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Lack of Matrix Metalloproteinase-9 Impairs Neutrophil Degranulation and Prevents Liver Damage in Mice

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Abstract

Hepatic ischemia and reperfusion injury (IRI) is an inflammatory condition that contributes to graft rejection and mortality after liver transplantation. Neutrophils are important mediators of these events and contribute to liver injury. Leukocyte transmigration across endothelial and extracellular matrix protein barriers is dependent on adhesion and focal matrix degradation. In this study, we investigated the importance of the relationship between MMP-9 and neutrophil migration and activation. C57/BL6 wildtype (WT) and MMP-9-/- mice were subjected to 60 minutes of ischemia followed by reperfusion. Livers and serum were collected for measurements of enzyme activities, chemokine/cytokine protein and mRNA levels by ELISA and qPCR and for histological analysis. Neutrophils in the liver sections were counted with the help of immunofluorescence detection. Neutrophils from WT and MMP-9-/- mice were isolated from bone marrow and cultured with different stimuli for evaluation of neutrophil degranulation. Fluorogenic Dye-quenched (DQ)™-gelatin was used as a substrate to evaluate gelatinolytic activity present in the secretions of isolated WT and MMP-9-/- neutrophils, in the presence or absence of MMP-2/9 or elastase inhibitors. Compared to WT mice, MMP-9-/- mice showed significantly reduced liver damage and inflammation, as evidenced by lower levels of alanine aminotransferase (ALT) and myeloperoxidase (MPO) activities, cytokine production and histological outcome. In contrast, no differences were observed in the production of chemokines. Ly-6G-positive neutrophils had migrated less into livers of MMP-9-/- than WT mice. We also showed that MMP-9 seems to be essential for neutrophil activation, since in MMP-9-/- neutrophils the release of other enzymes was decreased in comparison with WT neutrophils. In conclusion, we showed that MMP-9 regulates neutrophil migration and activation.

Introduction

Hepatic ischemia and reperfusion injury (IRI) remains a major clinical limitation in orthotopic liver transplantation [1]. Indeed, IRI is a biphasic phenomenon in which the ischemic damage is amplified during the reperfusion phase, a pathophysiological process that involves the activation of the immune system as the major contributor of liver damage [2-5]. Liver injury induced by IR is linked to intense immune leukocyte infiltration. In this pathology, neutrophils are the main producers of cytokines, chemokines, enzymes and free radicals [6]. Leukocyte migration across endothelial barriers to inflamed tissue depends on the coordination of cellular adhesion-release steps and focal matrix degradation [7]. Although adhesion molecules are critical for the successful promotion of neutrophil transmigration by providing attachment to the vascular endothelium, it has been suggested that matrix metalloproteinases (MMPs) are critical for facilitating leukocyte movement across vascular barriers [8].

Among MMP family, gelatinase B or MMP-9 is an inducible enzyme released primarily by neutrophils and associated to inflammatory processes [9-11]. In contrast, healthy tissues express MMP-2 constitutively [11]. The role of MMPs has been investigated in several pathological conditions, including liver IRI [12-19]. In previous publications, animal studies have demonstrated that MMP-9 is an important factor in liver IRI, since MMP-9 is highly expressed in liver transplants, which is associated with intense leukocyte migration into the organ [20]. For decades, researches have shown that MMP-9 is critical for the migration of neutrophils [21]. In the present study, we investigated

the importance of the relationship between MMP-9 and neutrophil migration and activation. Our findings suggest that MMP-9 can have a crucial role for neutrophil activation during liver ischemia and reperfusion injury.

Material and Methods

Mice

Male C57BL/6J mice (8–12 weeks old) were obtained from the Central Animal Facility of the Universidade Federal de Minas Gerais (UFMG, Brazil). Male MMP-9-deficient (MMP-9-/-) knockout (KO) mice, 8 to 10 weeks of age were obtained from the Animal Facility of KU Leuven (Belgium). The knockout mice were backcrossed to C57BL/6J background as detailed [22]. The animals were maintained with filtered water and food ad libitum in a 12-hour (h) dark–light cycle in the thermoneutral zone for mice. All experiments were approved by the animal ethics committee of UFMG (CETEA/UFMG 422/15) and the ethical committee for animal experiments from KU Leuven (P111/2016).

Hepatic IR Injury Model

The IR was performed as described [23]. Mice were anesthetized with an intraperitoneal injection of xylazine (4 mg/kg) and ketamine (80 mg/kg). After a midline laparotomy, mice underwent a sham control operation or IR. In the IR group, the pedicle of the left and median lobes of the liver, containing the bile duct, hepatic artery, and portal vein (comprising 70% of the liver) was occluded using an atraumatic clamp (Aleamed, Kontich, Belgium). After 60 min of ischemia, the clamp was removed and reperfusion was initiated. The following time points were examined after reperfusion: 6, 12, 24, and 48 h. The control operation was performed with the same protocol but without vascular occlusion. In this case, the sham group refers to animals operated in the earliest time-point evaluated in each experiment (6 h), since we observed no difference between sham groups at any time-point after surgery, in any of the parameters evaluated (data not shown). Mice were placed on a heating pad to maintain body temperature at 37°C throughout the procedure. Blood was collected for analysis of serum ALT as an index of hepatocellular injury using a kinetic test (Bioclin, Belo Horizonte, Brazil). Cytokines and chemokines were quantified by enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) in serum and tissues were collected for real-time quantitative polymerase chain reaction (RT-$_q$PCR) of livers. Fragments of liver were fixed and sectioned for histology as described below.

Zymography

Protein extraction and zymography analyses were performed as described [24]. Briefly, gelatinolytic enzymes were detected in 100 µg of total protein from liver extracts, prepared in non-reducing sample buffer. Separation of proteins was achieved on 10% sodium dodecyl-sulfate–poly-acrylamide gels containing 1mg/mL of gelatin. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 20 minutes each time, rinsed in water, and incubated overnight in 50 mmol/L Tris-HCl, 10 mmol/L $CaCl_2$, pH 7.5, at 37°C. The gels were stained with Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA), and destained with methanol (30%) and acetic acid (10%). Gelatinolytic enzymes will degrade the gelatin in the gel and appear as a clear zone on a blue background. These zones represent the presence of gelatinolytic enzymes, regardless of their complexation to non-covalent inhibitors or activation status. Positive controls for MMP-2 and MMP-9 (BIOMOL International, Plymouth, PA), and prestained molecular weight markers (Bio-Rad Laboratories) served as standards.

Neutrophil Accumulation in Liver

Neutrophil accumulation was determined by liver myeloperoxidase (MPO) content. 50mg of tissue was homogenized in a buffered solution containing antiproteases protease inhibitors, as previously described [25]. MPO levels were assessed using 25µl of the supernatant of the homogenized sample and 25 µl of a solution of 1.6 mM of 3,3'-5,5'-tetramethylbenzidine (Sigma—dissolved in dimethyl sulfoxide) and 0.01 mM H2O2, dissolved in phosphate buffer (pH 5.4) containing hexa trimethylammonium bromide. The reaction was started at 37°C for 5 minutes in a 96-well microplate by adding the supernatant and the TMB solution. After that, H_2O_2 was added and followed by a new incubation at 37°C for 5 minutes. The reaction was stopped by adding 100 ml of 1M H_2SO_4 and quantified at 450 nm in a spectrophotometer.

Measurement of Gene Expression by Real-Time qPCR

Relative changes in gene expression were evaluated by $_q$PCRs. Total RNA extraction was performed with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Afterwards, RNA quantification was achieved using the Nanodrop2000 (Thermo Scientific, Waltham, MA, USA) and for each sample 2µg of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Relative changes in gene expression were evaluated by using the TaqMan Fast Universal PCR master mix (Applied Biosystems). cDNA was combined with master mix in 96-well MicroAmp plates (Applied Biosystems) and the reaction was analyzed with the 7500 Fast Real-time PCR system. Obtained Ct values were processed following the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as a housekeeping gene [26].

Histological Analysis

The livers were washed with 0.9% NaCl and fixed in 4% buffered formalin. Subsequently, the samples were dehydrated in ethyl alcohol solutions, bathed in xylol and included in histological paraffin blocks. Tissue sections of 5µm thickness were obtained using a microtome and stained with hematoxylin & eosin. The slices were visualized using the BX41 (Olympus) optical microscope and images obtained using the Moticam 2500 camera (Motic) and Motic Image Plus 2.0ML software.

Immunofluorescence Microscopy Analysis

Study of histological changes was performed on 4-µm acetone-fixed frozen sections. To investigate neutrophil infiltration in inflamed liver, immunofluorescent labeling was performed using PE rat anti-Mouse Ly6G (Cat:551461, BD Bioscience, San Jose, CA, USA). Hoechst was used for nuclear counterstaining, and sections were coverslipped with Prolong Gold antifade reagent (Ref: P36934, Life Technologies, Eugene, OR). Images were taken with a Zeiss Axiovert 200M (Carl Zeiss AG, Oberkochen, Germany) and AxioVision Rel 4.8 acquisition software (Carl Zeiss AG, Oberkochen, Germany).

Isolation of Murine Neutrophils from Bone Marrow

Isolation of murine neutrophils was performed as described [27]. Briefly, femurs and tibias were harvested and stripped of all muscle and sinew, and bone marrow was flushed with 2.5 mL of RPMI-1640 containing 5% fetal bovine serum (FBS) on ice. Cells were pelleted, and erythrocytes were removed by hypotonic lysis. The entire bone marrow preparation was resuspended at 5 x 10^7 cells/mL in Hanks' balanced saline solution (HBSS). Cells were layered on a Percoll (Sigma–Aldrich) gradient (3 mL of 55% Percoll, top; 3 mL of 65% Percoll, middle; 4 mL of 80% Percoll, bottom) and centrifuged at 2000 rpm for 30 minutes at 10°C. Mature neutrophils were recovered at the interface of the 65% and 80% fractions and were more than 90% pure.

In Vitro Neutrophil Activation Assay

Isolated neutrophils from WT and MMP-9-/- mice were resuspended in FBS-free RPMI-1640 medium at a final concentration of 1.0 x 106 /mL. Neutrophils were incubated with CXCL1, CXCL6, C5a or fMLP (10 nM), and incubated at 37°C in 5% CO_2 for 2h. Neutrophil activity was then assessed by ELISA on the supernatant by the meas-

urement of MPO, elastase and Neutrophil Gelatinase B-Associated Lipocalin (NGAL) (R&D Systems, Minneapolis, MN, USA).

The Fluorogenic DQ™-Gelatin Assay

The following protocol was used as previously described to determine the gelatinolytic activity [28]. To a 96-well plate (chimney, 96-well, Greiner Bio-one, Frickenhausen, Germany), 50 µl of sample (unstimulated or stimulated WT or MMP-9-/- neutrophil supernatant) were incubated (30 min at 37°C) with 30 µl assay buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% Tween 20), 30 µl SB-3CT ((an inhibitor of MMP-2/MMP-9) or 30 µl ElaV (an elastase inhibitor) at a final concentration of 10 µM. Next, 20 µl fluorogenic dye-quenched gelatin (DQ-gelatin, Invitrogen) at a final concentration of 2.5 µg/ml was added to the mixture. Immediately thereafter, the plate was placed in the fluorescence reader (FL600 Microplate fluorescence reader, Biotek, Highland Park, IL, USA) and fluorescence was measured every 30 seconds for 30 min. The velocity of the gelatin degradation reaction was expressed as fluorescence units (FU) per minute and is indicative of the net proteolytic activity present in the samples. In each experiment, both positive (no inhibitor) and negative (no enzyme) controls were included. All data were corrected by subtraction of their respective negative controls.

Statistical Analysis

Experimental data analysis was performed with one-way analysis of variance (ANOVA with Tukey's post-hoc test) and Student t test provided by Prism 6.0 software (GraphPad). All data are given as the mean ± SEM. *In vivo* experimental groups had at least four mice per group. Data shown are representative of at least two independent experiments. Differences were considered significant at $p < 0.05$.

Results

Liver IRI is regulated by MMP-9 *in vivo*

It is known that extracellular matrix (ECM) regulates various cellular functions, such as adhesion, migration, differentiation, proliferation, and survival. Moreover, dysregulation of ECM production and proteolysis is often associated with the development of liver pathology [7]. The alterations of the ECM are in agreement with reports describing the potential implications MMPs in liver diseases [29]. In order to determine whether MMP-9 plays a role in liver IRI, expression of this gene was evaluated in the liver after different times of reperfusion. First, we performed zymography analysis to corroborate that MMP-9 was absent in MMP-9-/- mice. MMP-9 protein was detected in the liver of WT mice already after a reperfusion period of 6 hours. MMP-2 was also detected in both WT and MMP-9-/- mice, and appeared to be decreased in MMP-9-/- mice Figure 1A. Having hypothesized that MMP-9 would be relevant for the generation of sterile inflammation following liver IRI, we subjected WT and MMP-9-/- mice to 1h of ischemia followed by 6h and 12h of reperfusion. MMP-9 mRNA levels were significantly up-regulated in WT livers between 6h to 48h post reperfusion with a maximum after 12h Figure 1B. Then, we quantified serum levels of ALT and tissue levels of MPO and TIMP-1. We found that MMP-9-/- mice showed significantly less liver damage, as shown by the reduced ALT levels 6h and 12h after reperfusion when compared with WT mice Figure 1C. MPO, one of the most abundant proteins in neutrophils, has emerged as an enzyme critically involved in the pathogenesis of inflammatory diseases [30,31]. In our liver IRI model, MPO activity 6h and 12h after reperfusion was significantly reduced in MMP-9-/- mice Figure 1D. TIMP-1, which is the major endogenous regulator of MMP-9, was almost undetectable in the liver of sham mice. However, when compared with WT mice, hepatic TIMP-1 mRNA expression was markedly down-regulated in MMP-9-/- mice 6h and 12h after liver IRI Figure 1E. Furthermore, decreased liver ALT and MPO concentrations were associated with significantly better histological preservation. Elevated sinusoidal congestion and extensive areas of necrosis characterized livers from WT mice 12h after reperfusion. In contrast, MMP-9-/- mice showed preserved architecture after liver IRI Figure 1F.

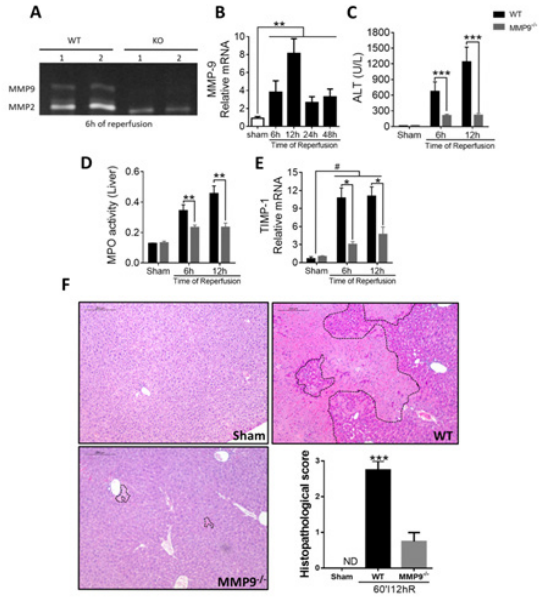

Figure 1: Effects of the lack of MMP-9 in the liver of mice subjected to IR.

(A) Representative gelatin zymography showed higher expression of MMP-9 in WT livers 6h after reperfusion when compared with MMP-9-/- mice.

(B) MMP-9 mRNA expression was almost undetectable in control livers, but significantly up-regulated in WT livers from 6h to 48h hours post reperfusion. **p< 0.05 vs sham-operated animals.

(C, D) ALT levels and MPO activity in MMP-9-/- mice were significantly lower than WT mice after 6 and 12 hours of reperfusion, ***p< 0.05 and **p < 0.05 vs WT.

(E) Expression of TIMP1 was significantly higher in WT mice 6 hours and 12 hours after IR injury when compared with MMP9-/- mice, * p<0.05 vs MMP9-/- mice.

(F) WT livers were characterized by elevated sinusoidal congestion and extensive necrosis after 12 hours. In contrast, MMP9-/- showed significant histological preservation (n=6).

Lack of MMP-9 Impaired Neutrophil Accumulation in the Liver IRI

Leukocyte migration across endothelial barriers depends on focal matrix degradation induced by matrix metalloproteinases and on chemotactic cues [21]. Ly6G is expressed primarily in neutrophils and correlates with the cellular level of differentiation and maturation [23]. To confirm that the observed reduction on MPO activity was associated with neutrophil numbers in the IR-injured liver, we next aimed to determine whether absence of MMP-9 could directly affect neutrophil numbers in the IR-injured liver. For this, we assessed the expression of Ly6G in the WT and MMP-9-/- tissues subjected to 1h ischemia followed by 12h reperfusion. Immunofluorescence analysis showed that liver sections of sham-operated mice were negative for Ly6G Figure 2, left upper panel. However, Ly6G positive cells were predominantly detected in the liver 12h after reperfusion in WT mice Figure 2, right upper panel. Of note, livers of MMP-9-/- mice showed significantly less Ly6G staining at 12h after reperfusion Figure 2, left bottom panel. Histological scores of migrating cells showed a higher number of Ly6G positive cells in WT tissues compared with MMP-9-/- Figure 2, right bottom panel. These results suggest that MMP-9 may contribute to neutrophil migration to the ischemic tissue.

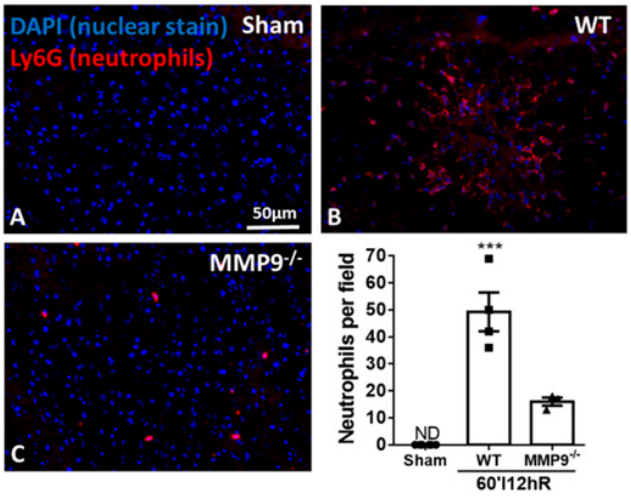

Figure 2: Effects of the lack of MMP-9 on neutrophil infiltration in Liver IRI.

(A) Representative photomicrographs of Ly6G staining (neutrophil marker) in sham.

(B) WT mice subjected to IR.

(C) MMP-9-/- mice subjected to IR.

Accumulation of neutrophil were increased in WT livers compared with sham livers. MMP-9-/- mice showed a significant reduction in the number of infiltrated neutrophils. Ly6G is shown in red and nuclei in blue (×200).

Proinflammatory Cytokine Production was Reduced in Mmp-9-Deficient Mice

TNF-α and IL-6 are cytokines capable of regulating MMPs expression [32,33]. To determine whether the lack of MMP-9 could alter the levels of cytokines, we quantified the concentration of these mediators in both serum and liver of WT and MMP-9-/- mice. As observed, the extent of leukocyte infiltration correlated with significantly increased expression of the proinflammatory cytokines TNF-α and IL-6 in livers of WT mice, which were significantly decreased in the MMP-9-/- mice 6h after reperfusion Figure 3A&3B. Moreover, while levels of TNF-α and IL-6 were also significantly increased in the serum of WT mice subjected to IR, they were markedly reduced in MMP-9-/- mice after 6h of and of reperfusion Figure 3C&3D.

MMP-9 Deficiency Did Not Alter the Expression of Major Chemokines in Hepatic IRI

Chemokines and MMPs play key roles in the migration of immune cells to sites of inflammation. In response to chemokine stimulation, leukocytes produce and secrete various proteolytic enzymes. In addition, MMPs can also regulate cytokine and chemokine activity by proteolytic processing [34-36]. To evaluate whether the observed decrease in neutrophil migration and less liver damage in MMP-9-/- mice were associated with possible modifications of the expression of chemokines, we determined the levels of major cell activating chemokines linked to liver IRI. Our data showed that decreased leukocyte migration, less liver damage and inflammation observed in MMP-9-/- mice were not associated with changes in the production of chemokines in post-ischemic livers. As observed, CXCL1, CXCL2 and CXCL6 were comparably expressed in both MMP-9-/- and WT serum of mice 6h after reperfusion Figure 4A-C.

Lack of MMP-9 Impaired Neutrophil Degranulation in Vitro

The granules secreted by neutrophils contain various molecules, such as MPO, defensins, elastase, proteinase 3, NGAL, which play an essential role in the antimicrobial activity of neutrophils and in the regulation of the inflammatory response [37]. Our in vivo observations demonstrated that lack of MMP-9 interfered with neutrophil migration and MPO activity, raising the possibility that MMPs regulate neutrophil activation or degranulation. This finding prompted us to verify whether MMP-9 was able to regulate the production or release of others enzymes by neutrophils through degranulation. For this, we cultured WT and MMP-9-/- neutrophils in the presence of different stimuli, including CXCL1, CXCL6, C5a and fMLP. As illustrated, MPO activity was increased in stimulated WT neutrophils. However, stimulated MMP-9-/- neutrophils showed reduced MPO activity Figure 5A. We also evaluated the presence of neutrophil elastase. Compared with stimulated WT neutrophils, MMP-9-/- neutrophils showed reduced release of elastase Figure 5B. Moreover, NGAL was shown upregulated in WT neutrophils stimulated with CXCL6 and fMLP when compared with stimulated MMP-9-/- neutrophils Figure 5C. Altogether, our results suggest that MMP-9 may control neutrophil activation.

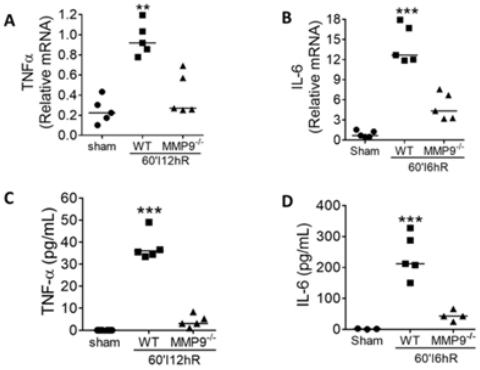

Figure 3: Effects of the lack of MMP-9 on the concentrations of cytokines in the serum and liver tissue levels of mice subjected to IR. MMP-9-/- mice showed reduced production of mRNA for the cytokines TNF-α (A) and IL-6 (B) in the tissue when compared with WT animals subjected to IR. Similarly, MMP-9-/- mice showed reduced production of TNF-α (C) and IL-6 (D) protein in serum when compared to WT animals subjected to IR. ** p<0.05 and *** p<0.05 vs. MMP-9-/- animals.

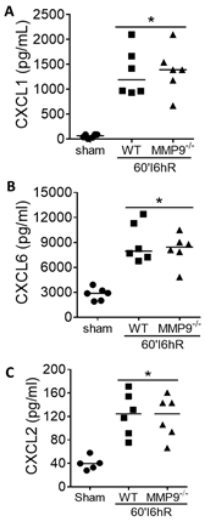

Figure 4: Effects of the lack of MMP-9 on the concentrations of chemokines in the serum of mice subjected to IR. MMP-9-/- and WT mice showed increased production of the chemokines CXCL1 (A), CXCL2 (B) and CXCL6 (C) 6h after reperfusion when compared with Sham animals. However, no differences were observed in the production of chemokines between MMP-9-/- and WT animals subjected to IR. * p<0.05 and vs sham mice.

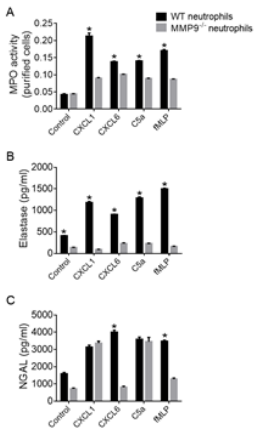

Figure 5: Inhibition of neutrophil degranulation in Mmp9-/- neutrophils in vitro. WT stimulated neutrophils showed high MPO activity, which was reduced in MMP-9-/- neutrophils (A). Elastase was measured by ELISA on supernatants of control or stimulated WT and MMP-9-/- neutrophils. Elastase in stimulated WT neutrophils was very high in supernatants, compared with MMP-9-/- neutrophils (B). NGAL in WT neutrophils was higher only in neutrophils stimulated with CXCL6 and fMLP *p<0.05 vs stimulated MMP-9-/- neutrophils (C).

Inhibition Gelatin Degradation in MMP-9-/- Neutrophils in Vitro

In contrast to all other MMPs, only gelatinases (MMP-2/9) have a gelatin-binding fibronectin domain and are therefore able to efficiently degrade gelatins (denatured collagen) [38]. Due to the lowered degranulation observed in MMP-9-/- neutrophils, we used DQ™-gelatin, which mimics the natural substrate of the extracellular matrix, to measure the gelatin-degrading capacity of neutrophil degranulation. Our data on in vitro gelatinolysis demonstrated that chemokines, fMLP and C5a enhanced the secretion of gelatinases by WT neutrophils Figure 6. However, gelatinolysis was lower when stimulated MMP-9-/- neutrophils were compared with WT neutrophils. As expected, in WT neutrophils, gelatinolytic activity derived from MMP-2 and MMP-9, as determined by addition of the MMP-2/MMP-9 inhibitor SB-3CT, presented a considerable amount of the signal Figure 6. Taken together, these results suggest that mainly inhibition of MMP-9 regulates the gelatinolytic activity of neutrophils.

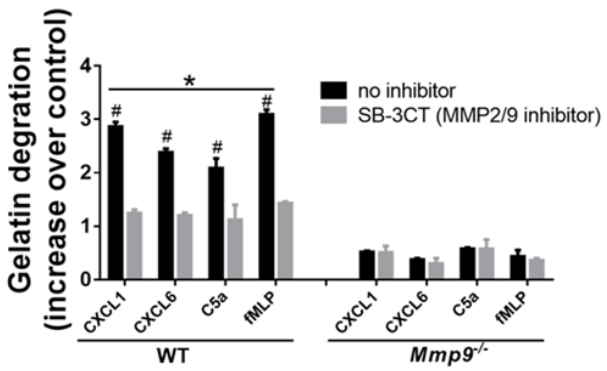

Figure 6: Inhibition of gelatin degradation in MMP-9-/- neutrophils in vitro. Gelatinolytic activity was decreased in MMP-9-/- neutrophils when compared to WT neutrophils (black bars), *p<0.05 vs stimulated MMP-9-/- neutrophils. Also in WT neutrophils, inhibition of MMP-9 decreased the gelatinolytic activity compared to cells treated with vehicle (grey bars), #p<0.05 vs SB-3CT.

Discussion

During IR injury, an intense inflammatory process occurs in the liver. In summary, IRI triggers an inflammatory process in the liver with recruitment of neutrophils into the vasculature, mainly in the sinusoids. Neutrophil recruitment involves a multistep process, which includes participation of DAMPs released from necrotic cells, chemokine production, expression of adhesion molecules by endothelial cells and leukocytes, and release of effector molecules by neutrophils, such as ROS and MMPs. Then, manipulation of the recruitment of excessive neutrophil and their behavior into tissue still hold promise as strategies to treat IRI and may improve overall graft success in transplantation. Understanding the mechanisms that allow neutrophils to respond to sterile tissue injury and cell death is fundamental to our understanding of both homeostatic innate immune functions and immune responses in disease.

The mechanisms by which neutrophils damage the liver during IR, the role of their granular content can be considered. The molecules present in the granules are variable, including receptors, adhesion molecules, MMPs, MPO, lysozyme and antimicrobial peptides. Among the different molecules within the granules of neutrophils, we investigated the functional significance of MMP-9 in our model. We found a role for MMP-9 in tissue damage and neutrophil activation. Previous reports showed that MMP-9 contributes to the recruitment of leukocytes and worsens liver IRI [19, 39]. Our experiments extend these findings and we demonstrated that can be an interaction among the neutrophil proteases, since the lack of MMP-9 interferes not only with tissue damage, but can also controls the release of other enzymes by neutrophils.

The contribution of MMP-9 to the pathogenesis of liver IRI was clearly demonstrated by the fact that liver damage of sham group was attenuated in MMP-9-/- mice 6h and 12h after reperfusion and was associated with improvement in serum aminotransferase levels and better liver histopathological outcomes. These results support previous publications and indicated that expression of MMP-9 increased the susceptibility to liver IRI [40]. Moreover, absence of MMP-9 contributes to accelerated liver recovery after IRI [41]. Recently, MMP-9 inhibition was shown to prevent hepatic IRI [42]. These results are also in line with many publications showing key roles for MMP-9 on leukocyte recruitment into inflamed tissues [8,21,43,44].

Proinflammatory cytokines are implicated in liver IRI [45]. We showed that, compared with WT, MMP-9-/- mice had less expression of cytokines TNF-α and IL-6 in both liver and serum 6h after reperfusion. TNF-α is often associated with neutrophil infiltration and liver damage [46]. Our data indicated that MMP-9 is important for inflammation after liver IRI. Interestingly, although MMP-9-/- mice were significantly protected against liver IR injury, the levels of major chemokines did not change. Previous reports have shown that MMPs can regulate cytokine and chemokine activity by proteolytic processing. Some of these proteases modify the biological activity of the chemokines [35,47,48]. So, although in our hands, the levels of chemokines between WT and MMP-9-/- mice were comparable, the chemokines produced in MMP-9-/- mice may be less activated because of the lack of the enzyme. However, at present it is not yet possible to define *in vivo* the local processing of chemokines and to support this hypothesis.

In this study, we also adopted *in vitro* tests to investigate a relationship between MMP-9 and neutrophil activation. In our model, MPO activity was profoundly reduced in MMP-9-/- mice after liver IRI, suggesting that in addition to mediating leukocyte recruitment, MMP-9 may also facilitate neutrophil degranulation/activation. Keeping this in mind, we wondered whether the reduced MPO activity would be due to lower injury or whether MMP-9 could also control the production of enzymes by neutrophils. Therefore, we cultured WT and MMP-9-/- neutrophils in the presence of different stimuli, including CXCL1, CXCL6, C5a and fMLP. Interestingly, we observed that MMP-

9 seems to slow down neutrophil degranulation, since MPO activity, elastase and NGAL were reduced in stimulated MMP-9-/- neutrophils. These data are in line with many publications suggesting that MMPs, originally discovered for their role in the breakdown of ECM proteins, have gained the status of regulatory proteases in signaling events inside the cell by ligating and processing hormones, enzymes, cytokines, chemokines, adhesion molecules and other MMPs [49-55]. These data demonstrated that MMP-9 seems to be essential for neutrophil activation. In our model, we also demonstrated that treatment of WT neutrophils with MMP-2/9 inhibitors decreased the gelatinolytic activity of neutrophils. These results demonstrate regulatory mechanisms of neutrophil MMP-9 and indicate that the proteolytic activity of neutrophils can be regulated by MMP-9. Since neutrophils are major players during liver IRI, the inhibition of MMP-9 can be an important factor on cell migration and protection against liver injury.

Conclusion

In conclusion, this study demonstrated that IR triggers an inflammatory process in the liver with recruitment of neutrophils into the parenchyma. Moreover, we documented that MMP-9 expressed by neutrophils is likely to be a key factor in cell migration and activation, since the lack of MMP-9 led to protection against liver IRI. Furthermore, we showed that MMP-9 regulates neutrophil migration and their activation. These results offer a new insight into the intracellular mechanisms responsible for neutrophil activation/degranulation and indicate that neutrophil proteases can control neutrophil degranulation. Thus, pharmacological manipulation or gene deletion of one of these pathways holds promise as a strategy to treat IR and may improve overall graft success in liver transplantation. We hope that this work contributes to the development of novel therapeutic approaches to prevent hepatic IRI and, consequently, to improve the outcome of liver transplantation.

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Conflict of Interest

All authors approved the manuscript and this submission and have no conflict of interest.

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