Th2 effector cytokine balance.

We considered the possibility that the divergence of disease severity between WT and MMP-12 null mice was the result of the differential generation of encephalogenic T cells after MOG immunization. Two factors argue against this. First, we did not find evidence of a difference in the onset of disease between both groups, suggesting that the impact of MMP-12 develops after signs of disease are evident in EAE. Second, when we used splenocytes and lymph node cells from WT or MMP-12 null mice at day 10 after MOG immunization, and before disease signs were obvious, the antigen-specific response of T cells was similar between both genotypes when evaluated using either IFN-γ (Fig. 7*A*) or proliferation.

It is possible that MMP-12 has other beneficial roles in EAE apart from modulating the Th1/Th2 response. MMP-12 may regulate repair processes within the CNS, including remyelination (33); MMP-9 has been implicated in this phenomenon (34). MMPs may provide for migratory cues for various neural cell types (1) or activate neurotrophins for neural cell survival (35). As well, there is the potential for MMPs to contribute to the termination of inflammation, although such studies have not focused on MMP-12. In this regard, IL-1β can be degraded by MMP-2, - 3, and -9 (36). Cytokine receptors, such as IL-2Rα, can be cleaved from T-cells by MMP-9 (37). MMPs also have the ability to process chemokines into antagonists at chemokine receptors. The N terminus truncation of monocyte chemoattractant protein (MCP)-3 by MMP-2, -13, and -14 generated an antagonist at several chemokine receptors (38). As well, stromal-cell derived factor-1 is processed by MMP-1, -2, -3, -9, -13, and –14 to result in alteration of its properties (39). Altogether, the expression of beneficial MMPs in EAE and MS may have several modes of action by which to produce a favorable recovery response from these diseases.

Previous studies have demonstrated that the use of metalloproteinase inhibitors in EAE or MS alleviated disease severity (12, 13, 15, 16). Thus, if MMP-12 is beneficial in EAE, which MMP is detrimental to the disorder? MMP-9 is a candidate since the MMP-9 null mice are less susceptible to the development of EAE (14). Conceivably, many of the up-regulated MMPs found in this study are possible candidates, either alone or in concert, as detrimental MMPs to the EAE disease process; a systematic study to address this is clearly warranted. Our results also caution against the indiscriminate use of nonselective MMP inhibitors in MS or EAE, as detrimental as well as beneficial properties of MMPs will be similarly impaired.

We have shown that a mechanism by which MMP-12 alters Th1/Th2 polarization is through the expression of the key transcriptional factors that control Th1/Th2 effector cell development, T-bet, and GATA-3. How MMP-12 accomplishes this precisely is unknown as there is still much to learn about the detailed mechanisms that regulate T effector cell formation. Another point to consider is whether MMP-12 deficiency causes a compensatory change in levels of other MMPs, which would then result in the phenotype described in the study. In a recent study of MMP-2 null mice, the higher susceptibility of MMP-2 null mice to EAE was attributed to a deficiency of MMP-9 (40). In MMP-12 null mice, however, we have found no evidence of altered expression of transcripts for other MMPs, either in the normal state or when afflicted with EAE (data not shown).

In summary, we demonstrate for the first time that a highly up-regulated MMP in EAE, MMP-12, is protective against the disease. The results also provide a new link between two important pathways of inflammation, that of MMPs and Th1/Th2 polarization.

ACKNOWLEDGMENTS

This study was funded by the Interdisciplinary Health Research Team (IHRT) program of the Canadian Institutes of Health Research. A. Weaver was funded through a studentship from the Multiple Sclerosis Society of Canada. We thank Tammy Wilson for skilled technical assistance and Fabrizio Giuliani for intellectual feedback.

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Received April 11, 2005; accepted June 21, 2005.

Fig. 1

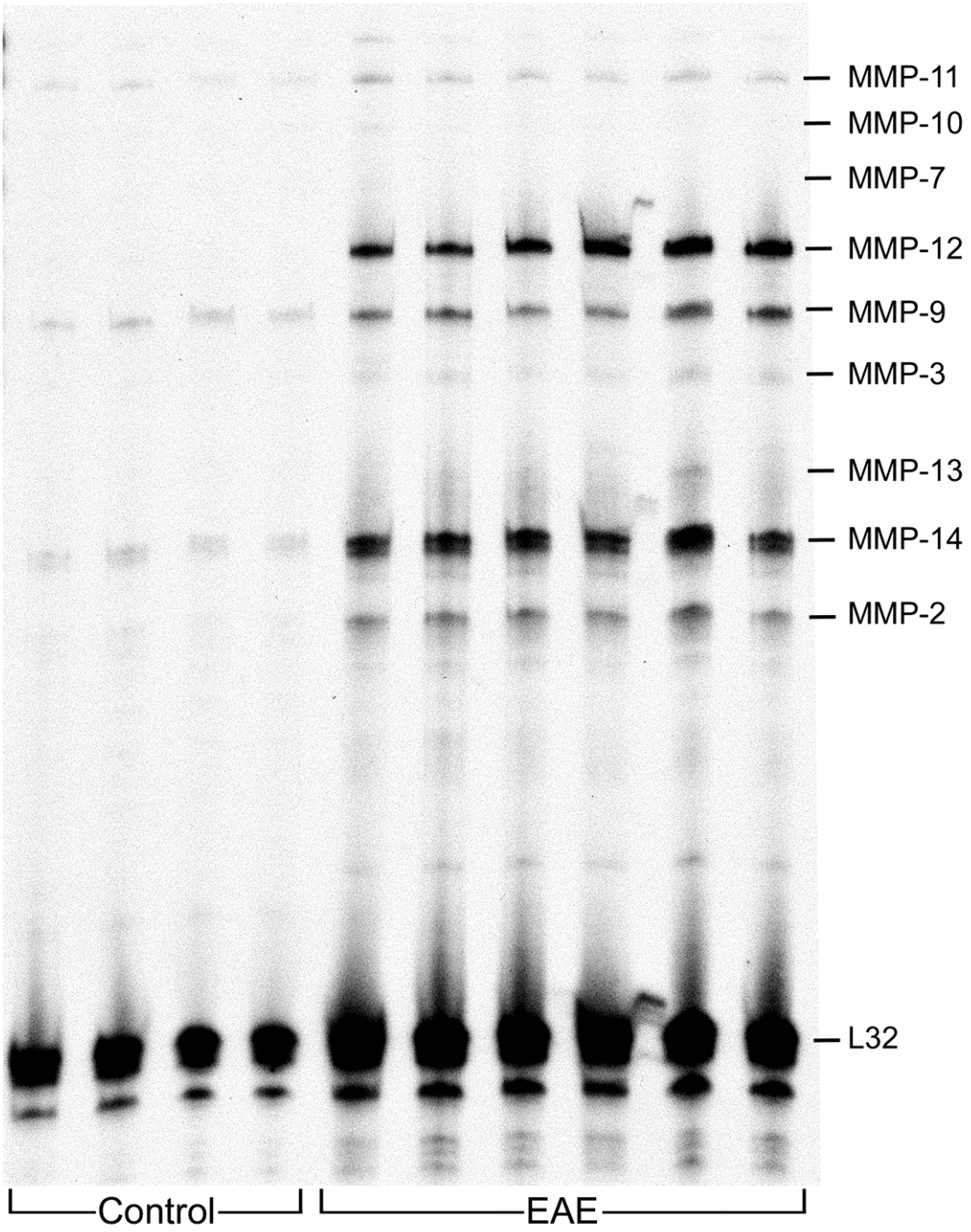

Figure 1. RNase protection assay for MMP mRNA expression at peak disease severity in 129/SvEv WT mice. Six EAE spinal cord specimens are displayed, along with 4 normal spinal cord samples as controls.

Fig. 2

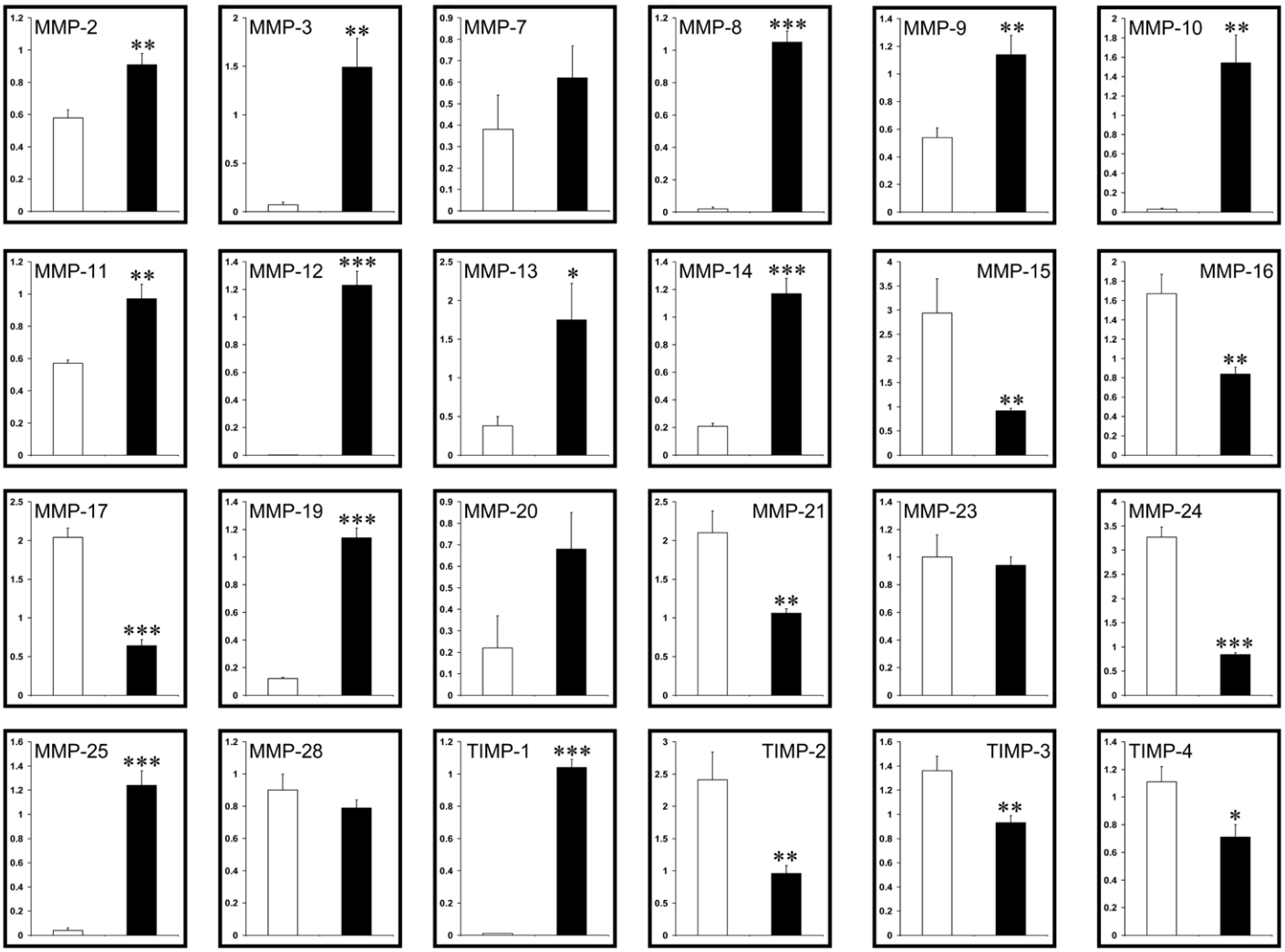

Figure 2. TaqMan real time PCR analyses of spinal cord samples from normal controls (*n* of 4, white bar) and from mice at peak EAE severity (black bar, *n* of 6). RNA levels on the *y*-axis are expressed relative to 18S rRNA levels. Values are means ± SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (2-tailed Student's *t* test). Both forms of MMP-1 were undetectable. There is no MMP-4 to -6, as members initially assigned these were subsequently found to be other MMPs. There is no human or mouse MMP-18 or -22. MMP-26 is only found in humans, and there is no mouse homologue. At the time of this analysis, the sequence for MMP-27 was unknown. Thus, of 23 MMPs in mouse, only MMP-27 was not examined.

Fig. 3

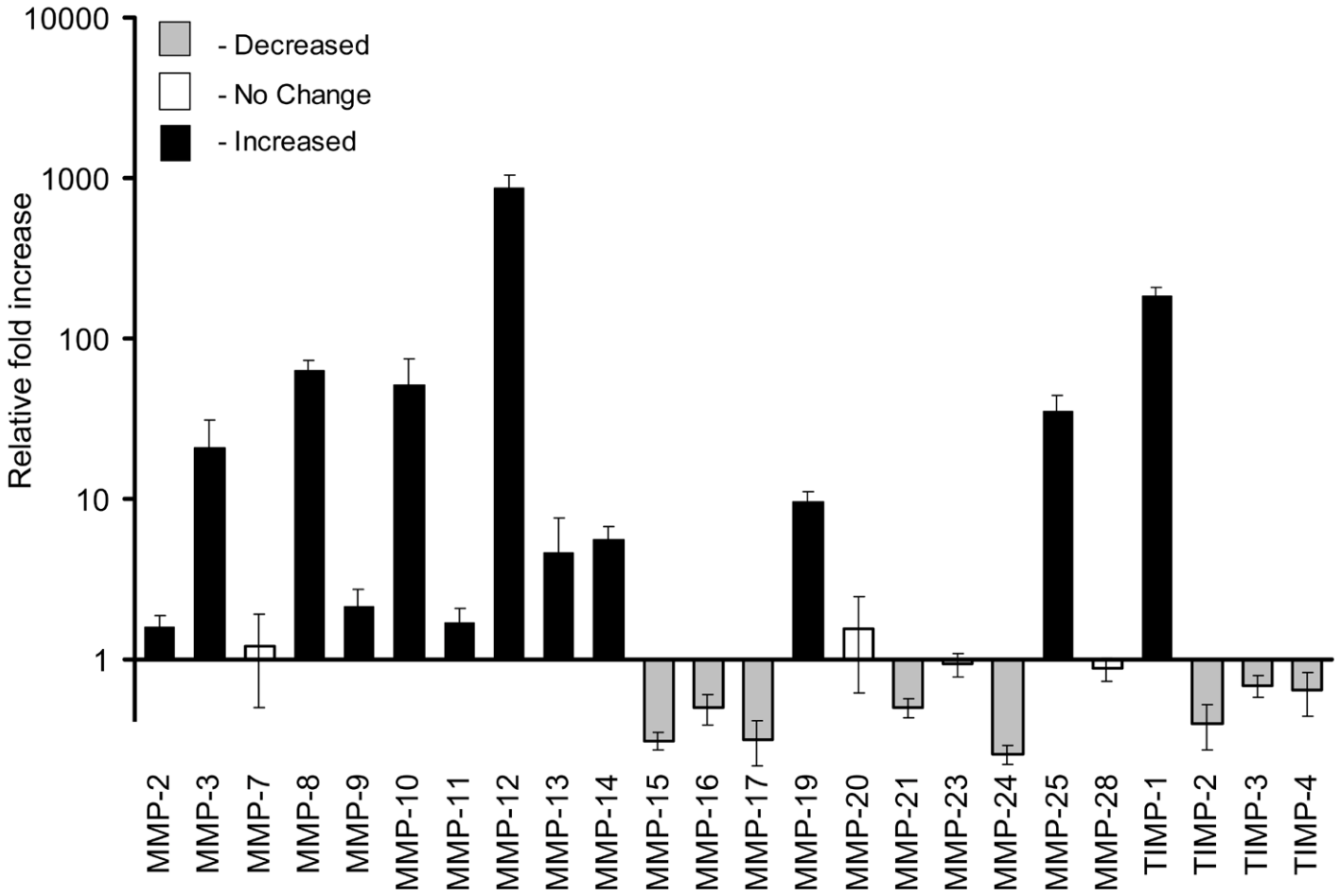

Figure 3. Summary of MMP and TIMP data from TaqMan analyses, displaying the relative fold increase of each individual MMP member compared with controls. Black bars denote significant increase, empty bars reflect no change, while gray bars denote transcripts that are down-regulated from normal controls. Statistical significance was determined at $P < 0.05$, using 2-tailed Student's *t* test.

Fig. 4

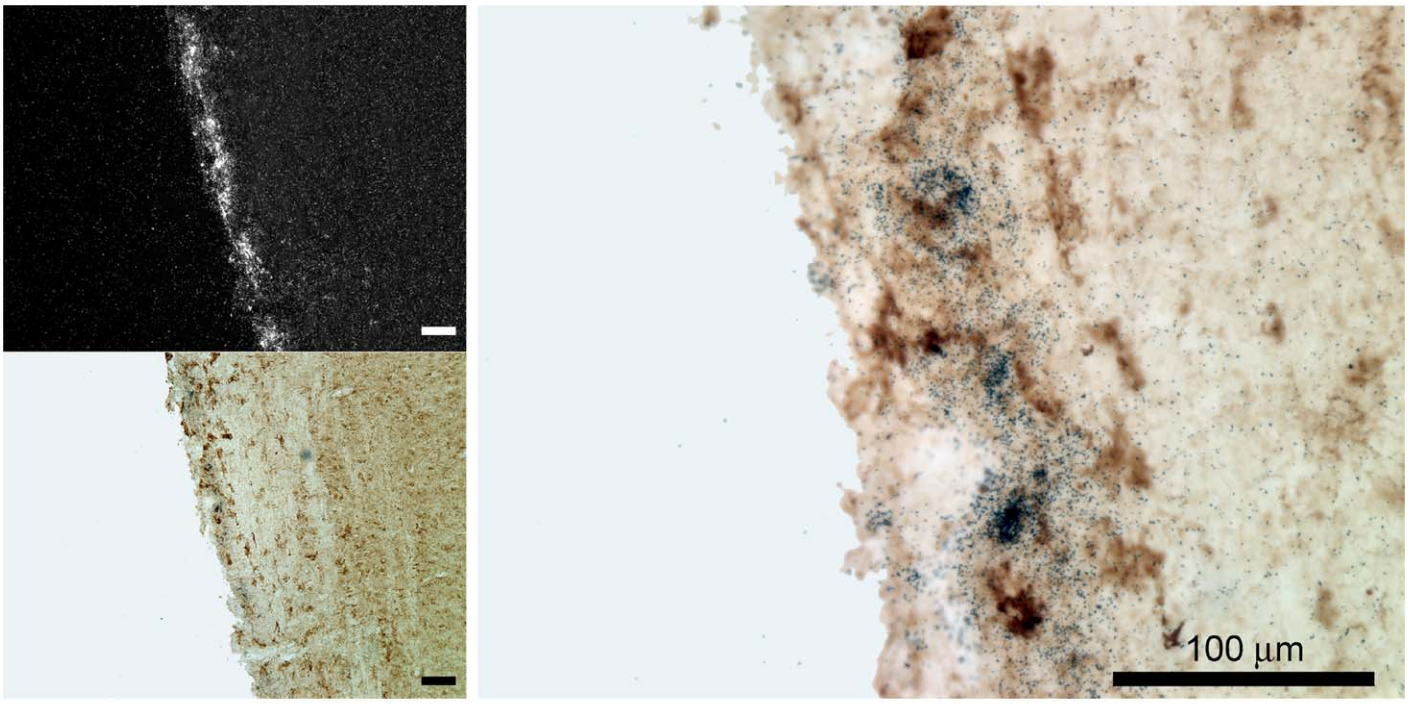

Figure 4. Expression of MMP-12 is largely in microglia/macrophages. By in situ hybridization, the signal for MMP-12 (***top left***, darkfield) is mostly at the subpial surface of the spinal cord and corresponds to cells that are microglia/macrophages as detected by Iba1 immunohistochemistry (***bottom left***, which is the same section as the darkfield image; positive Iba1 cells are brown). A higher magnification photomicrograph is displayed on the right. MMP-12 could not be localized to glial fibrillary acidic protein (GFAP)-positive astrocytes (data not shown).

Fig. 5

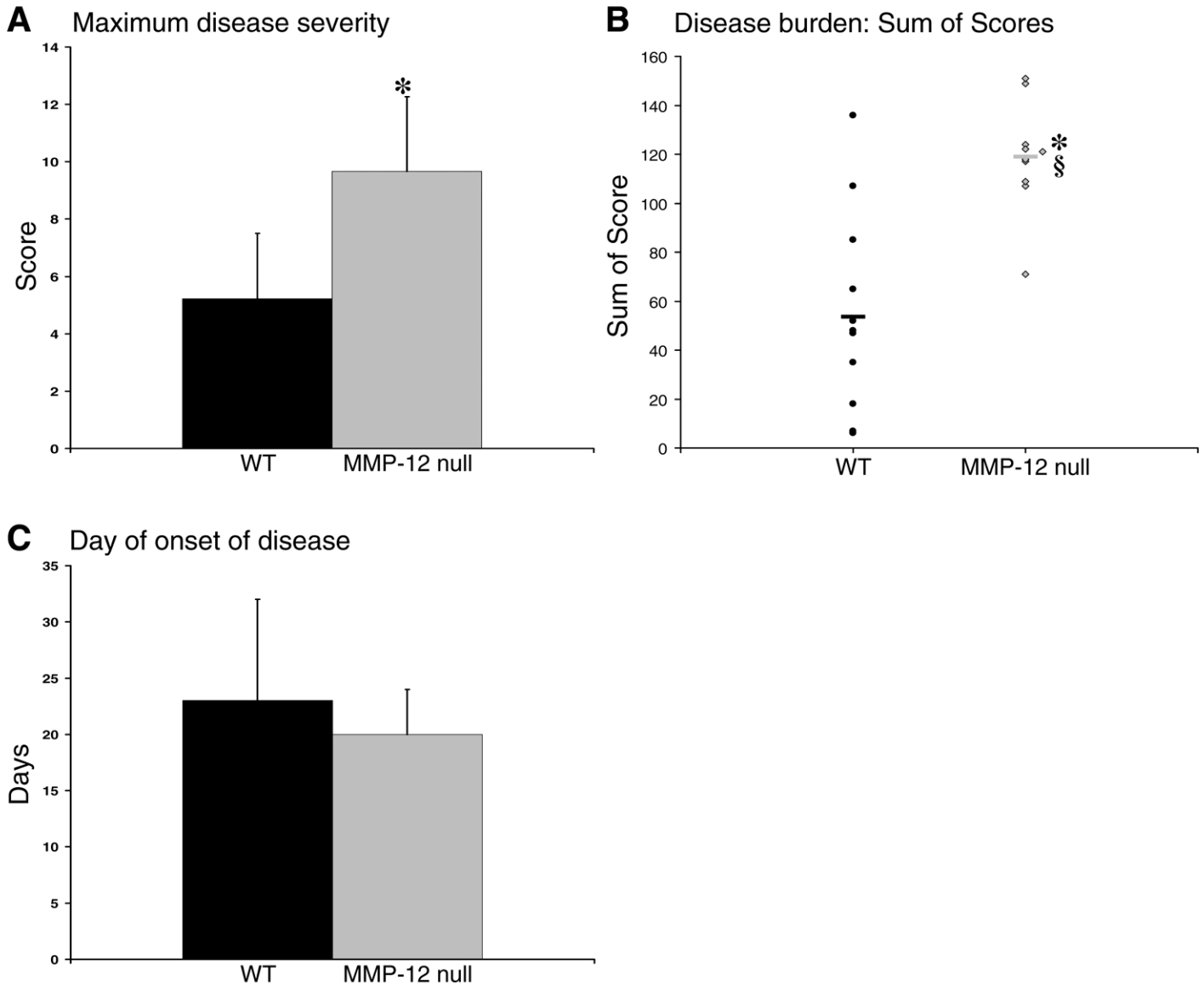

Figure 5. MMP-12 null mice have a worse disease course than WT animals. ***A*)** Maximum disease severity attained for each mouse over the course of experiment has been tabulated, and the group average (±SD) is displayed. MMP-12 null mice have a greater maximum disease severity than the WT group. ***B*)** Daily disease score for each mouse is added over the entire experiment to produce the sum of score for that mouse. Each point is of a single animal, while horizontal bar represents the group mean. [§]Note that 2 MMP-12 null mice died during the course of the experiment because of the severity of their EAE disease, and they have not been included in the analysis. In ***C***, means ± SD of the day of onset of EAE is displayed for both groups. $*P < 0.05$.

Fig. 6

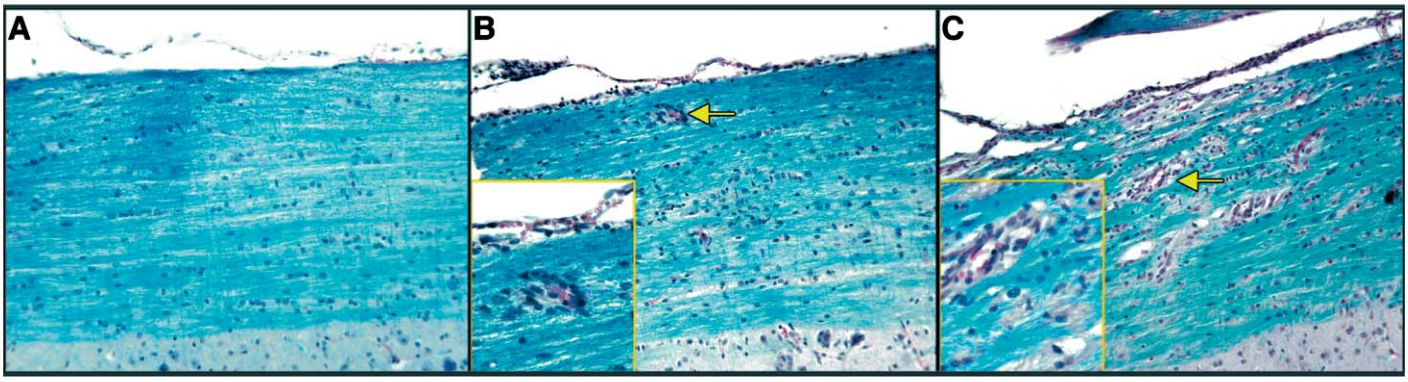

Figure 6. The degree of inflammation and demyelination in the spinal cord is increased in MMP-12 null mice. H&E and LFB were used to characterize inflammatory activity and demyelination, respectively, of the thoracic spinal cord. Displayed are sections from a normal noninduced mouse (***A***) and from a WT (***B***) or MMP-12 null mouse (***C***) at peak disease. Sections are representative of 5 EAE animals analyzed from each genotype; only the lateral columns are displayed. MMP-12 null mice, on average, had a large number of infiltrating cells that were more diffusely spread around blood vessels and away from the subarachnoid space than in WT. Pictures were taken at an original magnification of ×200, while the inserts were at ×400. An area of inflammatory infiltrate is indicated by each arrow.

Fig. 7

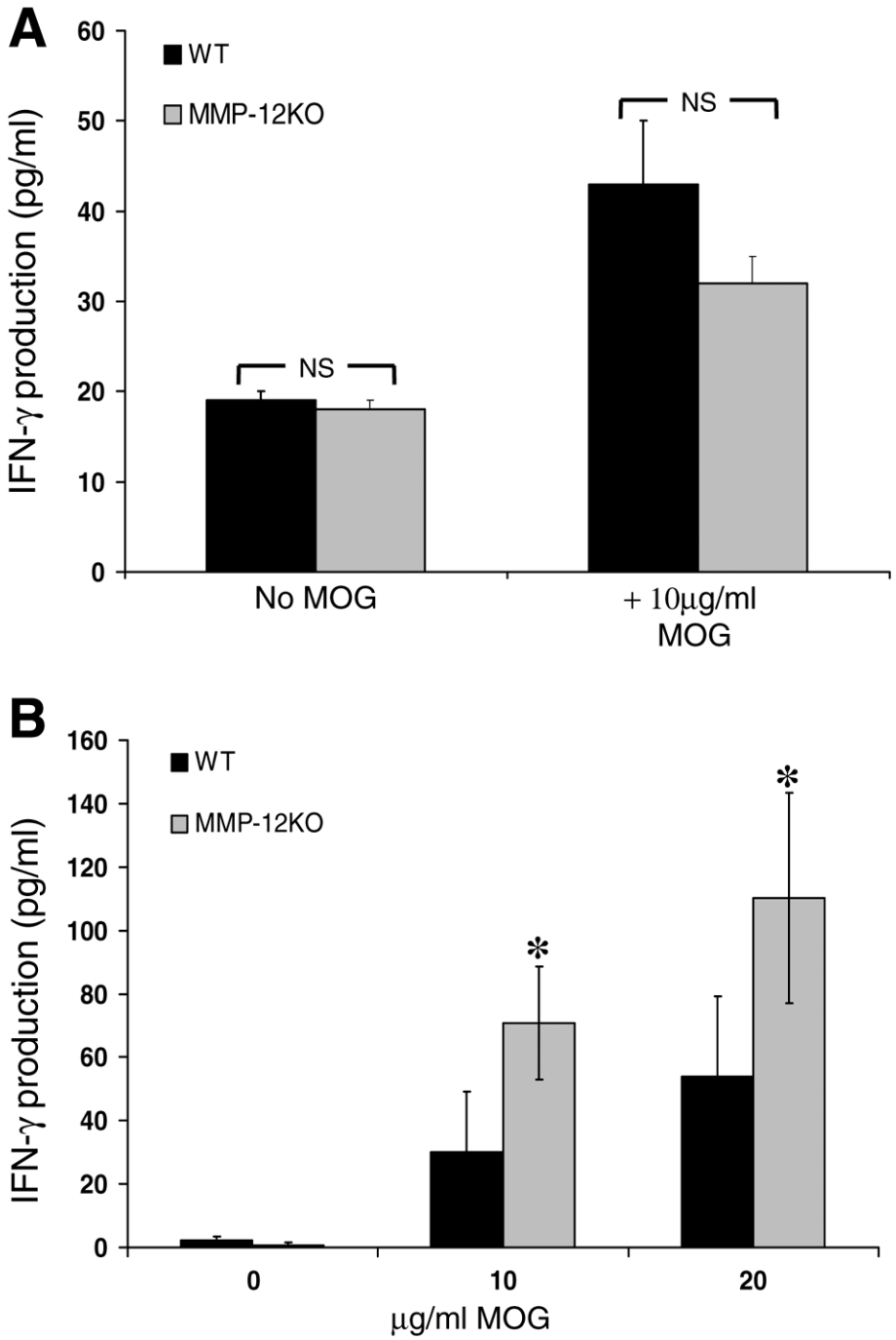

Figure 7. IFN-γ production to investigate the generation of MOG-specific T cells (***A***) and their polarization (***B***). ***A*)** Cells were obtained from mice 10 days after MOG immunization, before the appearance of symptoms, and were incubated with, or with MOG, in culture for 72 h. NS: not significant. ***B*)** Cells were taken from EAE-afflicted mice at a disease score of 4, just before the eventual divergence of disease severity. The secretion of the Th1 cytokine, IFN-γ, is increased in MMP-12 null mice compared with WT, following MOG stimulation. Each bar is means ± SE of 4 samples.

Fig. 8

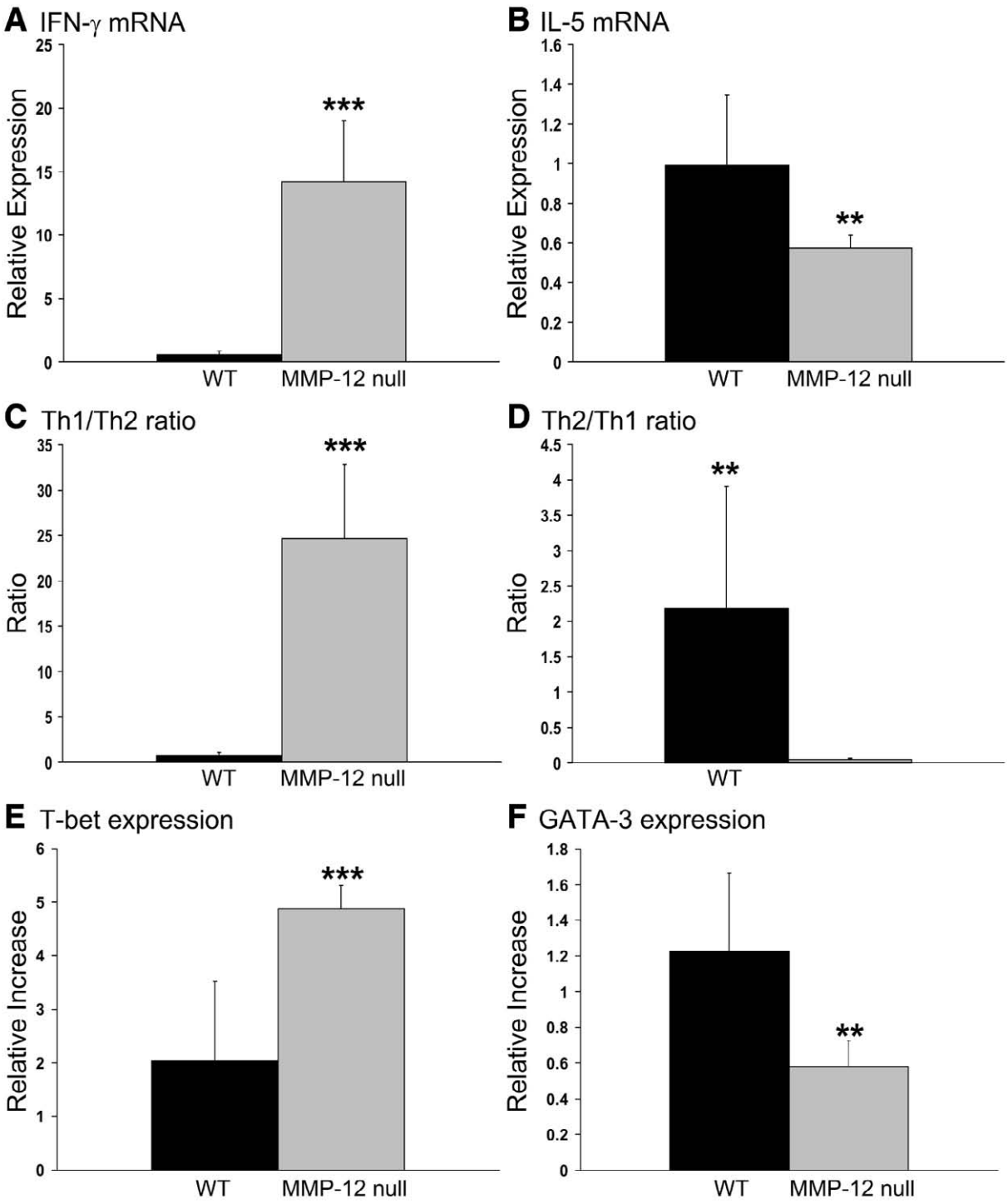

Figure 8. An increase in Th1 bias is evident in MMP-12 null lymphoid cells. ***A, B***) Each bar is the relative expression of the cytokine of interest in MOG stimulated vs. unstimulated condition, for either null or WT cells. ***C***) The Th1/Th2 ratio is obtained by analyzing the individual interferon-γ to IL-5 level for that mouse and group means ± SE is then displayed; ***D*** gives the opposite ratio. The level of statistical significance denotes comparison between WT and null cells. Finally, in ***E*** and ***F***, MMP-12 null mice have significantly higher expression of T-bet and lower expression of GATA-3, a condition of transcriptional regulation that favors a Th1 bias. All bars represent *n* of 4 samples. $^{**}P < 0.01$, $^{***}P < 0.001$. In control experiments, cells from normal WT mice that were not exposed to MOG were treated with or without MOG in culture. There was no relative change of T-bet or GATA-3 expression with MOG treatment compared with no MOG, and the relative ratio was 1.