

Resistance of Collagenase-2 (Matrix Metalloproteinase-8)-Deficient Mice to TNF-Induced Lethal Hepatitis¹

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Abstract: Acute fulminant liver failure is a serious worldwide health problem. Despite maximal supportive intensive care treatment, the disease offers a poor prognosis, with mortality rates of >80%. We have previously shown that a broad-spectrum inhibitor of matrix metalloproteinases (MMPs) confers complete protection in a mouse model of TNF-induced lethal hepatitis, thereby suggesting the possibility of protecting cancer patients against the deleterious side effects of TNF therapy. In our search for the individual matrix metalloproteinases involved, we found that the recently generated MMP-8-deficient mice are significantly protected against TNF-induced acute hepatitis. In contrast to their wild-type counterparts, MMP-8-null mice display very little hepatocyte necrosis and apoptosis, resulting in a much better survival outcome. We found that these animals clearly display impaired leukocyte influx into the liver and no release of the neutrophil-specific, LPS-induced CXC chemokine. Our findings provide evidence that MMP-8 plays an essential role in acute liver failure and might be a promising new target for the treatment for this illness.

Abbreviations used in this paper: ALF, acute liver failure; ActD, actinomycin D; ECM, extracellular matrix; GalN, D-(+)-galactosamine; IHC, immunohistochemistry; KO, knockout; LIX, LPS-induced CXC chemokine; MMP, matrix metalloproteinase; MPO, myeloperoxidase; PMN, polymorphonuclear neutrophil; WT, wild type.

Fulminant liver disease or acute liver failure (ALF)⁵ due to viral hepatitis, alcohol abuse, or hepatotoxic drugs is one of the most intriguing and challenging conditions in the field of internal medicine (1, 2). TNF, produced mainly by activated macrophages, plays a central role in the pathology of ALF (3). This proinflammatory cytokine was discovered through its ability to specifically kill tumor cells both in vitro (4) and in vivo (5). However, due to the systemic inflammatory response syndrome resulting from high dose TNF administration, TNF-based antitumor therapy has to date been limited to locoregional therapy (6). Moreover, TNF-induced systemic inflammation is characterized mainly by hemodynamic shock and liver toxicity (7).

TNF has also been shown to increase the activity of many members of the matrix metalloproteinase (MMP) family (8), which consists of >25 Zn²⁺-dependent endoproteases. These enzymes are very effective in breaking down the major protein components of the extracellular matrix (ECM) and basement membrane. In recent years, however, it has become evident that these enzymes also exert important regulatory functions by processing a wide range of nonmatrix substrates, thereby influencing important processes, such as angiogenesis and cell migration (9).

We have previously demonstrated that a broad-spectrum MMP inhibitor (BB94) could protect D-(+)-galactosamine (GalN)-sensitized mice against TNF-induced fulminant liver failure (10). In addition, we were able to demonstrate roles for gelatinase-A (MMP-2), gelatinase-B (MMP-9), and stromelysin-1 (MMP-3) in breaking down the ECM at the sinusoids. We have now extended the study by trying to identify the role of collagenase-2 (MMP-8, also known as neutrophil collagenase) using the recently generated MMP-8 knockout (KO) mouse (11). Our interest in MMP-8 stems from the important role it could play in regulating inflammatory processes by cleaving different CXC chemokines and thereby modulating their biological activities. These chemokines include IFN-inducible protein-10, monokine induced by IFN- γ , and LPS-induced CXC chemokine (LIX), also known as murine granulocyte chemotactic protein-2 or CXCL5 (12, 13).

We report that MMP-8 is a central mediator in acute lethal hepatitis, because MMP-8-deficient mice are markedly protected against TNF/GalN-induced lethal hepatitis. Compared with their wild-type (WT)

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counterparts, these mice display an impaired neutrophil influx accompanied by a lack of release of the neutrophil-specific chemokine LIX from the ECM.

Materials and Methods

Mice

MMP-8-deficient mice (mixed C57BL/6-129Sv background) were generated by gene targeting (11). These mice and the controls with matching genetic background were bred as homozygous lines in an air-conditioned, temperature-controlled, conventional animal house. Experiments were performed with mice aged 8-13 wk. Previous studies have ruled out any difference in the responses of C57BL/6 and 129/Sv mice in the TNF/GalN and TNF/actinomycin D (ActD) models. All experiments were approved by and performed according to the guidelines of the animal ethical committee of the faculty of sciences at Ghent University (Ghent, Belgium).

Disease models

TNF/galactosamine experiments were performed by injecting mice i.p. with a combination of 0.25 μ g of murine TNF and 20 mg of GalN (Sigma-Aldrich). The latter drug specifically blocks transcription in hepatocytes, thereby sensitizing them to low doses of TNF (14). Mice usually start dying 6-8 h after the injection due to acute hepatitis, which is the result of massive apoptosis, followed by secondary necrosis. Although data suggest that apoptosis (measured by DNA fragmentation) precedes secondary necrosis (measured by the release of massive amounts of intracellular transaminases into the circulation) (15), it remains to be proven whether both events happen independently of one another. The survival rate was followed over the course of 2 days, after which no further deaths occurred. Rectal body temperature was measured using an electronic thermometer (Comark Electronics).

For hepatitis induced by TNF/ActD, mice were injected i.p. with a combination of 20 μ g of ActD (Sigma-Aldrich) and increasing doses of murine TNF (0.1, 0.3, and 0.5 μ g). The animals were observed during the next 4 days, after which no further deaths occurred.

Alanine aminotransferase

Mice were anesthetized with Avertin (tribromoethanol; Sigma-Aldrich), and blood was taken by cardiac puncture. The blood was allowed to clot, and serum was prepared and stored at -20°C. Alanine aminotransferase levels in serum were analyzed using a colorimetric test (Sigma-Aldrich) according to the manufacturer's guidelines.

Histological analysis and immunohistochemistry (IHC)

Livers were washed for 5 min *in vivo* with a 0.9% NaCl solution, excised, and then fixed and paraffin embedded. Sections of 4 μ m were stained using a standard H&E procedure. The histopathology 6 h after TNF/GalN administration was assessed visually, which allowed clear identification of apoptotic and necrotic cells. Apoptotic hepatocytes display cell shrinkage and formation of apoptotic bodies; cells that show cytoplasmic eosinophilia, cell swelling, and cell lysis are considered necrotic cells (16).

IHC of paraffin sections was performed by incubation for 2 h at 37°C with either an anti-human myeloperoxidase (MPO) Ab (Prosan) or an anti-murine LIX Ab (PeproTech). The LIX signal was amplified using the tyramide signal amplification system (PerkinElmer). The MPO signal was quantified by counting the number of MPO-positive cells in eight randomly chosen fields of both WT and MMP-8-deficient livers in a blind fashion and taking the average of these counts.

For MMP-8 IHC, cryosections were used. After excision, livers were placed in cryoembedding compound (MICROM) and stored at -80°C. Cryosections of 5 μ m were cut, air-dried, and acetone-fixed. MMP-8 was visualized by incubation for 2 h at room temperature with an anti-murine MMP-8 Ab (Santa-Cruz Biotechnology). The signal was amplified using the Vectastain Elite ABC peroxidase system (Vector Laboratories).

Western blot

Livers were snap-frozen in liquid N₂ and stored at -80°C until use. Samples were homogenized and then centrifuged. The supernatant was normalized for protein content using a DC protein assay (Bio-Rad) before loading onto 16% precast Tris-glycine gels (Invitrogen Life Technologies). Proteins were subsequently transferred to a nitrocellulose membrane by semidry blotting. These membranes were probed with the same anti-murine LIX Ab (PeproTech) used in the IHC. An ECL substrate (PerkinElmer) was used in combination with a specific HRP-coupled anti-rabbit secondary Ab (DakoCytomation) to visualize LIX.

Semiquantitative RT-PCR

Liver samples were stored in RNAlater buffer (Ambion). Samples were homogenized, and RNA was extracted using an RNeasy kit (Qiagen). After cDNA synthesis, semiquantitative RT-PCR was performed using the following primers: 5'-CATCAACATTGCTTTCGTCTC-3' and 5'-TGAG GTCAACTGTTCTCAGC-3' for MMP-8, 5'-ATCTCGCCATTCATGC GGATG-3' and 5'-CTATTGAACACTGGCCGTTCT-3' for LIX, and 5'-TTCCGATGCCCTGAGGCTCT-3' and 5'-CAGGAGGAGCAATGATC TTG-3' for actin.

MPO quantification

For determining MPO concentrations, liver samples were homogenized, sonicated, freeze-thawed, and separated by centrifugation. Supernatants were analyzed by spectrophotometry with *o*-dianisidine dihydrochloride and hydrogen peroxide at 460/490 nm. The MPO concentration was normalized to the total protein content of the sample.

Statistical analysis

Mean values were compared using an unpaired Student's *t* test with Welch's correction. Survival curves were compared using a log-rank χ^2 test, and final outcomes were compared using a χ^2 test.

Results

Collagenase-2 deficiency confers protection against TNF/GalN-induced ALF

To study the possibility that MMP-8 plays a role in disease development in a model of ALF, we injected MMP-8-null animals and their WT counterparts i.p. with a combination of 0.25 μ g of TNF and 20 mg of GalN. The TNF/GalN model is a well-established model of inflammatory acute hepatitis; it is characterized by leukocyte influx, necrosis, and apoptosis (17) as described in *Materials and Methods*.

The survival rate clearly shows that MMP-8-null mice are significantly protected against the mortality induced by TNF/GalN compared with their WT counterparts (Fig. 1A; $p = 0.003$). These protected mice do not exhibit the hypothermia observed in WT animals 6 h after challenge (Fig. 1B; $p = 0.001$). Furthermore, the alanine aminotransferase concentrations in the circulation 6 h after challenge clearly show that the massive death of hepatocytes observed in WT animals was greatly reduced in the MMP-8-null mice (Fig. 1C; $p < 0.001$). This result was confirmed by histological examination of the liver 6 h after challenge (Fig. 2, A and B). Indeed, the livers of WT animals displayed apoptotic as well as necrotic hepatocytes, widespread hemorrhage, and loss of structural integrity, in contrast to the livers of MMP-8-deficient mice, which showed almost no signs of liver damage.

Collagenase-2 deficiency does not protect against ALF induced by TNF/ActD

To test whether the protection observed in the TNF/GalN model also persists in another model of ALF, we injected MMP-8-deficient and WT animals i.p. with a combination of different doses of TNF (0.1, 0.3, and 0.5 μ g) combined with 20 μ g of ActD. This model is dependent on direct induction of hepatocyte apoptosis, whereas in the TNF/GalN model, hepatocyte apoptosis is indirect and mediated by inflammation.

TNF/ActD induced lethal acute hepatitis, with deaths starting to occur 4 h after the challenge, and none occurring after 96 h (Fig. 1, D-F). We observed a clear dose-related response, with most of the animals that received the highest dose dying during the first 24 h. Survival time was longer at lower doses, but eventually no difference was seen in overall mortality. None of the doses used resulted in a significant difference between the MMP-8-null animals and their WT counterparts in either survival time or final outcome.

Collagenase-2 deficiency leads to impaired neutrophil migration in response to a TNF/GalN challenge

In contrast to the TNF/ActD model, inflammation is a central event in the development of ALF induced by TNF/GalN. Neutrophil infiltration is a critical step in disease development, because a neutrophil-depleting Ab protects mice against the lethal effects of TNF/GalN (10). We therefore compared the leukocyte influx in the livers of WT animals and MMP-8-null mice in response to TNF/ GalN treatment by staining liver slides with an MPO Ab and counting the number of MPO-positive cells per field.

Infiltration of MPO-positive cells into the liver parenchyma was observed in WT animals as early as 4 h after TNF/GalN injection, and the number of leukocytes increased over time. In MMP-8-null mice, some MPO-positive leukocytes did accumulate in the venular lumen, but almost none of them actually infiltrated the liver tissue (Fig. 2, C and D). A large and significant reduction in neutrophil infiltration was observed in MMP-8-deficient animals compared with WT mice when counting the number of MPO-positive cells (Fig. 2E), indicating that MMP-8 plays a crucial role in the extravasation and migration of MPO-positive leukocytes.

MMP-8 expression and localization after TNF/GalN challenge

After TNF/GalN challenge, MMP-8 mRNA was strongly induced by 1 h after the injection, as determined by semiquantitative RT-PCR (Fig. 3A). Although we repeatedly observed almost no MMP-8 mRNA 4-5 h after challenge, at later time points we again detected high levels of mRNA expression. Interestingly, this increase was associated with the increase in MPO levels in the liver (Fig. 3β), suggesting that this secondary MMP-8 up-regulation was due to infiltrating neutrophils.

Staining liver slides with a specific MMP-8 Ab revealed that MMP-8 is present in circulating neutrophils in unchallenged WT animals (Fig. 3C). After TNF/GalN challenge, MMP-8 staining was observed near endothelial cells around the veins (Fig. 3E). At the final stage of the disease model, MMP-8 could be seen in infiltrated leukocytes, scattered through the destroyed liver parenchyma (Fig. 3G). Livers of MMP-8-null mice served as negative controls and displayed little or no background staining (Fig. 3, D, F, and H).

LIX is not released in collagenase-2-deficient animals

To investigate the link between MMP-8 and neutrophil migration, we decided to focus on LIX, the only neutrophil-specific (CXC ELR⁺) chemokine whose biological activity can be modulated by MMP-8 (13). As shown in Fig. 4A, LIX mRNA was detectable as early as 1 h after TNF/GalN administration and reached its maximum 3 h after the challenge. To detect the location of LIX protein in liver sections, we used an LIX-specific Ab. LIX protein could be detected in all healthy unchallenged mice near the endothelial cells around the portal veins (Fig. 4, B and C). Because there was no detectable level of LIX mRNA in unchallenged livers, we propose that this is an accumulated ECM-associated reservoir of LIX protein. Although it has not yet been described specifically for LIX, it is known that many chemokines bind to ECM components through interactions involving glycosaminoglycans (18, 19).

Based on the ability of MMP-8 to cleave LIX both N- and C-terminally and in view of its up-regulation at early time points, we speculated that MMP-8 might play a role in the release of this ECM-associated chemokine pool. The observation that the LIX signal moves from the venular area into the sinusoids of the liver in WT animals 4 h after challenge, in contrast to KO animals, in which no such delocalization was observed and in which the LIX signal remained confined to the area around the portal veins even 6 h after challenge (Fig. 4, D-F), supports such an idea.

FIGURE 1: *MMP-8-null mice are significantly protected against TNF/GalN-induced ALF, but not against TNF/ActD-induced ALF.* A, Survival of MMP-8 WT and KO animals upon i.p. administration of a combination of 0.25 μ g of TNF and 20 mg of GalN (**, $p = 0.003$). B, Comparison of body temperatures of MMP-8 WT ($n = 8$) and KO ($n = 7$) animals, 6 h after TNF/GalN injection (**, $p = 0.001$). C, Alanine aminotransferase (ALT) levels in the circulation 6 h after TNF/GalN administration in MMP-8 WT ($n = 6$) and MMP-8 KO ($n = 6$) animals (***, $p < 0.001$). D-F, Survival of MMP-8 WT and KO animals upon i.p. administration of 20 μ g of ActD combined with 0.1 (D), 0.3 (E), or 0.5 (F) μ g of TNF ($p = NS$).

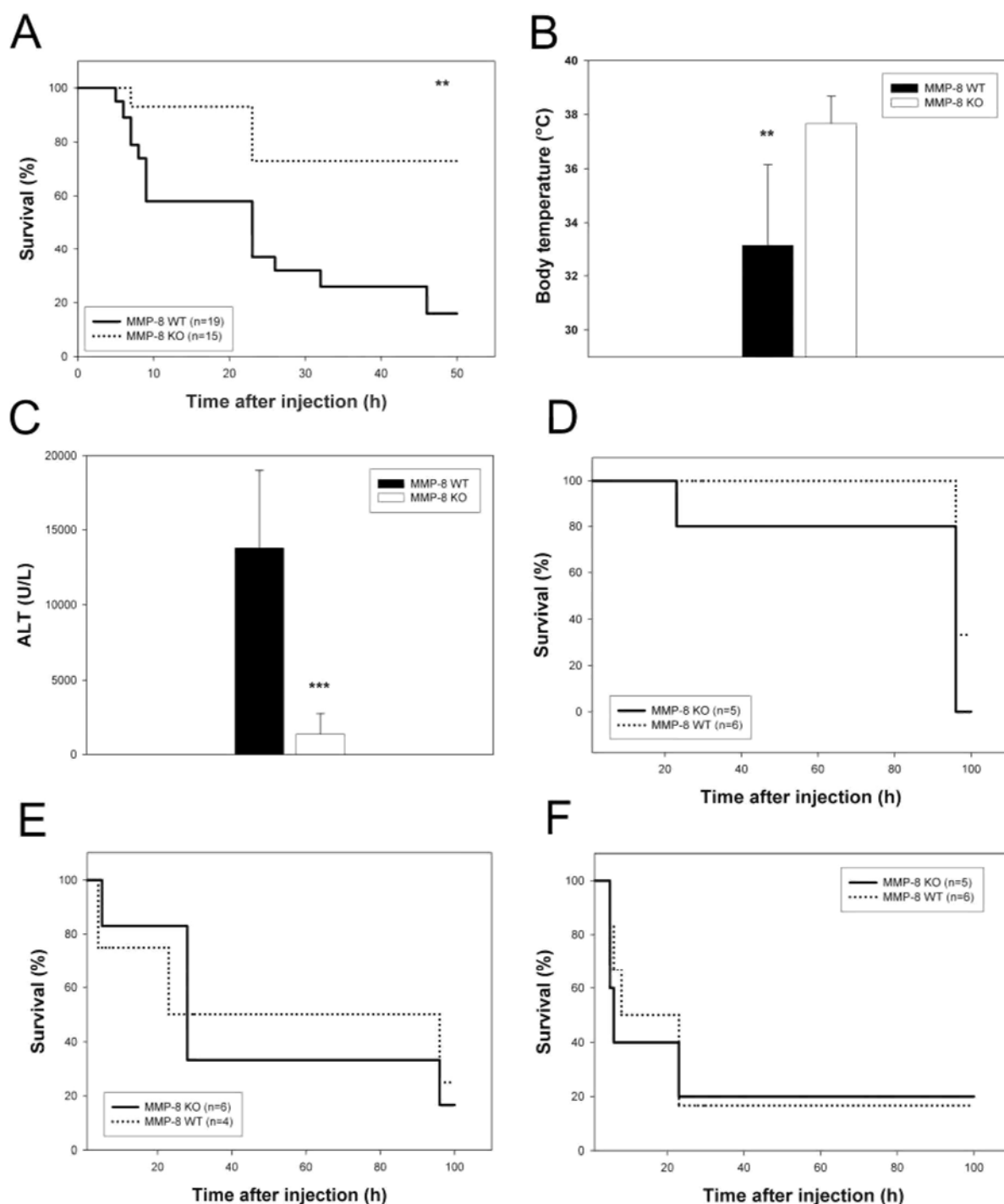
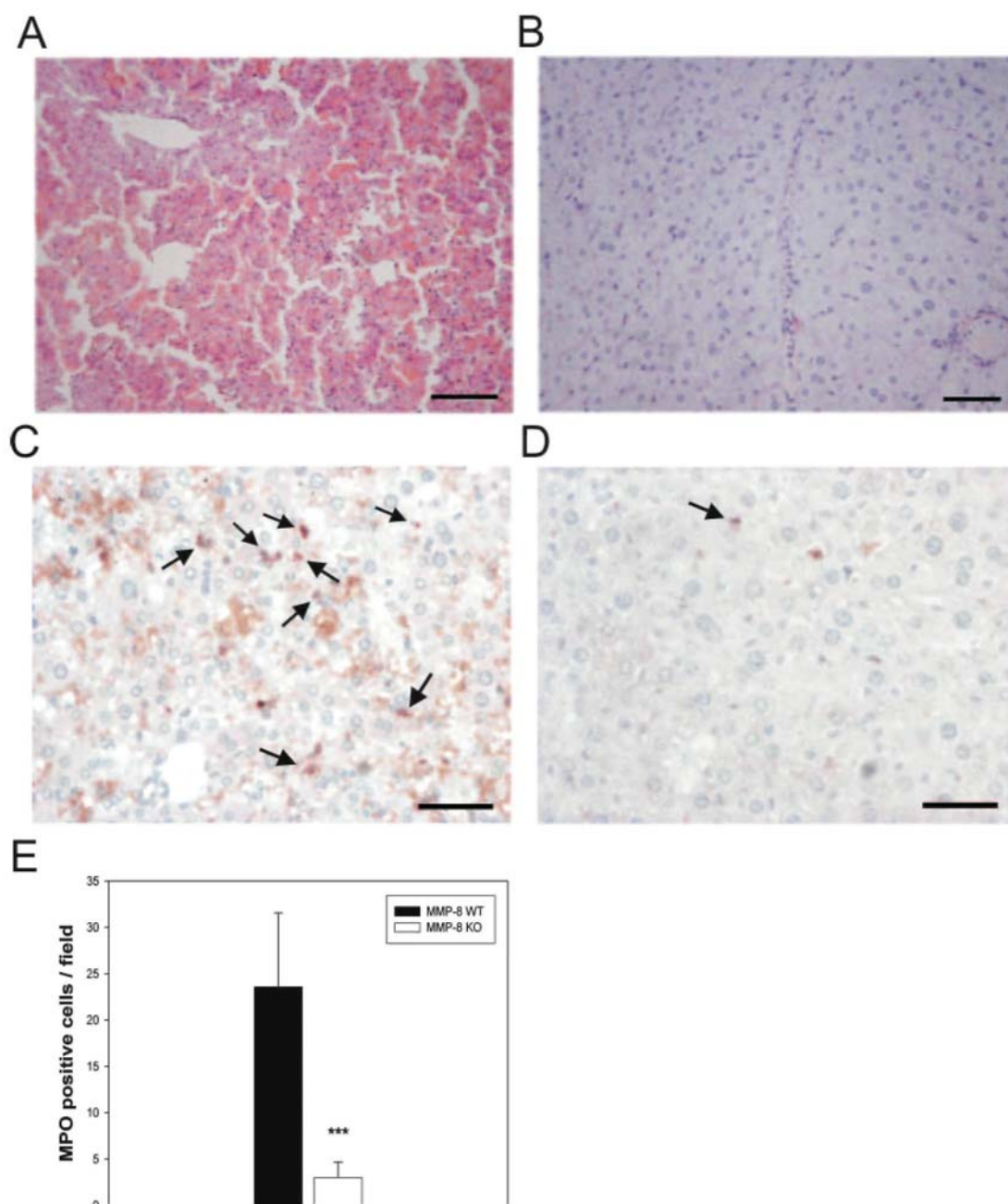


FIGURE 2: Neutrophil infiltration and liver damage after TNF/GalN challenge are reduced in MMP-8-null mice compared with WT animals. H&E staining of WT animals (A) and MMP-8-null mice (B) is shown. MPO IHC staining of livers of WT animals (C) and MMP-8-null mice (D) is shown, with arrows indicating MPO-positive cells. E, The number of MPO-positive cells per field 6 h after TNF/GalN challenge. Bars = 200 μ m (A), 125 μ m (B), and 100 μ m (C and D).



Balbin et al. (11) demonstrated that incubation of full-length LIX (aa 1-92) with MMP-8 generates either an N-terminally cleaved form (as 5-92) or a form that is both N- and C-terminally cleaved (as 5-72). Both truncated forms have been shown to have higher chemotactic activities than the full-length form (20). To investigate whether it is the full-length LIX protein that is being released from the ECM, we analyzed total liver protein extracts (excluding the ECM) by Western blot (Fig. AG). No LIX could be detected in either WT or MMP-8-null mice before the challenge, indicating that LIX is still associated with the ECM, as was also demonstrated by IHC. Also in line with our IHC data, LIX protein was observed in liver homogenates of WT animals starting 3 h after TNF/GalN challenge and reaching a maximum 3 h later. The m.w. of the LIX observed on these Western blots indicates that it is not the smallest form of LIX, which is cleaved both N- and C-terminally by MMP-8 (aa 5-72) (11). We have been unable to discriminate between the full-length LIX protein (aa 1-92) and the other

previously reported truncated form that lacks only its first four amino acids. In contrast, Western blot analysis of liver homogenates of KO animals shows little or no LIX signal at any time point after TNF/GalN administration, confirming that LIX remains associated with the ECM in the absence of MMP-8.

FIGURE 3: MMP-8 expression and IHC detection in livers of MMP-8-null mice and WT animals after TNF/GalN challenge. A, Semiquantitative RT-PCR on whole liver RNA extracts for MMP-8 after TNF/GalN challenge; actin was used as a normalization control. B, MPO content of whole liver; normalization was based on the protein content of each sample. IHC staining for MMP-8 on livers of unchallenged WT (C), unchallenged MMP-8-null (D), WT (E), and KO (F) mice 2 h after TNF/GalN challenge and of WT (G) and KO (H) livers 6 h after TNF/GalN injection. Bars = 15 μ m (C, E, and G) and 50 μ m (D, F, and H).

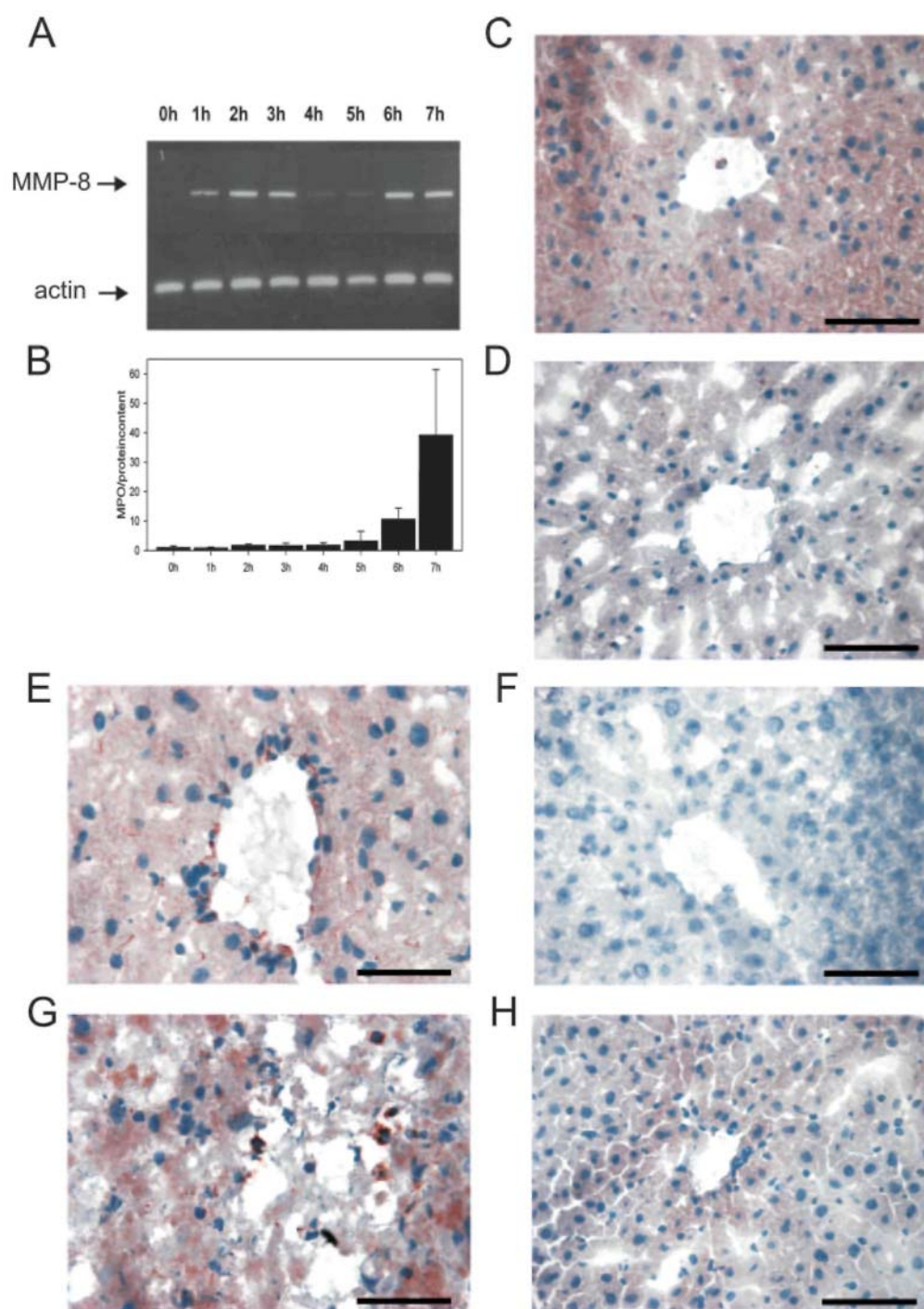
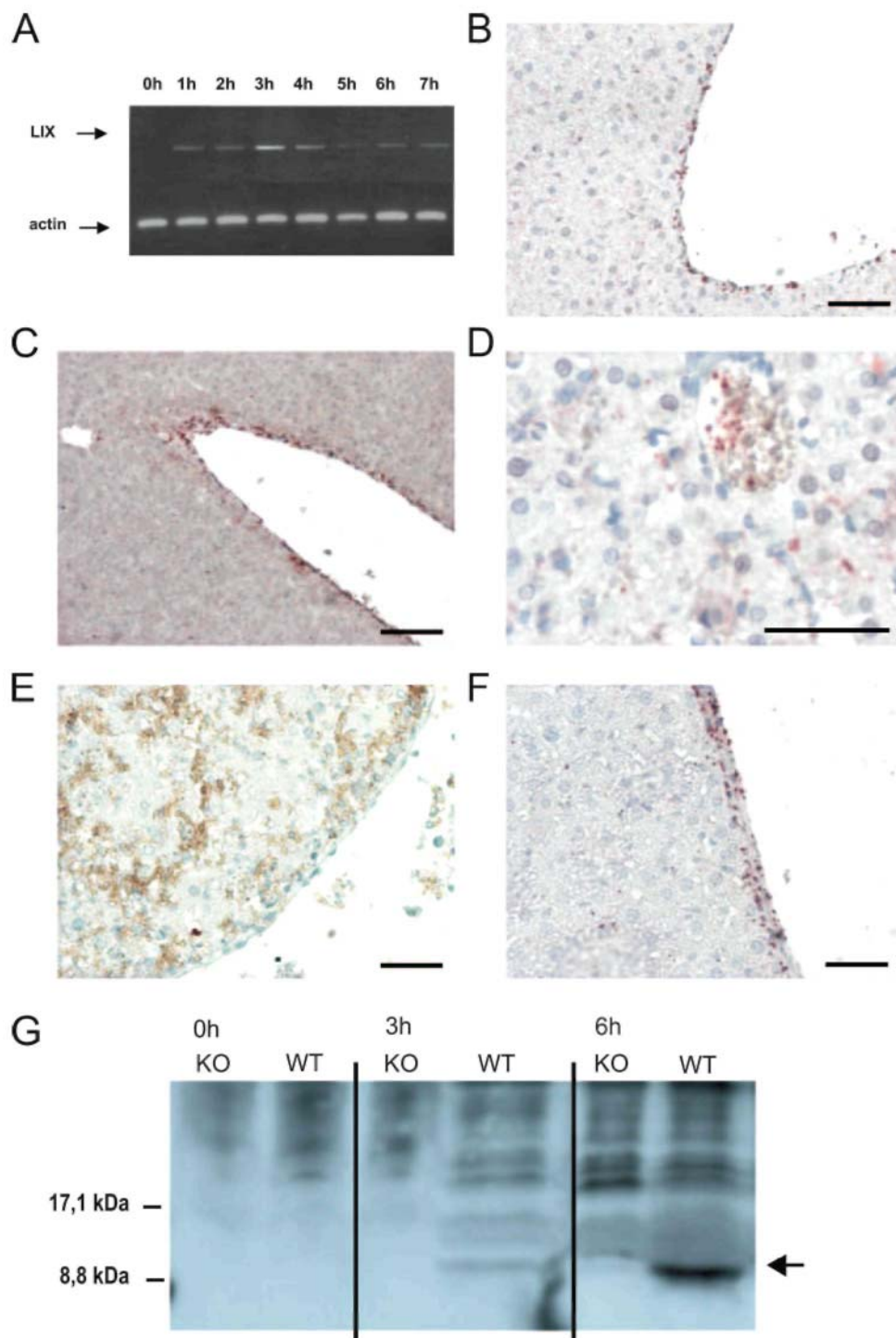


FIGURE 4: *LIX* expression and *LIX* protein localization in livers of *MMP-8*-null mice and *WT* animals after *TNF/GalN* challenge. A, Semiquantitative RT-PCR on whole liver RNA extracts for *LIX* after *TNF/GalN* challenge; actin was used as a normalization control. IHC staining for *LIX* on livers of unchallenged *WT* (B), unchallenged *MMP-8*-null (C), and *WT* livers 4 h (D) and 6 h (E) after *TNF/GalN* injection and *MMP-8*-deficient liver 6 h after *TNF/GalN* challenge (F). G, Liver protein extract of *WT* and *MMP-8*-null mice after *TNF/GalN* challenge, separated on a 16% Tris-glycine gel (125 µg/lane), and visualized using an anti-murine *LIX* polyclonal Ab. Bars = 100 µm (B, D, E, and F) and 200 µm (C).



Discussion

Few medical conditions are as dramatic and as devastating as ALF. The disease can affect previously healthy people unexpectedly, with mortality rates of >80% (2). Identifying and studying the actors responsible for the development of this aggressive acute hepatitis opens up new possibilities for future therapies. Our research group previously demonstrated that at least three different MMPs (MMP-2, -3, and -9) mediate the death of mice caused by TNF/GalN. Their role is supposedly linked to the breakdown of the ECM (mainly fibronectin) at the sinusoids, resulting in loss of tissue integrity (10). Because the first experiments performed with the recently generated KO mouse of another intriguing MMP, namely, collagenase-2 (MMP-8), indicate that it plays a crucial role in inflammatory processes (11), the goal of the present study was to investigate its contribution in this model. To this end, we injected MMP-8-null mice and matching WT animals with TNF in combination with GalN, a hepatotoxin that specifically blocks transcription in hepatocytes and leads to extreme apoptosis and secondary necrosis (14-16, 21).

MMP-8-deficient animals were significantly protected against TNF/GalN -induced lethal hepatitis compared with their WT counterparts. This protection is characterized by a large reduction of liver damage and significantly improved survival. In contrast, we did not observe any difference between the two types of mice in sensitivity to another model for ALF that is inflammation independent and uses TNF in combination with ActD. These data suggest that MMP-8 plays a role in the development of the inflammatory reaction itself, rather than influencing the sensitivity of hepatocytes for necrosis or apoptosis.

Looking at neutrophil migration after TNF/GalN challenge, MMP-8-null animals clearly lack the massive influx of MPO-positive cells into the liver parenchyma seen in WT animals after TNF/GalN challenge. This is an interesting observation, because previous studies pointed out that neutrophil migration is a crucial step in the development of this disease (10, 22).

It became clear from the MMP-8 expression pattern in the liver that MMP-8 is rapidly expressed upon initiation of the disease model. Interestingly, 4-5 h after the challenge, MMP-8 mRNA levels drop dramatically, only to rise again in the final stage of the disease. Based on the results of the MMP-8 IHC, we believe that this biphasic MMP-8 expression can be attributed to the expression of MMP-8 not only by the recruited neutrophils (23), but also by fibroblasts and endothelial cells in response to TNF (24). Hence, TNF-induced MMP-8 expression by endothelial cells and fibroblasts could be responsible for the early mRNA up-regulation observed by RT-PCR, whereas the later mRNA signal could be attributed to activated, infiltrating polymorphonuclear neutrophils (PMNs). This hypothesis is in line with our observation that the rise in MPO levels coincides with the up-regulation of MMP-8 expression at later time points.

We decided to investigate the link between MMP-8 and neutrophil migration by studying the role of LIX, a CXC (ELR⁺) chemokine. LIX is the only known murine PMN chemoattractant whose biological activity can be modulated by MMP-8, because MMP-8 generates truncated forms of LIX that are much more chemotactic than the full-length LIX (20). LIX is considered to be the murine counterpart of two closely related human chemokines, epithelial cell-derived neutrophil-activating peptide-78 and granulocyte chemotactic protein-2 (25).

When studying LIX mRNA expression levels, we found that the mRNA levels rapidly increased after TNF/GalN administration. Indeed, we were able to show that TNF can induce the expression of both MMP-8 and LIX in mouse embryonic fibroblasts (our unpublished observations). When studying LIX protein localization using IHC, it became evident that LIX is present in livers of healthy unchallenged animals around the portal veins. Because no LIX mRNA can be observed in unchallenged animals, we can assume that the LIX around the portal veins forms an ECM-associated reservoir, probably through interaction with glycosamino-glycans, as has been described for many other chemokines (18). Indeed, when we compare the amino acid sequence of LIX to that of human CXC chemokines, several residues considered to be critical in the interaction of the latter with heparan sulfate appear to be conserved in LIX, suggesting that LIX can interact with glycos-aminoglycans (26). The presence of a reservoir of chemotactic cytokines clearly has the advantage that, as soon as leukocyte extravasation is required, chemokines are promptly released. Interestingly, the LIX signal clearly moves from the portal venular area into the sinusoids of the liver after TNF/GalN challenge only in WT animals, not in MMP-8-null animals.

The release of ECM-bound LIX allows the soluble chemokine to bind the CXC receptors on rolling neutrophils, thereby initiating the actual neutrophil emigration (27). Soluble chemokines are carried by the blood flow toward the central veins into the sinusoids and space of Disse, stimulating further migration and activating the neutrophils present there. These activated neutrophils release the contents of their granules, leading to high

concentrations of PMN-derived MMP-8 and MMP-9 and creating a positive feedback loop in LIX activation (28). Their presence might also explain the secondary MMP-8 mRNA up-regulation detected by the quantitative RT-PCR analysis. Encounter of the activated neutrophils that migrate into the liver with GalN-sensitized hepatocytes leads to massive necrosis and apoptosis (29). Eventually, widespread hemorrhage and loss of liver integrity and functionality lead to rapid death (30).

A possible explanation for the LIX release from the ECM could be the ability of MMP-8 to cleave LIX both N- and C-terminally (11). To discriminate between full-length LIX and possible truncated forms, we used Western blot analysis. In line with our IHC observations, soluble LIX could be detected in livers of WT animals upon TNF/GalN challenge, in contrast to samples from MMP-8-null mice, which showed little or no LIX. Because the smallest form, which is both N- and C-terminally cleaved (aa 5-72), could not be detected in liver samples, and because Western blot analysis does not allow us to discriminate between the full-length form (aa 1-92) and the form that is only N-terminally cleaved (aa 5-92), two explanations of a possible MMP-8-mediated LIX release remain plausible. First, association of LIX with the ECM is at least partly dependent on the N-terminal domain, so N-terminal cleavage would lead to its release from the solid phase. Second, MMP-8 releases LIX through cleavage of a still unidentified anchor molecule linking LIX to the ECM. Interestingly, PMNs are indeed able to release cell surface-associated heparan sulfate from endothelial cells, although this activity was mainly linked to serine proteases rather than MMPs (31). However, because no LIX-deficient mouse or LIX-specific inhibitor has yet been generated to irrefutably prove this hypothesis, we cannot exclude that the increased LIX signal is merely a downstream consequence of an increased inflammatory response in MMP-8 WT animals. The fact that two other neutrophil-specific chemotactic cytokines, keratinocyte-derived chemokine and MIP-2, appear to be important regulators of leukocyte transmigration and extravascular accumulation in a similar (LPS/GalN) model, also indicates that LIX is not the sole CXC chemokine involved and might explain why some MMP-8-null mice still succumb to the TNF/GalN challenge (32).

In conclusion, our data illustrate that MMP-8 plays a critical role in the development of inflammation-dependent TNF-induced hepatitis. Other MMPs (MMP-2, -3, and -9) were previously found to be involved in this model of ALF. Their role is supposedly to break down the ECM at the sinusoids, thereby weakening liver structure and its integrity. We believe that MMP-8 plays a direct regulatory role in neutrophil recruitment, possibly through the release of ECM-associated LIX, a PMN-specific chemokine. Our findings might form an interesting step toward the development of new therapeutics for the treatment of acute and fulminant hepatitis.

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Disclosures

The authors have no financial conflict of interest.

References

1. Schiodt, F. V., and W. M. Lee. 2003. Fulminant liver disease. *Clin. Liver Dis.* 7: 331-349.
2. Lee, W. M. 1993. Acute liver failure. *N. Engl. J. Med.* 329: 1862-1872.
3. Muto, Y., K. T. Nouri-Aria, A. Meager, G. J. Alexander, A. L. Eddleston, and R. Williams. 1988. Enhanced tumour necrosis factor and interleukin-1 in fulminant hepatic failure. *Lancet* 2: 72-74.
4. Fransen, L., M. R. Ruyschaert, J. Van der Heyden, and W. Fiers. 1986. Recombinant tumor necrosis factor: species specificity for a variety of human and murine transformed cell lines. *Cell. Immunol.* 100: 260-267.

5. Brouckaert, P. G., G. G. Leroux-Roels, Y. Guisez, J. Tavernier, and W. Fiers. 1986. In vivo anti-tumour activity of recombinant human and murine TNF, alone and in combination with murine IFN- γ , on a syngeneic murine melanoma. *Int. J. Cancer* 38: 763-769.
6. Eggermont, A. M. 1998. TNF α in isolated perfusion systems: success in the limb, developments for the liver credits, debits and future perspectives. *Anticancer Res.* 18: 3899-3905.
7. Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey III, A. Zentella, and J. D. Albert. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science* 234: 470-474.
8. Knittel, T., M. Mehde, D. Kobold, B. Saile, C. Dinter, and G. Ramadori. 1999. Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF- α and TGF- β 1. *J. Hepatol.* 30: 48-60.
9. McCawley, L. J., and L. M. Matrisian. 2001. Matrix metalloproteinases: they're not just for matrix anymore! *Curr. Opin. Cell Biol.* 13: 534-540.
10. Wielockx, B., K. Lannoy, S. D. Shapiro, T. Itoh, S. Itoharu, J. Vandekerckhove, and C. Libert. 2001. Inhibition of matrix metalloproteinases blocks lethal hepatitis and apoptosis induced by tumor necrosis factor and allows safe antitumor therapy. *Nat. Med.* 7: 1202-1208.
11. Balbin, M., A. Fueyo, A. M. Tester, A. M. Pendas, A. S. Pitiot, A. Astudillo, C. M. Overall, S. D. Shapiro, and C. Lopez-Otin. 2003. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat. Genet.* 35: 252-257.
12. Van den Steen, P. E., S. J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Carboxyterminal cleavage of the chemokines MIG and IP-10 by gelatinase B and neutrophil collagenase. *Biochem. Biophys. Res. Commun.* 310: 889-896.
13. Van Den Steen, P. E., A. Wuyts, S. J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur. J. Biochem.* 270: 3739-3749.
14. Decker, K., and D. Keppler. 1974. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev. Physiol. Biochem. Pharmacol.* 71: 77-106.
15. Van Molle, W., G. Denecker, I. Rodriguez, P. Brouckaert, P. Vandenebeele, and C. Libert. 1999. Activation of caspases in lethal experimental hepatitis and prevention by acute phase proteins. *J. Immunol.* 163: 5235-5241.
16. Gujral, J. S., A. Farhood, and H. Jaeschke. 2003. Oncotic necrosis and caspase-dependent apoptosis during galactosamine-induced liver injury in rats. *Toxicol. Appl. Pharmacol.* 190: 37-46.
17. Jaeschke, H., Y. S. Ho, M. A. Fisher, J. A. Lawson, and A. Farhood. 1999. Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress. *Hepatology* 29: 443-450.
18. Patel, D. D., W. Koopmann, T. Imai, L. P. Whichard, O. Yoshie, and M. S. Krangel. 2001. Chemokines have diverse abilities to form solid phase gradients. *Clin. Immunol.* 99: 43-52.
19. Rot, A., and U. H. von Andrian. 2004. Chemokines in innate and adaptive host defense: basic chemokine grammar for immune cells. *Annu. Rev. Immunol.* 22: 891-928.
20. Wuyts, A., A. D'Haese, V. Cremers, P. Menten, J. P. Lenaerts, A. De Loof, H. Heremans, P. Proost, and J. Van Damme. 1999. NH₂- and COOH-terminal truncations of murine granulocyte chemotactic protein-2 augment the in vitro and in vivo neutrophil chemotactic potency. *J. Immunol.* 163: 6155-6163.
21. Silverstein, R. 2004. D-galactosamine lethality model: scope and limitations. *J. Endotoxin Res.* 10: 147-162.
22. Jaeschke, H., A. Farhood, and C. W. Smith. 1991. Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. *Am. J. Physiol.* 261: 1051-1056.
23. Devarajan, P., K. Mookhtiar, H. Van Wart, and N. Berliner. 1991. Structure and expression of the cDNA encoding human neutrophil collagenase. *Blood* 11: 2731-2738.
24. Hanemaaijer, R., T. Sorsa, Y. T. Konttinen, Y. Ding, M. Sutinen, H. Visser, V. W. van Hinsbergh, T. Helaakoski, T. Kainulainen, H. Ronka, et al. 1997. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells: regulation by tumor necrosis factor- α and doxycycline. *J. Biol. Chem.* 272: 31504-31509.
25. Smith, J. B., L. E. Rovai, and H. R. Herschman. 1997. Sequence similarities of a subgroup of CXC chemokines related to murine LIX: implications for the interpretation of evolutionary relationships among chemokines. *J. Leukocyte Biol.* 62: 598-603.
26. Lortat-Jacob, H., A. Grosdidier, and A. Imberty. 2002. Structural diversity of heparan sulfate binding domains in chemokines. *Proc. Natl. Acad. Sci. USA* 99: 1229-1234.

27. Jung, U., K. E. Norman, K. Scharffetter-Kochanek, A. L. Beaudet, and K. Ley. 1998. Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment in vivo. *J. Clin. Invest.* 102: 1526-1533.
28. Sternlicht, M. D., and Z. Werb. 2001. How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell. Dev. Biol.* 17: 463-516.
29. Chosay, J. G., N. A. Essani, C. J. Dunn, and H. Jaeschke. 1997. Neutrophil margination and extravasation in sinusoids and venules of liver during endotoxin-induced injury. *Am. J. Physiol.* 272: 1195-1200.
30. Bahjat, F. R., V. R. Dharnidharka, K. Fukuzuka, L. Morel, J. M. Crawford, M. J. Clare-Salzler, and L. L. Moldawer. 2000. Reduced susceptibility of nonobese diabetic mice to TNF- α and D-galactosamine-mediated hepatocellular apoptosis and lethality. *J. Immunol.* 165: 6559-6567.
31. Key, N. S., J. L. Platt, and G. M. Vercellotti. 1992. Vascular endothelial cell proteoglycans are susceptible to cleavage by neutrophils. *Arterioscler. Thromb.* 12: 836-842.
32. Li, X., D. Klintman, Q. Liu, T. Sato, B. Jeppsson, and H. Thorlacius. 2004. Critical role of CXC chemokines in endotoxemic liver injury in mice. *J. Leukocyte Biol.* 75: 443-452.