Open access • Journal Article • DOI:10.1002/GLIA.20478

Metalloproteinases are enriched in microglia compared with leukocytes and they regulate cytokine levels in activated microglia — Source link

Robert K. Nuttall, Claudia Silva, Walter Hader, Amit Bar-Or ...+3 more authors

Institutions: University of East Anglia, University of Calgary, Montreal Neurological Institute and Hospital

Published on: 01 Apr 2007 - Glia (Wiley Subscription Services, Inc., A Wiley Company)

Topics: Neuroinflammation, TIMP1, Microglia, Matrix metalloproteinase and Cytokine

GLIA 55:516–526 (2007)

Metalloproteinases Are Enriched in Microglia Compared with Leukocytes and They Regulate Cytokine Levels in Activated Microglia

ROBERT K. NUTTALL,[1] CLAUDIA SILVA,[2] WALTER HADER,[2] AMIT BAR-OR,[3] KAMALA D. PATEL,[4,5] DYLAN R. EDWARDS,[1] AND V. WEE YONG[2]*

[1]*School of Biological Sciences, University of East Anglia, Norwich, United Kingdom*
[2]*Department of Clinical Neurosciences, University of Calgary, Calgary, Alberta, Canada*
[3]*Montreal Neurological Institute, Montreal, Quebec, Canada*
[4]*Department f Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada*
[5]*Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta, Canada*

KEY WORDS
microglia; cytokines; metalloproteinases; cell activation

ABSTRACT
Microglia are resident immune cells within the central nervous system (CNS). They become activated following neurological insults and increase their expression of cytokines. Also elevated in CNS injuries are proteases, including matrix metalloproteinases (MMPs) and A disintegrin and metalloproteinases (ADAMs). The spectrum of metalloproteinase members expressed by microglia and by the systemic leukocytes that infiltrate the injured CNS is unknown, as are their functions. We determined the levels of transcripts encoding all 24 MMPs, nine ADAMs, and their four physiological antagonists, tissue inhibitor of metalloproteinases (TIMPs), in human microglia, B and T cells, monocytes, and neutrophils. We found a distinct pattern for each immune subset and an enrichment of metalloproteinases in microglia compared with leukocytes. When microglia were activated, there was an upregulation of transcripts for nine metalloproteinases, and reduction of TIMP3. Activation of microglia also resulted in increased levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-10 protein in the conditioned media of cells. The amount of secreted TNF-α, but not IL-1β or IL-10, was suppressed by BB94, a broad spectrum metalloproteinase inhibitor, and by TIMP3 but not TIMP1 or TIMP2. This inhibitory profile suggests the involvement of an ADAM member in TNF-α secretion. We conclude that microglia bear a metalloproteinase signature distinct from systemic cells, and that following activation, microglia upregulate TNF-α protein levels through a combination of elevated cytokine transcripts, increased metalloproteinase level and activity, and through the decrease of TIMP3. The results have implications for the regulation of neuroinflammation and its outcomes following CNS injuries.

INTRODUCTION

Microglia are resident immune cells within the central nervous system (CNS). In the uninjured adult brain, they are highly dynamic surveyors that continually send and retract processes into the local environment (Nimmerjahn et al., 2005). Microglia respond rapidly to all types of injuries to the CNS by becoming activated (Kreutzberg, 1996; Streit et al., 1999). In this regard, they change their morphology from ramified to large round cells resembling macrophages. They proliferate and become more motile, they phagocytose cellular debris, and they produce a number of cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-10 (Becher et al., 2000; Buttini et al., 1994; Chabot and Yong, 2000; Kreutzberg, 1996; Streit et al., 1999). The full spectrum of microglia activity imparts complex outcomes on the integrity of the CNS. While the persistent activation of microglia and the elaboration of a range of molecules are thought to be detrimental, some microglial responses such as the production of neurotrophic factors and removal of toxic products (Batchelor et al., 1999; Simard et al., 2006) may serve to limit injury and enhance repair. Understanding microglial activity and controlling their actions can have an important consequence on the recovery of the nervous system.

The metalloproteinase subfamilies of matrix metalloproteinases (MMPs) and A disintegrin and metalloproteinases (ADAMs) are proteolytic enzymes that are promptly expressed and elevated following all types of insults to the CNS (Cunningham et al., 2005; Yong, 2005; Yong et al., 2001; Zhao et al., 2006). There are 24 MMP members that are secreted or membrane-bound, and all have metalloproteinase activity. Of the 32 human membrane-associated ADAMs, many are restricted to the testes and only about half the family have the HExxHxxGxxH catalytic core required for metalloproteinase activity. There are only nine ADAMs that have both a broad tissue distribution and the capacity to be proteolytically active (Blobel, 2005). The activity of metalloproteinases is inhibited principally by the four physiological tissue inhibitors of metalloprotei-

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0894-1491/suppmat.

Grant sponsor: Canadian Institutes of Health Research.

*Correspondence to: V. Wee Yong, PhD, Hotchkiss Brain Institute and the Departments of Clinical Neurosciences and Oncology, University of Calgary, 3330 Hospital Drive, Calgary, Alberta T2N 4N1, Canada. E-mail: vyong@ucalgary.ca

Received 19 July 2006; Revised 26 November 2006; Accepted 28 November 2006

DOI 10.1002/glia.20478

Published online 10 January 2007 in Wiley InterScience (www.interscience.wiley.com).

nases (TIMPs). For the most part, each of the TIMPs can inhibit MMPs, although TIMP1 cannot block the membrane-type MMPs effectively (Baker et al., 2002). Selectivity for ADAMs is more pronounced: TIMP3 is the only inhibitor of ADAM17 (Amour et al., 1998), but cannot inhibit ADAM8 and ADAM9 (Amour et al., 2002); while TIMP1 and TIMP3 can also inhibit ADAM10 (Amour et al., 2000).

As with microglia actions, the functions of MMPs and ADAMs are complex and they not only have proinflammatory and neurotoxic properties but also reparative ones (Cunningham et al., 2005; Yong, 2005; Zhao et al., 2006). These differential functions are likely due to their cleavage of extracellular matrix proteins, growth factors, cytokines, chemokines, adhesion molecules, and cell surface receptors (McCawley and Matrisian, 2001). For example, TNF-α is actually synthesized as pro-TNF-α (26 kDa) that is converted by metalloproteinase activity to the mature 17 kDa cytokine TNF-α (Black et al., 1997; Moss et al., 1997).

In the adult CNS, most MMPs are expressed at low or undetectable levels, but several become upregulated in neurological diseases, such as multiple sclerosis, malignant glioma, and stroke (reviewed in Yong et al., 2001). The sources of the increase in MMP and ADAM members in response to an insult within the CNS are likely multiple, and they include neural cells as well as leukocytes that have infiltrated into the CNS. A systematic comparison of the relative capacity of different cell types to express the full spectrum of MMP and ADAM family members is nonexistent. In particular, a comparison between microglia and their systemic counterparts in immunity, leukocytes, is lacking. This is important since all these cell types mediate inflammatory responses within the CNS in response to an insult, and the roles of MMPs produced by these different cell types in modulating immune responses need clarification.

In this study, we have profiled all 24 MMP members and nine ADAMs expressed by microglia and leukocyte subsets. We found distinct profiles of metalloproteinases for each inflammatory cell subset, and the relative expression of metalloproteinase transcripts was higher in microglia compared with all systemic leukocyte subsets. Following activation, the levels of mRNA for many MMPs and ADAMs are further elevated in microglia. By using inhibitors, it was found that metalloproteinase activity has important roles in the regulation of levels of TNF-α in microglia. These results provide a mechanistic link between metalloproteinase activity and the activation of microglia that leads to the generation of inflammatory cytokines.

MATERIALS AND METHODS

Cultures

Microglia were derived from adult human brain tissue obtained at surgical resection to treat intractable epilepsy. These were tissues that were adjacent to, but did not encompass, the epileptic focus in cortical areas or in the hippocampus, and they comprise material that had to be resected enroute to the epileptic focus. In previous work, we had described that microglia so obtained were not different from microglia derived from other neural pathologies or from normal brain (Williams et al., 1992). We have also not found any obvious differences in the microglia characteristics in culture when these are obtained from different areas of the CNS. In addition, adult microglia are comparable to microglia obtained from fetal human brain with respect to their production of inflammatory cytokines (Chabot et al., 2002; Giuliani et al., 2005).

Microglia from human adult brain were derived using methods that were previously published (Chabot and Yong, 2000; Williams et al., 1992). In brief, fragmented brain tissue was incubated with 0.25% trypsin and 100 μg/mL DNase for 1 h at 37°C, and dissociated into single cells by forcing them through a mesh of 130 μm pore size. Following centrifugation through 30% Percoll, which allowed myelin, debris, and red blood cells to be separated from viable neural cells, the latter cell layer was removed, centrifuged, washed, and plated into T-25 uncoated flasks. Feeding medium was 10% fetal bovine serum in minimum essential medium supplemented with 0.5% dextrose. Microglia from adult human brain are very adherent cells, as opposed to other cell types (e.g. oligodendrocyte), on uncoated flasks. Thus, one day after plating, floating cells were removed to leave behind adherent microglia. The latter was removed by retrypsinization 2–3 days after, replated, and experiments (see below) were then initiated 1–2 days following replating. The purity of microglia was typically in excess of 95%, as evaluated using markers such as CD11b and Iba1 (Fig. 1).

In experiments to obtain microglia RNA, 500,000 cells were seeded per well of a six-well plate. In experiments to collect conditioned media for cytokine protein analyses, 5,000 microglia cells were seeded per well of a 96-well plate. The initial seeding medium consisted of minimum essential medium supplemented with 10% fetal bovine serum. After 24 h, the serum containing medium was removed and cells were fed with serum-free AIM-V medium (Invitrogen Life Technologies). Lipopolysaccharide (LPS, 100 ng/mL) was added to activate microglia, and RNA or conditioned medium was obtained 24 h later. In experiments using TIMPs (Chemicon) or BB94 (British Biotech), a broad spectrum metalloproteinase inhibitor, these were applied 30 min before LPS. For RNA analysis, cells were harvested into Trizol (Invitrogen Life Technologies). For determinations of protein levels of cytokines the conditioned medium was removed after 24 h.

Peripheral blood mononuclear cells (PBMCs) were collected from healthy human volunteers in accordance with the McGill Ethical Review Board and sorted into B cell (CD19+), T cell (CD3+), and monocyte (CD14+) cell populations as described previously (Bar-Or et al., 2003). The purity of monocytes, B cell, and T cell preparations was routinely >97%.

Neutrophils were isolated from healthy human donors by dextran sedimentation, hypotonic lysis of erythrocytes, and density centrifugation on Histopaque 1077 (Patel and

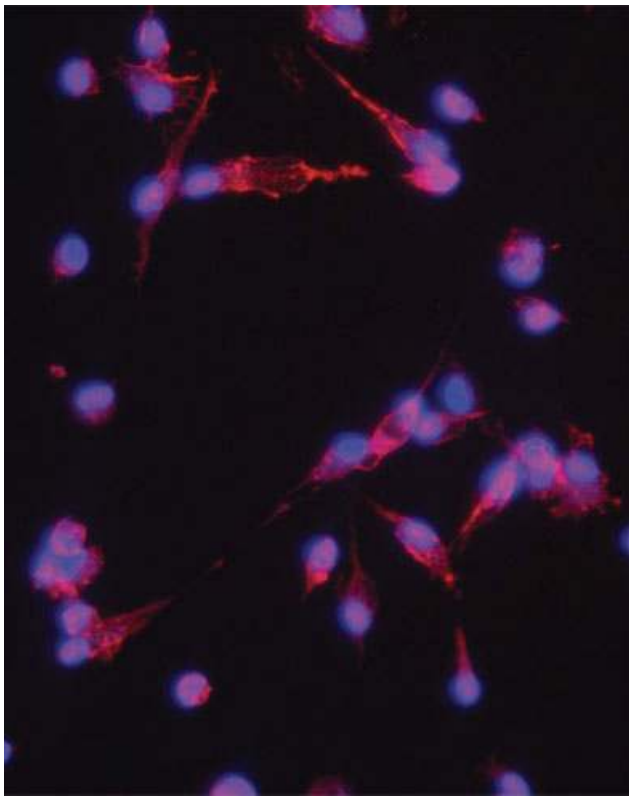

Fig. 1. Adult human microglia in culture. Cells were labeled using the Iba1 marker, which marks monocytoid cells, and were counterstained for nuclei with Hoescht stain. Most cells were double positive. Note that while some microglia are still rounded and undifferentiated, some have extended processes representative of ramified microglia. We counted the number of Iba1-positive cells across four wells to obtain the purity of microglia in culture. An average of 150 cells was evaluated per well. The purity of adult human microglia was (100 ± 0.5)% (mean ± SEM).

McEver, 1997). The granulocyte fractions were greater than 95% neutrophils, as assessed by Kimura staining.

Reverse Transcription, Real Time PCR

One microgram of total RNA was reverse transcribed using 2 μg random hexamers and 200 units of Superscript II reverse transcriptase (Invitrogen Life Technologies), according to the supplier's instructions. Quantitative PCR (qPCR) reactions were done, as previously described (Nuttall et al., 2003), with each reaction containing 5 ng of reverse transcribed RNA in 25 μL reaction volume. Sequences for primers and probes are described elsewhere (Elkington et al., 2005; Nuttall et al., 2003). To determine the relative RNA levels within the samples, standard curves for the PCR reaction were prepared by using the cDNA from one sample and making twofold serial dilutions covering the range equivalent to 20–0.625 ng of RNA; for 18S analyses, the range was 4–0.125 ng.

To determine relative expression levels between genes, the cycle threshold (C_T) at which amplification entered the exponential phase was determined and this number was used as an indicator of the amount of target RNA in each tissue, i.e., a lower C_T indicated a higher quantity of starting RNA.

Clustering of genes and cell samples was done using Gene Cluster version 3.0 and TreeView (Eisen et al., 1998). Relative expression levels of each gene (normalized to 18S rRNA) across all samples were adjusted by log transforming the data, then median centering, followed by normalization; sample data across genes were not adjusted. Hierarchical clustering of both genes and samples was done using average linkage clustering of Spearman rank correlations. A heat map of relative expression based on this clustering was done using TreeView.

To determine if LPS significantly changed RNA levels within microglia cells, analyses were done using the relative expression levels normalized to 18S rRNA. Each adult human preparation provided enough microglia for only a limited number of analyses so four separate microglia sources were used, with each treated either with LPS (100 ng/mL) or were left untreated as controls. A paired *t*-test was then performed between the control and LPS group of each microglia preparation across the four sets and a P-value <0.05 was considered significant.

Enzyme Linked-Immuno Sorbent Assay

Enzyme Linked-Immuno Sorbent Assay (ELISAs) for TNF-α, IL-1β, and IL-10 were performed using commercially available kits (Biosource International). Group differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison. Each experiment for cytokine protein analysis was reproduced across three sets of microglia donors.

RESULTS

Transcripts for MMPs and ADAMs Are Enriched in Microglia Compared with Leukocyte Subset

Metalloproteinase mRNA profiles by real time PCR were obtained from cells under basal culture condition. These involved four specimens each of microglia and neutrophils, nine samples of monocytes, and 10 specimens each of B and T cells. To compare the relative expression of individual genes in microglia and leukocytes, we classified the mean C_T values of each gene as a range from "very high expression" to "not detected" (Fig. 2). For the most part, microglial cells produce transcripts for all MMPs at much higher levels than circulating leukocytes, with the exceptions being MMP11, which is higher in B cells; MMP17, which is higher in monocytes, T cells, and B cells; and MMP25, which is higher in monocytes and neutrophils. Microglia and peripheral leukocytes produce mRNA for all ADAMs, with the exception of ADAM33, which is not detected in any cell type. ADAM9, ADAM12, and ADAM28 transcripts are highest in microglia. While all cell types produce TIMP1 and TIMP2 transcripts at high levels, TIMP3 and TIMP4 are highest in microglial cells.

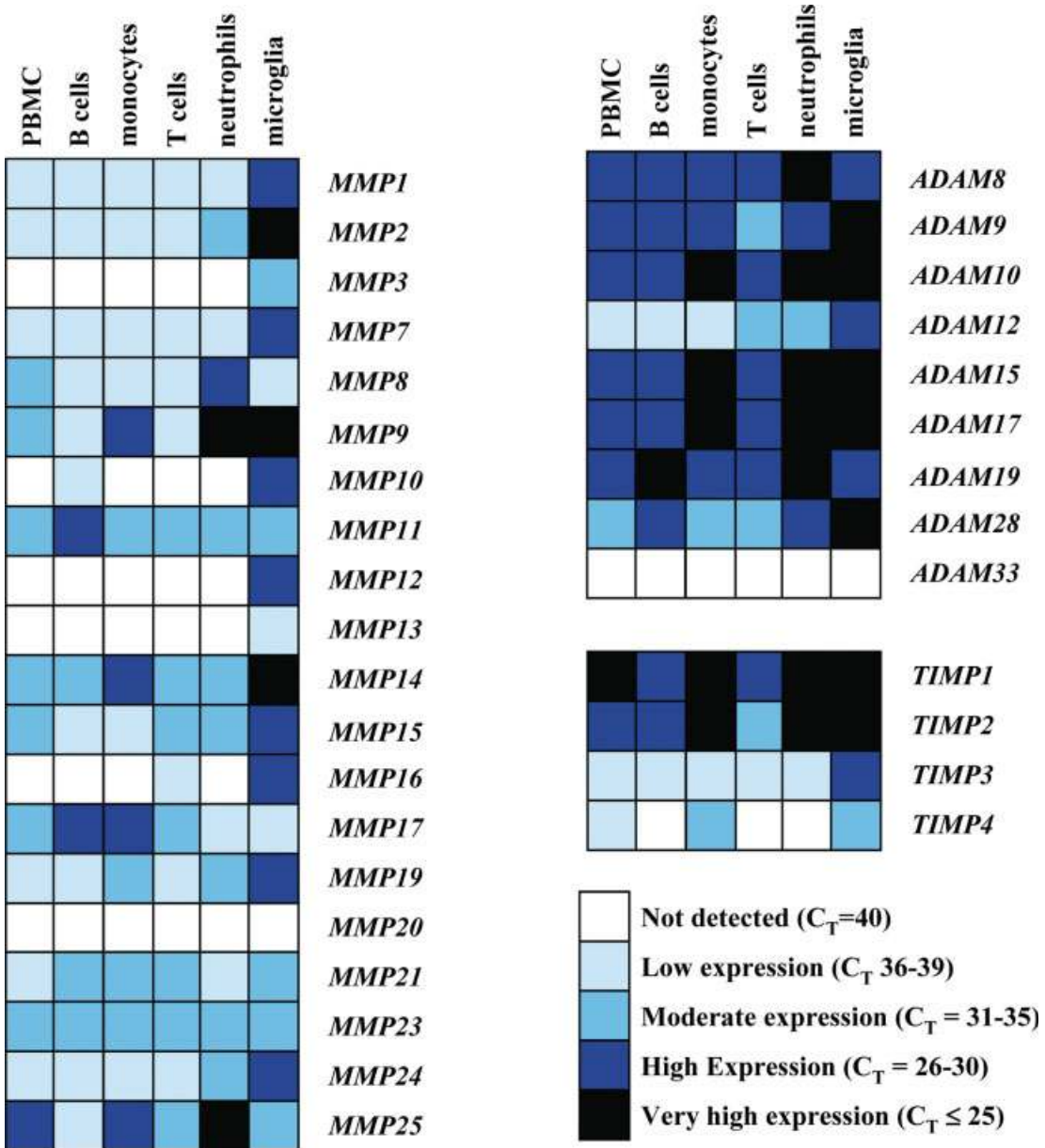

Fig. 2. RNA levels for MMPs and ADAMs are enriched in microglia compared with peripheral leukocytes. Classification of mRNA levels was determined from the C_T of each gene as either very high ($C_T \leq 25$), high (C_T = 26–30), moderate (C_T = 31–35), low (C_T = 36–39), or not detected (C_T = 40); see figure for color scheme. Boxes represent the mean of four preparations of PBMCs, neutrophils, and microglia, nine samples of monocytes, and ten preparations of B and T cells.

While classifying gene expression based on C_T allows one to compare levels of one gene with another, the broad and arbitrary values assigned to each group makes precise comparison of gene levels between samples difficult. Therefore, the C_T data were converted to quantitative relative expression levels using standard curves and these data were normalized to 18S rRNA levels. These expression data were analyzed by hierarchical clustering of both samples and genes (Fig. 3). Individual cell types clustered into distinct groups based on metalloproteinase and TIMP expression, with B cells being closely related to T cells, while microglial cells are distantly related to the neutrophils and the monocytes. Similar to the C_T analysis, microglial cells are characterized by high expression of several MPs and TIMPs.

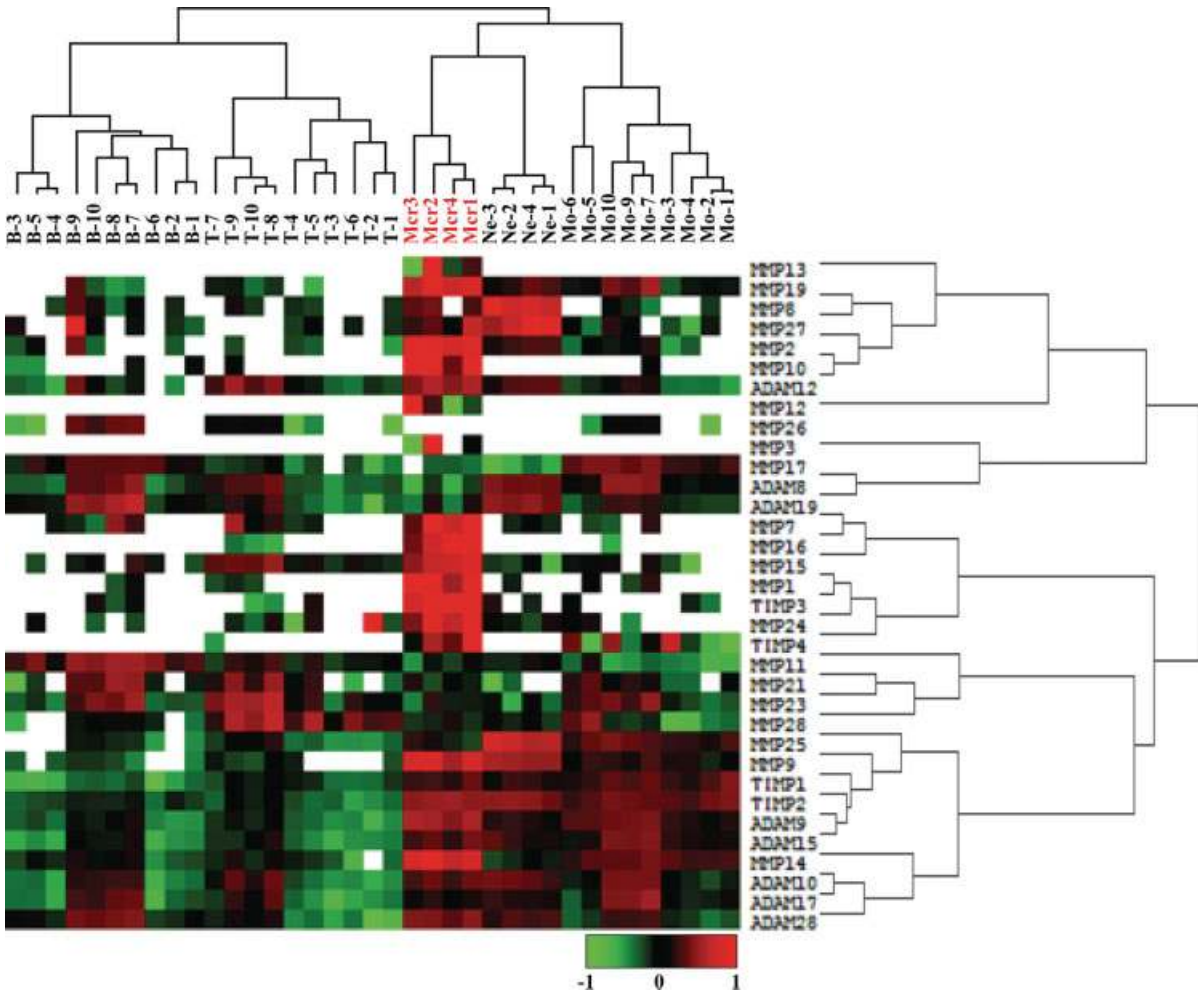

Fig. 3. Microglia have a gene expression signature of MMP and ADAM distinct from peripheral leukocytes. Relative expression levels of each gene (normalized to 18S rRNA) across all samples were adjusted by log transforming the data, and then median centering followed by normalization. Adjusted values within individual cells were clustered (average linkage of Spearman's rank correlation) by both gene and sample, with black being the median, red representing the samples above the median, and green representing values below the median (see legend). The white color indicates genes that were not detected in the indicated sample. Samples are in columns and gene levels are in rows. Samples include B cells from 10 donors (B-1 to B-10), T cells (T) from 10 subjects, microglia (Mcr) from four surgical specimens (highlighted in red), neutrophils (Ne) from four volunteers, and monocytes (Mo) from nine donors.

LPS Activation of Microglia Changes the mRNA Expression of Several MMPs and ADAMs

The LPS activation of microglia resulted in a significant increase in the mRNA levels of MMP1, 3, 8, 10, and 12 (Fig. 4A). The rise of MMP7, 13, 14, and 19 transcript levels was near level of significance after LPS treatment (Fig. 4A). In contrast, MMP15 and ADAM15 mRNA levels were reduced in microglia following activation, while values for MMP2, 16, and 21, and for ADAM12 were decreased by treatment but did not attain significance (Fig. 4B). RNA levels of eight MMPs and six ADAMs were unchanged by LPS activation (Supplemental Fig. 1), while levels of MMP20, MMP26, and ADAM33 were not detected following LPS activation, similar to the untreated samples.

With respect to endogenous MP inhibitors, LPS significantly increased the mRNA levels of TIMP1, but decreased the levels of TIMP3; TIMP2 mRNA level decreased in three of the four microglial preparations, while TIMP4 was elevated in three of the four preparations (Fig. 5).

Activation of Microglia with LPS Increases Protein Secretion of TNF-α, IL-1β, and IL-10

Microglial activation is characterized by increased production of cytokines. Using ELISA, TNF-α and IL-10 were

Fig. 4. The microglial mRNA levels of several MMPs and ADAMs change in response to LPS. **A:** Genes whose RNA levels increased in response to LPS; **B:** Genes whose RNA levels decreased in response to LPS. The *y*-axis for each gene is the relative mRNA levels normalized to 18S rRNA levels, and are arbitrary units. The data points on the left of each graph are relative expression levels of four unstimulated microglia samples (CON = controls), points on the right are the paired samples following LPS stimulation. Shown are those genes whose levels significantly change (paired *t*-test), or whose levels change in three of four sample pairs. $^{*}P < 0.05$; $^{**}P < 0.01$; *P*-values for genes that did not change significantly are shown. Genes whose levels do not change are in Supplemental Figure 1.

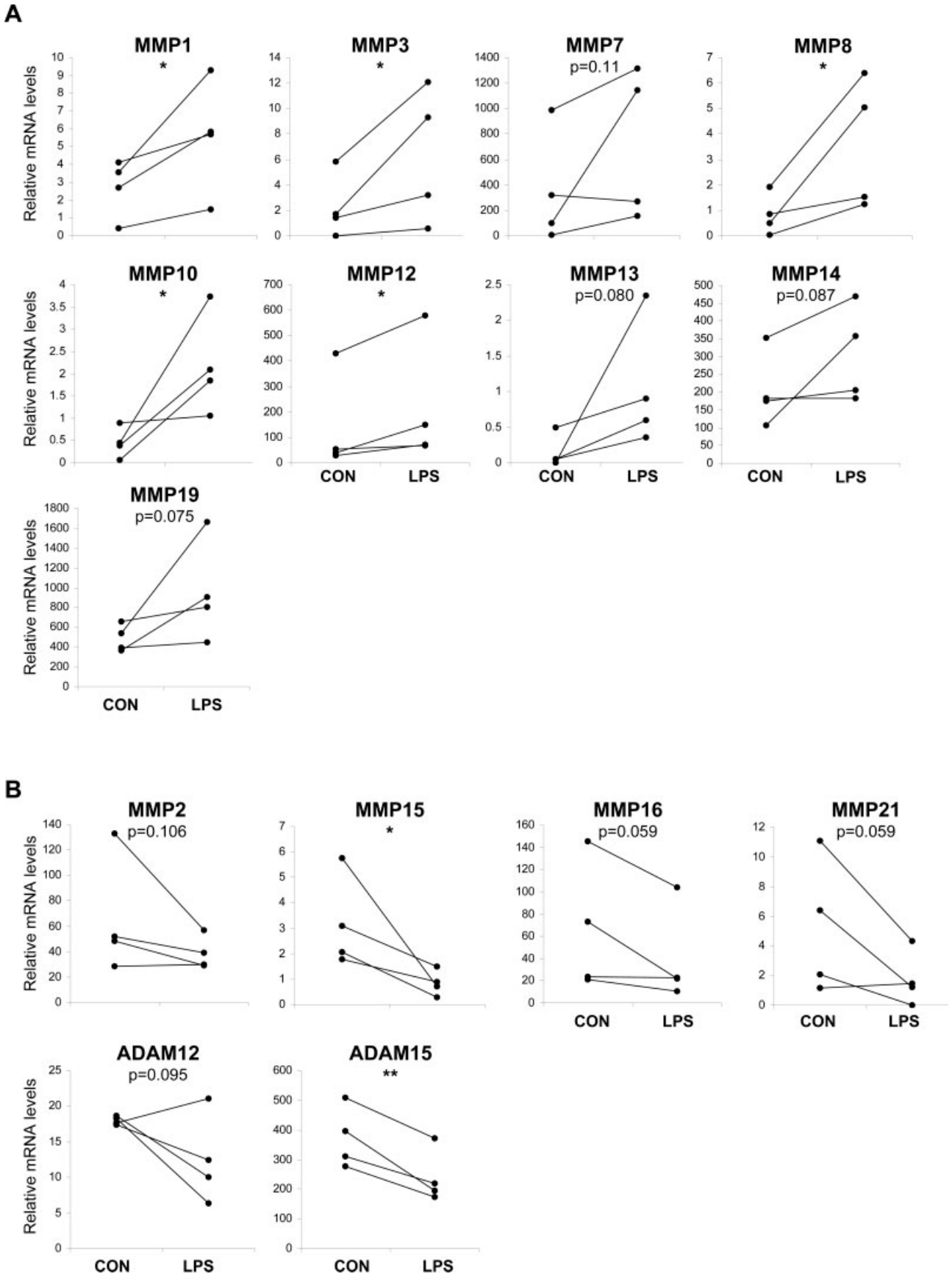
A
MMP1
*
MMP3
*
MMP7
p=0.11
MMP8
*
MMP10
*
MMP12
*
MMP13
p=0.080
MMP14
p=0.087
MMP19
p=0.075
Relative mRNA levels
CON
LPS
B
MMP2
p=0.106
MMP15
*
MMP16
p=0.059
MMP21
p=0.059
ADAM12
p=0.095
ADAM15
**

Figure 4.

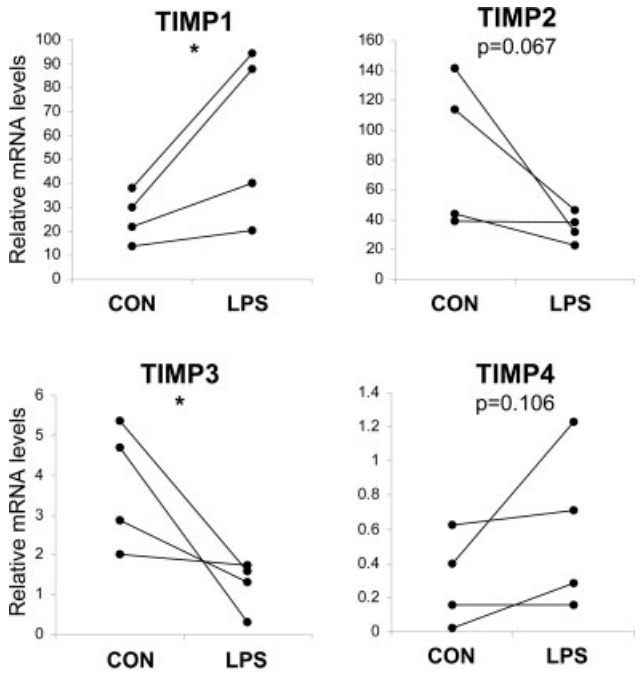

Fig. 5. The microglial mRNA levels of TIMPs change in response to LPS. The *y*-axis for each gene is the relative mRNA levels normalized to 18S rRNA levels, and are arbitrary units. The data points on the left of each graph are relative expression levels of four unstimulated microglia samples (CON = controls), points on the right are the paired samples following LPS stimulation. Data were analyzed by paired *t*-test; * = $P < 0.05$; *P*-values for genes that did not change significantly are shown.

virtually undetected in the conditioned medium of untreated microglia, but were substantially elevated by LPS treatment. IL-1β protein, detectable in the conditioned medium of control cells, was elevated by ~twofold following LPS stimulation.

Because the level of TNF-α protein in the conditioned medium of cultured cells reflects several events (Black et al., 1997; Idriss and Naismith, 2000; Moss et al., 1997) including elevation of transcript level; translation of transcript to pro-TNF-α protein, which becomes membrane associated; and then conversion of pro-TNF-α to secreted TNF-α, we examined whether cytokine transcripts were also altered by LPS treatment of microglia. Figure 6B shows that LPS activation resulted in an increase of only TNF-α and IL-1β mRNA levels, while transcripts for IL-10 were either unchanged or, paradoxically, suppressed in two of the four microglia preparations. Thus, the changes in cytokine protein levels (Fig. 6A) of microglia following LPS treatment were not necessarily matched by concurrent changes in cellular RNA levels.

Metalloproteinase Inhibitors Decrease Levels of TNF-α, but not IL-1β or IL-10 Protein

To test the involvement of metalloproteinases in regulating the levels of soluble cytokines, microglia were activated with LPS in the absence or presence of BB94, a broad-spectrum metalloproteinase inhibitor. Levels of secreted TNF-α protein were reduced as BB94 amounts were increased, yet this inhibitor had no effect on the protein levels of IL-1β or IL-10 (Fig. 7). BB94 did not affect the RNA levels of TNF-α, IL-1β, or IL-10 (data not shown), indicating that BB94 acted only at the level of regulating the amount of secreted TNF-α.

To further characterize the metalloproteinase subtype(s) involved in regulating TNF-α levels, microglial cells were exposed to exogenous TIMPs. In basal conditions, none of the TIMPs had a detectable effect on TNF-α protein level measured in the conditioned medium of control cells. However, when microglia were activated by LPS, the increase in TNF-α protein levels was selectively suppressed by TIMP3 but not by TIMP1 or TIMP2 (Fig. 8).

DISCUSSION

Microglia are resident immune cells in the CNS. In the normal state, they have important roles in maintaining homeostasis of the CNS through their continual surveillance of the brain microenvironment (Kreutzberg, 1996; Nimmerjahn et al., 2005). In pathologies of the CNS, activation of microglia occurs (Becher et al., 2000; Kim and de Vellis, 2005; Kreutzberg, 1996; Ladeby et al., 2005; Streit et al., 1999); the activated cell undergoes morphological changes, proliferates, becomes motile, and produces a number of cytokines and chemokines. Among the cytokines generated during microglial activation are TNF-α, IL-1β, and IL-10 (Chabot and Yong, 2000); TNF-α and IL-1β are proinflammatory cytokines which augment the immune response while IL-10 may have regulatory roles. It is thus important to identify factors that regulate microglia activation and their production of cytokines as the persistent rise of proinflammatory molecules within the CNS is likely to lead to detrimental outcomes.

Besides microglia-regulated neuroinflammation, the infiltration of systemic leukocytes is a consistent occurrence in CNS injuries. As these leukocytes bring inflammatory molecules into the CNS in addition to activating microglia (Chabot and Yong, 2000; Chabot et al., 2002; Giuliani et al., 2005), mechanisms that regulate the trafficking of leukocytes into the CNS are important to unravel. Given the vital role of metalloproteinases in regulating leukocyte transmigration across vessels in general (D'Haese et al., 2000; Lanone et al., 2002; Shipley et al., 1996), we measured levels of metalloproteinases expressed in peripheral immune cells and compared these to amounts in microglia; all cell types for this purpose were in basal culture conditions without any obvious activation stimulus. We find that microglia are distinguished by higher expression levels of mRNA for multiple metalloproteinases and TIMP3. Many of these genes are unique to microglia (MMP3, 10, 12, 13, and 16) while several are highest in microglia, but also expressed to varying degrees in other cell types.

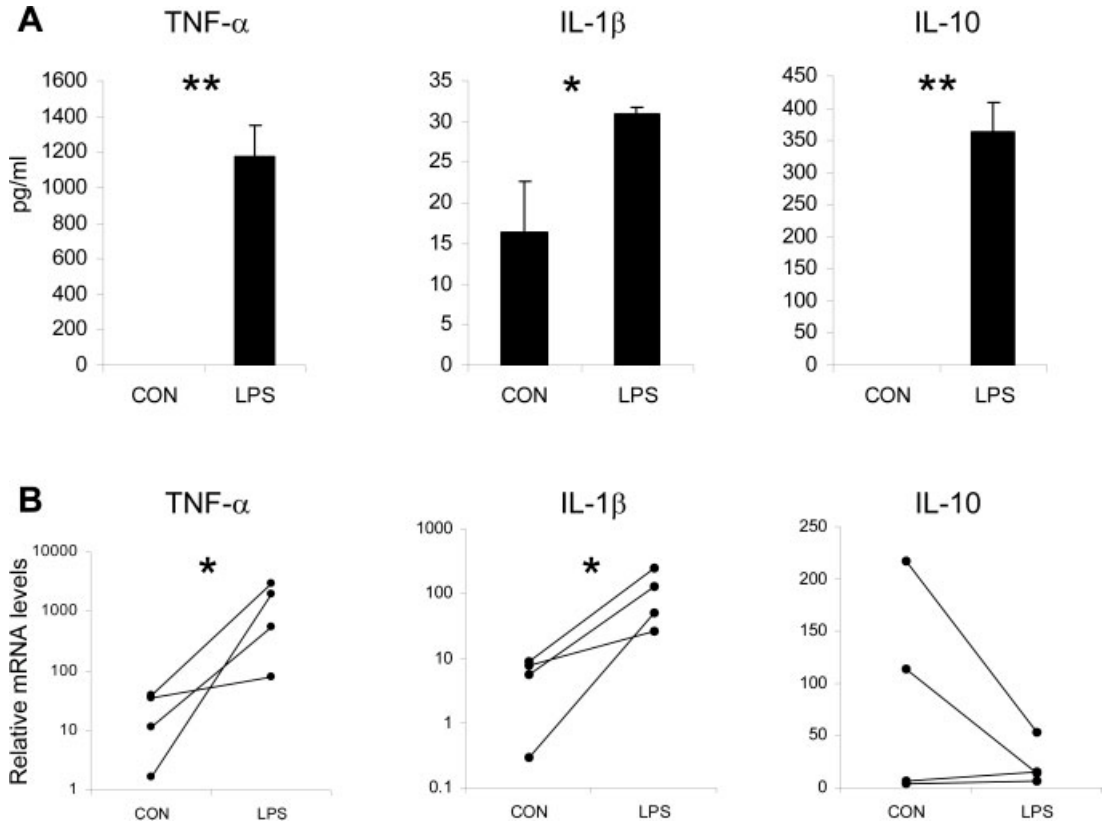

Fig. 6. Protein levels of TNF-α, IL-1β, and IL-10 increase in the conditioned media of microglial cells following stimulation with LPS. **A:** Protein levels for TNF-α, IL-1β, and IL-10 were determined by ELISA. Shown is one representative experiment, where each bar is the mean ± SEM of protein levels from three different wells. This result was reproduced in two other microglia samples. **B:** RNA levels for TNF-α, IL-1β, and IL-10 in paired microglial cells from four subjects were determined by quantitative PCR. The *y*-axis for each gene is the relative mRNA levels normalized to 18S rRNA levels, and are arbitrary units. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with controls.

Apart from enrichment of metalloproteinases in microglia, comparisons of a number of immune cell types reveal other noteworthy expression patterns. MMP8 transcript shows the highest expression in neutrophils, consistent with its alternate name of neutrophil collagenase. However, microglia also express this gene, and its mRNA level is elevated during activation. This gene is also elevated in the CNS of mice afflicted with a neuroinflammatory condition, experimental autoimmune encephalomyelitis (Toft-Hansen et al., 2004; Weaver et al., 2005). Also notable is the higher expression of transcripts for ADAMs in inflammatory cells compared with lower expression of MMPs. This might reflect the role of ADAMs as ectodomain sheddases, where they are known to cleave TNF-α, TNF receptors, epidermal growth factor receptors, amyloid precursor protein, and L-selectin (Blobel, 2005).

In a previous comparison of B cells, T cells, and monocytes, we showed that a number of MMPs are enriched in monocytes (Bar-Or et al., 2003). Here, we show that transcripts for MMPs are even more enriched in neutrophils and microglia. When metalloproteinase expression levels are used to cluster samples according to type, microglia are most closely related to neutrophils, followed by monocytes, with B and T cells in their own distinct cluster. This may reflect the shared lineage of these immune cells, with microglia, neutrophils, and monocytes deriving from myeloid progenitor cells and B and T cells from lymphoid progenitor cells.

The functions of metalloproteinases within immune cell subsets are likely to be multiple and complex (Parks et al., 2004). Given the enrichment of metalloproteinases in microglia compared with other immune subsets, and given our desire to understand mechanisms that regulate cytokine production in microglia, we examined whether metalloproteinases could regulate cytokine levels in microglia. We used LPS to activate microglia, which led to an increase in 11 protease/inhibitor genes and a reduction in eight others. Microglia activation by LPS also resulted in an increase of TNF-α transcripts and the secreted form of TNF-α protein. Unlike the ILs, this elevation requires metalloproteinase activity, since BB94, a broad spectrum protease inhibitor, and TIMP3 both suppress its increase. ADAM17 is the best studied protease in the generation of the secreted form of TNF-α and has the alternate name of TNF-α converting enzyme, or TACE (Black et al., 1997; Moss et al., 1997). However, ADAM9, ADAM10, and ADAM19 (Chesneau et al., 2003; Roghani et al., 1999; Rosendahl et al., 1997) can elevate TNF-α *in vitro*, although in mice lacking or overexpressing these sheddases, ADAM17 and ADAM19 are the only ones capable of

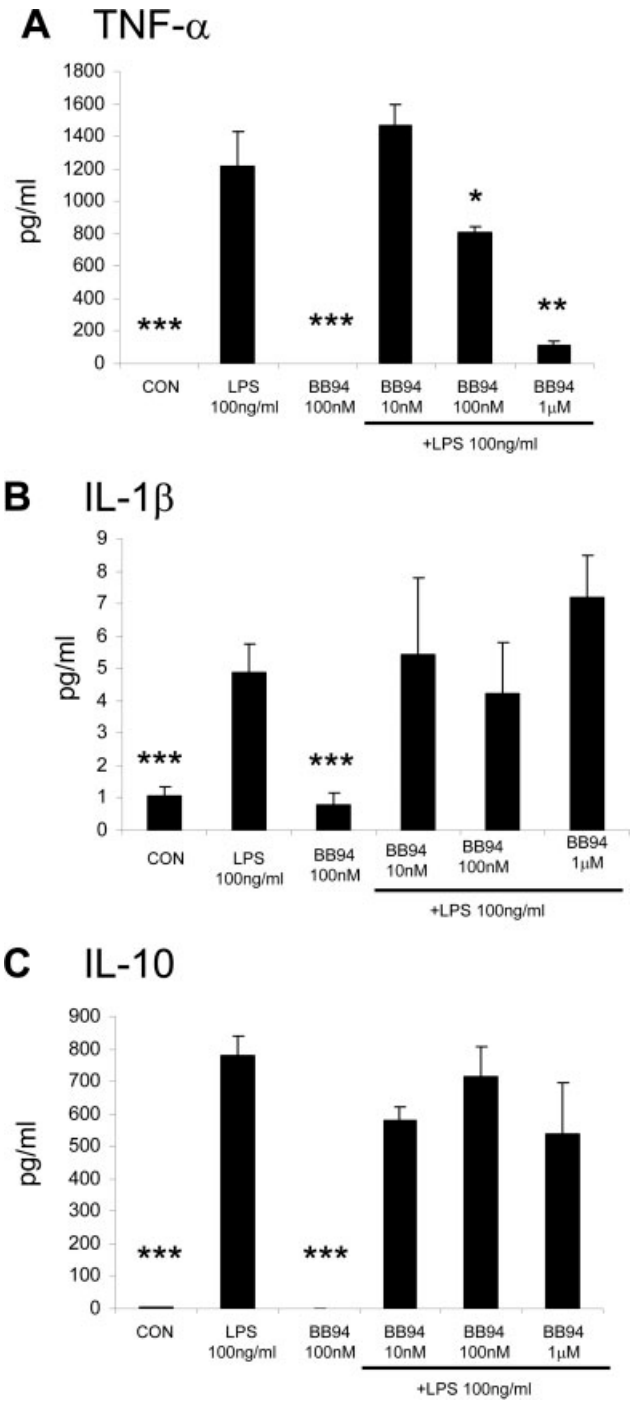

Fig. 7. Protein levels in conditioned media of TNF-α (**A**), but not IL-1β (**B**) or IL-10 (**C**), are affected by the metalloproteinase inhibitor BB94. Protein levels for cytokines were determined by ELISA. Shown is one representative experiment where each bar is the mean ± SEM of protein levels from three different wells. Data was analyzed by one-way ANOVA followed by Tukey's multiple comparison. Compared with LPS group: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. This result was reproduced in two other sets of microglia cultures.

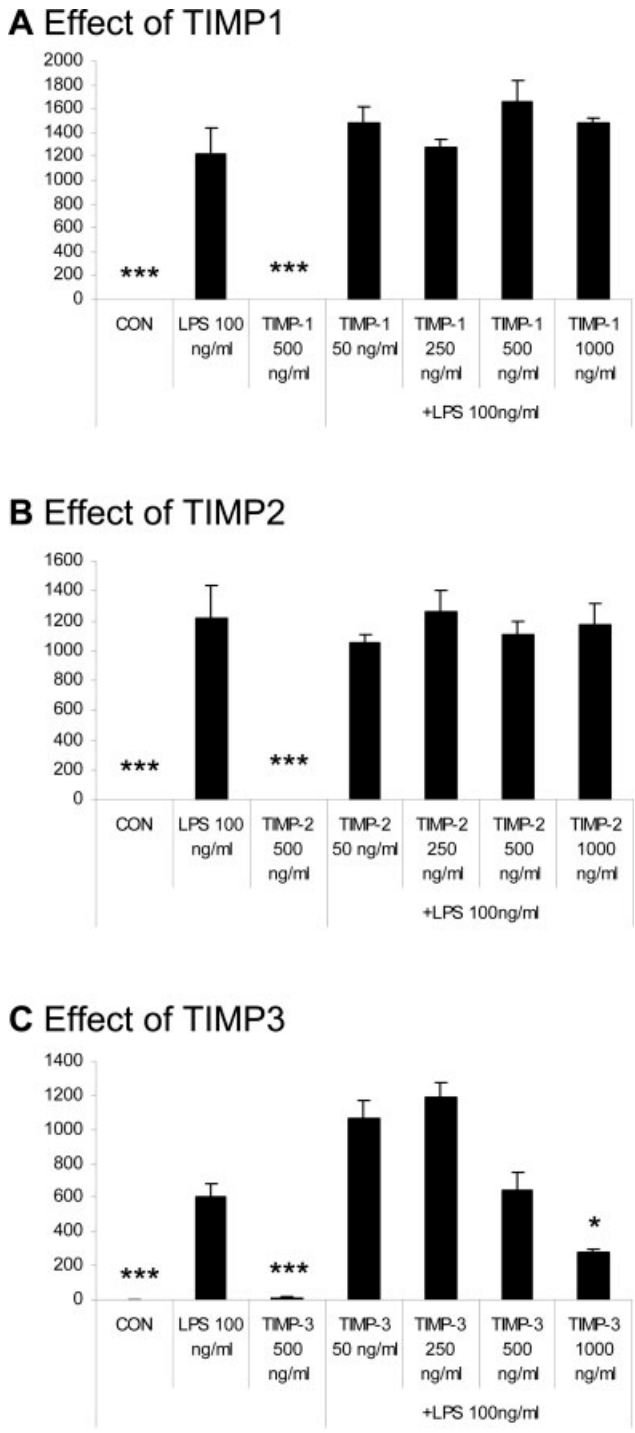

Fig. 8. Protein levels of TNF-α in conditioned media of activated microglia are reduced by TIMP3 (**C**) but not TIMP1 (**A**) or TIMP2 (**B**). Shown is one representative experiment where each bar is the mean ± SEM of TNF-α protein level (pg/mL) from three different wells. Note that TIMP1, 2, and 3 by themselves did not affect basal TNF-α levels. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison. Compared with LPS group: $^{*}P < 0.05$; $^{***}P < 0.001$. This result was reproduced in two other sets of microglia cultures.

producing the mature form of TNF-α (Zheng et al., 2004). Our observation that TIMP3, but not TIMP1 or TIMP2, block TNF-α processing during microglia activation supports the hypothesis that this is due to either ADAM17 or ADAM19, since ADAM10 is also inhibited by TIMP1 (Amour et al., 2000), while ADAM9 is not blocked by TIMP3 (Amour et al., 2002). Interestingly, the mechanism

by which LPS stimulation leads to elevated TNF-α levels in the culture medium involves increased RNA levels of TNF-α and decreased expression of TIMP3, but without affecting the RNA levels of ADAM17 or ADAM19.

The metalloproteinase response can differ depending on the immune subset being activated. In monocyte-derived macrophages, LPS activation resulted in an increase in the RNA levels of eight genes, six of which (MMPs 1, 3, 7, 10, 12, and TIMP1) were also elevated in microglia (Elkington et al., 2005). However, MMP2 and ADAM19 were elevated only in monocyte-derived-macrophages, while MMPs 13, 14, and 19 were elevated only in microglia. As for the genes suppressed by LPS, ADAM12, ADAM15, and TIMP3 were reduced in both cells types, while MMPs 2, 15, 16, 21, and TIMP2 were reduced only in microglia. This comparison emphasizes the fact that a shared activating stimulus can have different effects depending on the responsive cell type.

LPS activation in microglia led not only to elevated RNA and protein levels of TNF-α, but also increased amounts of IL-1β and IL-10 proteins detected in the culture medium. However, TNF-α was the only one whose protein levels were affected by the metalloproteinase inhibitors. This is likely due to findings that in other immune cell types, IL-1β is processed by caspase-1 (also called IL-1β converting enzyme, or ICE) and caspase-5, which are both cysteine proteases (Burns et al., 2003). IL-10, on the other hand, is not processed by any protease and is produced and secreted as an active cytokine (Hsu et al., 1990). While we have not completely determined the mechanisms by which LPS elevates secreted IL-1β and IL-10 proteins, our results do suggest that IL-1β is being regulated partly at the RNA level, while IL-10 is likely regulated post-transcriptionally.

The enrichment of metalloproteinases in microglia deserves further comments on their functions apart from the regulation of cytokine levels. Microglia are highly motile cells in the normal CNS and in response to injury (Nimmerjahn et al., 2005). The well described role of metalloproteinases in regulating cellular motility (Sternlicht and Werb, 2001), and the elevated expression of metalloproteinases in microglia that our results show, may underlie the high motility of microglia in CNS pathology. Furthermore, the persistence and survival of activated microglia in CNS pathologies may be related to the reduced TIMP3 upon cellular activation, since TIMP3 can induce apoptosis of many cell types (Baker et al., 1998). Overall, the high expression of many metalloproteinases in microglia invites further investigation of their mechanistic interactions.

In summary, this study reveals that microglia bear a metalloproteinase signature that is more abundant and distinct from systemic leukocytes. Following activation, microglial cells augment the immune response by altering the metalloproteinases that are produced, and these contribute to alterations of TNF-α levels. One mechanism highlighted by this study is that TNF-α is secreted by microglia upon activation not through upregulation of ADAM17 (TACE) expression, but through increase of TNF-α transcripts, elevation of BB94- and TIMP3-inhibitable metalloproteinase activity, and by downregulation of TIMP3 mRNA levels. Our results highlight the possibility that metalloproteinase inhibitors have the potential as therapeutics to decrease microglial inflammation and its consequences within the nervous system.

REFERENCES

Amour A, Knight CG, English WR, Webster A, Slocombe PM, Knäuper V, Docherty AJ, Becherer JD, Blobel CP, Murphy G. 2002. The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs. FEBS Lett 524:154–158.

Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, Stephens PE, Shelley C, Hutton M, Knäuper V, Docherty AJ, Murphy G. 1998. TNF-α converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett 435:39–44.

Amour ACG, Knight CG, Webster A, Slocombe PM, Stephens PE, Knäuper V, Docherty AJ, Murphy G. 2000. The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. FEBS Lett 473:275–279.

Baker AH, Edwards DR, Murphy G. 2002. Metalloproteinase inhibitors: Biological actions and therapeutic opportunities. J Cell Sci 115:3719–3727.

Baker AH, Zaltsman AB, George SJ, Newby AC. 1998. Divergent effects of tissue inhibitor of metalloproteinase-1 -2 or -3 overexpression on rat vascular smooth muscle cell invasion proliferation and death in vitro. TIMP-3 promotes apoptosis. J Clin Invest 101:1478–1487.

Bar-Or A, Nuttall RK, Duddy M, Alter A, Pennington CJ, Bourgoin P, Edwards DR, Yong VW. 2003. Analyses of all matrix metalloproteinase members in leukocytes emphasize monocytes as major inflammatory mediators in multiple sclerosis. Brain 126:2738–2749.

Batchelor PE, Liberatore GT, Wong JT, Porritt MJ, Frerichs F, Donnan GA, Howells DW. 1999. Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci 19:1708–1716.

Becher B, Prat A, Antel JP. 2000. Brain-immune connection: Immunoregulatory properties of CNS-resident cells. Glia 29:293–304.

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. Nature 385:729–733.

Blobel CP. 2005. ADAMs: Key components in EGFR signaling and development. Nat Rev Mol Cell Biol 6:32–43.

Burns K, Martinon F, Tschopp J. 2003. New insights into the mechanism of IL-1β maturation. Curr Opin Immunol 15:26–30.

Chabot S, Le DW, Yong FP, Metz LM, Myles T, Yong VW. 2002. Cytokine production in T lymphocyte-microglia interaction is attenuated by glatiramer acetate: A mechanism for therapeutic efficacy in multiple sclerosis. Mult Scler 8:229–306.

Chabot S, Yong VW. 2000. Interferon β-1b increases interleukin-10 in a model of T cell-microglia interaction: Relevance to MS. Neurology 55: 1497–1505.

Chesneau V, Becherer JD, Zheng Y, Erdjument-Bromage H, Tempst P, Blobel CP. 2003. Catalytic properties of ADAM19. J Biol Chem 278: 22331–22340.

Cunningham LA, Wetzel M, Rosenberg GA. 2005. Multiple roles for MMPs and TIMPs in cerebral ischemia. Glia 50:329–339.

D'Haese A, Wuyts A, Dillen C, Dubois B, Billiau A, Heremans H, Van Damme J, Arnold B, Opdenakker G. 2000. In vivo neutrophil recruitment by granulocyte chemotactic protein-2 is assisted by gelatinase B/MMP-9 in the mouse. J Interferon Cytokine Res 20:667–674.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868.

Elkington PT, Nuttall RK, Boyle JJ, O'Kane CM, Horncastle DE, Edwards DR, Friedland JS. 2005. Mycobacterium tuberculosis but not vaccine BCG specifically upregulates matrix metalloproteinase-1. Am J Respir Crit Care Med 172:1596–1604.

Giuliani F, Hader W, Yong VW. 2005. Minocycline attenuates both T cell and microglia activity to impair cytokine production in T cell–microglia interaction. J Leukoc Biol 78:135–143.

Hsu DH, de Waal Malefyt R, Fiorentino DF, Dang MN, Vieira P, de Vries J, Spits H, Mosman TR, Moore KW. 1990. Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. Science 250:830–832.

Idriss HT, Naismith JH. 2000. TNFα and the TNF receptor superfamily: Structure–function relationships. Microsc Res Tech 50:184–195.

Kim SU, de Vellis J. 2005. Microglia in health and disease. J Neurosci Res 81:302–313.

Kreutzberg GW. 1996. Microglia: A sensor for pathological events in the CNS. Trends Neurosci 19:312–318.

Ladeby R, Wirenfeldt M, Garcia-Ovejero D, Fenger C, Dissing-Olesen L, Dalmau I, Finsen B. 2005. Microglial cell population dynamics in the injured adult central nervous system. Brain Res Brain Res Rev 48: 196–206.

Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B, Chen Q, Homer RJ, Wang J, Rabach LA, Rabach ME, Shipley JM, Shapiro SD, Senior RM, Elias JA. 2002. Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. J Clin Invest 110:463–474.

McCawley LJ, Matrisian LM. 2001. Matrix metalloproteinases: They're not just for matrix anymore! Curr Opin Cell Biol 13:534–540.

Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton LK, Schoenen F, Seaton T, Su JL, Becherer JD. 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. Nature 385:733–736.

Nimmerjahn A, Kirchhoff F, Helmchen F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314–1318.

Nuttall RK, Pennington CJ, Taplin J, Wheal A, Yong VW, Forsyth PA, Edwards DR. 2003. Elevated membrane-type matrix metalloproteinases in gliomas revealed by profiling proteases and inhibitors in human cancer cells. Mol Cancer Res 1:333–345.

Nuttall RK, Sampieri CL, Pennington CJ, Gill SE, Schultz GA, Edwards DR. 2004. Expression analysis of the entire MMP, TIMP gene families during mouse tissue development. FEBS Lett 563:129–134.

Parks WC, Wilson CL, López-Boado YS. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4:617–629.

Patel KD, McEver RP. 1997. Comparison of tethering and rolling of eosinophils and neutrophils through selectins and P-selectin glycoprotein ligand-1. J Immunol 159:4555–4565.

Roghani M, Becherer JD, Moss ML, Atherton RE, Erdjument-Bromage H, Arribas J, Blackburn RK, Weskamp G, Tempst P, Blobel CP. 1999. Metalloprotease-disintegrin MDC9: Intracellular maturation and catalytic activity. J Biol Chem 274:3531–3540.

Rosendahl MS, Ko SC, Long DL, Brewer MT, Rosenzweig B, Hedl E, Anderson L, Pyle SM, Moreland J, Meyers MA, Kohno T, Lyons D, Lichenstein HS. 1997. Identification and characterization of a protumor necrosis factor-α-processing enzyme from the ADAM family of zinc metalloproteases. J Biol Chem 272:24588–24593.

Shipley JM, Wesselschmidt RL, Kobayashi DK, Ley TJ, Shapiro SD. 1996. Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. Proc Natl Acad Sci USA 93:3942–3946.

Simard AR, Soulet D, Gowing G, Julien JP, Rivest S. 2006. Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. Neuron 49:489–502.

Sternlicht MD, Werb Z. 2001. How matrix metalloproteinases regulate cell behavior? Ann Rev Cell Dev Biol 17:463–516.

Streit WJ, Walter SA, Pennell NA. 1999. Reactive microgliosis. Prog Neurobiol 57:563–581.

Toft-Hansen H, Nuttall RK, Edwards DR, Owens T. 2004. Key metalloproteinases are expressed by specific cell types in experimental autoimmune encephalomyelitis. J Immunol 173:5209–5218.

Weaver A, Goncalves da Silva A, Nuttall RK, Edwards DR, Shapiro SD, Rivest S, Yong VW. 2005. An elevated matrix metalloproteinase (MMP) in an animal model of multiple sclerosis is protective by affecting Th1/Th2 polarization. FASEB J 19:1668–1670.

Williams K, Bar-Or A, Ulvestad E, Olivier A, Antel JP, Yong VW. 1992. Biology of adult human microglia in culture: Comparisons with peripheral blood monocytes and astrocytes. J Neuropathol Exp Neurol 51: 538–549.

Yong VW. 2005. Metalloproteinases: Mediators of pathology and regeneration in the CNS. Nat Rev Neurosci 6:931–944.

Yong VW, Power C, Forsyth P, Edwards DR. 2001. Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci 2:502–511.

Zhao BQ, Wang S, Kim HY, Storrie H, Rosen BR, Mooney DJ, Wang X, Lo EH. 2006. Role of matrix metalloproteinases in delayed cortical responses after stroke. Nat Med 12:441–445.

Zheng Y, Saftig P, Hartmann D, Blobel CP. 2004. Evaluation of the contribution of different ADAMs to tumor necrosis factor-α (TNF-α) shedding and of the function of the TNF-α ectodomain in ensuring selective stimulated shedding by the TNF-α convertase (TACE/ADAM17). J Biol Chem 279:42898–42906.