

# Matrix metalloproteinases, tissue inhibitors of MMPs and TACE in experimental cerebral malaria

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Cerebral malaria (CM) is a life-threatening disorder and a major medical problem in developing countries. It is caused by the sequestration of malaria-infected erythrocytes onto brain endothelia, followed by blood–brain barrier (BBB) damage and neurological deficit. In the present study, matrix metalloproteinases (MMPs) were analysed in a mouse model of CM with *Plasmodium berghei* ANKA. Increased numbers of gelatinase B (MMP-9)-positive cells, which were also CD11b<sup>+</sup>, were detected in the brain. In addition, activation of gelatinase B occurred in CM brains, and not in brains of mice with non-CM. However, selective genetic knockout of gelatinase B did not alter the clinical evolution of experimental CM. To study other protease balances, the mRNA expression levels of nine matrix metalloproteinases (MMPs), five membrane-type MMPs, TNF- $\alpha$  converting enzyme (TACE) and the four tissue inhibitors of metalloproteinases (TIMPs) were analysed during CM in different organs. Significant alterations in expression were observed, including increases of the mRNAs of MMP-3, -8, -13 and -14 in the spleen, MMP-8, -12, -13 and -14 in the liver and MMP-8 and -13 in the brain. Net gelatinolytic activity, independent of gelatinase B and inhibitable with EDTA, was detected *in situ* in the endothelia of blood vessels in CM brains, but not in brains of mice with non-CM, suggesting that metalloproteases, different from gelatinase B, are active in the BBB environment in CM. The increase in MMP expression in the brain was significantly less pronounced after infection of C57Bl/6 mice with the noncerebral strain *P. berghei* NK65, but it was similar in CM-susceptible C57Bl/6 and CM-resistant Balb/C mice upon infection with *P. berghei* ANKA. Furthermore, in comparison with C57Bl/6 mice, a larger increase in TIMP-1 and a marked, > 30-fold induction in MMP-3 were found in the brains of Balb/C mice, suggesting possible protective roles for TIMP-1 and MMP-3.

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Malaria is caused by protozoa of the genus *Plasmodium*, of which four species can infect humans. Although the genetics of the parasite<sup>1</sup> and the vector<sup>2</sup> have been well studied, insights into crucial mammalian host genes that may be useful targets for therapy are lacking. *P. falciparum* is the most virulent species in humans, and *P. vivax*, *P. ovale* and *P. malariae* are less a cause of lethality. World-wide, every year about 300 million acute illnesses

and more than one million deaths are reported, most of which occur in sub-Saharan Africa (www.who.int).

One of the major causes of lethality is a complication called cerebral malaria (CM). To escape the macrophages in the spleen, the late stages (late trophozoites and schizonts) of the parasite in the erythrocyte express proteins (eg erythrocyte membrane protein-1, PfEMP-1, in *P. falciparum*) at the surface of the erythrocyte, which mediate attachment of the infected erythrocyte to endothelia, in particular to those in the brain. This phenomenon leads to congestion of the blood vessels and to local inflammation. Subsequent neurological symptoms develop, ending in coma and death.<sup>3</sup> The exact causes of the neurological problems are not fully

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understood, but it is generally accepted that both metabolic insufficiencies (eg hypoxia, due to the congestion) and immunological reactions play a role.<sup>4,5</sup> A considerable number of leukocytes also marginate and participate in a local immune response.<sup>6</sup> Platelets also adhere to the activated brain endothelium in CM and play a role in the pathogenesis.<sup>7</sup> *In vitro*, *P. falciparum*-infected erythrocytes induce caspase-activation and apoptosis of human endothelial cells, indicating another possible pathogenic mechanism.<sup>8</sup> Both in humans and in mice, it was shown that the blood–brain barrier (BBB) is disrupted, which leads to mild to severe brain oedema.<sup>9</sup> Oedema formation further worsens the hypoxia by compressing cerebral blood vessels, leading to a collapsed blood flow, which is probably the ultimate cause of death in mice.<sup>10</sup>

In a widely used mouse model with the parasite *P. berghei* ANKA, several proinflammatory cytokines and cytokine-receptors play an essential role in the development of CM.<sup>6</sup> These include, for example, interferon- $\gamma$  (IFN- $\gamma$ ), IFN- $\gamma$  receptor, lymphotoxin- $\alpha$  and tumor necrosis factor receptor-2.<sup>11–14</sup> Also, TNF- $\alpha$  has been associated with CM (for a review, see Hunt and Grau<sup>6</sup> and Gimenez *et al*<sup>15</sup>). Until now, mainly regulatory molecules of the immune system have been investigated, with less attention to effector molecules. One exception is perforin from CD8<sup>+</sup> T-cells, which is also essential for the neurological symptoms in mice.<sup>16–18</sup>

Since the BBB is disrupted during CM, it is tempting to speculate that proteases of the extracellular matrix are involved. One such class of proteases are the matrix metalloproteinases (MMPs). MMPs are neutral Zn<sup>2+</sup>-dependent endopeptidases with a number of conserved domains, including the pro-, catalytic and Zn<sup>2+</sup>-binding domains and most have a carboxyterminal hemopexin domain.<sup>19,20</sup> Some MMPs have additional domains, for example, membrane type (MT)-MMPs have a carboxyterminal membrane anchor, and gelatinases have a gelatin-binding fibronectin-like domain. MMPs have distinct but overlapping substrate specificities and degrade highly efficiently components of the extracellular matrix. It is generally accepted that overproduction of these MMPs leads to tissue destruction and pathology, when not counterbalanced by their physiological inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). One particular MMP, gelatinase B or MMP-9, has been implicated in the degradation of the BBB in various diseases.<sup>21–27</sup> In addition, malaria-derived hemozoin has recently been shown to induce gelatinase B in a TNF- $\alpha$  dependent manner.<sup>28</sup> Furthermore, MMPs have been suggested to be involved in the shedding of membrane-bound TNF- $\alpha$ .<sup>29,30</sup> However, the main activator of pro-TNF- $\alpha$  is a non-MMP metalloproteinase, namely TNF- $\alpha$  converting enzyme (TACE), also called A Disintegrin and Metalloproteinase (ADAM)-17.<sup>31,32</sup> It was therefore relevant to analyse MMPs, TACE and TIMPs during CM.

## Materials and methods

### Infection of Mice with *P. berghei*

C57Bl/6 J mice or gelatinase B knockout mice, which were backcrossed to a C57Bl/6 J background for 10 generations, were injected intraperitoneally (i.p.) with 10<sup>4</sup> *P. berghei* ANKA parasites (a kind gift of Professor Dr A Waters and Professor Dr C Jansse, Leiden University Medical Centre, The Netherlands). For the analysis of non-CM, C57Bl/6 mice were infected with the parasite strain *P. berghei* NK65 (a kind gift of Professor Dr D Walliker, University of Edinburgh, Scotland, UK)<sup>33</sup> or Balb/C mice were infected with *P. berghei* ANKA. Mice with non-CM displayed no signs of CM, and died 2–3 weeks after infection with elevated (>30%) parasitemia. The percentage of infected erythrocytes in the peripheral blood was determined by microscopical analysis after Giemsa-staining. Statistical analyses of Kaplan–Meyer mortality curves were performed with the log-rank test software on the website of the Walter and Eliza Hall Institute (WEHI). All experiments were approved by the local ethical committee (Licence LA121251, Belgium).

### Ribonuclease Protection Assays (RPAs) and RT-PCR

At the indicated time intervals, inoculated mice or the corresponding controls were killed. Heparinised blood was collected by heart puncture and organs were removed and homogenised in TRIzol extraction reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted according to the procedure of the manufacturer and quantified by UV absorption. RPAs were performed as previously described.<sup>34</sup> Quantitative RT-PCR on 1  $\mu$ g RNA/sample was performed with the corresponding MMP/TACE primer and probe sets from Applied Biosystems (Foster City, CA, USA) on an ABI Prism 7000 Sequence Detection System apparatus (Applied Biosystems). As a control for the mRNA quantification, ribosomal 18S mRNA was also quantified by RT-PCR and found to remain constant in the different organs during the course of infection.

### Zymography

At the indicated time intervals, inoculated mice or the corresponding controls were killed. Heparinised blood was collected by heart puncture and organs were removed and homogenised in organ lysis buffer (50 mM Tris-HCl pH 7.4, 10 mM CaCl<sub>2</sub>, 5 mM *o*-phenantroline, 2 mM DTE, 0.1% Triton X-100, protease inhibitor cocktail (Complete, EDTA-free, Roche Diagnostics, Mannheim, Germany)). After centrifugation to remove insoluble debris, gelatinases in the extracts were prepurified by binding to gelatin-Sepharose, as described.<sup>35</sup> Subsequently, the prepurified gelatinases were analysed by gelatin

zymography.<sup>36</sup> The amounts loaded on zymography are the equivalent of 2 mg original tissue for lungs, kidneys, livers and spleens and of 30 mg brain tissue. As a molecular weight standard, we used a mixture of 1 ng recombinant human full-length progelatinase B (92 and 200 kDa) and 1 ng of two different recombinant gelatinase B mutants, lacking the hemopexin domain (72 and 144 kDa) or both the hemopexin and collagen V-like domains (48 kDa), all produced in Sf9 insect cells.

### **In Situ Zymography**

Gelatin *in situ* zymography was performed on 5- $\mu$ m-thick frozen brain sections, according to a modified protocol.<sup>37,38</sup> After cutting, the sections were allowed to dry and subsequently incubated for 3 h at 37°C with 20  $\mu$ g/ml quenched FITC-labelled gelatin (DQ gelatin, Molecular Probes, The Netherlands) in the following buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.2 mM NaN<sub>3</sub>, to which 20  $\mu$ g/ml unlabelled gelatin and a protease-inhibitor cocktail (Complete, EDTA-free from Roche) was added. This cocktail contains inhibitors against all classes of proteases except metalloproteases. After incubation, the sections were examined under the fluorescence microscope to localise the sites at which gelatinolysis occurred. As a control, 15 mM EDTA was added to inhibit metalloproteases.

### **Immunohistochemistry**

Frozen sections of 10  $\mu$ m were fixed in 10% neutral formalin (Sigma), washed in PBS and blocked with 1% BSA for 30 min. The sections were incubated with polyclonal goat anti-mouse MMP-9 antiserum (R&D Systems, Abingdon, UK; 6  $\mu$ g/ml) for 2 h, washed and incubated with FITC-labelled donkey anti-goat IgG (Jackson ImmunoResearch, Cambridge-shire, UK). In the case of double staining, phycoerythrin(PE)-labelled anti-CD11b (Beckton-Dickinson, Erembodegem, Belgium) was added together with the FITC-labelled donkey anti-goat IgG. For staining of neutrophils, fixed and blocked sections were incubated with PE-labelled anti-mouse Gr-1 antibody (eBioscience, San Diego, CA, USA). For this staining, sections of a spleen of an uninfected wild-type mouse were used as a position control. After a final wash, the sections were mounted with ProLong Gold Antifade (Invitrogen) and examined under the fluorescence microscope. Bright-green gelatinase B<sup>+</sup> cells were counted in 20 fields (20  $\times$  magnification) of cerebral cortex or cerebellum.

### **Treatment with the MMP-Inhibitor BB-94**

Six days after infection, mice were injected i.p. with a single dose of 50 mg/kg BB-94 (Batimastat, British Biotech, UK), resuspended at 3 mg/ml in

PBS + 0.01% Tween-20. Control mice were injected with the corresponding volume of PBS + 0.01% Tween-20.

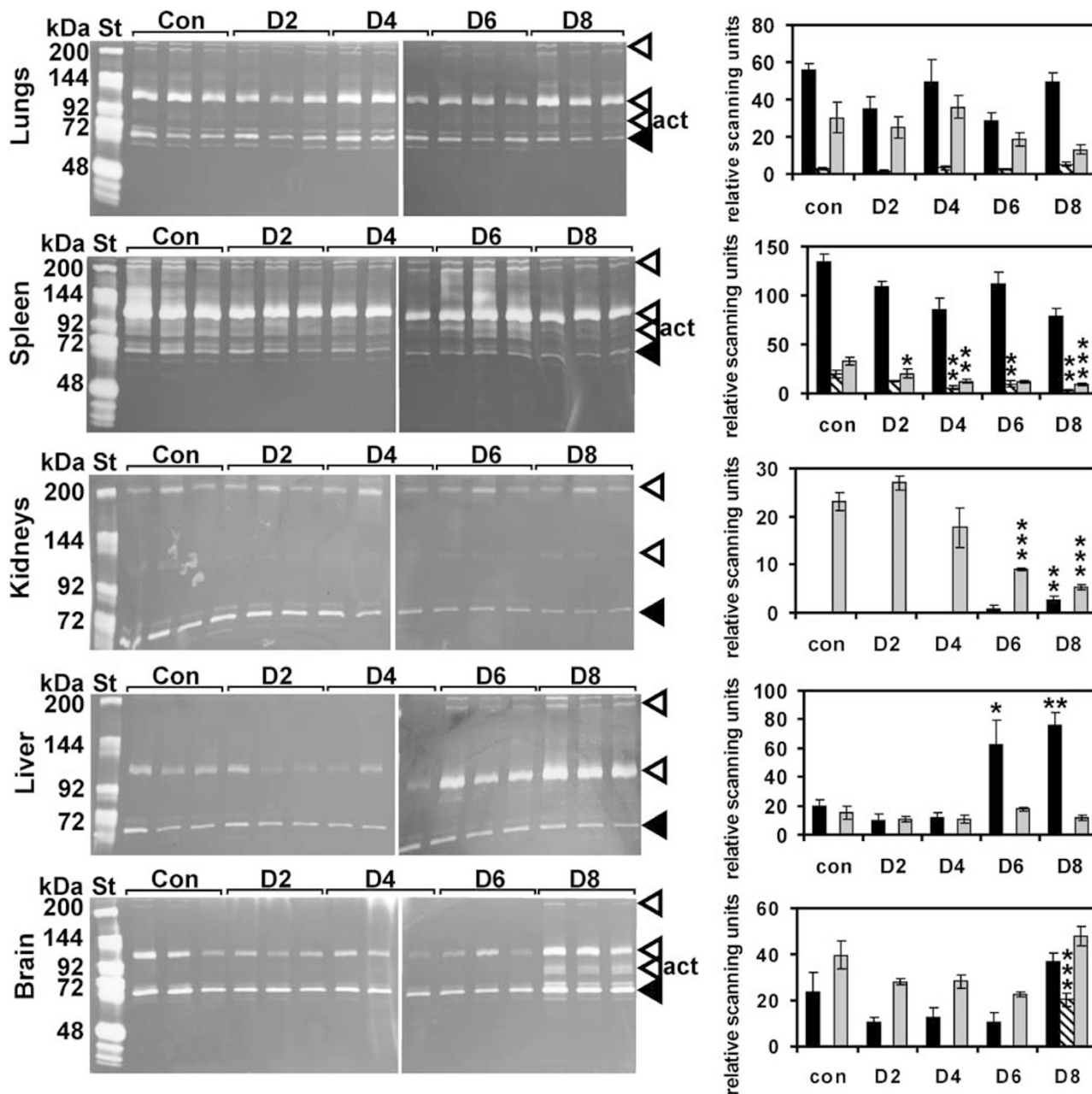
## **Results**

### **Zymography Analysis of Gelatinases A and B during Experimental CM**

Gelatinase B/MMP-9 has been shown to be crucial for the disruption of the BBB in several diseases and is also an important modulator of immune reactions, in particular by processing cytokines and chemokines.<sup>39,24</sup> Therefore, it was relevant to compare the expression of gelatinases by gelatin zymography analysis during the course of experimental CM in the brain and in organs important for immune reactions against the parasite, including spleen and liver.

Mice were inoculated with *P. berghei* ANKA-infected red blood cells. Infected erythrocytes (0.5–2%) were detected in the peripheral blood after 6 days, and the parasitemia increased and reached 7–10% infected erythrocytes after 8 days. Most mice (~95%) died of CM after 8–10 days. Organ extracts at different intervals during the disease were prepared, prepurified on gelatin-Sepharose and analysed by gelatin zymography (Figure 1). Levels of gelatinase A (MMP-2), which is expressed relatively constitutively by many cell types, decreased slightly in the serum (data not shown), kidneys and spleen, and increased slightly in the brain 8 days after infection (Figures 1 and 2). In contrast, a marked increase of progelatinase B was noticed in the liver.

In addition to the highly sensitive detection of different amounts of gelatinases, gelatin zymography is a useful tool to analyse the various molecular forms of gelatinases. In particular, activated forms are easily distinguished from the inactive proforms because activation results in a decrease of the molecular weight. Interestingly, activation of (pro)-gelatinase B was best observed in the brain after 8 days, concomitantly with the occurrence of the cerebral symptoms (Figures 1 and 2). The identities of progelatinase B and of the activated gelatinase B band were confirmed by their absence in gelatinase B knockout mice (see Figure 2). To evaluate whether the activation of gelatinase B occurs in malarial infections without cerebral complications, C57Bl/6 mice were infected with *P. berghei* NK65, a similar parasite strain that does not cause CM. Also, Balb/C mice, which are resistant to CM, were infected with *P. berghei* ANKA. In both cases, similar degrees of parasitemia (7–10%) as in mice with CM were obtained after 8 days, but the symptoms of CM were completely absent and the mice appeared healthy. Gelatin zymography analysis of brain extracts of these mice indicated that the activation of gelatinase B was significantly less apparent ( $P < 0.003$  in both cases) than in mice with CM, suggesting that this activation is specific for CM (Figure 2).

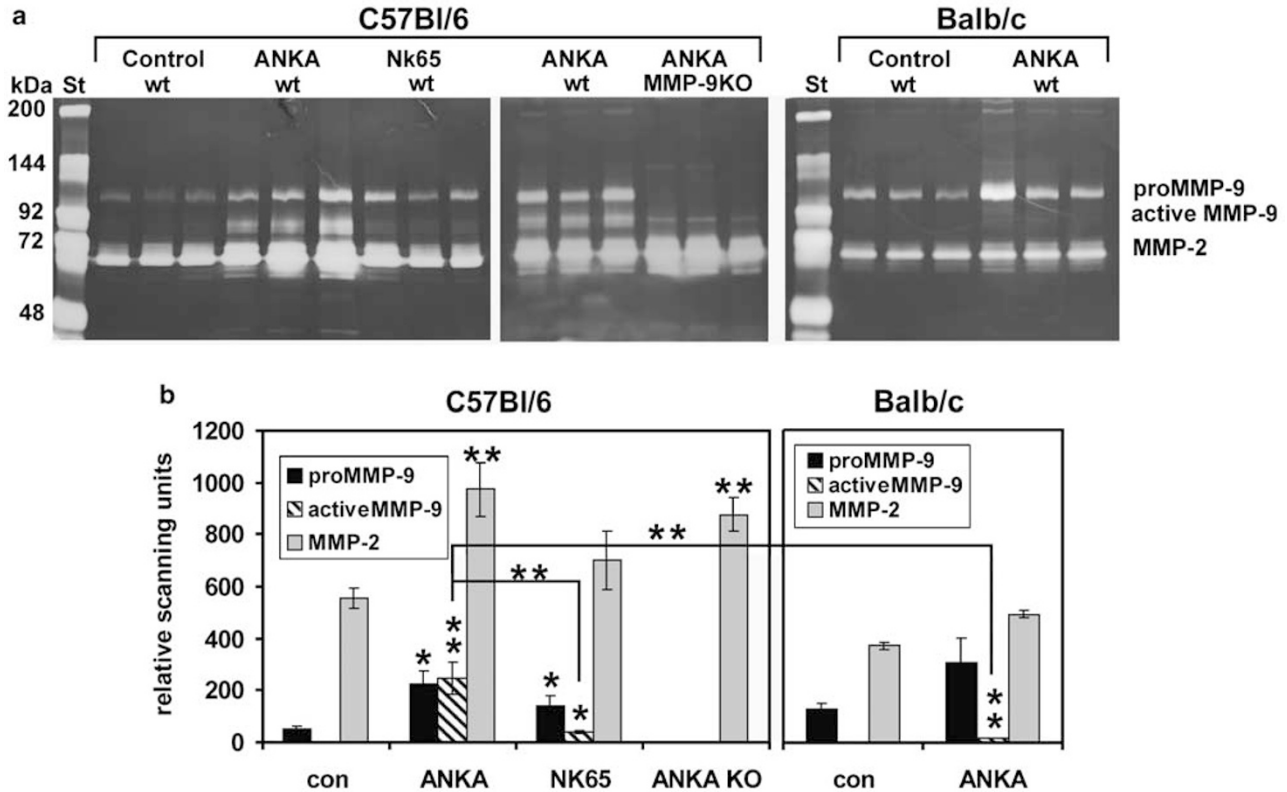


**Figure 1** Gelatin-zymography analysis of organ extracts of mice infected with *P. berghei ANKA*. C57Bl/6 mice were infected with *P. berghei ANKA* and killed at the indicated days after infection. Extracts of the organs of three mice at each time point were analysed by gelatin zymography to measure the amounts and the molecular forms of gelatinase A and B (MMP-2 and -9). The gelatinase B monomers (110 kDa) and dimers (220 kDa) are indicated with open arrowheads and activated monomers (95 kDa) with open arrowheads annotated with 'act'. Gelatinase A is indicated with filled arrowheads. The molecular weights of a standard gelatinase preparation are indicated in kDa. Quantification by scanning densitometry of the zymolytic bands of progelatinase B (black bars), activated gelatinase B (hatched bars) and gelatinase A (grey bars) is shown in the graphs at the right, and the statistical differences with the uninfected controls are indicated (\* $P < 0.05$ ; \*\* $P < 0.02$ ; \*\*\* $P < 0.002$ ). St, standard gelatinase preparation; Con, organ extracts of uninfected control mice; D2, 2 days after infection.

**Gelatinase B in the Brain of Infected Mice is Associated with CD11b<sup>+</sup> Cells**

To localise gelatinase B in the brain of mice with CM, immunohistochemistry on frozen sections was performed (Figure 3). Bright gelatinase B<sup>+</sup> cells were detected in wild-type mice and were absent in

gelatinase B knockout mice. The numbers of gelatinase B<sup>+</sup> cells were 10-fold increased in brains (cerebral cortex and cerebellum) of mice with CM vs uninfected animals. This is apparently in contradiction with the relatively modest increase in gelatinase B as detected by zymography (Figures 1 and 2), but in total brain extracts the local increase



**Figure 2** Gelatin-zymography analysis of brain extracts of mice infected with *P. berghei* ANKA and *P. berghei* NK65. (a) C57Bl/6 wild type or gelatinase B knockout or Balb/C wild-type mice were infected with *P. berghei* ANKA or with *P. berghei* NK65 and killed after 8 days. Extracts of the brains of these mice were analysed by gelatin zymography and compared with brain extracts of three uninfected control mice (Con) to measure the amounts and the molecular forms of gelatinase A and B (MMP-2 and -9), which are indicated. The faint and sharply delineated zymolytic band around 85 kDa in the brain extracts of the gelatinase B knockout mice is unrelated to gelatinase B. The molecular weights of a standard gelatinase preparation are indicated in kDa. St, standard gelatinase preparation. (b) The zymolytic bands shown in panel a were quantified by scanning densitometry. Significant differences with the uninfected controls and between ANKA- and NK65-infected C57Bl/6 mice and ANKA-infected Balb/c mice are indicated (\* $P < 0.05$ ; \*\* $P < 0.02$ ). Histogram colour code is as in Figure 1.

in these scattered gelatinase B<sup>+</sup> cells might not be detectable. Double immunostaining showed that all these gelatinase B-expressing cells are CD11<sup>+</sup>, indicating a myeloid origin of these cells.

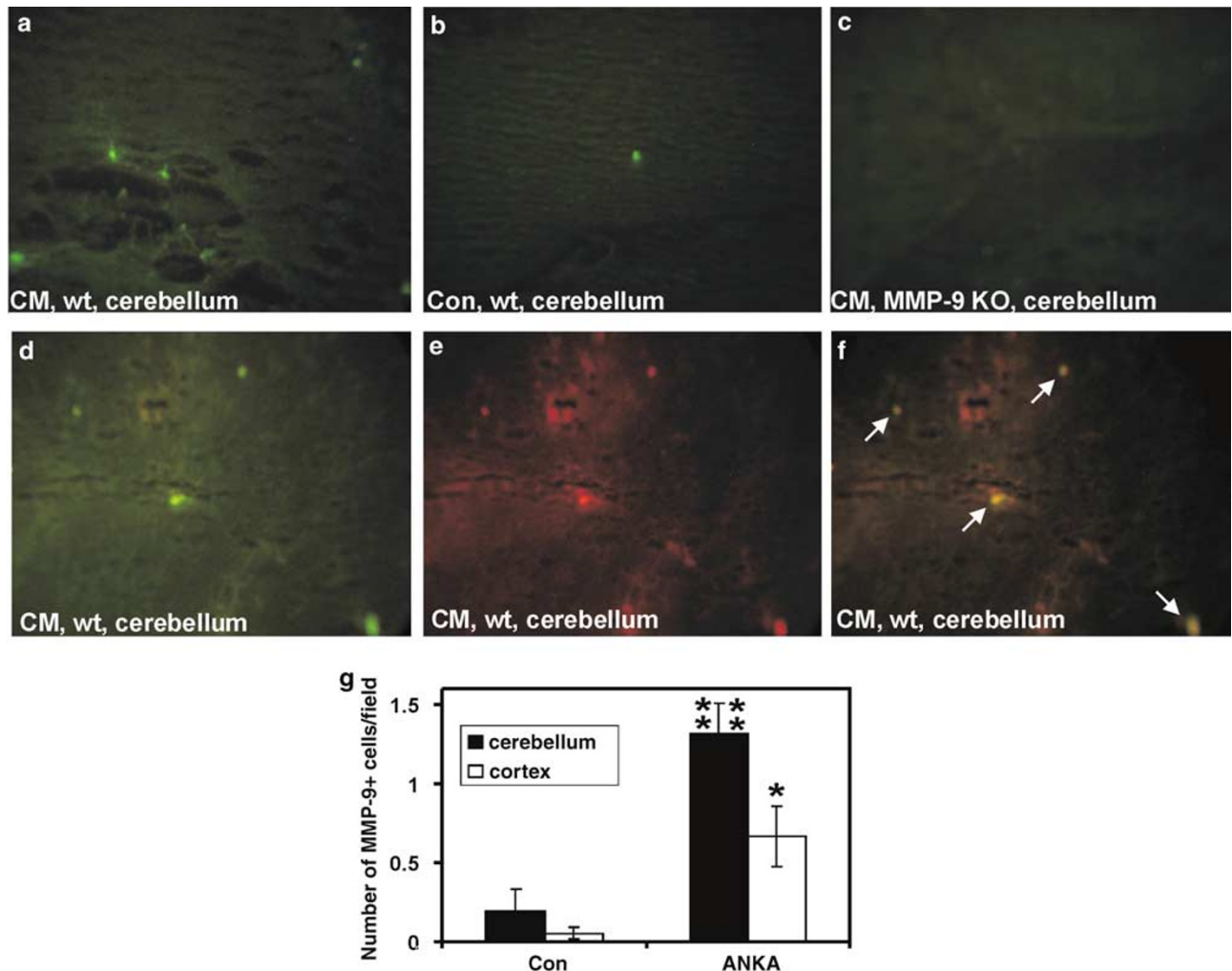
### CM in Gelatinase B Knockout Mice

The destruction of the BBB and the associated brain pathology may be a cause of mortality during CM. Since we observed expression and activation of gelatinase B in the brain and since gelatinase B is involved in the destruction of the BBB, we compared the mortality caused by CM in gelatinase B knockout and corresponding wild-type mice. As indicated in Figure 4a, no difference in lethality was observed in the gelatinase B knockout mice vs wild-type mice. As an internal control of infection, no significant differences in parasitemia were observed between the two animal populations during the course of disease (data not shown). Since some induced phenotypes in gelatinase B knockout mice are different in young mice compared to adult mice,<sup>40,41</sup> we performed a similar analysis in young

gelatinase B knockout and corresponding wild type mice of 3 weeks. No significant differences of mortality and parasitemia were observed (Figure 4b). As a conclusion, gelatinase B does not play an indispensable role in the pathogenesis of CM.

### Expression of MMPs during the Course of Experimental CM

MMP-9 acts together with other MMPs in disease processes in a so-called MMP cascade.<sup>25</sup> In order to study the expression and involvement of other MMPs during the disease development, the mRNAs of a large number of MMPs and MT-MMPs were analysed by RPA at different time intervals and in various organs. After 6–8 days, the mRNAs of several MMPs were increased in liver, spleen and lungs (Figure 5). In particular, marked increases of MMP-8, -12 and -13 in the liver, of MMP-3, -8, -9, and -13 in the spleen and MMP-8 and -9 in the lungs were noticed. Also, a decrease of MMP-11 in kidneys and lungs was observed at 6 and 8 days of disease development. In the brain, the mRNAs of



**Figure 3** Immunohistochemical localisation of gelatinase B in the brain of mice with CM. Frozen sections of mice with CM (a, c–f) or uninfected mice (b) were stained with an antiserum specific for gelatinase B (green). Sections of gelatinase B knockout mice (c) were used as negative controls. To further characterise the gelatinase B<sup>+</sup> cells, double immunostaining with CD11b (red) was performed. In (d), the green fluorescence of gelatinase B<sup>+</sup> cells in a double-stained section are shown, and in (e) the red fluorescence of CD11b<sup>+</sup> cells is obvious in the same field. In (f), panels d and e were merged and colocalisation is indicated by the white arrows. (g) The number of gelatinase B<sup>+</sup> cells were counted in 20 fields of cerebral cortex or cerebellum and compared between uninfected mice and mice with CM. \* $P < 0.05$ ; \*\*\*\* $P < 2 \times 10^{-6}$ .

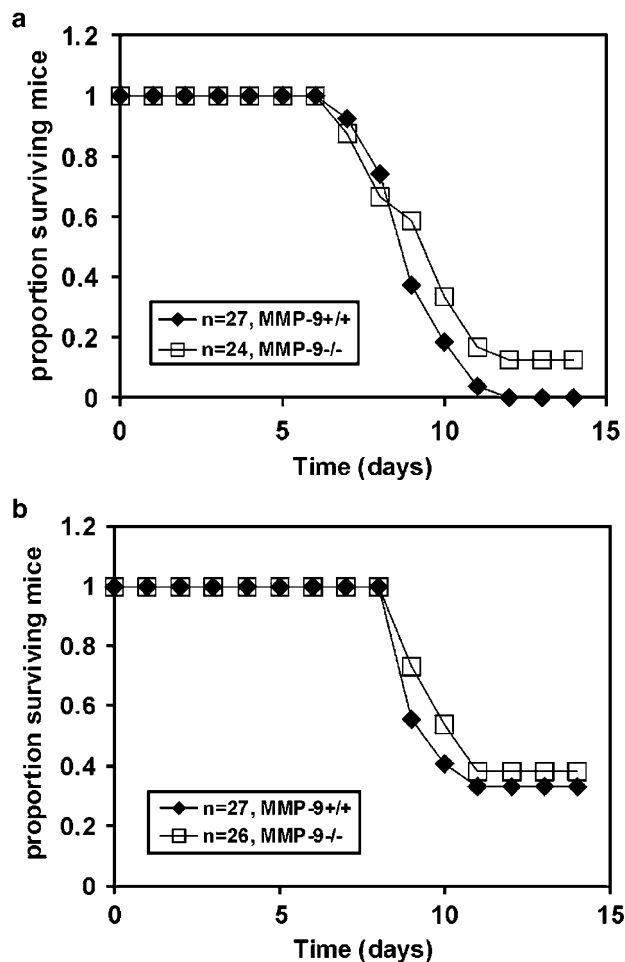
most MMPs were below the detection limit of the RPA. Therefore, RT-PCR for MMP-2, -3, -7, -8, -9, -11, -12 and -13 was performed on the brain mRNA samples (Figure 6). Marked increases of MMP-8 and MMP-13 at day 6 and day 8 were detected, and also an increase of MMP-3 at day 8. In accordance with the zymography data in Figures 1 and 2, gelatinase B/MMP-9 mRNA was only modestly increased. MMP-12 mRNA was significantly decreased at day 8, and MMP-11 remained unchanged during the disease course. MMP-2 and MMP-7 mRNAs remained below the detection limits. Since MMP-8, which is a main MMP from neutrophils, was strongly increased in the brain, it was analysed by immunohistochemistry for the neutrophil marker Gr-1 whether neutrophils were trapped in the vessels. No immunoreactivity with anti-Gr-1 was found, indicating that MMP-8 is presumably pro-

duced by other cells than neutrophils (data not shown).

For the MT-MMPs, an increase of MT1-MMP (MMP-14) mRNA in liver, spleen and kidneys was detected, and MT2-MMP (MMP-15) decreased in the liver and kidneys while the disease progressed (Figure 5). In the brain, MT-MMPs were detected and remained unchanged (data not shown). These alterations in expression of MMPs during the course of CM are summarised as a part of Table 1.

#### Expression of TACE/ADAM-17 during the Course of Experimental CM

TACE, also named A Disintegrin And Metalloproteinase (ADAM)-17, is the most important sheddase of membrane-bound TNF- $\alpha$ .<sup>31,32</sup> Therefore, the



**Figure 4** Disease course of CM in adult and young gelatinase B knockout and wild-type mice. (a) Adult (8–10 weeks old) gelatinase B knockout ( $\square$ ,  $n=24$ ) and corresponding wild-type ( $\blacklozenge$ ,  $n=27$ ) mice were infected with *P. berghei* ANKA. The mortality of both groups was followed after infection. The difference between the gelatinase B knockout and wild-type mice was not significant. (b) The mortality of young (3 weeks old) gelatinase B knock-out ( $\square$ ,  $n=26$ ) and corresponding wild-type ( $\blacklozenge$ ,  $n=27$ ) mice was followed after infection with *P. berghei* ANKA. The difference between the gelatinase B knockout and wild-type mice was not significant.

expression of TACE was analysed by RT-PCR in the mRNA samples of the different organs during the course of experimental CM (Figure 7). TACE mRNA was significantly increased at day 6 in liver and spleen, and returned to basal levels at day 8.

#### Expression of TIMPs in the Course of Experimental CM

An important regulation mechanism for the activity of MMPs and MT-MMPs in the extracellular space is inhibition by TIMPs. In fact, the biological activity of any MMP is regulated by the balance between the activated MMP and the different TIMPs.<sup>42</sup> Also, TACE is regulated by inhibition by TIMP-3 (but not

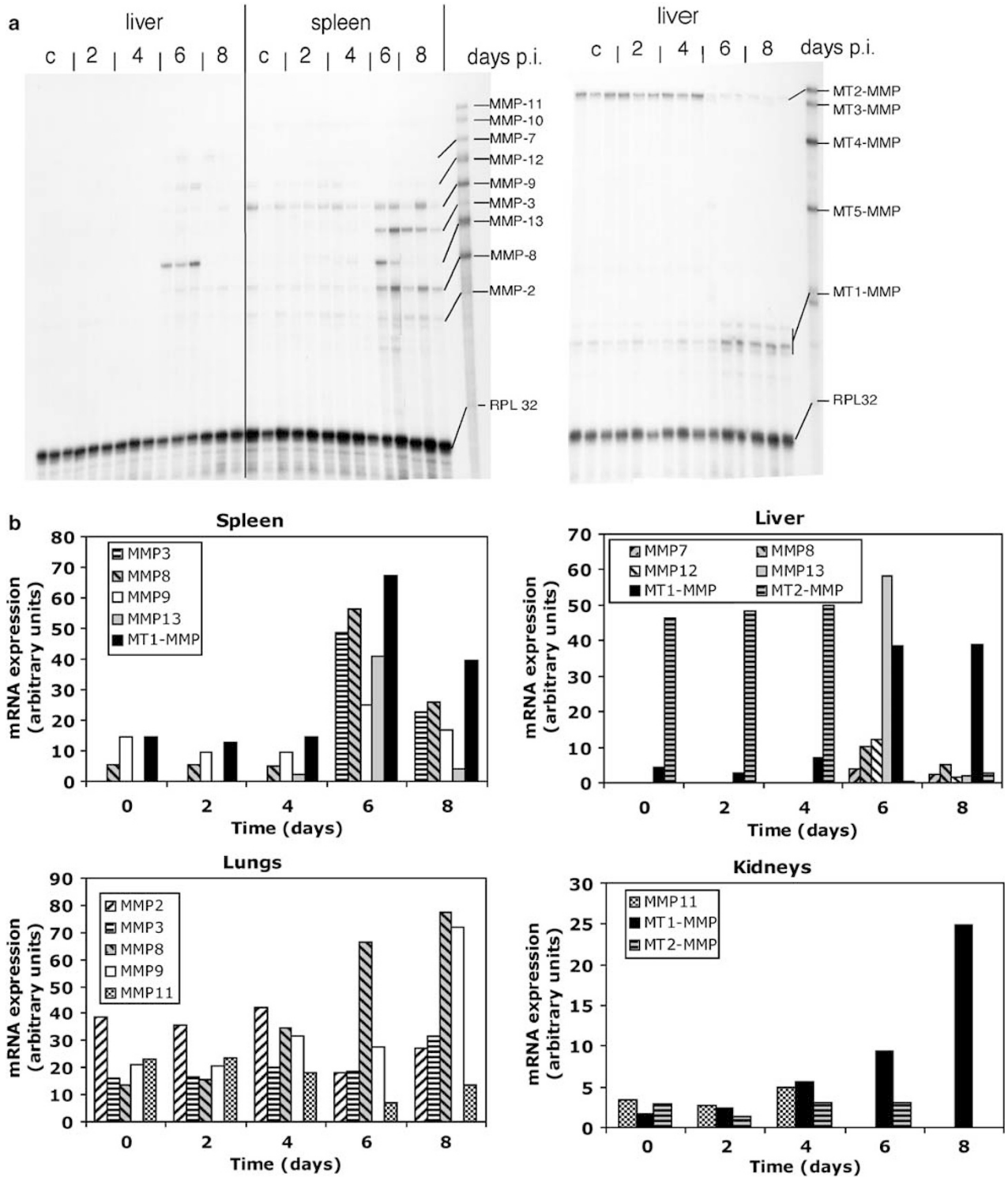
by TIMP-1, -2 or -4).<sup>43</sup> Therefore, the mRNAs for TIMPs were also analysed by RPA. A marked increase of TIMP-3 and TIMP-1 was observed in liver and spleen at days 6 and 8 after infection (Figure 8a and b), while no changes in the expression of TIMPs were detected in lungs and kidneys (summarised in Table 1). Since TIMP-1 remained below the detection limit of the RPA in the brain, RT-PCR was performed, and a four-fold increase in TIMP-1 mRNA expression was documented (Figure 8c).

#### Localisation of Net Proteolytic Activity in Brains from Mice with CM by *In Situ* Zymography

The observations of increased expression of MMPs and the activated forms of MMP-9, concomitant with a limited increase in the expression of TIMP-1, raises the question whether uninhibited, ‘net’ proteolytic activity might be present in brains from mice with CM. To test this hypothesis, proteolytic activity was measured *in situ* in brain sections using quenched FITC-labelled gelatin (Figure 9). In brains from uninfected control mice, only a low level of activity was associated with the meninges and septum and its large blood vessels, and also with cell bodies throughout the brain. In the brains from mice with CM, bright fluorescence was detected, indicating strong net gelatinolytic activity. This activity was mainly associated with blood vessels, including both small capillaries and larger blood vessels, and was particularly obvious in the granular layer and white matter in the cerebellum. In the cortex, a large number of capillaries contained gelatinolytic activity and also some larger vessels in the septum showed bright fluorescence. Similar patterns were observed in gelatinase B knockout mice and were unaffected by a general protease inhibitor against all classes of proteases except metalloproteases. However, the activity was completely disrupted in the presence of EDTA. Furthermore, little gelatinolytic activity was observed in the brains of infected Balb/C mice, which are resistant to CM. This shows for the first time that intense, metalloprotease-dependent proteolytic activity in small and large blood vessels is associated with CM.

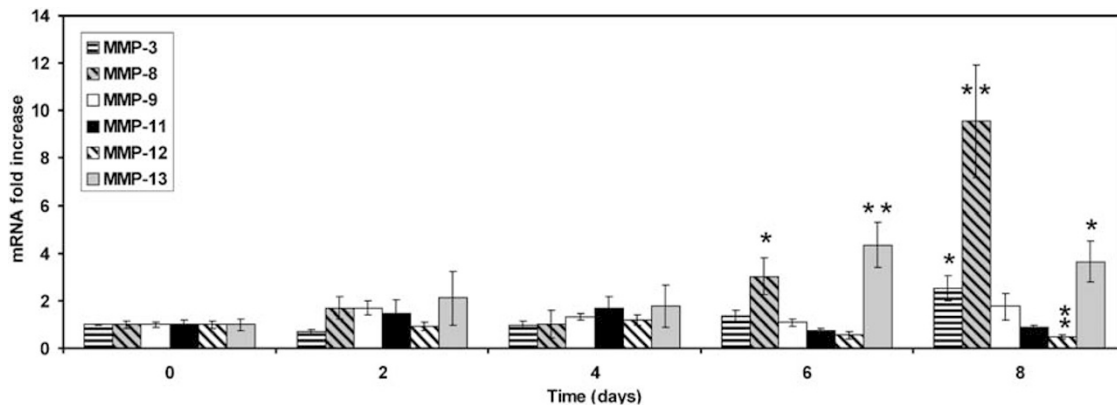
#### Inhibition of MMPs with BB-94 Delays Experimental CM

To determine whether MMPs and/or TACE play a role in experimental CM, infected mice were injected with the broad-spectrum MMP- and TACE-inhibitor BB-94. We choose to inject the inhibitor i.p. at day 6 during the course of infection, when the expression of MMPs and TACE starts to increase. At the used dosage, sufficient levels of the inhibitor remain present in the organism to inhibit most MMPs during at least 3 days.<sup>44</sup> By this treatment, the mice died only 1 day later, but this



**Figure 5** Detection of organ-specific mRNA expression of MMPs and MT-MMPs during the development of CM in mice. C57Bl/6 mice were infected with *P. berghei* ANKA and spleen, lungs, kidneys and liver were dissected at the indicated times (three mice for each time point) and used for mRNA preparation. After electrophoresis, the mRNAs of different MMPs and MT-MMPs were detected using radiolabelled probes. The house-keeping gene RPL32 was used as a reference control. (a) A limited set of autoradiographs of some organs are shown as examples. (b) Autoradiographs for the expression of MMPs and MT-MMPs were scanned, quantified by densitometry ( $n = 3$  for each time point) and normalised with the RPL32 mRNA as reference. A selected set of averaged data, containing all observed alterations as a function of time, are shown. C, uninfected control; p.i., postinfection.





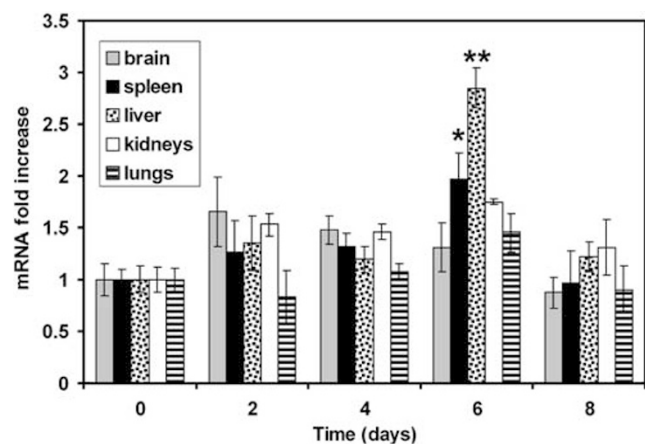
**Figure 6** Detection of the mRNA of MMPs by RT-PCR in the brain during development of CM in mice. Brains from C57Bl/6 mice (three mice per indicated time point) infected with *P. berghei* ANKA were dissected and homogenised at the indicated time points during the course of the disease. mRNA was isolated, quantified and subjected to RT-PCR for soluble MMPs. Relative mRNA levels are indicated as fold increases compared to mRNA from uninfected mice. MMP-2 and -7 remained below the detection limit. 18S mRNA was used as a control for the quantification of total mRNA. \* $P < 0.05$ ; \*\* $P < 0.02$ .

**Table 1** Changes in expression of MMPs, TACE and TIMPs during CM

	Brain	Liver	Spleen	Lung	Kidney
MMP-2	↑d8 <sup>a</sup>	- <sup>a</sup>	↓d2-8 <sup>a</sup>	-	↓d6,8 <sup>a</sup>
MMP-3	↑d8	ND	↑d6,8	-	ND
MMP-7	ND	↑d6,8	ND	ND	ND
MMP-8	↑d6,8	↑d6,8	↑d6,8	↑d6,8	ND
MMP-9	↑A d8 <sup>a</sup>	↑d6,8	-	↑rd8	↑d8
MMP-11	-	ND	ND	↓d6,8	↓d6,8
MMP-12	↓d8	↑d6	ND	ND	ND
MMP-13	↑d6,8	↑d6	↑d6	ND	ND
MT1	-	↑d6,8	↑d6,8	-	↑d6,8
MT2	-	↓d6,8	ND	-	↓d8
MT3	-	ND	ND	ND	ND
MT4	-	ND	ND	ND	ND
MT5	-	ND	ND	ND	ND
TACE	-	↑d6	↑d6	-	-
TIMP-1	↑d6,8	↑d6,8	↑d6,8	-	-
TIMP-2	-	ND	-	-	ND
TIMP-3	-	↑d6,8	↑d6,8	-	-
TIMP-4	-	ND	ND	ND	ND

<sup>a</sup>Detected only by zymography. ND, not detected; -, no change in expression levels compared to uninfected control; ↑, increase in expression; ↓, decrease in expression; ↑A, activation of the proenzyme; MTn, MTn-MMP; d, days after infection

delay in mortality was statistically significant by Kaplan–Meier analysis ( $P = 0.009$ ) (Figure 10). Since no differences in parasitemia were observed between controls and BB-94 treated animals, indicating that BB-94 has no effect on parasite growth (data not shown), these data are in line with the idea that



**Figure 7** Detection of the mRNA of TACE by RT-PCR in different organs at different times during CM development. Samples of mRNA from the indicated organs of *P. berghei* ANKA-infected mice (3 mice per indicated time point) were isolated, quantified and analysed by RT-PCR with a primer and probe set specific for TACE. 18S was used as a control for the quantification of the total mRNA. \* $P < 0.02$ ; \*\* $P < 0.001$ .

the balance between MMPs and inhibitors may influence the course of CM.

### Comparison of the Expression of MMPs, TACE and TIMPs in Cerebral vs Non-CM

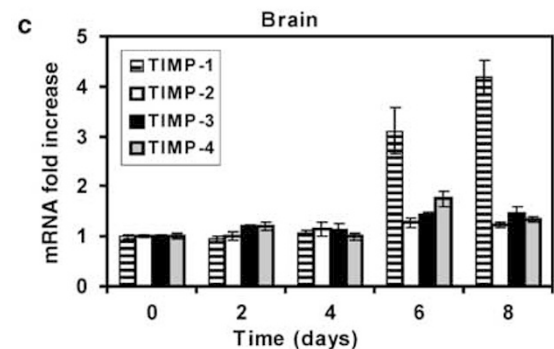
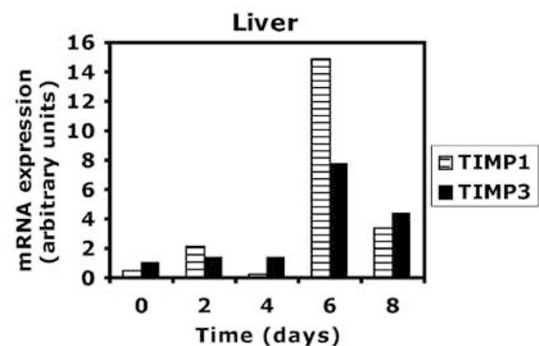
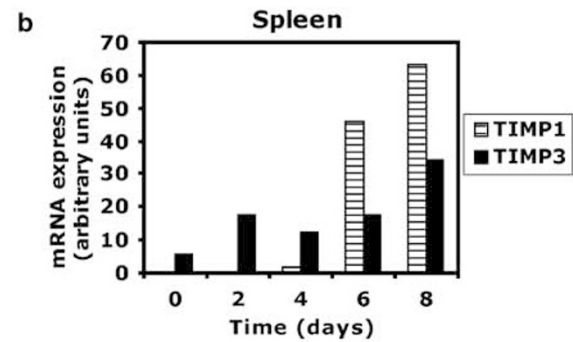
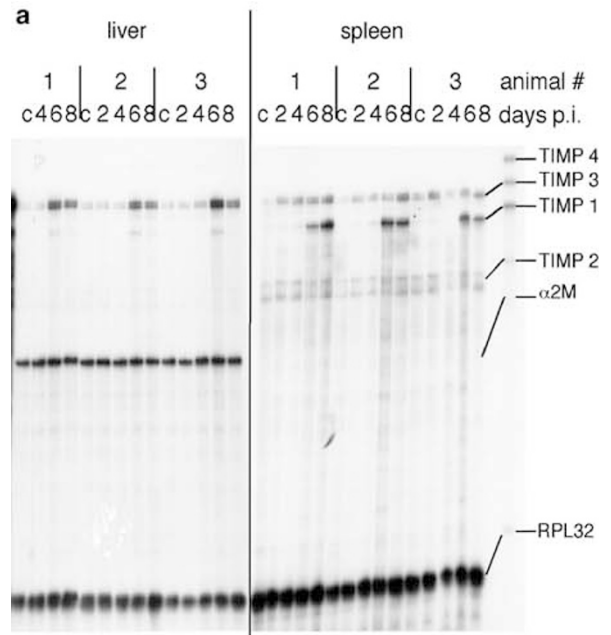
While it is tempting to speculate that the increased expression of several MMPs plays a role in the pathogenesis of CM, inhibition of MMPs with the broad-spectrum MMP-inhibitor BB-94 resulted in a delay of CM of only 1 day. The limited effect of this inhibitor might perhaps be explained by the possibility that some MMPs may be disease-promoting, while others could be protective. Therefore, we compared the expression of MMPs, TACE and TIMPs in the brain between CM and non-CM (Figure 11).

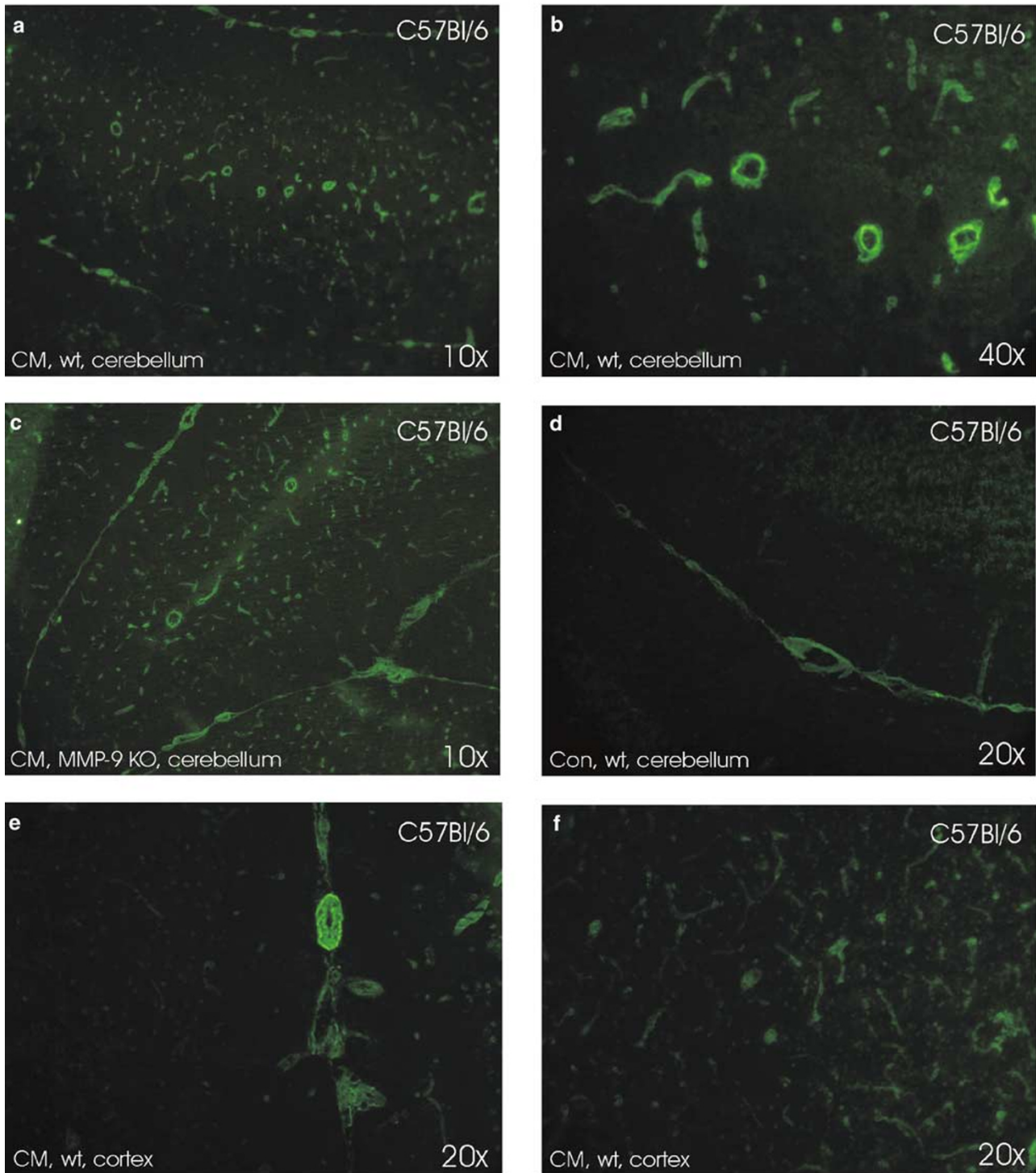
In the brains of C57Bl/6 mice infected with *P. berghei* NK65, which does not cause CM, the increases in expression of MMP-8 and -13 were significantly attenuated ( $P=0.02$  for MMP-8,  $P=0.04$  for MMP-13) when compared to mice with CM (infected with *P. berghei* ANKA). In contrast, after infection of CM-resistant Balb/C mice with *P. berghei* ANKA, the expression of MMP-3 was 30-fold enhanced, compared to 2.5-fold in mice with CM ( $P=0.017$  for the difference in increase), and MMP-8 and -13 were similarly increased as in mice with CM. Furthermore, the increase in expression of TIMP-1 was higher in the Balb/C mice compared to the C57Bl/6 mice with CM.

## Discussion

In the present study, we first analysed the expression of gelatinase B in an experimental *in vivo* model for CM. Zymography analyses of total extracts of different organs were performed, and this revealed a marked increase of gelatinase B in the liver. In the brain of mice with CM, increased numbers of gelatinase B<sup>+</sup> cells, which were also CD11b<sup>+</sup>, were found. In addition, activated forms of gelatinase B were detected, which are rather specific for CM, since these are significantly less apparent in non-CM (in C57Bl/6 mice infected with *P. berghei* NK65 or Balb/C mice infected with *P. berghei* ANKA). Activated forms of gelatinase B are usually difficult to observe, because they are highly unstable and subject to rapid turnover. The detection of these forms probably reflects that a continuous activation reaction is occurring. This observation is interesting, as gelatinase B has been implicated in several diseases of the central nervous system. However, no difference in mortality and parasitemia in gelatinase B knockout mice vs wild-type animals was observed, indicating that gelatinase B is not essential in the pathogenesis of CM. This is in contrast with the essential role of gelatinase B in other pathological conditions with BBB breakdown, such as, for example, ischemia and multiple sclerosis.<sup>21–27,40</sup> Therefore, the role of gelatinase B during CM is not yet clear. Several MMPs may act in concert, and elimination of one particular MMP may not necessarily result in a significant effect.

**Figure 8** Alterations of the expression of TIMPs during the development of CM. After collection of organs of mice infected with *P. berghei* ANKA (three mice for each indicated time point), mRNA was isolated and used to document the expression of TIMP-1, -2, -3 and -4 and  $\alpha$ 2-macroglobulin by RPA and/or RT-PCR. (a) A limited set of autoradiographs of the RPA of some organs are shown. (b) Autoradiographs for the expression of TIMPs were scanned, quantified by densitometry and normalised with the RPL32 mRNA as reference. A selected set of averaged data, containing all observed alterations as a function of time, are shown. (c) RT-PCR analysis of the expression of TIMPs in the brain. 18S was used as a control for the mRNA concentration. C, uninfected control; p.i., post-infection;  $\alpha$ 2M,  $\alpha$ 2-macroglobulin.





**Figure 9** Localisation of gelatinolytic activity in the brain by *in situ* zymography. Frozen sections of C57Bl/6 mice with CM (8 days after infection) and of different control mice were incubated with quenched FITC-labelled gelatin. Local gelatinolytic activity resulted in dequenching and was visualised by fluorescence microscopy. **(a)** Cerebellum of wild-type mouse with CM; **(b)** higher magnification of panel a showing strongly staining blood vessels. **(c)** Cerebellum from a gelatinase B knockout mouse with CM. **(d)** Cerebellum from an uninfected control mouse. **(e and f)** Cortex (with a strongly staining blood vessel in the septum in **e**) from a wild-type mouse with CM; **(g)** Cortex from an uninfected control mouse. **(h)** In the presence of EDTA, no gelatinolytic activity was observed (the cerebellum from a wild-type mouse with CM is shown). **(i)** Cerebellum of uninfected Balb/C mouse and **(j)** cerebellum from a CM-resistant Balb/C mouse 8 days after infection with *P. berghei* ANKA.

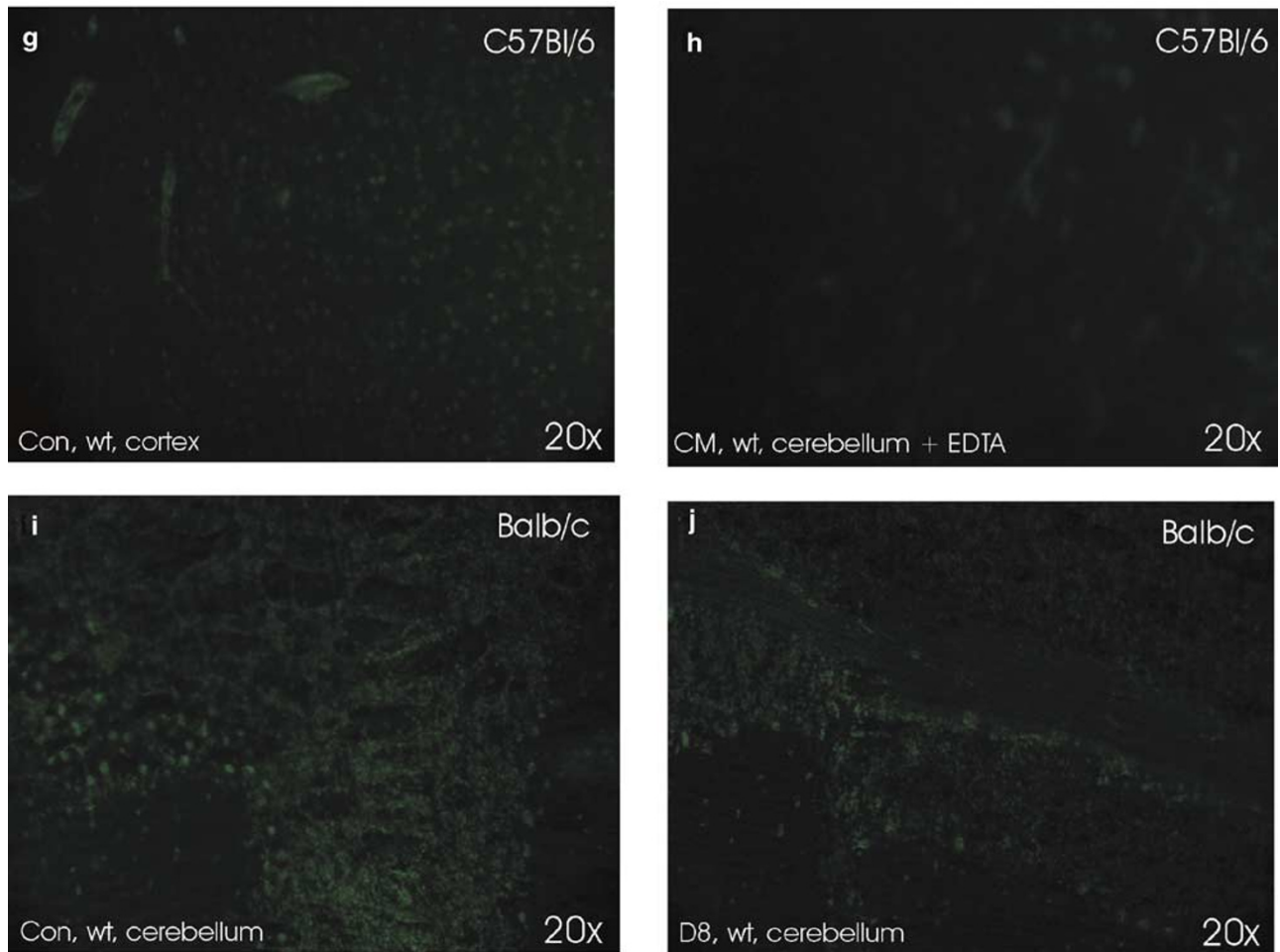
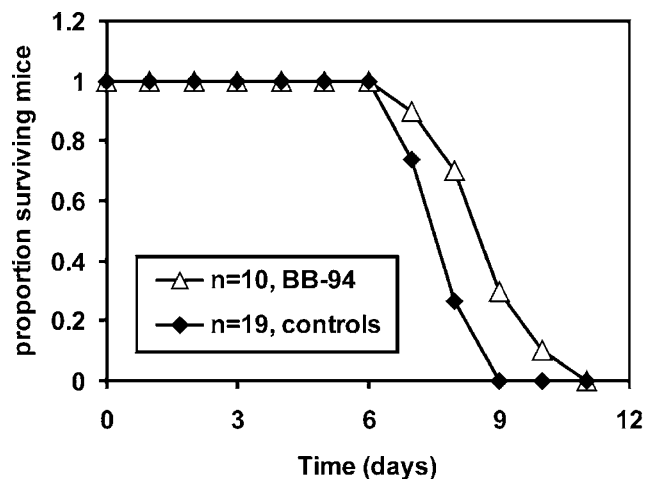


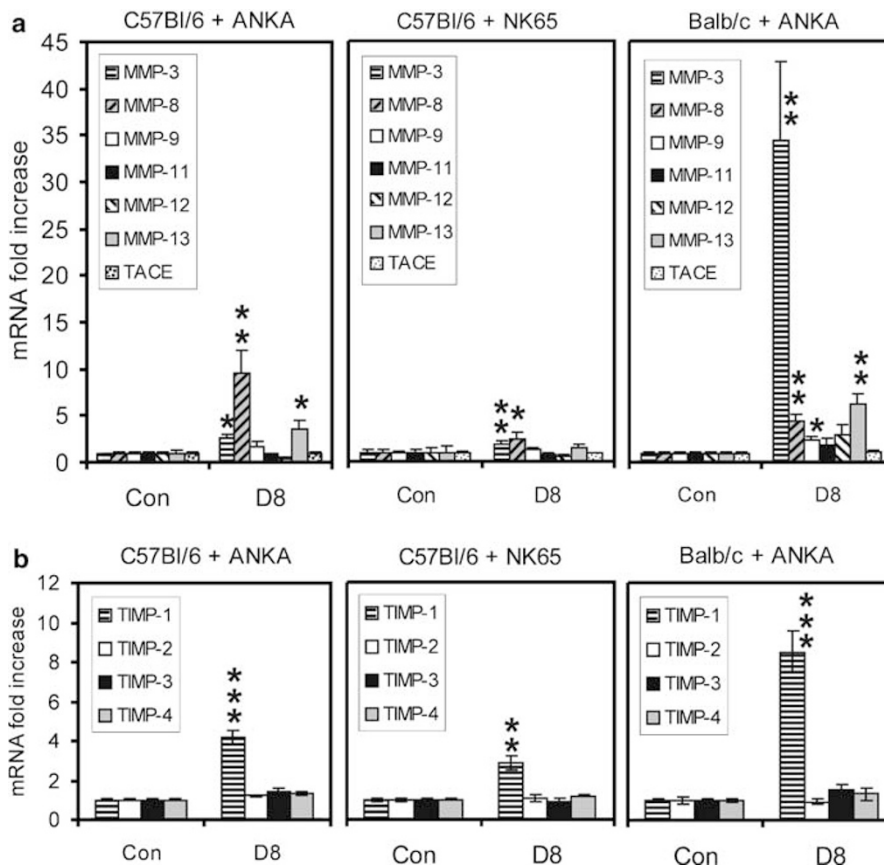
Figure 9 Continued.



**Figure 10** Disease course of CM in mice treated with BB-94. Adult (8 weeks old) C57Bl/6 mice were infected with *P. berghei* ANKA and treated with BB-94 ( $\Delta$ ,  $n=10$ ) or with control solution ( $\blacklozenge$ ,  $n=19$ ) 6 days after infection. The mortality was followed during the course of the disease and the difference between BB-94 treated and control-treated mice was found to be significant ( $P=0.009$ , log-rank test). The data are a compilation of two independent experiments, each with a similar and significant ( $P<0.05$ ) outcome.

Furthermore, compensatory mechanisms in MMP knockout mice cannot be excluded.<sup>45,46</sup>

As a follow-up, we show in the second part of our study that experimental CM in mice is associated with an increase in the expression of various MMPs and TACE in different organs (summarised in Table 1). The most considerable increases were noticed for the collagenases MMP-8 (neutrophil collagenase) and MMP-13 (collagenase 3) in several organs, including brain. These inductions were pronounced mainly at the end of the disease course when the cerebral symptoms appear. Interestingly, interstitial collagenase (MMP-1) is upregulated in human post-mortem brain samples from patients who died from CM.<sup>47</sup> Also, MMP-3 increased during mouse CM, particularly in the spleen. TACE was increased in spleen and liver before the cerebral symptoms occurred. TACE is considered as the most important sheddase of TNF- $\alpha$ ,<sup>31,32</sup> a cytokine involved in CM, but several MMPs are also able to shed TNF- $\alpha$ .<sup>29,30</sup> Furthermore, concomitant increases in the expression of the endogenous MMP-inhibitors TIMP-1 and -3 were observed. The exact role of the different MMPs and TIMPs in organs such as spleen, lungs,



**Figure 11** Comparison of the expression of MMPs, TACE and TIMPs in CM and non-CM. C57Bl/6 mice were infected with *P. berghei* ANKA (resulting in CM) or with *P. berghei* NK65 (non-CM), and Balb/C mice were infected with *P. berghei* ANKA (non-CM). RNA was extracted from the brains ( $n = 3$  for each condition) and RT-PCR was performed to analyse the expression of MMPs and TACE (a) and of TIMPs (b). 18S mRNA was used as a control for the quantification of total mRNA. \* $P < 0.05$ ; \*\* $P < 0.02$ ; \*\*\* $P < 0.001$ . Con, uninfected control; D8, 8 days after infection.

liver and kidney in malaria infection is not yet clear. MMPs contribute to organ damage in, for example, the liver or the lungs in other disease models,<sup>48–50</sup> but also contribute to antimicrobial responses.<sup>51,52</sup>

In the third part of this study, the significance of the expression of MMPs in brain was further examined. Using *in situ* zymography, intense, ‘net’ gelatinolytic activity was found to be associated with small capillaries and larger blood vessels in the brains of mice with CM. This activity was EDTA-inhibitable and was not found in mice with non-CM. However, it was not abrogated in gelatinase B knockout mice. Therefore, it is likely that this activity is the result of the combined action of several (other) metalloproteases. It is certainly also an indication for the activation of brain endothelial cells, in accordance with the increased expression of intercellular adhesion molecule-1.<sup>53</sup>

In view of the localisation of metalloprotease-dependent gelatinolytic activity at the brain endothelium and since MMPs are capable of degrading several components of the extracellular matrix, it was tempting to speculate that MMPs would participate in the pathogenesis of CM. Inhibition of

MMPs and TACE with the broad-spectrum MMP inhibitor BB-94 resulted in a significant but limited delay of mortality by only 1 day. A possible explanation is that MMPs might fulfil dual roles: while some MMPs may play a disease-promoting role during experimental CM, other MMPs possibly have protective roles, as was also found in other cerebral pathologies.<sup>23</sup> A further complication is that a particular MMP may have different roles in different organs. It is, for example, conceivable that an MMP participates in the degradation of the BBB and at the same time may participate in the immune response in the spleen or liver. Protective roles have also been observed in other diseases: several MMPs are important in wound healing,<sup>54</sup> remyelination in the brain<sup>55</sup> and in antimicrobial defence.<sup>51,52</sup>

In this context, the analysis of the expression of MMPs in the brains of mice with non-CM yielded interesting results. In comparison with the brains of mice with CM, the MMP expression was significantly less increased in brains of mice infected with the malaria parasite *P. berghei* NK65, consistent with the absence of cerebral pathology. However, in brains of CM-resistant Balb/C infected with

*P. berghei* ANKA, similar increases in MMP expression were noted as in the susceptible C57Bl/6 mice with CM, and additionally, a more than 30-fold increase in the expression of MMP-3 (to be compared with a 2.5-fold increase in the CM-susceptible mice) was found. Furthermore, the expression of TIMP-1 was also more increased in the brains of Balb/C mice compared to the C57Bl/6 mice. This could possibly be consistent with protective functions of both TIMP-1 and MMP-3 in the brain.

All together, the present data open new perspectives in the research on the immunopathological mechanisms in malaria infections. MMPs play important immunoregulatory roles. MMPs influence apoptosis by cleaving Fas-ligand into a soluble form,<sup>56</sup> and apoptosis of brain endothelial cells was shown to have a role in CM.<sup>8</sup> Particularly interesting, MMPs modulate immune responses by cleaving a variety of cytokines, cytokine receptors and chemokines, leading to either activation or inactivation of the cytokine.<sup>57–60</sup> This includes the activation of membrane-bound TNF- $\alpha$  into its soluble form.<sup>29</sup> Another metalloprotease important for the shedding of TNF- $\alpha$  is TACE or ADAM17, which is shown here to be increased in liver and spleen of infected mice.

Since specific inhibitors against most of the individual MMPs are lacking, it will be necessary to analyse the disease in the different MMP knock-out mice to determine the exact role (protective or detrimental) of each MMP during malaria infections. In addition, malaria parasites synthesise also metalloproteases, which are important, for example, in hemoglobin degradation.<sup>61</sup> Therefore, the use of particular MMP inhibitors may not only influence host but also parasite pathways. A detailed analysis of the role of each protease together with the development of specific inhibitors might yield novel insights and will indicate whether specific MMP-inhibition might be considered for novel therapies.

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## Conflicts of interest

The authors declare to have no conflicts of interest.

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