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Mechanisms of neutrophil recruitment in a model of hepatic ischemia and reperfusion injury

Belo Horizonte
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Thesis presented to the program of Physiology and Pharmacology, Institute of Biological Sciences, Federal University of Minas Gerais, Brazil and to the doctoral school of Biomedical Sciences, KU Leuven, Belgium as a partial fulfillment of the requirements to obtain the degree of Doctor in Physiology and Pharmacology from the Federal University of Minas Gerais, Brazil and the degree of Doctor in Biomedical Sciences from KU Leuven.

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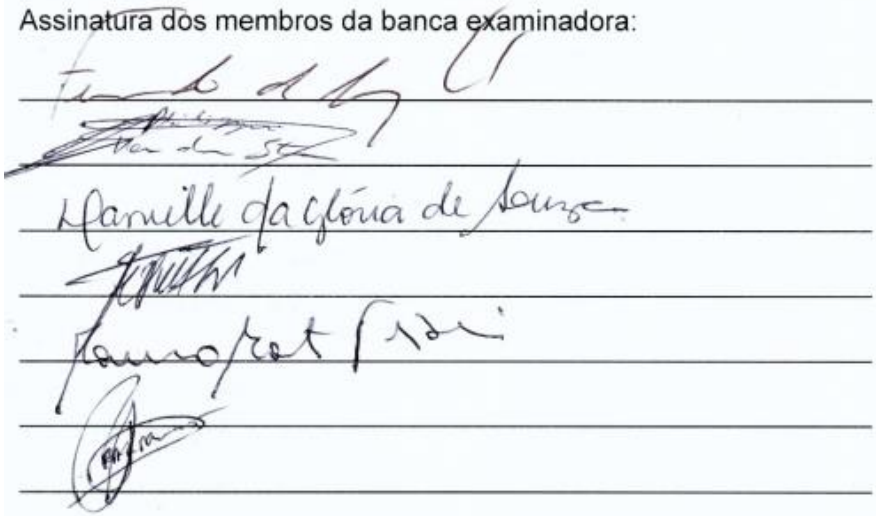
ATA DA DEFESA DA TESE DE DOUTORADO N° 282 DE THIAGO HENRIQUE CALDEIRA DE OLIVEIRA

Às 8:30 horas do dia 24 do mês de agosto de 2017, na Sala Prof. Nelo Rangel, 172 - Bloco K3 - ICB, realizou-se a sessão pública para a defesa da Tese de **Thiago Henrique Caldeira de Oliveira.** A presidência da sessão coube ao **Prof. Dr. Mauro Martins Teixeira,** orientador. Inicialmente, o presidente fez a apresentação da Comissão Examinadora assim constituída: **PROF. DR. STEYNER DE FRANÇA CÔRTES,** UNIVERSIDADE FEDERAL DE MINAS GERAIS,**Prof. Dr. Fernando de Queiroz Cunha,** Faculdade de Medicina/USP, **Prof. Dr. Philippe Van den Steen,** Rega Institute/KU Leuven, **Profa. Drª. Danielle da Glória de Souza,** ICB/Universidade Federal de Minas Gerais, **Prof. Dr. Paul Proost ,** Rega Institute/KU Leuven, orientador e **Prof. Dr. Mauro Martins Teixeira,** ICB/Universidade Federal de Minas Gerais, orientador. Em seguida, o candidato fez a apresentação do trabalho que constitui sua **Tese de Doutorado**, intitulada: **"MECANISMOS DE RECRUTAMENTO DE NEUTRÓFILOS EM MODELO DE LESÃO HEPÁTICA INDUZIDA POR ISQUEMIA E REPERFUSÃO".** Seguiu-se a arguição pelos examinadores e logo após, a Comissão reuniu-se, sem a presença do candidato e do público e decidiu considerar ....APROVADO..... a Tese de Doutorado. O resultado final foi comunicado publicamente ao candidato pelo presidente da Comissão. Nada mais havendo a tratar, o presidente encerrou a sessão e lavrou a presente ata que, depois de lida, se aprovada, será assinada pela Comissão Examinadora.

Belo Horizonte, 24 de agosto de 2017.

Assinatura dos membros da banca examinadora:



*** De acordo com as Normas Gerais de Pós-Graduação da UFMG o grau de Doutor só será concedido ao aluno que entregar ao Colegiado do Curso, no prazo máximo de 60 dias, a versão final da tese, em conformidade com as indicações da Comissão Examinadora. Após a entrega da versão final com a documentação exigida para emissão de diploma, a secretaria emitirá certificado de conclusão do doutorado.**

"The stimulus for the lobster to be able to grow is that it feels uncomfortable. I think that we have to realize that times of stress are also times that are signals for growth and if we use adversity properly we can grow through adversity"

(Rabbi Dr. Abraham Twerski)

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SUMMARY

LIST OF ABBREVIATIONS

ALT (alanine aminotransferase)

ATP (adenosine triphosphate)

C3a (complement 3a)

C5a (complement 5a)

C5aR (complement 5a receptor)

CC or CXC (Cys-Cys or Cys-Xaa-Cys motif)

CCL3 (CC chemokine ligand 3) MIP1α (macrophage inflammatory protein 1-alpha)

CLR (C-type lectin receptor)

CXCR1 (C-X-C chemokine receptor 1)

CXCR2 (C-X-C chemokine receptor 2)

CXCL1 (CXC chemokine ligand 1) or KC (Keratinocyte chemoattractant)

CXCL2 (CXC chemokine ligand 2) or MIP-2 (macrophage inflammatory protein 2)

CXCL6 (CXC chemokine ligand 6) or GCP-2 (granulocyte chemotactic protein 2)

DAMP (danger-associated molecular pattern)

DC (dendritic cell)

DNA (deoxyribonucleic acid)

ECM (extracellular matrix)

FPR1 (formyl peptide receptor 1)

fMLP (N-formyl-Met-Leu-Phe)

GAG (glycosaminoglycan)

GPCR (G protein-coupled receptor)

GSH (reduced glutathione)

HMGB1 (high mobility group box 1)

H_2O_2 (hydrogen peroxide)

(HOCl/OCl−) hypochlorous acid/hypochlorite

ICAM-1 (intercellular adhesion molecule-1)

IL-8 (Interleukin-8)

iNOS (inducible nitric oxide synthase)

IRI (ischemia-reperfusion injury)

MAC (membrane attack complex)

MAC-1 (macrophage antigen–1)

MIG 30 (the 30 C-terminal amino acids of CXCL9)

MMP (metalloproteinase)

MPO (myeloperoxidase)

NADPH (nicotinamide adenine dinucleotide phosphate)

NGAL (neutrophil gelatinase B-associated lipocalin)

NOD (nucleotide-binding oligomerization domain)

NF-κB (nuclear factor kappa B)

NK (natural killer)

NKT (natural killer T)

PAMP (pathogen-associated molecular pattern)

PRR (pattern recognition receptor)

RIG-I (Retinoic Acid-Inducible Gene I-like receptors)

ROS (reactive oxygen species)

SOD (superoxide dismutase)

TIMP (Tissue inhibitors of metalloproteinase)

TLR (Toll-like receptor)

TNF-α (tumor necrosis factor alpha)

LIST OF ILLUSTRATIONS

LIST OF FIGURES

ABSTRACT

Ischemia and reperfusion injury (IRI) may facilitate graft rejection is the main cause of morbidity and mortality after liver transplantation. During IRI, an intense inflammatory process occurs in the liver. Hepatic inflammation is initiated by the ischemic period but occurs mainly during the reperfusion time, and is characterized by marked neutrophil recruitment to the liver. The role of neutrophils as the main amplifiers of liver injury has been recognized in many publications. However, the mechanisms involved in neutrophil recruitment during liver IRI are not well known. Additionally, the molecules necessary for this type of migration are poorly defined. Here, we investigated different pathways used by neutrophils to infiltrate the liver and cause damage during inflammation induced by a model of liver IRI. We showed that IR induced significant liver injury, as observed by high levels of hepatic transaminases, neutrophil recruitment and tissue damage. We used intravital microscopy to show that neutrophil behavior changes during liver injury. During IRI, the number of neutrophils increased between 6h and 24h of reperfusion, whereas the distance traveled, velocity, neutrophil size, cluster formation and neutrophil shape reached maximum 6h after reperfusion. Neutrophil migration correlated with liver injury and was dependent on the chemokine receptors CXCR1/2, since mice treated with Reparixin had reduced liver injury and inflammation. *In vivo* imaging also revealed that Reparixin decreased neutrophil infiltration, migration and displacement. Moreover, neutrophils had smaller size and less elongated shape, indicating reduced activation. Moreover, we documented that MMP-9 expressed by neutrophils is likely to be a key factor in cell transmigration and activation. The lack of *Mmp-9* led to protection against liver IRI, as observed by reduced levels of transaminases, cytokines and histological evaluation. Interestingly, we documented that MMP-9 seems to control neutrophil degranulation. We showed that lack of *Mmp-9* impaired neutrophil release of others enzymes, including myeloperoxidase (MPO), elastase and neutrophil gelatinase B-associated lipocalin (NGAL). Finally, we also demonstrated that disruption of the interaction between glycosaminoglycans (GAG) and chemokines protected mice against liver IRI. Mice treated with a peptide containing the 30 C-terminal amino acids of CXCL9 (MIG 30) showed reduced liver injury and inflammation. We conclude that pharmacological manipulation or gene deletion of one of these pathways hold promise as strategies to treat IR and improve overall graft success in liver transplantation.

Key words: Hepatic IRI, chemokines, glycosaminoglycans, metalloproteinases, neutrophil-mediated liver injury

1. INTRODUCTION

The interruption of blood flow (***ischemia***) with consequent lack of oxygen and nutrient supply is an inherent phenomenon during various surgical procedures (Bellanti 2016). In liver surgery, clinical situations exist in which periods of ischemia can be particularly long, such as during removal of liver tumors, trauma, vascular reconstruction and transplantation (Chouillard, Gumbs et al. 2010, Zhai, Petrowsky et al. 2013, Zhang, Liu et al. 2016). Once the blood flow, oxygen tension and nutrients are restored (***reperfusion***), there is an increase in injury initiated by ischemia, aggravating the overall damage. This phenomenon is known as ***ischemia-reperfusion injury*** (IRI), which affects liver viability and directly correlates to graft rejection (Serracino-Inglott, Habib et al. 2001, Romanque, Uribe et al. 2005, Montalvo-Jave, Escalante-Tattersfield et al. 2008). IRI remains the major problem in clinical transplantation. IRI causes up to 10% of early transplant failures and can lead to a higher incidence of both acute and chronic rejection (Busuttil and Tanaka 2003, Mendes-Braz, Elias-Miro et al. 2012, Papadopoulos, Siempis et al. 2013). In an ischemic liver, when the oxygen levels fall, adenosine triphosphate (ATP) production is impaired (Selzner, Selzner et al. 2007). The deleterious effects of ATP reduction are further enhanced by the production of reactive oxygen species (ROS), cytokines, vasoactive agents and increased expression of adhesion molecules (Montalvo-Jave, Escalante-Tattersfield et al. 2008). Generation of ROS results in an increase of the intracellular calcium concentration and causes intracellular pH changes, resulting in organelle damage and apoptotic or necrotic cell death (Guan, Fu et al. 2014).

However, liver damage and failure are caused mainly during the reperfusion period, when there is a shift from metabolic distress caused by ischemia to an excessive innate immune response triggered by reperfusion. Liver IRI shows a significant inflammatory component and neutrophils are considered central players in the events leading to injury after reperfusion (Honda, Takeichi et al. 2013). Indeed, neutrophils are recruited and activated during reperfusion and, as discussed below, overwhelming evidence suggests that they contribute to the pathogenesis of IRI, including in the liver. Neutrophil-induced liver injury is a multistep process that includes neutrophil activation, recruitment of these cells from the vasculature, transendothelial neutrophil migration and contact to parenchymal cells. Chemokines are potent chemoattractants for neutrophils in this context and have been shown to contribute to hepatic neutrophil recruitment and liver IRI (Lentsch, Yoshidome et al. 1998). Moreover, neutrophil recruitment depends on the interaction of chemokines with G protein-coupled receptors

(GPCRs) on leukocytes and GAGs expressed on endothelial vessels, creating a concentration-dependent gradient of chemokines produced at the inflammatory site (Adage, Piccinini et al. 2012). For over 30 years, experimental models of hepatic IRI have provided significant knowledge of the disease pathogenesis, and have led to significant improvement of our understanding of the molecular mechanisms underlying reperfusion lesions. However, this complex process has led to several controversies in the field and needs better analysis. Here, we showed different mechanisms triggered by neutrophils that contribute to the pathogenesis of liver IRI.

1.1 Pathogenesis of liver IRI: innate immune response

As any reperfusion-associated injury, liver IRI is characterized by intense neutrophil infiltration in post-ischemic tissues. However, before this stage, resident cells act to create an inflammatory milieu, which contributes to neutrophil recruitment. First, IRI of the liver induces cell damage. This damage results in the release of endogenous molecules named danger-associated molecular patterns (DAMPs), in analogy to the term pathogen-associated molecular patterns (PAMPs) (Land 2015). The list of DAMPs includes high mobility group box 1 (HMGB1), deoxyribonucleic acid (DNA), ATP, urate, mitochondrial formyl peptides and S100 proteins. These molecules are detected by a variety of immune receptors, and have an important role in the initial (over)activation of the immune response (van Golen, van Gulik et al. 2012). During reperfusion, DAMPs released from apoptotic and necrotic cells stimulate Kupffer cells (liver resident macrophages) to produce inflammatory mediators, such as chemokines, cytokines and ROS. This process incites the next phase of reperfusion injury by orchestrating the homing, activation and adhesion of neutrophils (van Golen, van Gulik et al. 2012).

All cells of the innate immune system, including neutrophils, express pattern recognition receptors (PRRs), which can recognize DAMPs. These DAMP receptors include Toll-like receptors (TLRs), Retinoic Acid-Inducible Gene I-like receptors (RIG-I-), nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), and C-type lectin receptor (CLRs). By sensing DAMPs, these receptors promote an inflammatory response following reperfusion (Takeuchi and Akira 2010). TLRs are expressed in all cell types in the liver and their signaling leads to production of cytokines and chemokines through the activation of nuclear factor kappa B (NF-κB), promoting the recruitment of leukocytes (Land 2015). One of these endogenous ligands, DNA, is released from damaged hepatocytes and binds to TLR9

expressed on neutrophils, promoting further neutrophil recruitment and activation and subsequent hepatotoxicity. TLR9 expression on neutrophils is up-regulated upon liver damage and these cells sense and react to extracellular DNA by activating the TLR9/NF-κB pathway (Bamboat, Balachandran et al. 2010, Marques, Oliveira et al. 2015). *In vitro* experiments show that DNA released from necrotic hepatocytes increases cytokine expression by neutrophils through a TLR9-dependent mechanism. In addition, $TLR9^{-/-}$ mice subjected to 1 hour of ischemia and 12 hours of reperfusion show reduced hepatocellular necrosis, serum alanine aminotransferase (ALT) levels and lower production of inflammatory cytokines, showing that TLR9 signaling in neutrophils increases hepatic injury induced by IRI. (Bamboat, Balachandran et al. 2010). Following liver IRI, damaged hepatocytes have been shown to release the DAMP HMGB1, which exacerbates the hepatic injury through activation of TLR4 (Tsung, Sahai et al. 2005). The result of this activation creates an inflammatory environment that further incites the influx of inflammatory cells, including neutrophils.

In addition, complement activation plays a key role in post-ischemic inflammation and injury once complement inhibition is recognized as a potential therapeutic strategy for reducing IRI (Diepenhorst, van Gulik et al. 2009). This system consists of around 30 soluble and membrane-bound proteins which are activated by one of three pathways: the antibody dependent classical pathway, the alternate pathway and the mannose binding lectin pathway (Datta, Fuller et al. 2013). Activation of complement leads to the sequential production of the effector molecules complement 3a (C3a), complement 5a (C5a), and the membrane attack complex (MAC). C3a and C5a are soluble bioactive peptides that are cleaved from their parent proteins by enzymatic convertases, and the MAC is a terminal cytolytic protein complex assembled in cell membranes after cleavage of C5 (Marshall, He et al. 2014). Activation of this cascade of soluble plasma proteins results in the recruitment of a large number of inflammatory cells, ultimately leading to cell injury and death (Diepenhorst, van Gulik et al. 2009). Jaeschke and colleagues have shown that depletion of serum complement before liver ischemia prevents neutrophil accumulation during reperfusion (Jaeschke, Farhood et al. 1993). Furthermore, in partial hepatic IRI in rats, treatment with the complement 5a receptor (C5aR) antagonist attenuates the increases in liver enzymes, serum and tissue tumor necrosis factor alpha (TNF-α), infiltrating neutrophils, and also reduces liver histopathology (Arumugam, Woodruff et al. 2004).

1.2 Neutrophils

In liver inflammation, the defense against initial environmental challenges and injury is driven by a complex set of leukocytes, including natural killer cells (NKs), natural killer T cells (NKTs), dendritic cells (DCs), neutrophils, eosinophils and complement components (Xu, Huang et al. 2014). Of these cell subsets, neutrophils are known to induce liver injury, and are the largest circulating fraction of leukocytes and the first cells to arrive at the site of injury. The process of neutrophil recruitment into the liver during IRI-induced sterile inflammation is particularly different from the mechanism in other organs and to the one induced by pathogens. The multi-step process for neutrophils to reach the liver parenchyma and resulting in tissue damage includes neutrophil activation, adherence within the hepatic vasculature, neutrophil transmigration to the liver parenchyma and release of granule enzymes (Lee and Kubes 2008, Adams, Ju et al. 2010).

1.2.1 Role of Chemokines and Glycosaminoglycans in Liver IRI

The role of neutrophils in liver IRI has been shown in several publications (Ramaiah and Jaeschke 2007, Kubes and Mehal 2012). A range of inflammatory mediators, including chemokines, the complement proteins C3a and C5a, mitochondrial derived formyl peptides and leukotriene B4, can induce neutrophil influx in the hepatic microvasculature. Among these mediators, the role of chemokines is worth highlighting. Chemokines constitute a group of small structurally related chemotactic proteins indispensable for the coordination of leukocyte migration during inflammation (Rot and von Andrian 2004). Previous studies have demonstrated that Cys-Xaa-Cys (CXC) chemokines mediate neutrophil infiltration during liver IRI and other inflammatory conditions (Roh, Zhang et al. 2015, Su and Richmond 2015, Wilson, Freeman et al. 2015).

After tissue damage, chemokines are locally secreted by parenchymal cells and resident leukocytes, thereby creating a gradient along which neutrophils can migrate from the blood vessels to the site of inflammation. In mice, CXC chemokine 1 (CXCL1) and CXCL2, homologues of human interleukin-8 (IL-8 or CXCL8) are important chemoattractants of neutrophils. By recruiting and activating neutrophils, human CXCL8 has been implicated in a wide range of diseases, including liver IRI (Mosher, Dean et al. 2001, Li, Klintman et al. 2004). These chemokine ligands are recognized by CXC chemokine receptor 2 (CXCR2) expressed

on the neutrophil surface and mediate neutrophil recruitment (Kuboki, Shin et al. 2008). Human CXCL8 also bind to CXC chemokine receptor 1 (CXCR1). It was demonstrated that after liver IRI, expression of CXCL1 and CXCL2 in the ischemic lobes increases 100-1000-fold (Wilson, Kuboki et al. 2015). Moreover, Reparixin (DF1681B), an allosteric antagonist of the CXCR2 receptor, is able to prevent neutrophil influx and liver damage (Bertini, Allegretti et al. 2004). Wengner and colleagues demonstrated the process of neutrophil responses to chemokines in a murine model of peritonitis (Wengner, Pitchford et al. 2008). After two hours of a single intraperitoneal (i.p.) injection of thioglycolate a significant increase in the circulating numbers of neutrophils is noted. This is inhibited by 84% when mice are pretreated with neutralizing monoclonal antibodies to CXCL1 and CXCL2.

Chemokine production only is not sufficient to lead to neutrophil recruitment. For chemokines to have an effect on leukocyte trafficking, besides being produced, they need to be present at a defined concentration range and in the right place to be able to induce chemotaxis. This process requires interaction of chemokines with GAGs expressed on the vasculature (Verkaar, van Offenbeek et al. 2014). Thus, to create a gradient and to induce neutrophil migration *in vivo*, it is necessary for chemokines to interact with two essential receptors. First, they need to bind to their particular GPCRs, which are expressed by specific leukocytes. Chemokine – GPCR interaction induces the endothelial adhesion of leukocyte subtypes followed by their extravasation and directional migration toward the site of inflammation (Ley, Laudanna et al. 2007). In addition to binding to specific GPCRs, chemokines also bind cell surface GAGs at the vascular endothelium or GAGs in the extracellular matrix. (Handel, Johnson et al. 2005, Johnson, Proudfoot et al. 2005). Endothelial cells present GAGs such as heparan sulfate on their cell surface to which chemokines bind via their GAG binding site. Due to the chemokine-GAG interaction, a stable chemokine gradient is formed on the surface of the endothelial cells and in tissues and activated neutrophils migrate along this gradient toward the site of inflammation (Handel, Johnson et al. 2005). This interaction and formation of immobilized chemical concentration gradients is also called haptotaxis (Rink, Rink et al. 2015). During recent years, the importance of GAG-chemokine interactions in leukocyte migration has been investigated in more detail. Several GAGs, such as hyaluronan, dermatan sulfate and heparan sulfate have been implicated in events associated with inflammation, such as cytokine/chemokine production and presentation and leukocyte recruitment (Trowbridge and Gallo 2002, Taylor and Gallo 2006). More recently, *in vivo*, chemokines have been shown to localize within capillary venules in a GAG-dependent way. In these studies, an intravascular

gradient of chemokines was sequestered by heparan sulfate and immobilized on the endothelium (Massena, Christoffersson et al. 2010). This retention of chemokines on the endothelial layers occurs by interactions between sulfated domains of GAGs and basic amino acid motifs on chemokines. Chemokine presentation by the GAGs on the endothelial cell wall prevents their diffusion and degradation and retains high local concentrations of the produced chemokines (Middleton, Patterson et al. 2002).

Chemokine binding to GAGs has been proven to be indispensable for chemokine activity and neutrophil recruitment *in vivo* (Massena, Christoffersson et al. 2010, Severin, Gaudry et al. 2010, Sarris, Masson et al. 2012, Dyer, Thomson et al. 2014). The local concentration of chemokines induces crawling of neutrophils toward an inflammation site (Massena, Christoffersson et al. 2010). Chemokine production at sites of inflammation also results in the generation of GAG-mediated chemokine gradients in the extracellular matrix. Reduced chemokine-induced neutrophil migration is seen in mice with disturbed heparan sulfate (Bao, Moseman et al. 2010). Moreover, a study by Vanheule and colleagues shows that the blockade of GAG-chemokine interactions is able to inhibit neutrophil extravasation in a murine model of monosodium urate-induced gout (Vanheule, Janssens et al. 2015). Importantly, removing hyaluronan from the sinusoidal endothelium, or blocking its interaction with its principal receptor (CD44), reduces neutrophil recruitment as well (McDonald, McAvoy et al. 2008). Although therapeutic intervention in the chemokine system has long been focused on the development of chemokine and chemokine receptor antagonists (Allegretti, Cesta et al. 2012), more recently, it has been suggested that interruption of chemokine-GAG interactions might represent an innovative and useful way of interfering with chemokine action which may result in a decrease of inflammation (Adage, Piccinini et al. 2012).

1.2.2 Intrahepatic Neutrophil Migration

In sterile inflammation, there is a multistep hierarchy of directional cues that guide neutrophil localization to sites of damage. Neutrophils migrate intravascularly through the sinusoid channels toward the stressed tissue, ultimately infiltrating directly into the area of necrosis. These findings suggest there is an intravascular gradient that guides neutrophils to the site of inflammation (McDonald, Pittman et al. 2010). During liver IRI, direct cellular damage from oxidative stress during ischemia results in the passive release of DAMPs from necrotic cells. DAMPs induce sterile inflammation through stimulation of chemokine production by

resident cells and consequently neutrophil recruitment. When injected into mice, purified DAMPs or necrotic cells mobilize neutrophils to the site of inoculation (Chen, Kono et al. 2007). Using *in vivo* imaging to reveal a multistep event that guides the recruitment of neutrophils to locations of sterile injury, McDonald and colleagues demonstrated CXCL2 expression on the luminal surface of the liver sinusoids that was maximal at about 150 µm from the injury and gradually decreased out to 650 µm, demonstrating the presence of an intravascular gradient that leads toward the injured area. However, despite the intravascular gradient of CXCL2 was consistently observed to abruptly end at about 100 to 150 µm proximal to the border of necrotic tissue, neutrophils continue to migrate into the area of necrosis, beyond the maximum expression of CXCL2. Furthermore, within this proximal 150 µm surrounding the injury, directional neutrophil migration was independent of CXCR2 (McDonald, Pittman et al. 2010). This indicates that from a certain point, neutrophils migrate to the area of necrosis guided by other factors, which are independent of chemokines and CXCR2 **(Illustration 1)**.

This chemotactic stimulus was identified as mitochondrial N-formyl peptides (Zhang, Raoof et al. 2010). During necrosis, the mitochondrial content is spilled out into the extracellular milieu and mitochondrial N-formyl peptides can attract and activate neutrophils through the specific receptor formyl peptide receptor 1 (FPR1), which is as chemokine receptors a GPCR (Chen and Nunez 2010). In this way, neutrophils migrate first towards an intravascular chemokine gradient, and then switch to a formyl-peptide gradient near the injury site, where the FPR1 signal works in the absence of a chemokine gradient (Kolaczkowska and Kubes 2013). Studies in other animal models such as acetaminophen-induced liver injury have shown that antagonization of FPR1 causes partial reduction of neutrophil recruitment. However, the strategy to block both FPR1 and CXCR2 receptors simultaneously significantly reduces neutrophil migration to the liver and liver damage (Marques, Amaral et al. 2012). This shows that chemokines and formyl peptides work together in a sequential manner to guide neutrophils to sites of sterile inflammation (McDonald, Pittman et al. 2010).

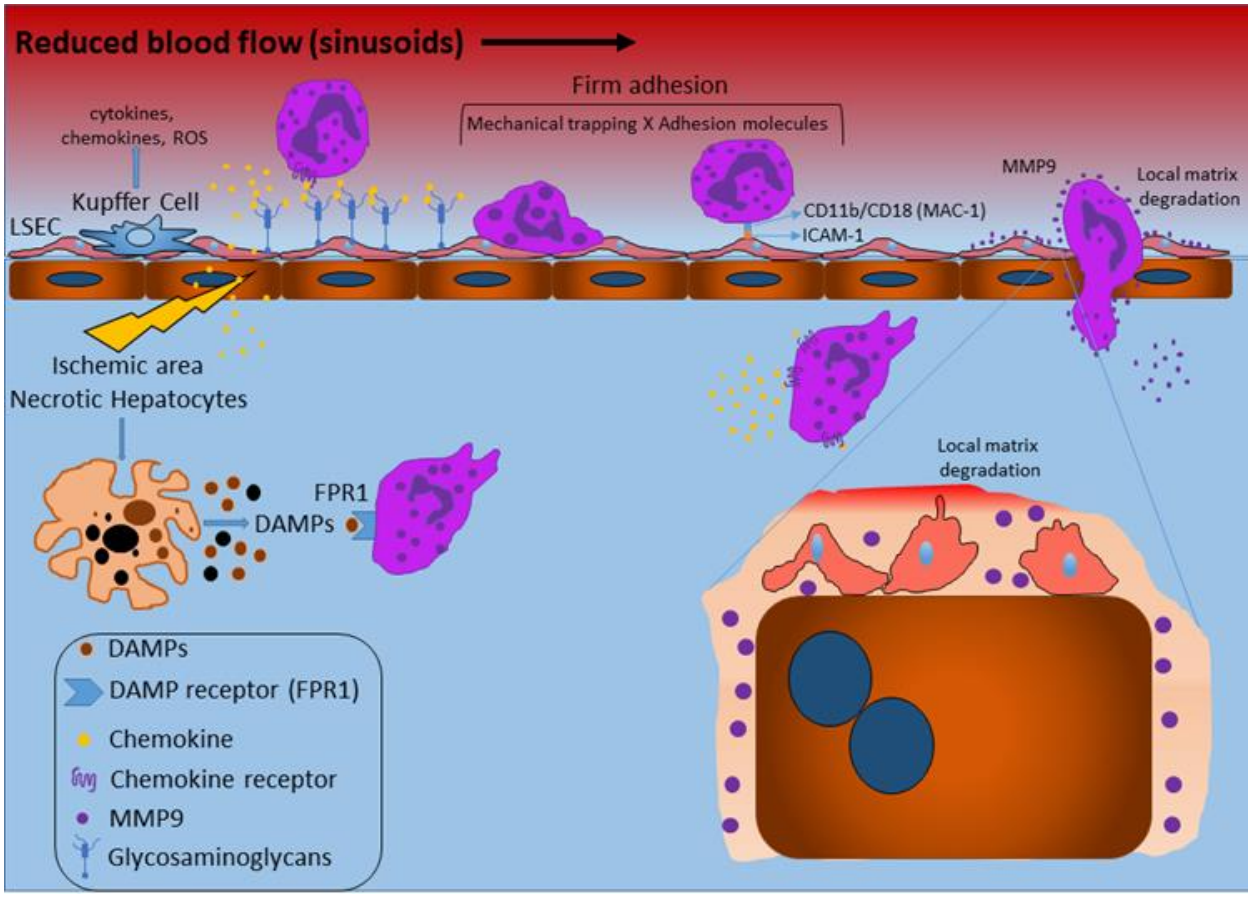

Illustration 1: Chemokine-, GAG- and MMP-dependent liver damage after ischemia-reperfusion injury

Due to the unusual hepatic microvasculature, which has a reduced diameter and blood flow, neutrophils are in intimate contact with the sinusoidal endothelial cells, and may be mechanically trapped in the sinusoids independent of selectins. Cell death by necrosis releases multiple DAMPs. Kupffer cells are the primary sentinel cells that sense cell death and generate pro-inflammatory cytokines and chemokines. Chemokines bind to GAGs on the surface of endothelial cells, forming a chemotactic gradient in the inflamed tissue. They guide intravascular neutrophil recruitment to the injury area. Interaction of leukocytes with chemokines on the endothelium leads to integrin activation, firm adhesion, transmigration across the endothelial barrier and accumulation of leukocytes at the site of inflammation. Neutrophils migrate directly into the area of cell death, where DAMPs induce direct neutrophil chemotaxis via mitochondrial formyl peptide-signaling through FPR1. In parallel, MMPs, particularly MMP-9, facilitate local matrix degradation and neutrophil extravasation across vascular barriers. Besides neutrophil recruitment, MMP-mediated loss of endothelial barrier integrity interferes with the liver's ability to regenerate after hepatic ischemia-reperfusion. Additionally, MMPs contribute to tissue injury by promoting parenchyma cell detachment from ECM, resulting in additional tissue necrosis.

1.2.3 Role of Matrix Metalloproteases (MMPs) in Liver IRI

The extracellular matrix (ECM), formed by the complex network of proteins and glycans surrounding cells in all solid tissues, is among the most important regulators of cellular and tissue functions in the body. The ECM regulates various cellular functions, such as adhesion, migration, differentiation, proliferation, and survival. Degradation of ECM is essential in many physiological processes, including angiogenesis and wound healing. However, dysregulation of ECM production and proteolysis is often associated with the development of liver pathology (Cox and Erler 2011). The alterations of the ECM are in agreement with reports describing the potential implications of metalloproteinases (MMPs) in liver diseases (Kuyvenhoven, Verspaget et al. 2004, Dechene, Sowa et al. 2010). Using a model of partial liver IRI in rats, Cursio and colleagues showed high expression of MMPs in a specific time-dependent pattern, which was associated with the release of liver enzymes and a large area of tissue necrosis by histology. In the same work, intensity of liver necrosis was significantly reduced in rats treated with specific MMP inhibitor, (Cursio, Mari et al. 2002). Leukocyte migration across endothelial and ECM barriers is dependent on cellular adhesion/release and focal matrix degradation mechanisms (Coito 2011). Although adhesion molecules are critical for the successful promotion of neutrophil transmigration by providing attachment to the vascular endothelium, there is a growing body of evidence suggesting that MMPs are critical for facilitating leukocyte movement across vascular barriers **(Illustration 1)** (Keck, Balcom et al. 2002, Coito 2011).

The MMPs are a family of 24 proteases using zinc-dependent catalysis to break/down ECM components, allowing cell movement and tissue reorganization (Hu, Van den Steen et al. 2007). Although this step is important in physiological processes such as tissue repair, the inappropriate, prolonged or excessive expression of these enzymes has harmful consequences for the liver (Palladini, Ferrigno et al. 2015). The role of MMPs has been investigated in several pathological conditions, including cancer (Morini, Mottolese et al. 2000, Bjorklund and Koivunen 2005), autoimmune diseases such as rheumatoid arthritis, lupus erythematosus and multiple sclerosis (Posthumus, Limburg et al. 1999, Faber-Elmann, Sthoeger et al. 2002, Ram, Sherer et al. 2006, Yong, Zabad et al. 2007, Gerwien, Hermann et al. 2016), chronic inflammation including inflammatory bowel diseases (Garg, Vijay-Kumar et al. 2009, de Bruyn, Vandooren et al. 2016), lung disease (Craig, Zhang et al. 2015) and liver IRI

(Kuyvenhoven, Ringers et al. 2003, Hamada, Fondevila et al. 2008, Kato, Kuriyama et al. 2014).

MMPs have been categorized into five major groups according to their ECM substrate specificity: collagenases, gelatinases, membrane-type enzymes, stromelysins and matrilysins (Bourboulia and Stetler-Stevenson 2010). Among the different MMPs, gelatinases (MMP-2 and MMP-9) are within the most prominent MMPs detected during liver IRI (Nagase and Woessner 1999, Kato, Duarte et al. 2015). MMP-2 and MMP-9 have gelatin-binding domains that resemble a similar motif in fibronectin. This motif is involved in the binding of fibronectin to denatured collagen (Parks, Wilson et al. 2004). Fibronectin is a large extracellular matrix glycoprotein with a recognized central role in cellular adhesion and migration, and it is likely an important ECM protein involved in leukocyte adhesion during liver IRI. The expression of cellular (EIIIA+) fibronectin by liver sinusoidal endothelial cells is an early feature after injury and it precedes leukocyte recruitment in hepatic IRI (Amersi, Shen et al. 2003). A study of Moore and colleagues shows that the interactions between fibronectin and its integrin receptor α4β1, expressed on leukocytes, specifically up-regulate the expression and activation of MMP-9 by infiltrating leukocytes in steatotic livers subjected to IRI. The high expression of MMP-9 is accompanied by massive neutrophil infiltration, increased levels of proinflammatory cytokines and impaired liver function. Moreover, the blockade of fibronectin-α4β1-integrin interactions inhibits the activation of MMP-9 by leukocytes and prevents cytokine release and cellular migration to the liver after IRI (Moore, Shen et al. 2007). In healthy tissues, MMP-2 is constitutively expressed in fibroblasts, endothelial cells and epithelial cells, and may be modestly up- or down-regulated by inflammatory stimuli (Nascimento, Rizzi et al. 2013). In contrast, MMP-9 is inducible and its expression is observed primarily in leukocytes including monocytes, macrophages and neutrophils (Moore, Shen et al. 2007). MMP-9 is released by neutrophils, and once produced, this enzyme is capable of digesting components of connective tissue matrix, such fibronectin and collagen type IV, facilitating the movement of neutrophils towards the site of damage (Kuyvenhoven, Ringers et al. 2003, Ram, Sherer et al. 2006).

The role of MMP-9 in the mechanism of neutrophil infiltration has been examined using *Mmp-9*$^{-/-}$ mice and mice treated with MMP-9-blocking antibodies or less specific small molecule MMP inhibitors (Hu, Van den Steen et al. 2007). In a model of liver IRI, Hamada and colleagues show significantly reduced liver damage by histological assessment with lowered circulating levels of aminotransferases, reduced hepatocyte necrosis, and improved sinusoidal blood flow (Hamada, Fondevila et al. 2008). Improved liver function correlates with reduced

parenchymal infiltration of neutrophils and reduced liver content of neutrophil myeloperoxidase (MPO). In addition, *Mmp-9*$^{-/-}$ neutrophils have impaired ability to migrate across fibronectin relative to normal cells. MMP-9 is mostly detected in Ly-6G and macrophage antigen–1 (MAC-1) leukocytes adherent to the vessel walls and infiltrating the damaged livers of wild-type mice after liver IRI. These findings have not been limited to the liver, but also to other organs such as heart and brain (Cheung, Sawicki et al. 2000, Martin, Garofalakis et al. 2012).

While the release of MMP-9 by neutrophils seems to have a harmful effect, MMP-2 seems to have an opposite role. Recent reports have shown direct evidence of a protective role for MMP-2 expression in the liver. For instance, mice treated with an anti-MMP-2 neutralizing antibody are characterized by intensified liver damage after IRI (Hamada, Fondevila et al. 2008). Moreover, *Mmp*-2$^{-/-}$ mice subjected to hepatic IRI show exacerbated liver damage compared to WT mice. Furthermore, *Mmp*-2 deficiency results in upregulation of MMP-9 activity, spontaneous neutrophil infiltration in naïve livers, and amplifies MMP-9-dependent transmigration of neutrophils *in vitro* and after hepatic IRI (Kato, Duarte et al. 2015). Tissue inhibitors of metalloproteinases (TIMPs) regulate the activity of MMPs. Alterations in the MMP-TIMP balance have been linked to pathological conditions that require disruption of the basement membrane, such as tumor invasion, angiogenesis, and wound healing (Chirco, Liu et al. 2006). There are at least four identified members (TIMP-1 to TIMP-4) in the TIMP family, varying in tissue-specific expression and in their ability to inhibit various MMPs (Egeblad and Werb 2002).

Among the different TIMPs, TIMP-1 is of particular interest. TIMP-1 expression is very low in naive livers and it is induced after liver IRI. However, it is still insufficient to prevent an elevated MMP activity in liver IRI (Moore, Shen et al. 2007). Duarte and coworkers have shown that animals lacking TIMP-1 show further impaired liver function and histological preservation after IRI. In this model, TIMP-1 deficiency leads to lethal liver IRI, as over 60% of the TIMP-1$^{-/-}$ mice died post-reperfusion, whereas all TIMP-1$^{+/+}$ mice recovered and survived surgery. In addition, lack of TIMP-1 expression is accompanied by markedly high levels of MMP-9 activity, which facilitates leukocyte transmigration across vascular barriers in hepatic IRI. Indeed, TIMP-1$^{-/-}$ livers are characterized by massive neutrophil infiltration and by up-regulation of proinflammatory mediators, including TNF-α, IFN-γ and inducible nitric oxide synthase (iNOS) post-IRI (Duarte, Hamada et al. 2012). Altogether, MMPs and TIMPs seem

to have important roles in the preservation of liver homeostasis and it is suggested that they might be targeted to ameliorate liver damage.

1.2.4 Neutrophil adhesion and migration

As the inflammatory process develops, leukocytes are recruited to the liver. In hepatic IRI and many other acute injuries, neutrophils are the first to arrive and usually in large numbers. Neutrophil recruitment during inflammation is normally attributed to a multi-step cascade involving initial tethering and rolling by selectins along the vessel wall, followed by firm adhesion to the vascular endothelium and emigration out of the vasculature (Sanz and Kubes 2012). This classical paradigm is well characterized for a number of organ microvasculatures including mesentery, peritoneum, skeletal muscle, and skin **(Illustration 2)** (Kim, Lee et al. 2011).

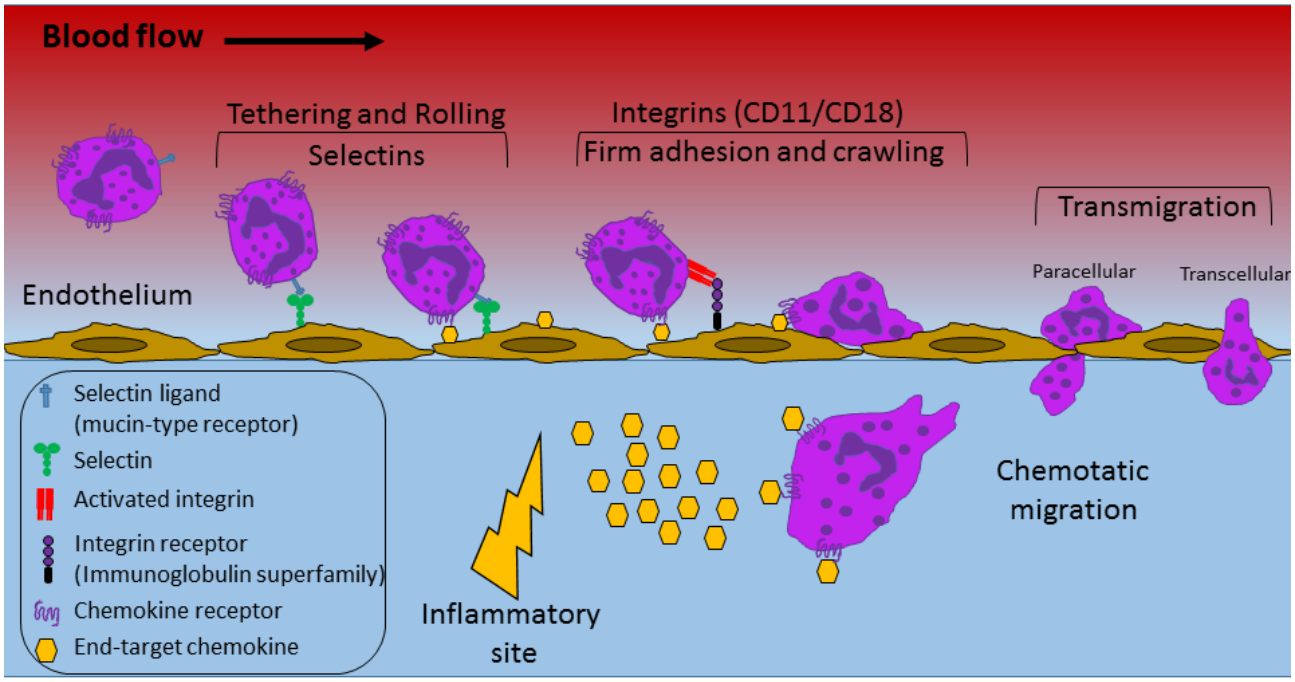

Illustration 2: Classical mechanism of leukocyte recruitment

In most tissues, leukocytes (e.g. neutrophils) initially tether to and roll on the endothelium adjacent to the inflammatory focus through selectin interactions with mucin-like selectin receptors. Engagement of chemokine receptors with their ligands presented on the endothelium stimulates activation of integrins on the leukocyte cell surface, enabling their interaction with their respective receptors to facilitate arrest, adhesion, and subsequent transmigration by paracellular or transcellular routes. Following a chemotactic gradient, they reach the extravascular compartment to accumulate in areas rich in chemoattractants and release lytic enzymes through degranulation.

Most tissues require selectin-mediated tethering and rolling in post-capillary venules, followed by firm adhesion mediated by integrins for proper neutrophil recruitment. Even though neutrophils follow the intravascular recruitment paradigm in most tissues, as skin and muscle, they use a very different strategy in the liver. For example, there is little evidence for the requirement of selectin-mediated rolling or β2 integrin-mediated adhesion for neutrophil migration in the liver sinusoids. In the liver sinusoids, the adhesive response to N-formyl-Met-Leu-Phe (fMLP) is not dependent upon selectins since adhesion is not reduced in the sinusoidal vessels of P-selectin-deficient mice or E-selectin/P-selectin-deficient mice treated with L-selectin antibody (Wong, Johnston et al. 1997, Jaeschke and Hasegawa 2006).

Due to the unusual hepatic microvasculature, which has a reduced diameter and blood flow, it is likely that neutrophils would be mechanically trapped in the inflamed sinusoids (McDonald, Pittman et al. 2010, Marques, Oliveira et al. 2015). Moreover, hepatic sinusoids are devoid of selectins and neutrophils move directly by integrin-mediated adhesion (van Golen, van Gulik et al. 2012). Thus, in these narrow capillaries, the rolling process is likely to be unnecessary (Hickey and Westhorpe 2013). This may explain why clinical trials of anti-adhesion therapy, in an attempt to reduce liver injury associated with traumatic shock and reperfusion injury, fail to show a significant benefit (Harlan and Winn 2002).

Using intravital microscopy to visualize the liver microvasculature, Wong and colleagues show that the majority of leukocytes (80%) adhere within the sinusoids in response to a chemotactic stimulus such as fMLP while 20% adhere to post-sinusoidal venules. This is in marked contrast to leukocytes adhering exclusively to post-capillary venules in tissues as the mouse cremaster muscle (Wong, Johnston et al. 1997). In hepatic IRI, the main sinusoidal ligand for neutrophils is intercellular adhesion molecule-1 (ICAM-1) (Farhood, McGuire et al. 1995, Benkoel, Dodero et al. 2003). Around sites of focal hepatic necrosis, neutrophil adhesion to sinusoidal endothelium is mediated by the binding of the integrin Mac-1 (also known as CD11b/CD18) to ICAM-1. The absence of ICAM-1 reduces neutrophil recruitment to the liver following acute hepatocellular necrosis induced by IRI (Nakano, Kuzume et al. 1995, Nishimura, Takei et al. 1996). Subsequently, neutrophils migrate through the intravascular channels toward the site of injury, penetrating directly into the area of damage. Menezes and colleagues show that fMLP induces recruitment of neutrophils similar to focal necrosis by adhesion and crawling dependent on Mac-1 and ICAM-1 (Menezes, Lee et al. 2009). Furthermore, ICAM-1 has been shown upregulated after hepatic IRI (Meyer, Brown et al. 1998). Data from the literature has shown that blocking of Mac-1 has protective effects against

hepatic reperfusion injury *in vivo* (Jaeschke, Farhood et al. 1993, Marubayashi, Oshiro et al. 1997).

Once outside the vessel, individual neutrophils often show extremely coordinated chemotaxis and cluster formation reminiscent of the swarming behavior of insects. This behavior was shown recently by Lammermann and coworkers in a model of focal ear skin damage and infected lymph nodes (Lammermann and Sixt 2009, Lammermann, Afonso et al. 2013, Lammermann 2016). They observed that once neutrophils have crossed the endothelium, arriving at the interstitial space, these cells adopt an amoeboid migration strategy, which relies mainly on contraction and protrusion of the cytoplasm, independently of adhesion molecules. This phenomenon happens in the 3D environment of the interstitium, usually composed of a meshwork of fibrillar extracellular matrix, such as collagen fibers. In this scenario, leukocyte migration is completely dependent on the acto-myosin cytoskeleton, in which leukocyte migration would happen by contraction of the cell posteriorly coupled to frontal protusion of lamellipodia, propelling the cell through the fibrillar ECM at high speed and without integrin requirement (Lammermann and Sixt 2009, Lammermann and Germain 2014). Interestingly, these concepts have not been applied to the liver so far, a tissue in which the reduced extravascular space is tightly packed with hepatocytes and non-parenchymal cells.

1.3 Mechanisms of neutrophil-mediated liver injury

As we described above, during organ transplantation, the liver is exposed to prolonged times of ischemia, which results in cell stress and cell death. This early damage is essential to liver injury during the reperfusion. Neutrophils are considered crucial effector cells in the pathophysiology of liver IRI. Because of their high mobility and the capability to release potent cytotoxic mediators, neutrophils primarily work to eliminate invading microorganisms and/or remove dead cells at the site of inflammation (Jaeschke 2003). However, in the liver and others organs, an excessive inflammatory response carries the risk of additional tissue damage as demonstrated during liver IRI and other conditions (Jaeschke and Smith 1997, Gujral, Farhood et al. 2003).

Interestingly, activation and accumulation of neutrophils in the sinusoids do not cause tissue damage to the epithelium, as it does in other organs. Only after migrating across the endothelium and in close proximity to the hepatocytes can neutrophils cause damage by oxidative stress, triggered by two major cytotoxic mediators, i.e., ROS and proteases (Schofield, Woodruff et al. 2013). During the first hours of reperfusion, Kupffer cells, the resident

macrophages of the liver, release ROS and proinflammatory cytokines, aggravating the initial damage caused by ischemia (Jaeschke 2003). The later phase includes a cascade of inflammatory events culminating in neutrophil infiltration into the liver, and consequently amplification of the tissue lesion (Ramaiah and Jaeschke 2007, Kolaczkowska and Kubes 2013). The adhesion of neutrophils to the targets induces their degranulation with release of proteases and ROS, which can diffuse into hepatocytes, inducing an intracellular oxidant stress, mitochondrial dysfunction and damage (Jaeschke 2006). Neutrophil extravasation into the parenchyma is a prerequisite for cytotoxicity, since neutrophils are recruited into the sinusoids without causing damage (Jaeschke and Smith 1997).

A critical role for ROS is observed in the pathophysiology of the liver IRI. Normally, ROS production is useful to eliminate circulating pathogens and is the mechanism responsible for the "respiratory burst" observed when cells are activated. However, excessive ROS after an ischemic insult is detrimental (Evankovich, Billiar et al. 2010). ROS formation is dangerous for cells due to the presence of polyunsaturated fatty acids in cellular membranes, the substantial number of unprotected protein sulfhydryl groups and DNA bases (Jaeschke and Ramachandran 2011). Despite this, hepatic cells have sophisticated defense systems, such as expression of superoxide dismutase (SOD 1 in the cytosol, SOD 2 in the mitochondria), and reduced glutathione (GSH), which are responsible for the detoxification of organic and inorganic peroxides, forming part of the cellular enzymatic antioxidant defense system (Jaeschke 2011). The protective role of GSH against hepatic injury has been demonstrated for decades (Bilzer, Paumgartner et al. 1999, Bilzer, Baron et al. 2002, Schauer, Gerbes et al. 2004). However, increased ROS production causes disturbances of the cellular homeostasis and, if not counteracted, this can lead to cell death. Excessive ROS formation results in its diffusion into hepatocytes, where ROS can lead to lysosomal iron mobilization and translocation to the mitochondria, which triggers a mitochondrial oxidant stress and eventually necrotic cell death (Uchiyama, Kim et al. 2008, Jaeschke and Ramachandran 2011). Consistent with this, inhibition of oxidant stress through blocking nicotinamide adenine dinucleotide phosphate (NADPH) oxidase reduces liver injury induced by neutrophils (Hasegawa, Malle et al. 2005). Cahova and colleagues show that the antioxidant metformin is able to reduce mitochondrial damage (markers: cytochrome C release, citrate synthase activity, mitochondrial DNA copy number, mitochondrial respiration), mitochondrial ROS production and liver inflammation in rats subjected to IRI (Cahova, Palenickova et al. 2015). In addition, Xue and colleagues show an example where the combination of increased ROS formation and impaired defense systems

causes cell death. In this work, in rats subjected to liver IRI, administration of GSH by means of the portal vein before ischemia increases the 7-day survival rates of rats after liver IRI from 38% to 75%. In addition, GSH pretreatment decreases intrahepatic neutrophil accumulation (Xue, Wang et al. 2008). ROS from Kupffer cells also contributes to the activation of inflammatory pathways that lead to neutrophil accumulation in the liver, resulting in additional, prolonged injury. Thus, Kupffer cell-derived ROS are involved in the pathogenesis of liver IRI injury through direct oxidant-mediated damage and by augmenting the local activation of proinflammatory pathways (Evankovich, Billiar et al. 2010)

Neutrophil-derived proteases also seem to play an important role in the cause of direct cell death of hepatocytes. Once neutrophils adhere to hepatocytes, full degranulation occurs, with consequent release into the area around the cell of several proteases, such elastases, MMP-9, cathepsin G, myeloperoxidase and proteinase-3 (Faurschou and Borregaard 2003). Neutrophil elastase is a serine protease found in the azurophil granules of neutrophils. The requirement for neutrophils to migrate out of the vasculature and through the basement membrane, as well as the potent proteolytic function of neutrophil elastase, have led to the theory that neutrophil elastase might be involved in the pathogenesis of inflammatory tissue injury such as that exemplified by liver IRI (Uchida, Freitas et al. 2010).

Neutrophil elastase is stored in azurophil granules in its active form until it is released following neutrophil exposure to the inflammatory stimuli. As a result, excessive release of neutrophil elastase degrades elastin, collagens, laminins, and other ECM components, thereby leading to subsequent organ damage through endothelial cell injury (Mainardi, Dixit et al. 1980, Uchida, Freitas et al. 2010). Consistent with this, a study of human liver damage identified a neutrophil elastase inhibitor that has therapeutic potential, and this inhibitor is associated with a reduced release of HMGB1 and reduced IL-6 levels (Tsujii, Okabayashi et al. 2012). In addition, in mice subjected to 90 minutes of ischemia and 6h and 24h of reperfusion, treatment with Sivelestat, a neutrophil elastase inhibitor, ameliorates the hepatocellular damage and decreases local neutrophil activity and infiltration (Uchida, Freitas et al. 2010).

One of the main molecules released after neutrophil recruitment and activation is MPO. This enzyme is used as a neutrophil marker, due to its high expression in these cells, then its activity correlates with neutrophil accumulation in tissues. In the presence of physiological chloride concentrations, MPO reacts with hydrogen peroxide (H_2O_2) to catalyze formation of hypochlorous acid/hypochlorite (HOCl/OCl−) and other oxidizing species (Klebanoff 2005). The participation of MPO has been shown in a range of pathophysiological conditions,

including liver IRI (Jaeschke, Farhood et al. 1990, Kawachi, Hines et al. 2000). Kato and colleagues have shown an increased MPO activity, which was consistent with increased neutrophil accumulation and hepatocyte necrosis (Kato, Edwards et al. 2002).

Although it is clear that neutrophils can cause cell death and necrosis, their phagocytic actions are believed to be relevant for proper tissue repair and protection. Therefore, it is not simple to say that the effects of neutrophils are entirely harmful or may contribute to resolution of inflammatory responses (Borregaard 2010, Mayadas, Cullere et al. 2014). The relevance of neutrophils in any particular setting is clearly context-dependent. For example, Reber and colleagues have shown that neutrophils can contribute to optimal host protection against endotoxemia, since neutrophil-depleted mice suffered from increased mortality. This beneficial role was induced through MPO activity (Reber, Gillis et al. 2017). On the other hand, Marques and colleagues showed that depletion of neutrophils with anti-Ly-6G reduced acetaminophen-mediated toxicity and liver injury (Marques, Oliveira et al. 2015). In the latter context, neutrophils enhanced liver injury and inflammation. In the context of liver IRI, there is overwhelming evidence for a deleterious role of neutrophil migration and function, as discussed above, and the activity of the neutrophil proteases together with ROS production appears to mediate most of the deleterious actions of neutrophils.

2. OBJECTIVES

2.1 Main Objective

To evaluate the role of neutrophils in liver ischemia and reperfusion injury

2.2 Specific Objectives

1) To evaluate the kinetics of hepatic injury induced by IRI *in vivo*
2) To study the behavior of neutrophils during inflammation induced by IRI using intravital microscopy
3) To determine the effect of the CXCR1/2 inhibitor DF1681B/Reparixin in a model of liver IRI
4) To investigate the role of MMP-9 in neutrophil function during liver IRI
5) To study the potential of GAG binding peptides for treatment in an experimental model of liver IRI.

3. MATERIALS AND METHODS

3.1 Experimental Animals

Male C57BL/6J and Lysm-eGFP mice (8-12 weeks old) were obtained from the Central Animal Facility of the Universidade Federal de Minas Gerais (UFMG, Brazil). Male *Mmp-9*$^{+/+}$ and *Mmp-9*$^{-/-}$ mice were obtained from Central Animal Facility of the Rega Institute (KU Leuven, Belgium). The animals were maintained with filtered water and food ad libitum in a 12-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).

3.2 Hepatic ischemia-reperfusion model

The IR was performed as described (Honda, Takeichi et al. 2013). 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 minutes of ischemia, the clamp was removed and reperfusion was initiated. The following time points were examined after reperfusion: 1h, 3h, 6h, 12h, 24h, and 48h. The control operation was performed using the same protocol but without vascular occlusion. Mice were placed on a heating pad to maintain body temperature at 37ºC throughout the procedure. Blood was obtained for analysis of serum ALT as an index of hepatocellular injury using a kinetic test (Bioclin, Belo Horizonte, Brazil). The kinetic assay is the method recommended by the International Federation of Clinical Chemistry. Cytokines and chemokines were quantified by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) both in serum and tissues and real-time polymerase chain reaction (qPCR) of livers. Fragments of liver were fixed and sectioned for histology as described below. Indocyanine green (ICG; Sigma) clearance by the liver was estimated in serum after injecting a single dose of 20 mg per kg intravenously. Blood was collected 20 minutes after injection and the amount of ICG was determined by spectrophotometry (absorbance in 800 nm).

3.3 Neutrophil accumulation in liver and lungs

Neutrophil accumulation was determined by the liver MPO content. 50 mg of tissue was homogenized in a buffered solution containing antiproteases, as previously described (Russo, Guabiraba et al. 2009). MPO levels were accessed using 25 µl of the supernatant of the homogenized sample and 25 µl of a solution of 1.6 mM of 3,3'-5,5'-tetramethylbenzidine (TMB; Sigma – dissolved in dimethyl sulfoxide) and 0.01 mM of H_2O_2, dissolved in phosphate buffer (pH 5.4) containing hexa trimethylammonium bromide (HTAB) (Russo, Guabiraba et al. 2009). The reaction was started at 378C 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 378C for 5 minutes. The reaction was stopped by adding 100 ml of 1M H_2 SO_4 and quantified at 450 nm in a spectrophotometer (Emax; Molecular Devices, Sunnyvale, CA).

3.4 Measurement of Gene Expression by Real-Time Polymerase Chain Reaction (qPCR).

Relative changes in gene expression were evaluated by quantitative polymerase chain reactions (qPCR). 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). Relative changes in gene expression were evaluated by qPCR using the TaqMan Fast Universal PCR master mix (Applied Biosystems). Sample mixes were loaded on a 96-well MicroAmp plate (Applied Biosystems) and were analyzed on the 7500 Fast Real-time PCR system. Obtained Ct values were processed following the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as housekeeping gene (Livak and Schmittgen 2001).

3.5 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.

3.6 Imaging of the Liver Using Intravital Confocal Microscopy

Parameters of neutrophil accumulation in the liver were observed using intravital microscopy as previously described, using a Nikon Ti C2 confocal microscope equipped with a 10x objective in male mice expressing the green fluorescent protein eGFP only in their neutrophils (Lysm-eGFP) (Marques, Oliveira et al. 2015). After different reperfusion times, the mice were anesthetized and the liver exposed on an acrylic support compatible with the microscope. Before image acquisition, the mice were injected i.v. with 100µl of the fluorophore Sytox Orange (1µM, Invitrogen) to stain DNA. Neutrophils were counted and their tracking parameters set using the Volocity program (PerkinElmer), which allows to identify and count neutrophils in the videos, frame by frame. This provides quantitative information about neutrophil location, migration distance, velocity, total displacement, and meandering. This information was plotted as mean ± standard error of the events in the video, and 1 video was made per mouse. Each video took 30 minutes and was recorded at the rate of 1 frame/minute.

3.7 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).

3.8 Effect of Reparixin on IR-Induced Liver Injury

The animals were treated with Reparixin (DF1681B) provided by Dompé Pharmaceutical S.p.A. The drug was diluted in sterile 0.9% NaCl solution at a dose of 15 mg/Kg. The treatment started 15 minutes before the reperfusion via intravenous injection and was continued subcutaneously every 2 h until 12 h after the reperfusion.

3.9 Competition of MIG 30 Peptide for Binding of chemokines to GAGs *in vitro* and Effect of MIG 30 peptide on IR-Induced *in vivo*.

The ability of the COOH-terminal peptides of CXCL9 or monokine induced by interferon-γ (MIG) to compete for GAG binding with the inflammatory chemokine CXCL6 was evaluated on heparin-binding plates [BD Biosciences or kindly provided by Dr. Jason Whittle (School of Engineering/Future Industries Institute, University of South Australia, Australia)] (Robinson, Buttle et al. 2012). In brief, GAGs (heparan sulfate; 25 µg/ml) were coated overnight at room temperature. Dilutions of MIG 30 peptide (a synthetic peptide covering the 30 C-terminal amino acids of CXCL9) combined with recombinant murine CXCL6(9-72) (Peprotech, Rocky Hill, NJ, USA) were added in duplicate and incubated for 2h at 37°C. Subsequently, bound CXCL6 was detected with biotinylated polyclonal goat anti-mouse CXCL6 (Peprotech) and peroxidase-labeled streptavidin. Finally, the peroxidase activity was quantified at 450 nm. Subsequently, mice subjected to liver IRI were injected intravenously with either saline or MIG 30 peptide at 100µg per dose 15 minutes before the reperfusion and 6 hours later.

3.10 The fluorogenic DQ™-gelatin assay

The following protocol was used as previously described (Vandooren, Geurts et al. 2011). To a 96-well plate (chimney, 96-well, Greiner Bio-one, Frickenhausen, Germany), 50 µl of sample (unstimulated or stimulated WT or *Mmp-9*$^{-/-}$ neutrophils) was 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 (MMP-2/9 inhibitor) or 30 µl ElaV (Elastase inhibitor) at a final concentration of 10 µM. Next, 20 µl fluorogenic 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. 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.

3.11 In vitro Neutrophils Degranulation Assay

Isolated neutrophils were resuspended in FBS-free RPMI-1640 medium at a final concentration of 1.0 X 10^6/mL. Neutrophils were activated with different chemoattractants, such as CXCL1, CXCL6, fMLP and C5a (10 nM) an incubated at 37°C in 5% CO_2 for 2 hours MPO activity, Elastase and NGAL were then measured in the cell supernatants according to the standard protocol and expressed as change in absorbance per minute per milliliter of supernatant.

3.12 Statistical Analyses

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 $P < 0.05$.

CHAPTER 1: Intravital Microscopic Evaluation of the Effects of a CXCR2 Antagonist in a Model of Liver Ischemia and Reperfusion Injury in Mice

4. RESULTS

4.1 Ischemia-reperfusion induces liver injury and inflammation

In order to investigate the kinetics of liver inflammation and neutrophil infiltration in liver IR, mice were subjected to 1h of ischemia and different times of reperfusion, upon which parameters of liver injury and inflammation were evaluated. Hepatic ischemia and reperfusion caused significant liver damage, as shown by increased ALT in serum, reaching a peak 12h after reperfusion and returning to almost base-line after 24h (Fig 1A). Furthermore, MPO, one of the main components of neutrophil primary granules, was significantly increased in the liver (Fig 1B). The MPO activity continued to increase, reaching a maximal level 48 h after reperfusion. The liver IR also triggered remote lung inflammation, shown by increased MPO levels in the tissue (Fig 1C). To better define the clinical relevance of our procedures, we evaluated the liver metabolic function through the hepatic clearance of indocyanine green (ICG), which is a test used to evaluate liver function in the clinic. Delay in clearance of ICG is correlated with loss of liver function. As observed, IR led to evident liver dysfunction in a time-dependent way, increasing up to 48h of reperfusion in comparison with sham animals (Fig 1D). Moreover, increased ALT level and MPO activity were associated with significant parenchymal cell damage, as observed by elevated sinusoidal congestion and extensive areas of necrosis, mainly 48 h after reperfusion when compared with the sham group (Fig 1E). Meanwhile, control livers showed normal architecture and perfusion.

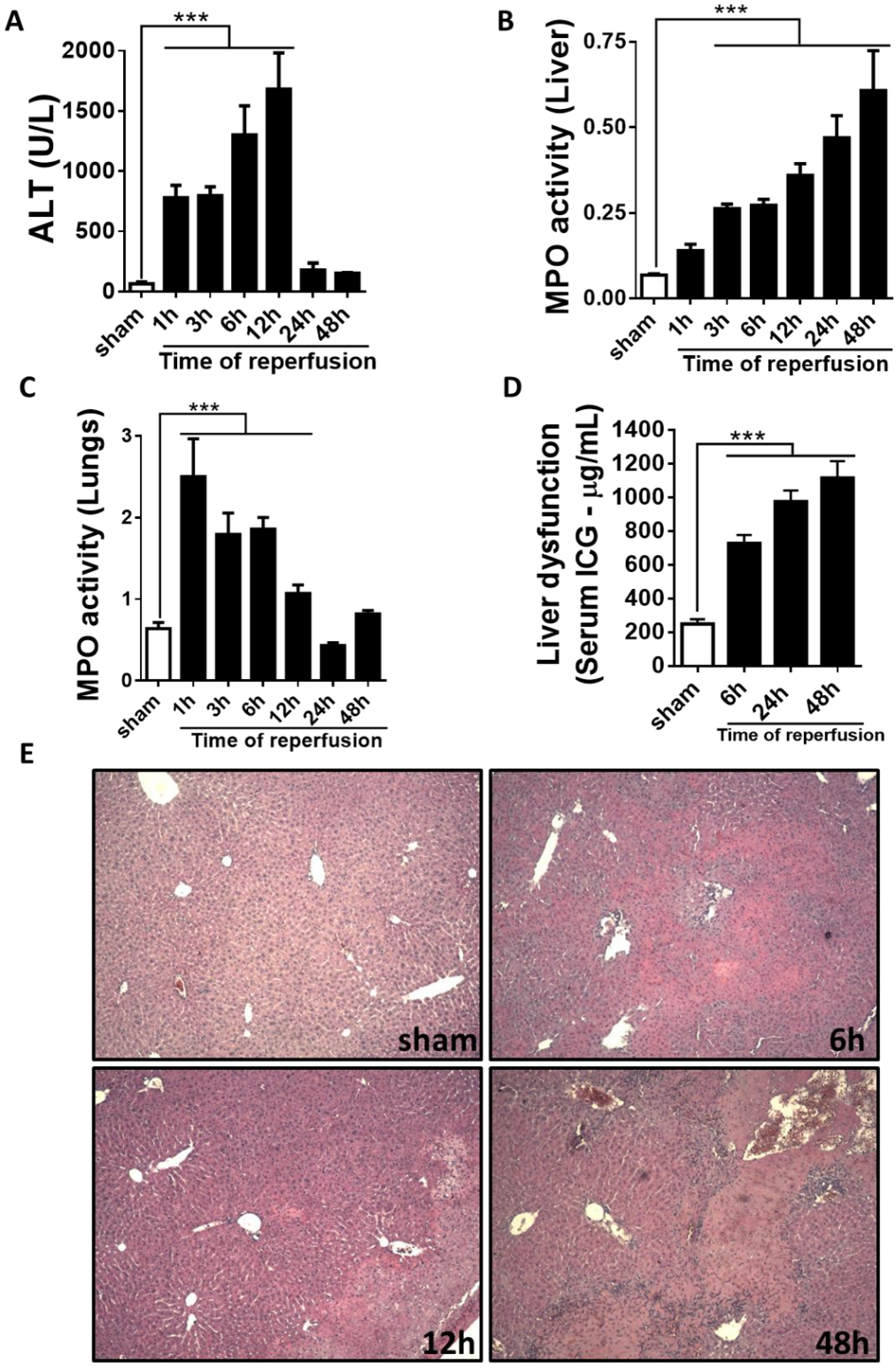

Fig 1. Parameters of liver injury and inflammation were evaluated at different times after reperfusion. The IR induced a significant increase in ALT in serum (A) and MPO activity in the liver and lungs (B, C) and a significant liver dysfunction in a time-dependent way, increasing up to 48 h after the start of the reperfusion (D). The histological sections showed a significant increased lesion in the liver of animals subjected to IR, mainly at 48h of reperfusion when compared to sham-operated animals (D). ***$p < 0.01$ vs with sham-operated animals. Sham animals were euthanized in different times of reperfusion.

4.2 Liver injury caused intense chemokine and cytokine production

Proinflammatory cytokines and chemokines have been implicated in liver IR injury (Jaeschke 2006). Here, we wished to determine which CXC chemokines with neutrophil attractant properties were being produced in the liver, as well as how IR altered the systemic levels of these mediators. We quantified chemokines in serum and liver extracts along different times of reperfusion. IR induced higher levels of CXCL1, CXCL2 and CXCL6 in mice. Tissue levels of CXCL1 and CXCL2 were maximal 12 h after reperfusion (Fig 2A, 2B), while CXCL6 was found elevated since the earliest timepoint and remained higher up to 24h after reperfusion (Fig 2C). In serum, the production of CXCL1, CXCL2 and CXCL6 reached the highest level between 6 h and 12 h of reperfusion, returning to almost baseline levels after 24 h (Fig 2D-F). Moreover, IL-6 and TNF-α, typical proinflammatory cytokines, were significantly up-regulated in serum of mice subjected to IR at 6 h and 12 h after reperfusion, respectively (Fig 2G, 2H).

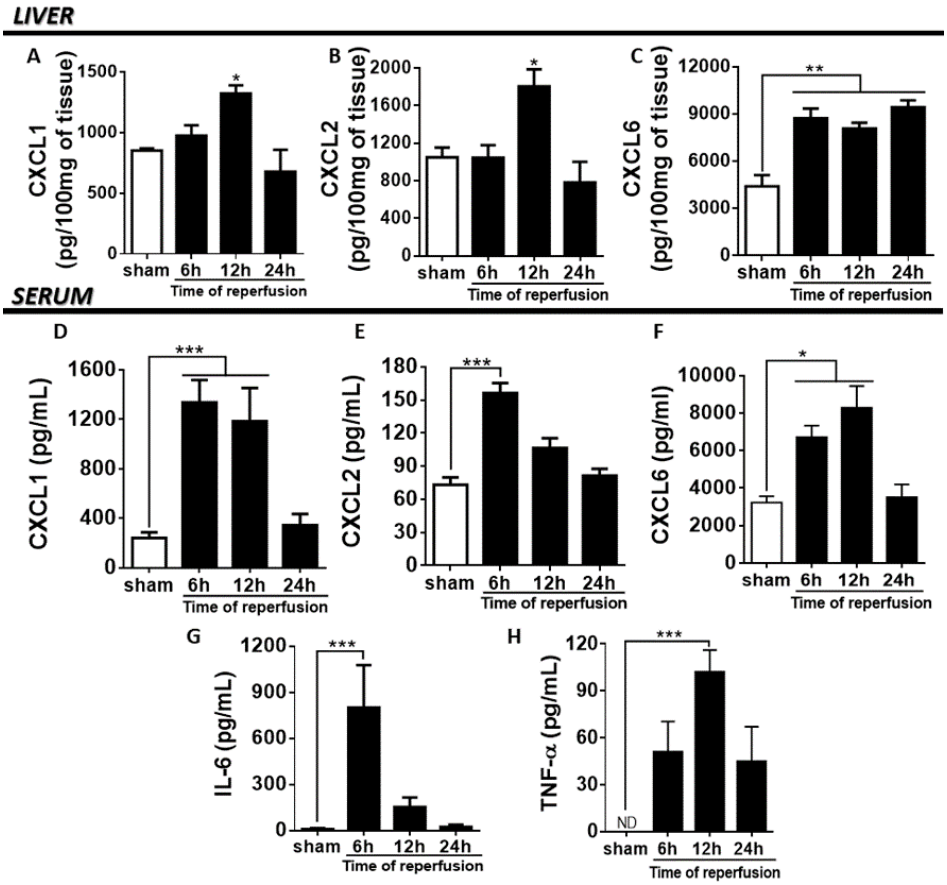

Fig 2. IR induced a significant production of proinflammatory markers (detected by ELISA) during different times of reperfusion. The liver injury was associated with a high production of CXCL1 (A), CXCL2 (B) and CXCL6 (C) in the liver tissue. Similarly, a significant production of CXCL1 (D), CXCL2 (E), CXCL6 (F), IL-6 (G) and TNF-α was detected in serum, mainly between 6h and 12h of reperfusion when compared to sham-operated animals. *$p < 0.05$ and *** < 0.001 vs with sham-operated animals.

4.3 Neutrophil influx during liver injury observed by confocal intravital microscopy

In order to establish a general view of neutrophil recruitment to the injured liver, we used mice expressing GFP in neutrophils (lysme-eGFP). To simultaneously evaluate areas of dead cells we injected mice with the extracellular DNA dye Sytox orange. As shown in figure 3, in the sham group only a few neutrophils are visible. Furthermore, there was no tissue damage as demonstrated by the absence of extracellular DNA labeling (Fig 3A). However, a large neutrophil infiltrate was observed in the IR groups after 6h, 12h and 24h of reperfusion. In addition, neutrophils could be seen forming clusters in the liver mainly 6h after reperfusion. The presence of dead cells (in orange) was observed at all time points, indicating IR induced liver cell death (Fig 3B, C, D).

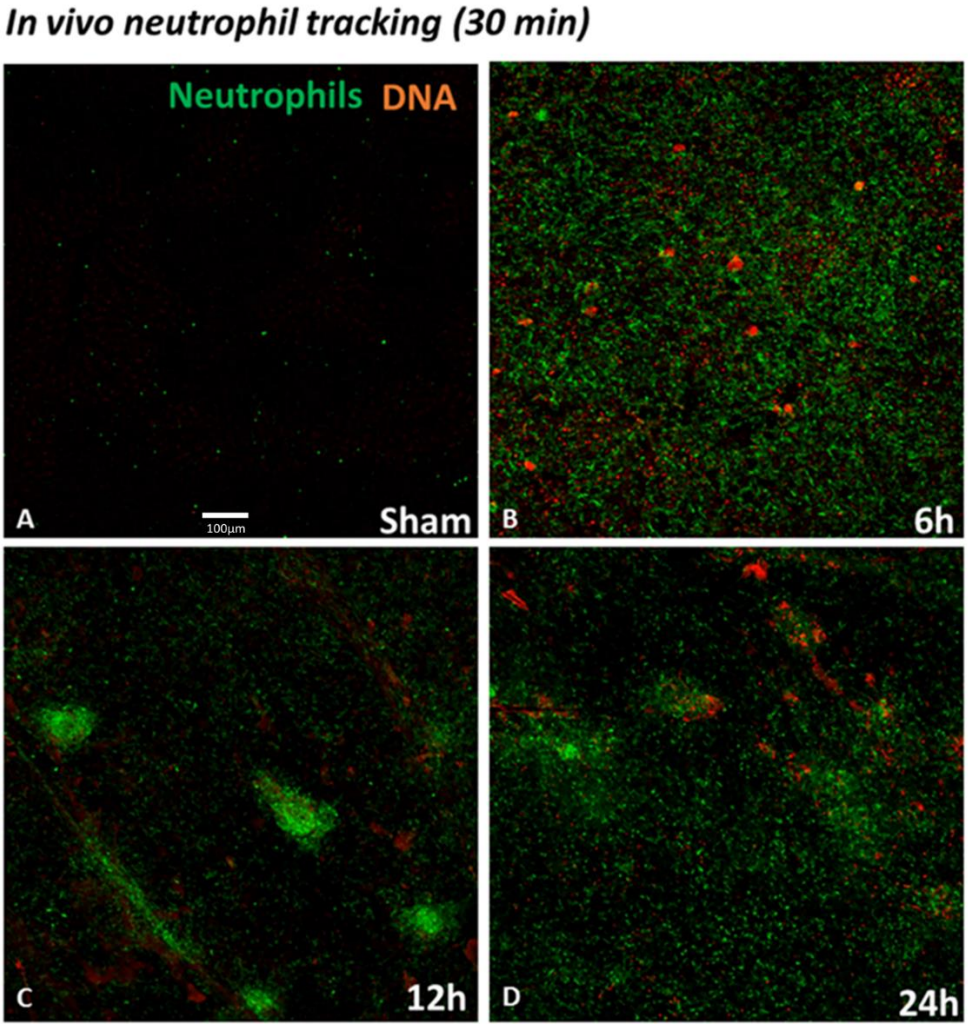

Fig 3. Neutrophil tracking during liver injury development observed by confocal intravital microscopy. Sytox orange was injected i.v to observe the presence of DNA as an indicator of cell death. Low-magnification intravital microscopy showed that IR induced a huge infiltration of neutrophils in the liver between 6h and 24h of reperfusion (Lysm-eGFP-expressing cells). In addition, IR caused cell death, observed by hepatic DNA accumulation (orange), in comparison with sham-operated mice. Scale bars: 100 µm.

4.4 Neutrophil activation and polarization in inflamed tissue

We next studied neutrophil movement in the liver parenchyma to evaluate how the neutrophils behaved during liver injury development. As previously noted, there was neutrophil accumulation in the liver parenchyma of IR mice when compared to sham controls (Panel 1). There was no difference in the number of neutrophils per field between 6h, 12h and 24h of reperfusion (Fig 4A). However, hepatic neutrophils traveled longer distances and at a higher crawling velocity in the livers 6h after reperfusion (Fig 4B, C). Moreover, IR promoted neutrophil activation as observed by their larger size, neutrophil cluster formation, and polarization, which yielded more elongated neutrophils. This activation phenotype, occurring mainly at 6h of reperfusion, preceded the peak of liver inflammation and necrosis (Fig 4D-F). Interestingly, all neutrophil movement parameters were decreased after 12h of reperfusion, indicating that the process of neutrophil activation and recruitment is a very early phenomenon and started to resolve at later time points.

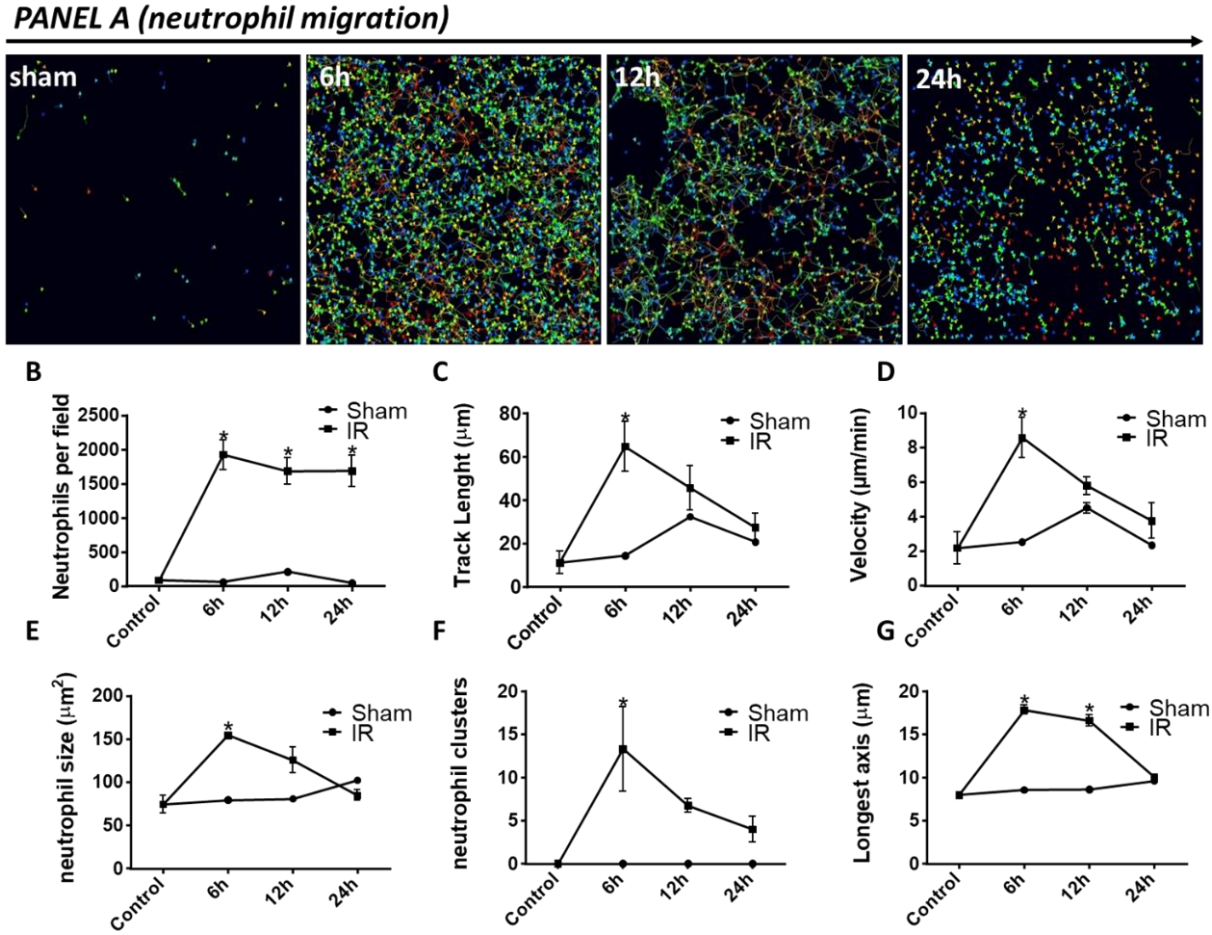

Fig 4. Neutrophils behavior during injury development observed by confocal intravital microscopy. There was neutrophil accumulation in the liver parenchyma of IR mice when compared to sham controls (Panel 1). scale bars: 100µm. Digital cell tracking showed that IR induced a huge infiltration of neutrophils into the liver (A). Furthermore, mainly after 6h of reperfusion, the total distance traveled by neutrophils was significantly higher (B) and these cells moved faster (C). Indeed IR promoted neutrophil activation as observed by their larger size (D), number of clusters of neutrophils (E), and finally, alteration in shape resulting in more elongated neutrophils (F), when compared to sham-operated animals (tracking of neutrophils during 30 minutes). *$p < 0.05$ vs sham-operated animals.

4.5 Reparixin significantly reduces liver IR injury

The main chemokine receptors on neutrophils regulating their recruitment to sites of injury are CXCR1 and CXCR2. To evaluate the role of CXCR1 and CXCR2 in liver IR injury, mice were treated with an allosteric antagonist for these receptors, Reparixin (DF1681B; 15mg/Kg/iv), 15 minutes before the reperfusion and subsequently every two hours. As noted, pharmacological inhibition of CXCR1/2 by Reparixin significantly reduced liver damage, which can be observed by reduced ALT in serum after 6h of reperfusion and an even further reduction after 12h (Fig 5A). Moreover, Reparixin reduced neutrophil recruitment in the liver and lungs by approximately 50% as shown by MPO activity (Fig 5B, 5C). Also, decreased liver ALT and MPO in Reparixin-treated mice were associated with better histological preservation, contrasting with saline-treated mice, which showed elevated sinusoidal congestion and extensive areas of necrosis followed by infiltration of inflammatory cells (Fig 5D).

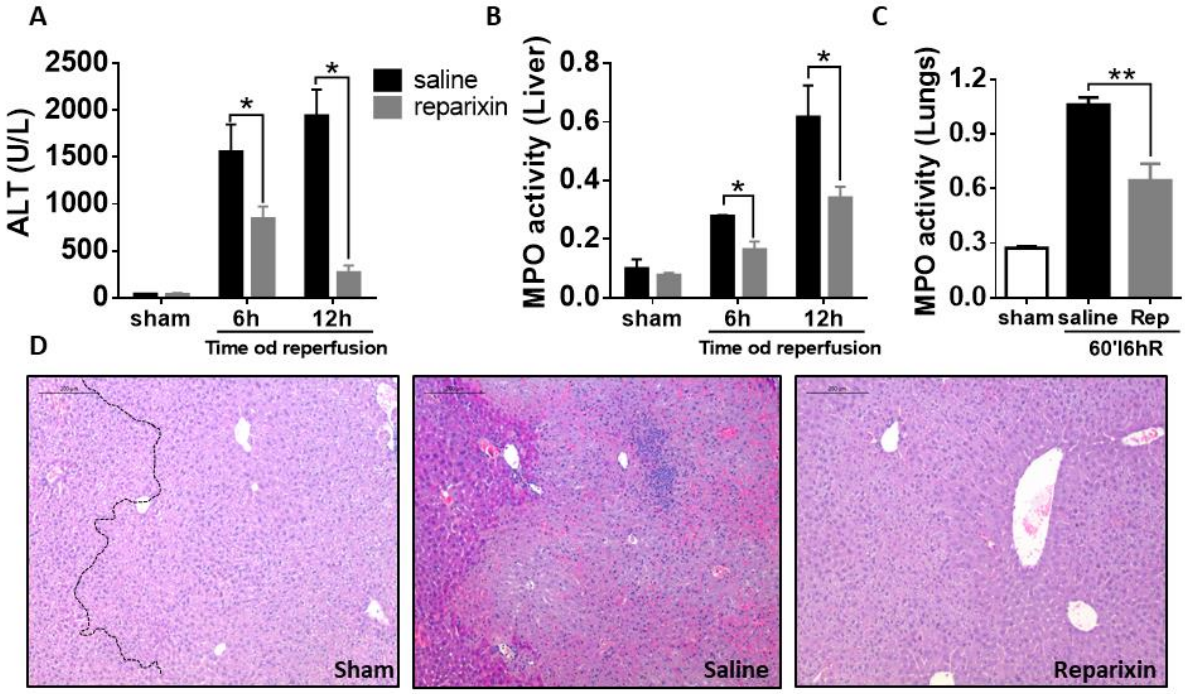

Fig 5. Effects of the treatment with Reparixin on levels of ALT and MPO in the liver and lungs of mice subjected to IR. Mice received a dose (i.v) of Reparixin (15 mg/kg) 15 minutes before the reperfusion and every two hours later (s.c). Reparixin reduced ALT release (A) and MPO activity in the liver (B) and lungs (C) when compared with animals subjected to IR and treated with saline. *p< 0.05 and ** p<0.01 vs saline-treated livers. (D) Representative H&E staining of livers 12 hours post-IR injury. Reparixin-treated animals showed significant histological preservation, contrasting with saline-treated animals, which showed elevated signs of necrosis and intense infiltration of leukocytes. Scale bars: 200μm.

4.6 Blocking of CXCR2 impaired neutrophil accumulation during liver IR injury

Ly6G is expressed primarily in neutrophils and correlates with the cellular level of differentiation and maturation (Ellis and Beaman 2002). We next aimed to determine whether CXCR1/2 antagonism could directly affect neutrophil numbers in the injured liver IR. Liver sections were negative for Ly6G in sham mice (Fig 6A). Ly6G positive cells were predominantly detected 12h after reperfusion in saline-treated mice (Fig 6B). However, livers of mice treated with Reparixin showed significantly less Ly6G staining 12 h after reperfusion (Fig 6C).

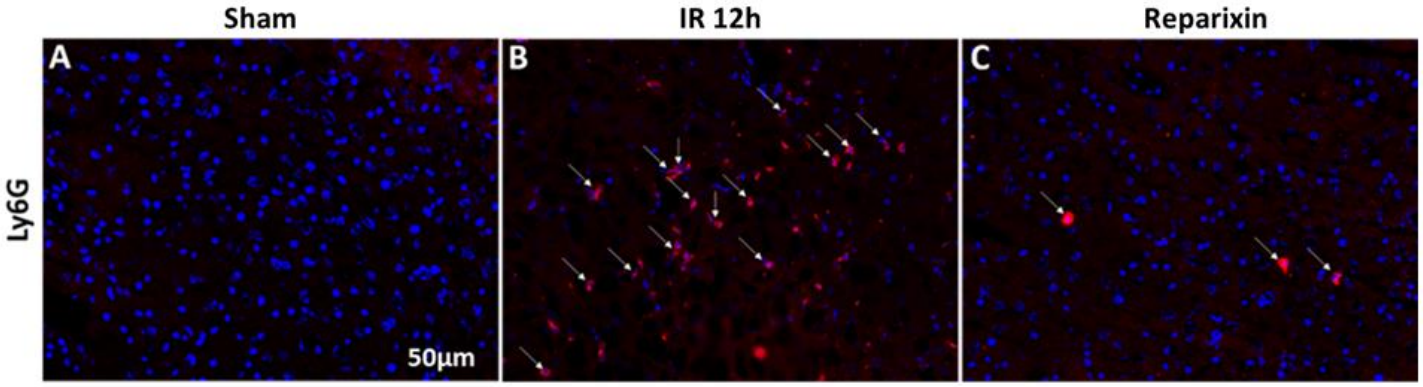

Fig 6. Effects of the Reparixin treatment on Neutrophil infiltration in Liver IR Injury. Representative photomicrographs of Ly6G staining (as neutrophil marker) in sham (A), wild type mice subjected to IR (B) or IR injured mice treated with Reparixin (C). Accumulation of neutrophils was increased in the IR liver compared with sham livers. Mice receiving Reparixin showed a significant reduction in the number of infiltrated neutrophils. Ly6G is shown in red and nuclei in blue (×200). Arrows point to neutrophils.

4.7 The production of inflammatory mediators was significantly inhibited in Reparixin-treated mice

As previously shown, important inflammatory mediators as TNF-α and IL-6 are responsible for driving the acute inflammatory response triggered by IR in the liver (Colletti, Remick et al. 1990, Hong, Radaeva et al. 2004). To determine if the inhibition of CXCR1/2 could alter the production of these cytokines, we quantified them in serum of Reparixin-treated mice. The serum levels of TNF-α were significantly increased after 12h of IR, while IL-6 and CCL3 were significantly elevated after 6h of IR. However, treatment with Reparixin significantly inhibited their production (Fig 7A, 7B, 7E). The neutrophil attractants CXCL1 and CXCL6 were also significantly increased in the serum of mice subjected to IR. However, Reparixin failed to reduce the levels of these chemokines (Fig 7B, 7C). Overall, treatment with Reparixin significantly reduced local and systemic inflammation induced by IR.

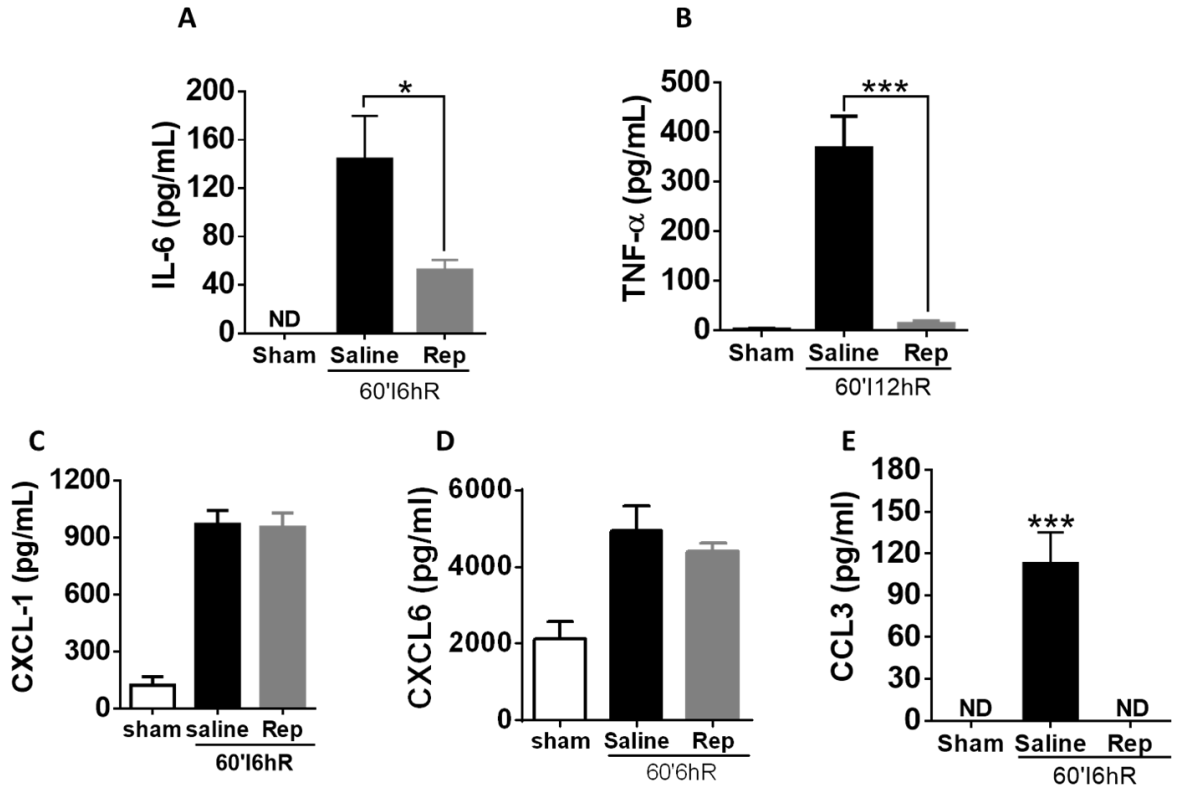

Fig 7. Effects of the treatment with Reparixin on the concentrations of cytokines and chemokines in the serum of mice subjected to IR. Mice received a dose of Reparixin (15 mg/kg) 15 minutes before the reperfusion (i.v) and every two hours later (s.c). Reparixin reduced the production of the cytokines TNF-α (A) and IL-6 (B) when compared with animals subjected to IR and treated with saline. No differences in the concentration of CXCL1 (C) and CXCL6 (D) between mice treated with saline and Reparixin was detected. However, mice treated with Reparixin showed lower production of the chemokine CCL3 (E) *p< 0.05 and *** < 0.001 vs relative to saline-treated livers.

4.8 Reparixin interferes with neutrophil activation and polarization

We used intravital microscopy to perform a detailed investigation of parameters of leukocyte recruitment under inflammatory conditions. As Reparixin treatment was able to reduce neutrophil migration and consequently liver injury, we wondered whether the lower liver injury would be associated with altered behavior of neutrophils during the course of disease. *In vivo* imaging revealed that Reparixin was able to decrease neutrophil infiltration 6h after reperfusion (Fig 8A). Moreover, neutrophils in Reparixin-treated mice moved over shorter distances (Fig 8B) and showed reduced displacement (Fig 8C). However, there was no difference in the velocity of these cells (Fig 8D). Interestingly, neutrophils had smaller size (Fig 8E) and significantly less elongated shape (Fig 8F) when mice were treated with Reparixin, indicating that these cells were significantly less activated than in untreated mice. Overall, Reparixin impaired neutrophil activation and reduced their ability to accumulate and migrate, subsequently leading to reduced liver IR injury.

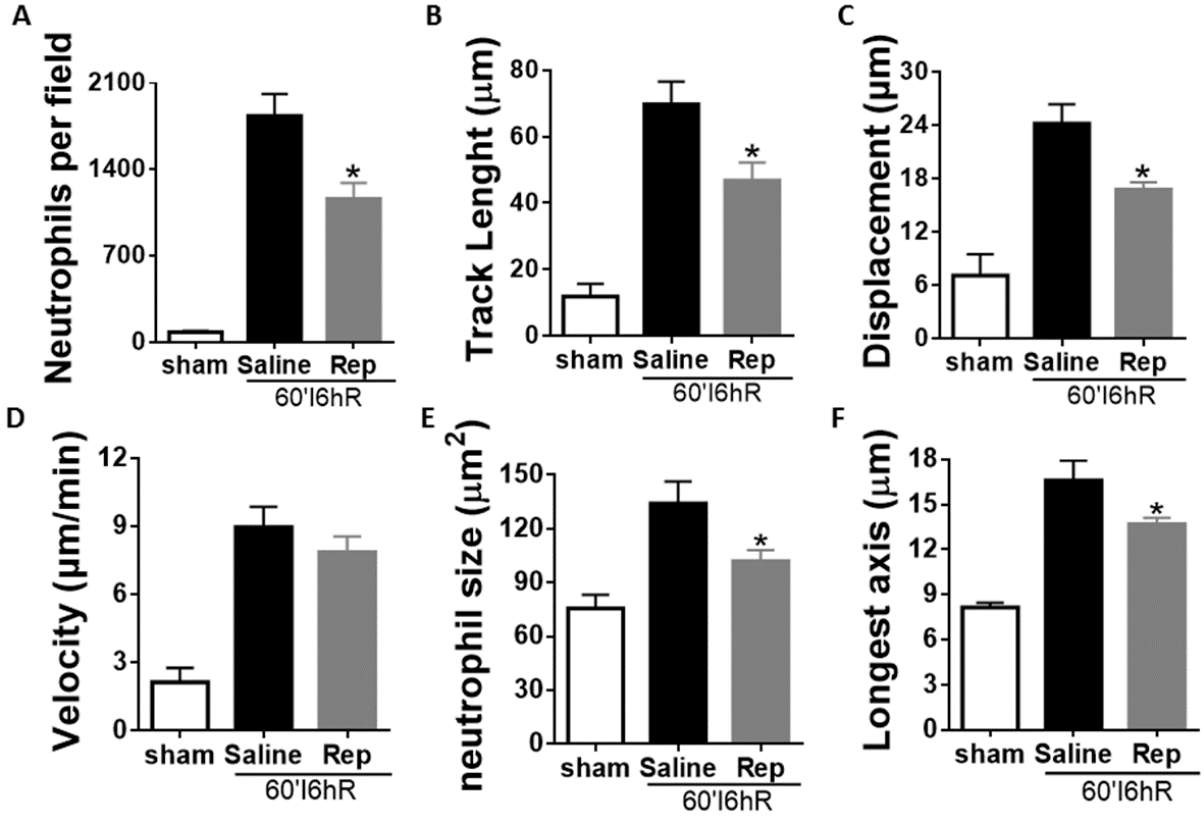

Fig 8. Effects of the treatment with Reparixin on neutrophil tracking in mice subjected to IR. Reparixin reduced neutrophil infiltration into the liver (A) and changed the behavior of these cells. The path length (track length) (B) and final displacement of neutrophils were decreased compared with animals subjected to IR and treated with saline (C). There was no difference in the velocity (D) but Reparixin reduced the size (E) and the longest diameter (F) of the neutrophils. *p < 0,05 vs relative to saline-treated livers.

CHAPTER 2: Matrix Metalloproteinase-9 Gene Deletion Attenuates Liver Ischemia and Reperfusion Injury in Mice

4.9 *Mmp-9* deficiency impaired neutrophil accumulation in the liver IR injury

To determine whether MMP-9 plays a role in liver IRI, expression of this gene was evaluated in the liver by real time RT-PCR after different times of reperfusion. MMP-9 messenger RNA (mRNA) was almost undetectable in control livers, however, it was significantly up-regulated in WT livers from 6 hours to 48h hours post reperfusion (Fig 9A). To examine whether a lack of MMP-9 activity would confer protection against hepatic IRI, we used *Mmp*-9-deficient mice in our model. *Mmp-9*$^{-/-}$ mice showed significantly less liver damage, as shown by the reduced aminotransferase levels 6 and 12 hours after reperfusion (Fig 9B). MPO, one of the most abundant proteins in neutrophils, has emerged as an enzyme critically involved in the pathogenesis of inflammatory diseases. In our liver IRI model, MPO activity 6 hours and 12 hours after reperfusion was significantly reduced in *Mmp-9*$^{-/-}$ mice (Fig 9C). Moreover, 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 12 hours after reperfusion. In contrast, *Mmp-9*$^{-/-}$ mice showed preserved architecture after liver IRI (Fig 9D). Immunofluorescence analysis showed that Ly6G (as neutrophil marker), absent in sham livers, was predominantly detected in WT livers after 12 hours of reperfusion. Ly6G was almost undetectable in *Mmp-9*$^{-/-}$ mice (Fig 9E).

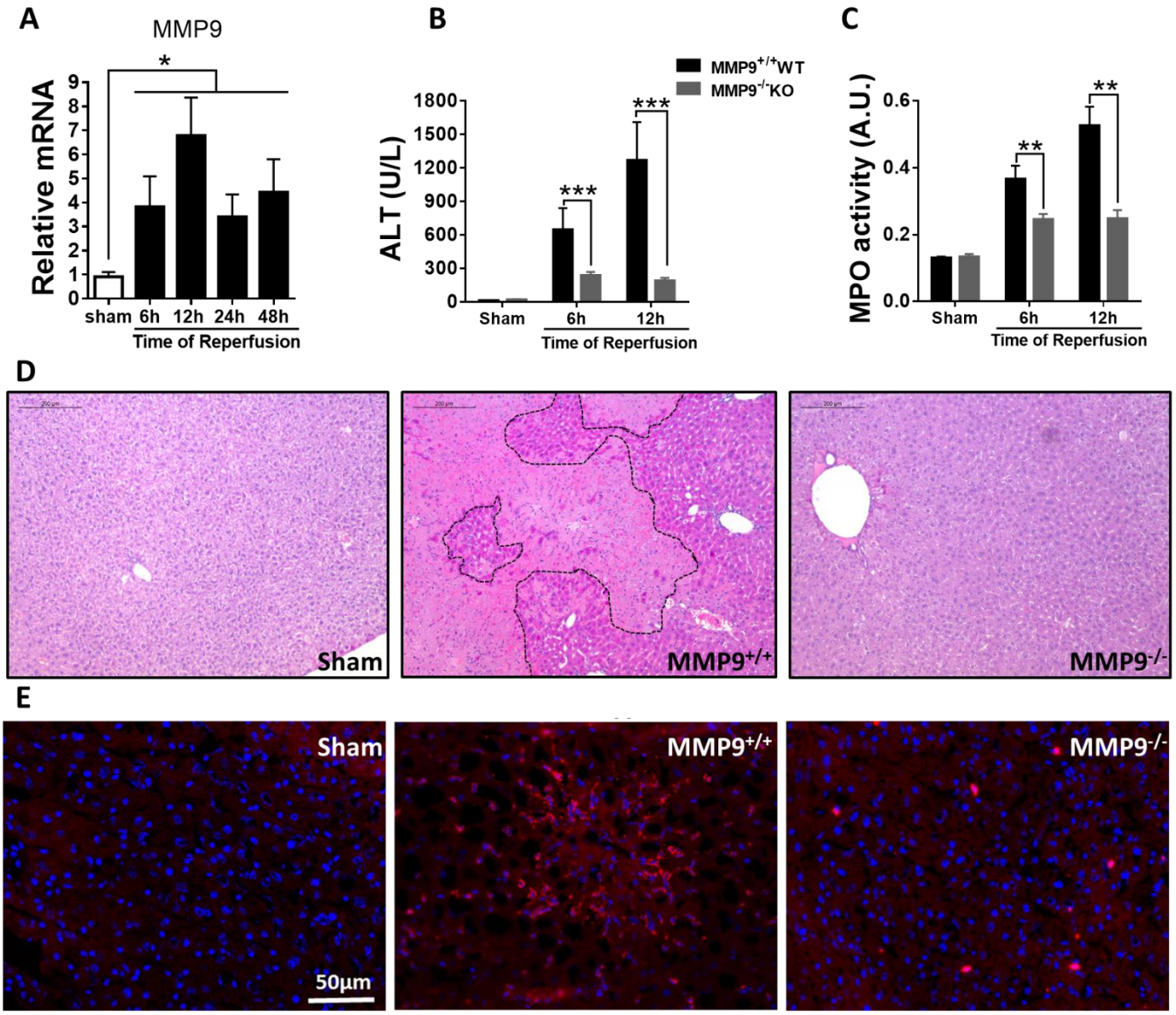

Fig 9. Effects of the lack of *Mmp-9* on levels of ALT, MPO and histological preservation in the liver of mice subjected to IR. MMP-9 mRNA expression was almost undetectable in control livers. The IR significantly up-regulated the MMP-9 expression in WT livers from 6 hours to 48h hours post reperfusion. *p < 0.05 vs sham-operated animals (A). ALT levels and MPO activity were, respectively, measured in blood samples and tissue taken 6 or 12 hours after IR injury. ALT levels and MPO activity in *Mmp-9*$^{-/-}$ mice were significantly lower than those in their respective WT controls after 6 or 12 hours of reperfusion (B, C). ***p < 0.01 and **p < 0.05 vs 6h and 12h of reperfusion. Control WT livers were characterized by elevated sinusoidal congestion and extensive necrosis after 12 hours. In contrast, *Mmp-9*$^{-/-}$ livers showed significant histological preservation (D) (n=6). Scale bars: 200µm. Representative photomicrographs of Ly6G staining (neutrophil marker) in sham, WT mice subjected to IR or *Mmp-9*$^{-/-}$ mice subjected to IR. Accumulation of neutrophils was 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) (E).

4.10 Proinflammatory cytokine production was inhibited in *Mmp-9*-deficient mice after IRI

Among the cytokines capable of regulating MMP-9 expression, an important role is assigned to TNF-α and IL-6, and their expression is associated with neutrophil infiltration and liver damage (Pryhuber, Huyck et al. 2005, Lee, Lan Tran et al. 2009). As observed, the extent of leukocyte infiltration shown above correlated with the expression of the proinflammatory cytokines TNF-α and IL-6 in livers of WT mice, which were significantly decreased in the *Mmp-9*$^{-/-}$ mice after reperfusion (Fig 10A, B). Moreover, serum levels of TNF-α and IL-6 were markedly reduced in *Mmp-9*$^{-/-}$ mice compared to WT after 6 hours and 12 hours of reperfusion (Fig 10C, D).

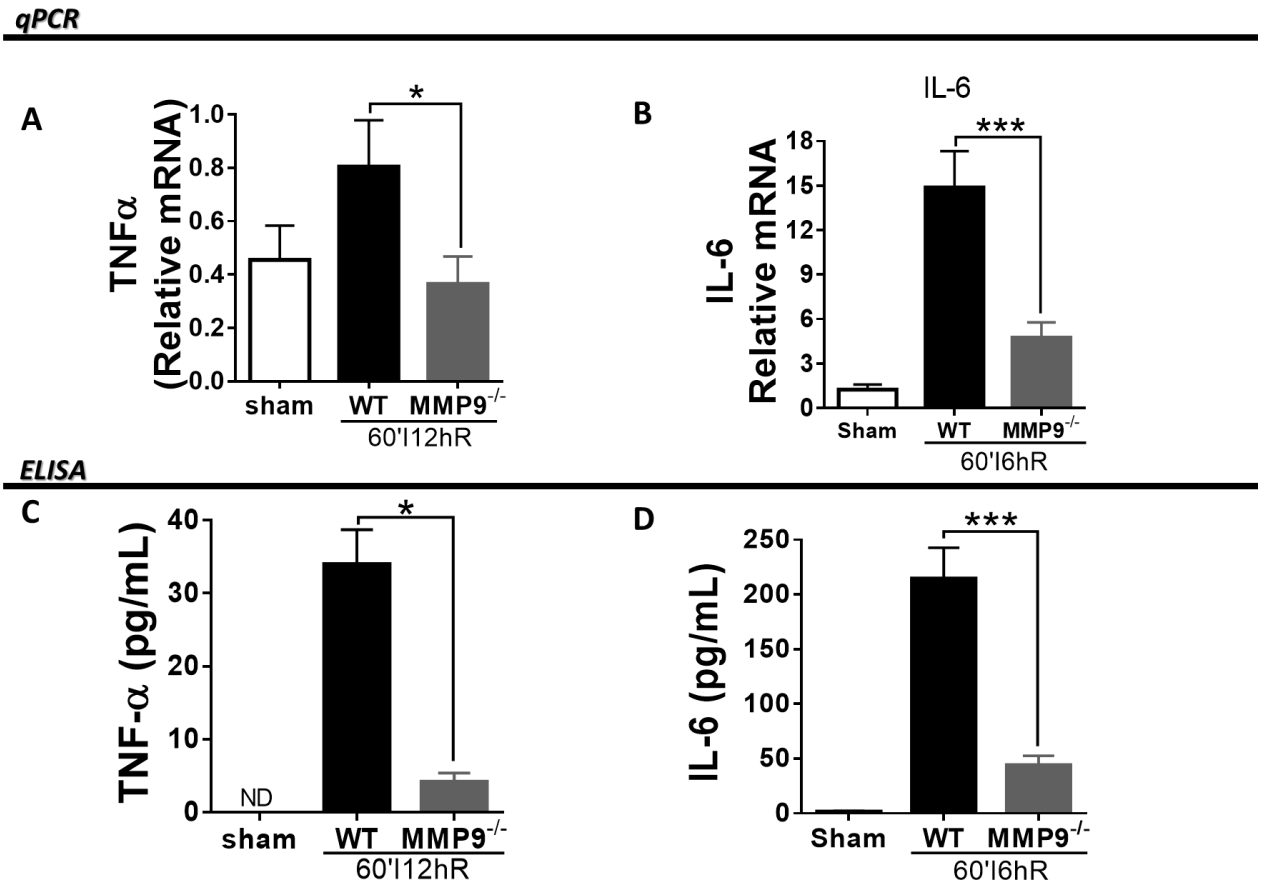

Fig 10. Effects *Mmp-9* absence on the concentrations of cytokines in the serum and liver tissue of mice subjected to IR. *Mmp-9*$^{-/-}$ mice showed reduced production of the cytokines TNF-α (A) and IL-6 (B) in the tissue (qPCR) when compared with WT animals subjected to IR. Similarly, *Mmp-9*$^{-/-}$ mice showed reduced production of TNF-α (C) and IL-6 (D) in the serum (ELISA) when compared to WT animals subjected to IR. * $p<0.05$ and *** $p<0.01$ vs WT livers.

4.11 *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. 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 assessed the levels of major cell activating chemokines linked to liver IRI. As observed, CXCL1, CXCL2 and CXCL6 were comparably expressed in both WT and *Mmp-9*$^{-/-}$ serum of mice 6 hours after reperfusion.

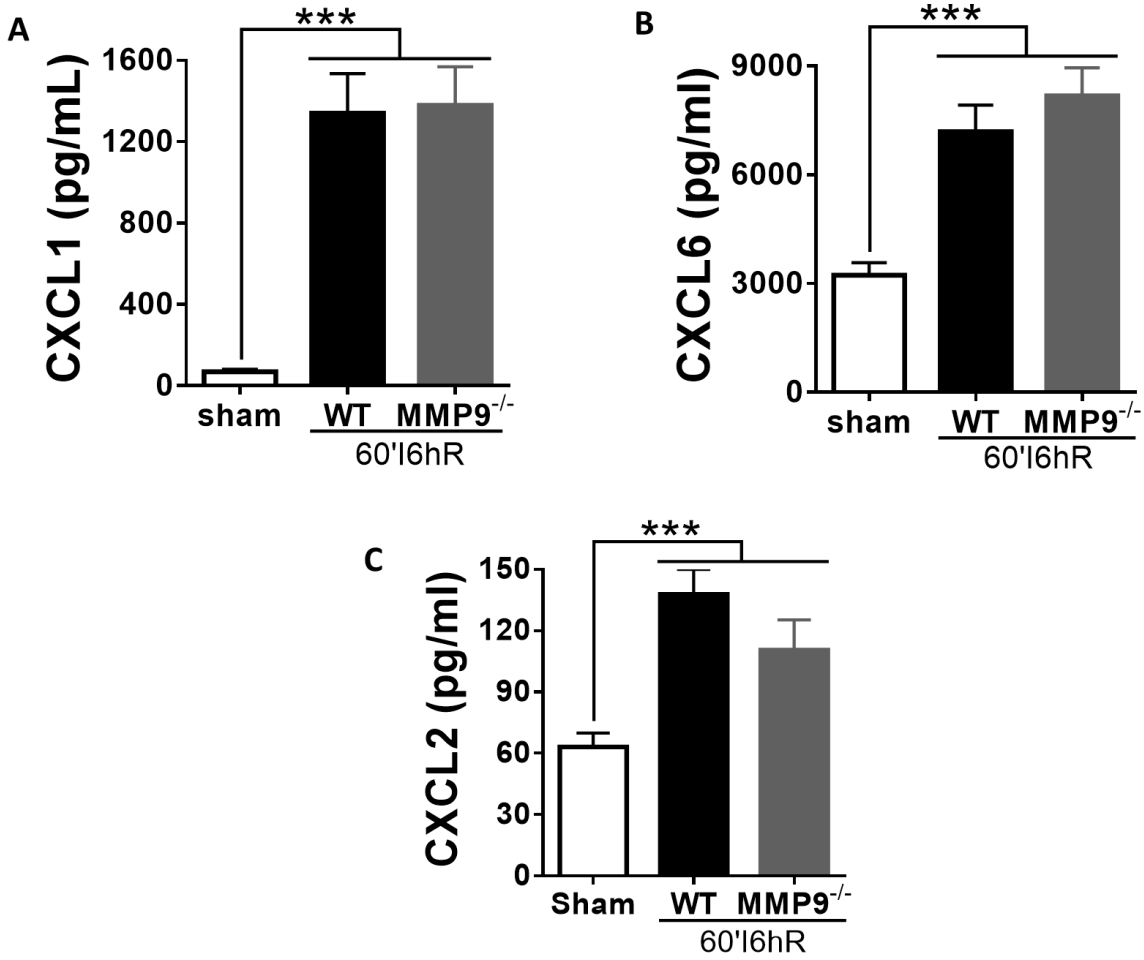

Fig 11. Effects of the lack of *Mmp-9* on the concentrations of chemokines in the serum of mice subjected to IR. *Mmp-9*$^{-/-}$ and WT mice showed increased production of the chemokines CXCL1 (A), CXCL2 (B) and CXCL6 (C) 6h after reperfusion when compared with Sham animals. However, no differences were observed in the production of chemokines between *Mmp-9*$^{-/-}$ and WT animals subjected to IR. * $p<0.05$ and vs sham mice.

4.12 Timp-1 expression was up-regulated in WT livers after IRI

TIMP-1 is the major endogenous regulator of MMP-9. To determine the expression of TIMP-1 during the course of liver injury, real-time PCR was performed on liver samples. TIMP-1 was almost undetectable in sham mice. Compared with WT, hepatic TIMP-1 mRNA expression was markedly down-regulated in *Mmp-9*$^{-/-}$ mice 6 hours and 12 hours after liver IRI (Fig 12).

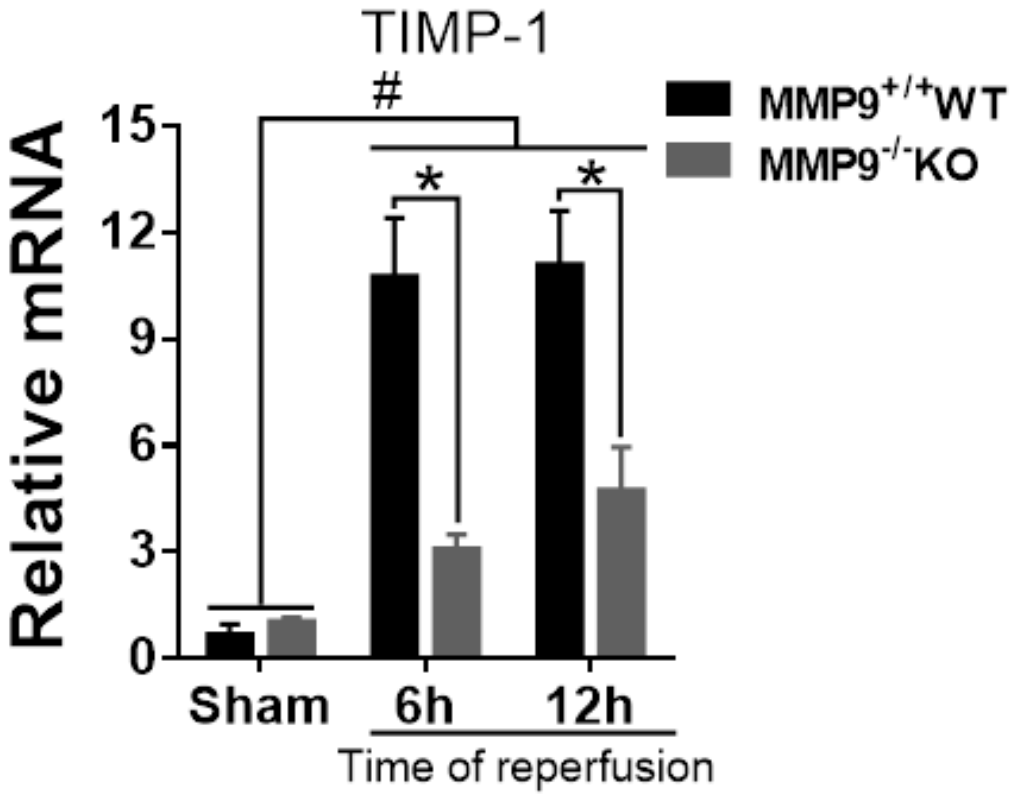

Fig 12. TIMP-1 expression in *Mmp-9* $^{-/-}$ and *Mmp-9* $^{+/+}$ mice subjected to IR. TIMP-1 expression, as detected by qPCR analysis was significantly up-regulated in WT and *Mmp-9*$^{-/-}$ mice 6 hours and 12 hours after IR injury as compared to the respective sham mice. # $p<0.05$ and vs sham mice. Expression of TIMP-1 was significantly higher in *Mmp-9*$^{+/+}$ mice 6 hours and 12 hours after IR injury when compared with *Mmp-9*$^{-/-}$ mice. * $p<0.05$ vs *Mmp-9*$^{-/-}$ mice.

4.13 Lack of *Mmp-9* impaired neutrophil degranulation *in vitro*

To verify whether MMP-9 was able to regulate the production or release of other enzymes by neutrophils through degranulation, 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 (Fig 13A). We also evaluated the presence of neutrophil elastase. Compared with stimulated WT neutrophils, *Mmp-9*$^{-/-}$ neutrophils showed reduced release of elastase (Fig 13B). Moreover, neutrophil gelatinase B-associated lipocalin (NGAL) was shown upregulated in WT neutrophils stimulated with CXCL6 and fMLP when compared with stimulated *Mmp-9*$^{-/-}$ neutrophils (Fig 13C). Altogether, our results indicate that MMP-9 may control neutrophil degranulation.

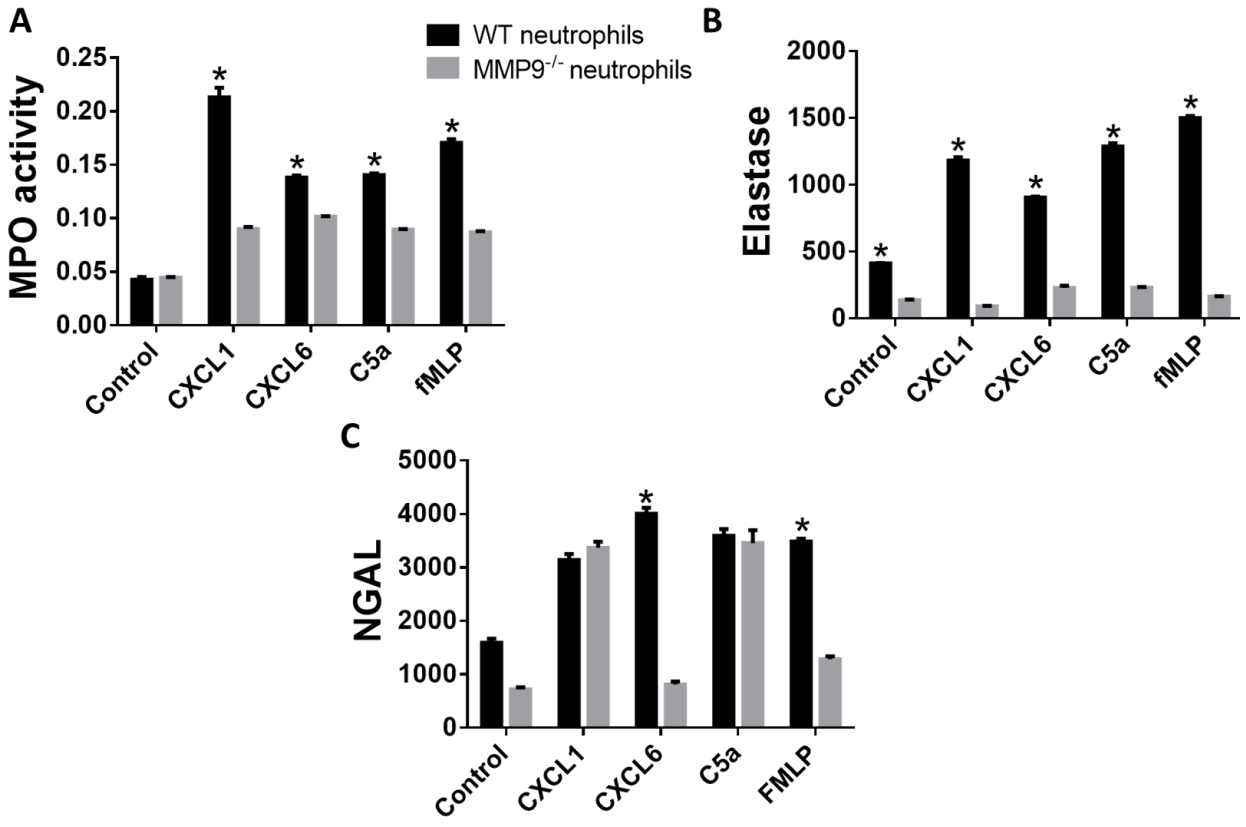

Fig 13. Inhibition of neutrophil degranulation in *Mmp-9*$^{-/-}$ neutrophils *in vitro*. (A) WT stimulated neutrophils showed high MPO activity, which was reduced in MMP-9$^{-/-}$ neutrophils. *p<0.05 vs stimulated MMP-9$^{-/-}$ neutrophils. (B) Elastase was measured by ELISA. Elastase in stimulated WT neutrophils was very high, compared with stimulated MMP-9$^{-/-}$ neutrophils. *p<0.05 vs stimulated MMP-9$^{-/-}$ neutrophils. (C) NGAL was high in neutrophils stimulated with CXCL6 and fMLP compared with MMP-9$^{-/-}$ neutrophils. *p<0.05 vs stimulated MMP-9$^{-/-}$ neutrophils. This is only one experiment of different measurements of the same sample in the assays.

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 (Sela-Passwell, Rosenblum et al. 2010). Here, we used DQ™-gelatin, which mimics the natural substrate, to measure the gelatinolytic activity with high sensitivity. Our data on *in vitro* gelatinolysis demonstrated that when compared with WT neutrophils, stimulated *Mmp-9*$^{-/-}$ neutrophils secreted lower amounts of gelatinolytic activity. In WT neutrophils, combined inhibition of MMP-2 and MMP-9 or inhibition of elastase decreased the gelatinolytic activity. The impaired gelatinolytic activity was most significant with elastase inhibitor indicating that elastase represents the major enzyme in this assay (Fig 14).

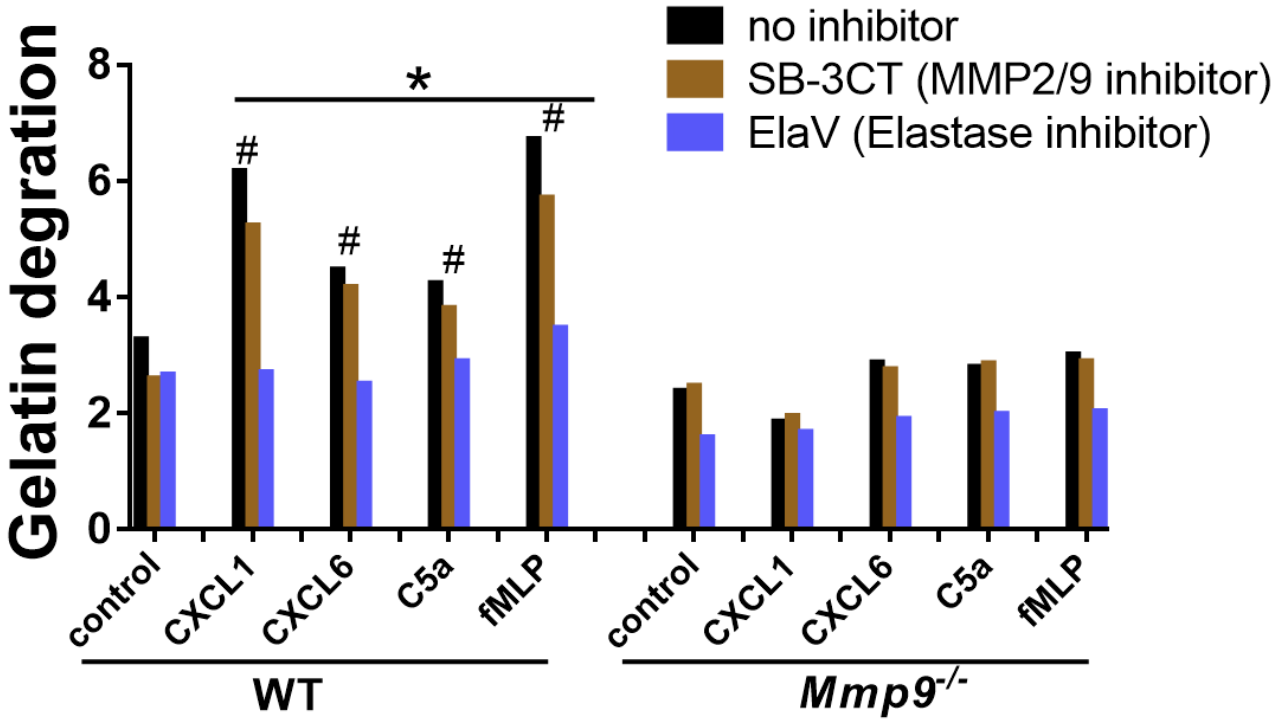

Fig 14. Inhibition of elastase and MMP-9 activity in WT versus MMP-9$^{-/-}$ neutrophils *in vitro*. MMP-9 activity is decreased in MMP-9$^{-/-}$ neutrophils when compared with WT neutrophils. *p<0.05 vs stimulated MMP-9$^{-/}$ neutrophils Also, in WT neutrophils, inhibition of MMP-9 or elastase decreased the gelatinolytic activity of these enzymes. The reduction in gelatinolytic activity was more significant upon treatment of the neutrophils with the elastase inhibitor. #p<0.05 vs SB-3CT and ElaV.

CHAPTER 3: Disruption of Glycosaminoglycan-Chemokine Interactions Protects Against Liver Ischemia Reperfusion Injury in Mice

4.14 MIG 30 peptide competes with CXCL6 for binding to gags (heparan sulfate)

In addition to receptor binding, chemokine activity largely depends on interactions with GAGs, such as heparin, heparan sulfate (HS), and dermatan sulfate (DS) (Johnson, Proudfoot et al. 2005). Previous reports have shown that the main human neutrophil attractant, CXCL8, has been demonstrated to be a relatively strong heparin-binding molecule (Kuschert, Hoogewerf et al. 1998). Here, we tested the ability of the MIG 30 peptide (corresponding to amino acids 74 to 103 of the chemokine CXCL9) to inhibit binding of CXCL6 to HS using GAG binding plates in an ELISA-like assay. More than half of the 30 amino acids of this MIG 30 peptide are positively charged (mainly Lys). As expected, given its highly positively charged nature, MIG 30 peptide competed with CXCL6 for binding to negatively charged HS very efficiently. Interestingly, even using the lowest dose of MIG 30 peptide, CXCL6 binding to HS was reduced more than 60%. If the molar concentration of MIG 30 exceeded the concentration of CXCL8 by a factor more than 10, CXCL6 binding to HS was almost completely abolished (Fig 15).

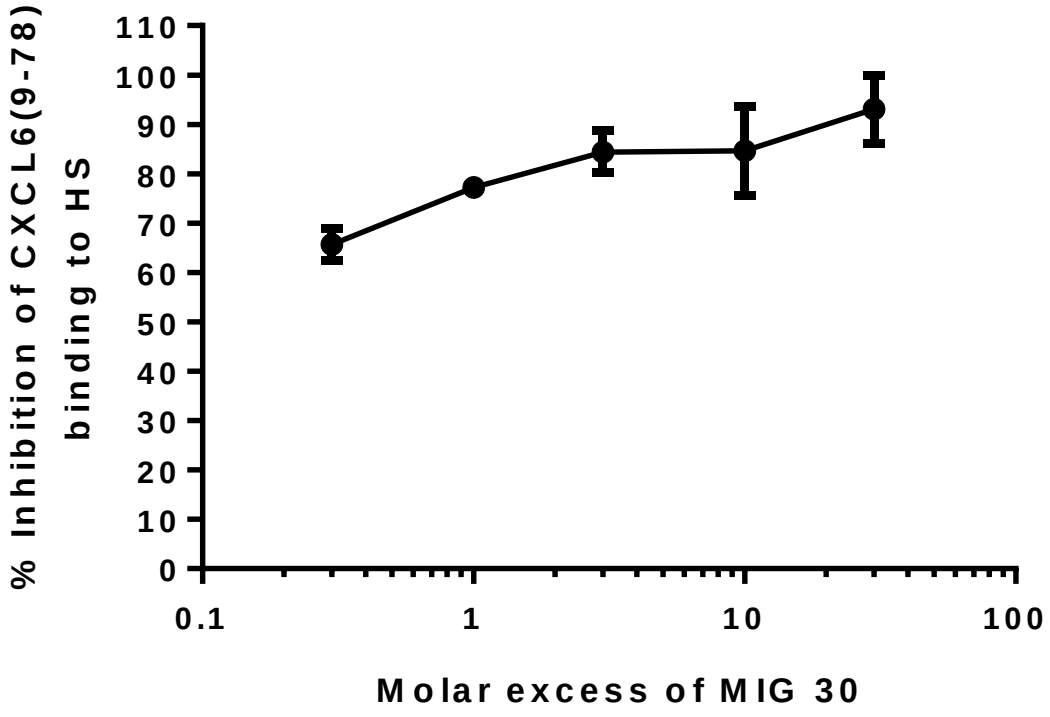

Fig 15. MIG 30 peptides compete with CXCL6 binding to GAGs. Competition between CXCL6 and MIG 30 peptides for binding to heparan sulfate was evaluated on GAG-binding plates. CXCL6 (300, 100, 30, and 10 nM) was added to heparan sulfate (immobilized in 96-well plates) in the presence/absence of the MIG 30 [CXCL9(74–103)]. Bound CXCL6 was detected with biotinylated anti-human CXCL6 antibodies. The mean + S.E. (error bars) (n >4) percentage inhibition of binding of CXCL6 to heparan sulfate is indicated on the y axis.

4.15 MIG 30 peptide protects mice from liver injury

GAGs are large molecules involved in chemokine retention in the vasculature. Chemokines form a gradient by binding to GAGs on endothelial cells. This gradient drives leukocyte activation and migration (Kufareva, Salanga et al. 2015). Based on its *in vitro* competition for GAG binding (Fig. 15), MIG 30 might possess promising anti-inflammatory properties. By competing with chemokines for GAG binding, this peptide decreased CXCL8's capacity to recruit neutrophils to a site of inflammation (Vanheule, Boff et al. 2017). Therefore, the potential anti-inflammatory capacity of MIG 30 was evaluated also in our model of liver IRI. Mice were treated with MIG 30 (100µg per dose) intravenously 15 minutes before the reperfusion and 6 hours later. MIG 30 peptide treatment significantly reduced liver damage, as observed by reduced ALT in serum (Fig 16A). ALT levels in treated mice were almost as low as in Sham control mice. Moreover, MIG 30 significantly reduced neutrophil recruitment in the liver, as evidenced by the evaluation of MPO activity, and prevented liver dysfunction, as observed by the comparable clearance of serum ICG as in sham operated mice (Fig 16B, 16C). The decreased liver ALT and MPO concentrations in MIG 30-treated mice were associated with better histological preservation, contrasting with saline-treated mice, which showed elevated sinusoidal congestion and extensive areas of necrosis followed by infiltration of inflammatory cells (Fig 16D).

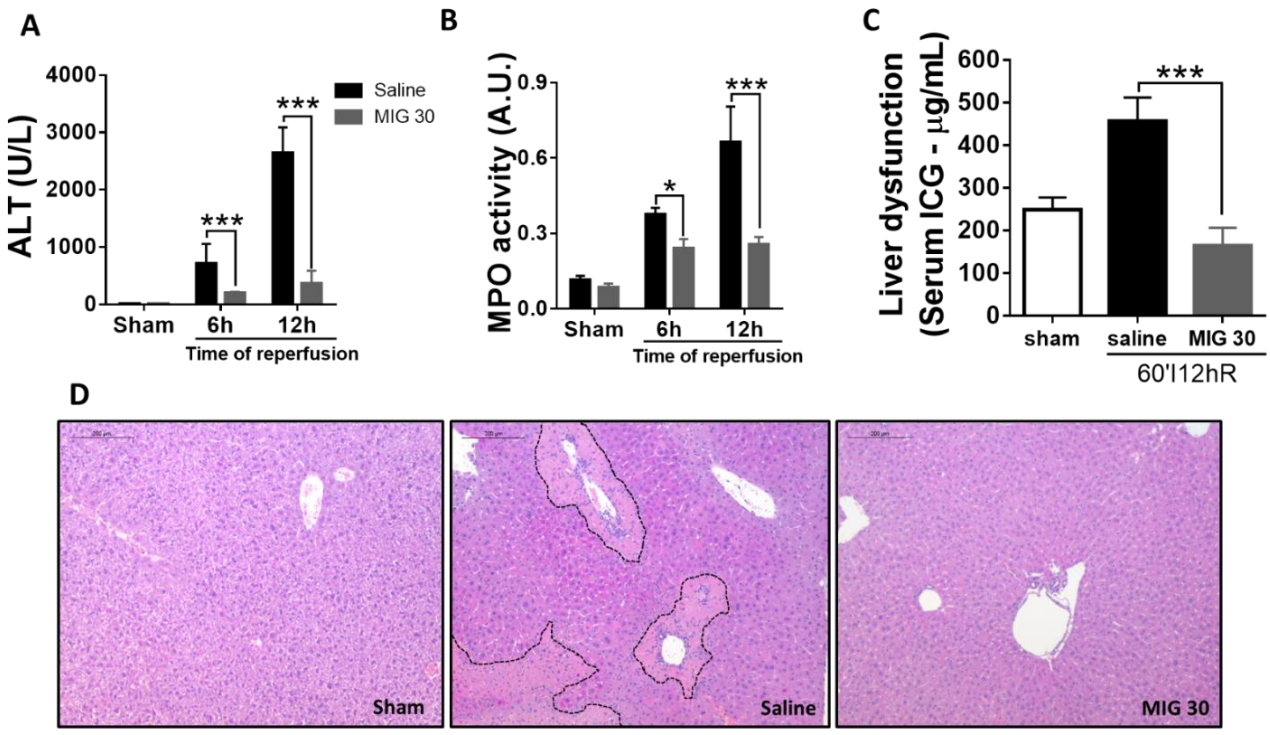

Fig 16. Effects of the treatment with MIG 30 on levels of ALT, MPO and ICG in the liver of mice subjected to IR. Mice were subjected to 60 min ischemia (60'I) and 12h of reperfusion (12hR). They received a dose (i.v) of MIG 30 (100µg) 15 minutes before the reperfusion and every six hours later (i.v). MIG 30 reduced ALT release (A), MPO activity (B) and prevented liver dysfunction (C) when compared with animals subjected to IR and treated with saline. ***p< 0.01 and * p<0.05 vs saline-treated livers. (D) Representative H&E staining of livers 12 hours post-IR injury. MIG 30-treated animals showed significant histological preservation, contrasting with saline-treated animals, which showed elevated signs of necrosis and intense infiltration of leucocytes. Scale bars: 200µm.

4.16 Proinflammatory chemokines were significantly inhibited in MIG-treated mice

Excessive expression of chemokines has been associated with inflammatory disorders, characterized by high levels of leukocyte infiltration. Among the inflammatory chemokines, the ELR^+ CXC chemokines are responsible for neutrophil chemotaxis (Li, Pettersson et al. 2014). To evaluate whether the observed decrease in neutrophil migration and reduced liver damage in MIG 30 treated mice were associated with possible modifications in the expression of chemokines, we assessed the levels of the major murine neutrophil activating chemokines linked to liver IRI. As observed, the production of CXCL1, CXCL2 and CXCL6 was increased in mice subjected to liver IRI and treated with saline. Treatment with MIG 30 significantly reduced the concentration of all three chemokines 6 hours after reperfusion (Fig 17A, B, C).

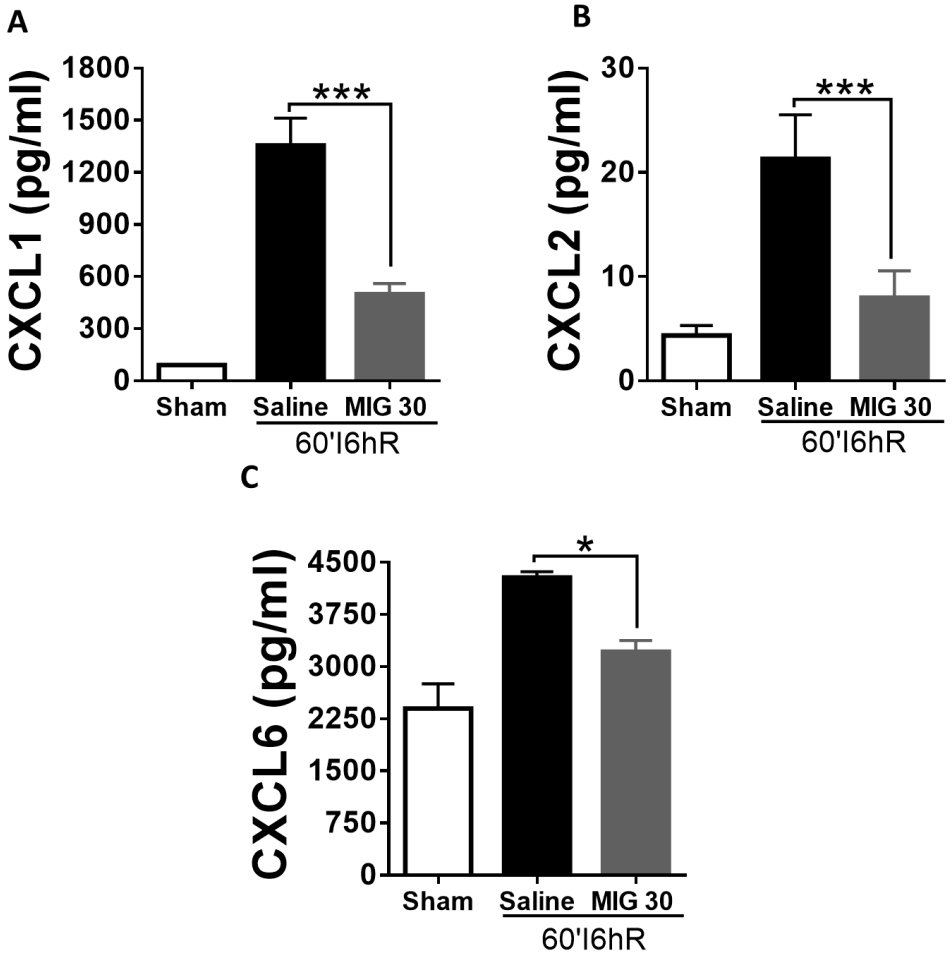

Fig 17. Effects of the treatment with MIG 30 on the concentrations of chemokines in the serum of mice subjected to IR. Mice were subjected to 60 min ischemia (60'I) followed by 6h reperfusion (6hR) and received a dose (i.v) of MIG 30 (100µg) 15 minutes before the reperfusion and every six hours later (i.v). MIG 30 reduced the production of the chemokines CXCL1 (A), CXCL2 (B) and CXCL6 (C) when compared with animals subjected to IR and treated with saline. ***$p< 0.01$ and * $p<0.05$ vs saline-treated livers.

4.17 MIG treatment significantly inhibits cytokine production in injured mice

We also quantified inflammatory cytokines responsible for driving the acute inflammatory response triggered by IR in the liver to evaluate whether MIG 30 could alter also their production in the serum and tissue. As observed, the liver damage was associated with the expression of the proinflammatory cytokines TNF-α and IL-6 in saline treated mice, which were significantly decreased in the MIG 30 treated mice (Fig 18A, B). Moreover, serum levels of TNF-α and IL-6 were markedly reduced in mice treated with MIG 30 peptide between 6 hours and 12 hours of reperfusion (Fig 18C, D).

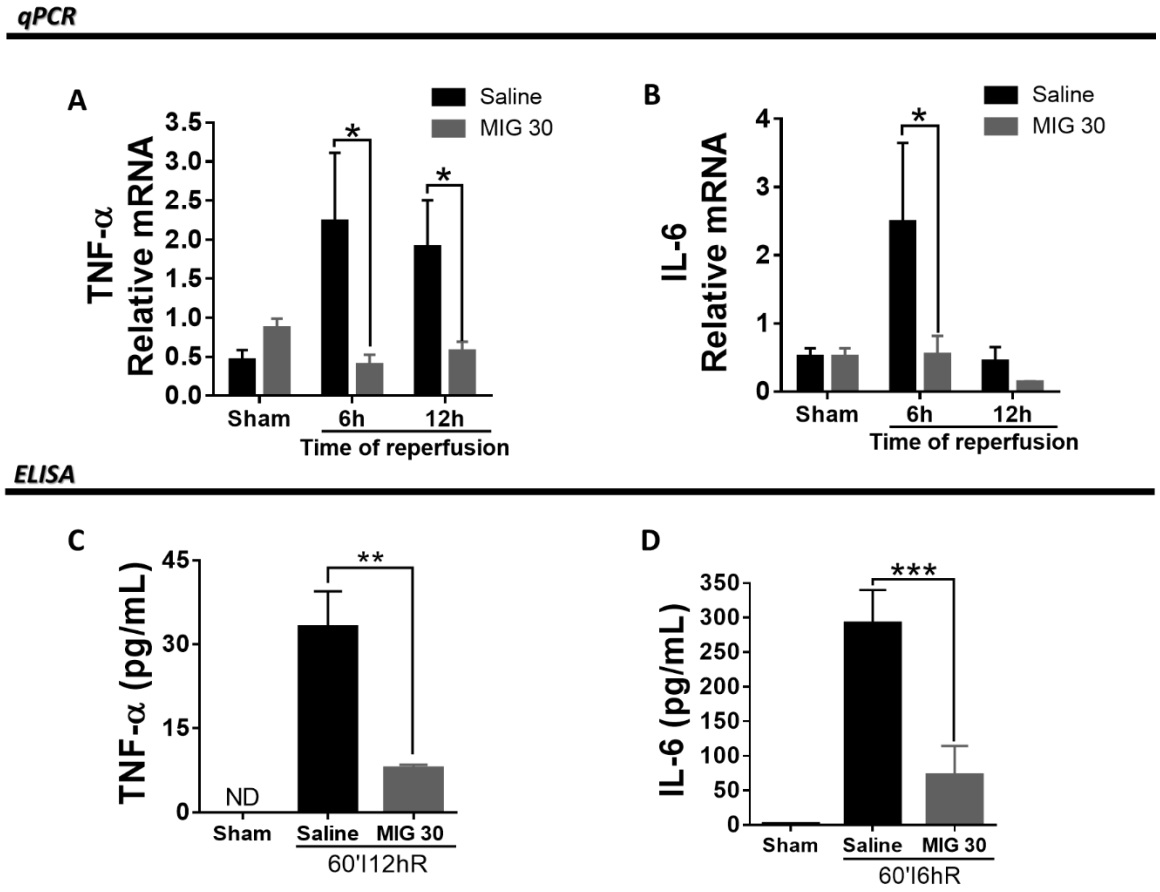

Fig 18. Effects of the treatment with MIG 30 on the concentrations of cytokines in the serum and liver tissue levels of mice subjected to IR. Mice were subjected to 60 min ischemia (60'I) and 6h or 12h of reperfusion and received a dose (i.v) of MIG 30 (100µg) 15 minutes before the reperfusion and every six hours later (i.v). MIG 30 reduced the production of the cytokines TNF-α (A) and IL-6 (B) in the tissue (qPCR) when compared with animals subjected to IR and treated with saline. * $p<0.05$ vs saline-treated livers. Similarly, MIG 30 reduced the production of TNF-α (C) and IL-6 (D) in the serum (ELISA) when compared with animals subjected to IR and treated with saline. * $p<0.05$ and *** $p<0.01$ vs saline-treated livers.

4.18 MIG 30 impaired neutrophil accumulation in liver IR injury

We next aimed to determine whether MIG 30 peptide could directly affect neutrophil migration in the injured liver after IR. Liver sections were negative for Ly6G in sham mice (Fig 19A), however, Ly6G positive cells were predominantly detected 12h after reperfusion in saline-treated mice (Fig 19B). Moreover, livers of mice treated with MIG 30 showed significantly less Ly6G staining 12 h after reperfusion (Fig 19C).

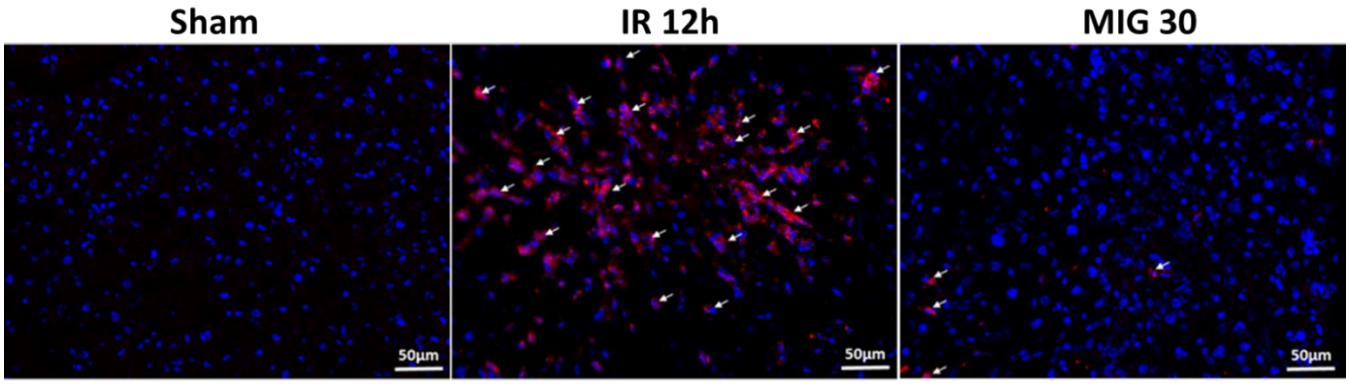

Fig 19. Effects of the MIG 30 treatment on Neutrophil infiltration in Liver IR Injury. Representative photomicrographs of Ly6G staining (neutrophil marker) in sham (A), wild type mice subjected to IR (B) or IR injured mice treated with MIG 30 peptide (C). Accumulation of neutrophils was increased in IR livers compared with sham livers. Mice receiving MIG 30 showed a significant reduction in the number of infiltrated neutrophils. Ly6G is shown in red and nuclei in blue (×200). Arrows point to neutrophils.

5. DISCUSSION

Liver transplantation is a common intervention for patients with advanced liver disease. This liver surgery requires clamping of the vascular portal triad, and thus induces ischemia, followed by reperfusion when the clamp is removed. Despite being the best method of choice, this surgical procedure may cause cell injury and organ damage. Hepatic IR injury is a complex phenomenon which involves numerous metabolic pathways. Although the pathophysiology has been extensively studied for more than 40 years, its mechanisms have not yet been completely clarified (van Golen, Reiniers et al. 2013, Zhai, Petrowsky et al. 2013). Even if the survival has increased in recent years due to improved surgical techniques, as well as the development of new immunosuppressive drugs, primary graft dysfunction still represents an important cause of morbidity and mortality for transplanted patients (Jaeschke and Woolbright 2012). All transplanted livers have some extent of dysfunction, since the IR lesion is an inevitable phenomenon for transplanted organs (Montalvo-Jave, Escalante-Tattersfield et al. 2008, Peralta, Jimenez-Castro et al. 2013). During IR injury, an intense inflammatory process occurs in the liver. This hepatic inflammation occurs mainly during the reperfusion and is characterized by a large neutrophil recruitment to the liver, as well as production of cytokines and activation of resident leukocytes.

Regarding liver inflammation, the defense against initial environmental challenges and injury is driven by a complex set of leukocytes. In this context, neutrophils are considered crucial effector cells in the pathophysiology of liver IR. Because of their high mobility and the capability to release potent cytotoxic mediators, neutrophils primarily work to eliminate invading microorganisms and/or remove dead cells at the site of inflammation (Jaeschke 2003). Also, during sterile injury, neutrophils migrate to sites of stress or necrosis where they can contribute in the healing and removal of cell debris, although the *in vivo* mechanisms of death cell removal are not well understood (Kubes and Mehal 2012, McDonald and Kubes 2016). Studies using models of acute injury, which generally lead to physiological repair mechanisms, have shown that inhibiting neutrophil recruitment leads to poor repair, so neutrophils appear to be required for the normal healing process (clearance of debris, production of extracellular matrix, normal revascularization) (Christoffersson, Henriksnas et al. 2010, Slaba, Wang et al. 2015). However, in the liver and others organs, an excessive inflammatory response triggered by an uncontrolled neutrophil migration and activation carries the risk of additional tissue damage as demonstrated during liver IR injury and other conditions (Jaeschke and Smith 1997,

Gujral, Farhood et al. 2003). Moreover, the mechanisms involved in neutrophil recruitment during liver IR are not well known. Additionally, the molecules necessary for this type of migration are not defined, since the liver presents an atypical sinusoidal vasculature in which the current leukocyte migration paradigm does not apply. Thus, the objective of this work was to evaluate the role of neutrophils by studying different mechanisms used by these cells during hepatic IR injury, such as chemokine receptors, metalloproteinases and interaction between chemokines and GAGs.

Our studies were initiated through the standardization of the IR model in order to establish the best time for further analysis. The animals were submitted to 60 minutes of ischemia, followed by different reperfusion times. In our hands, the IR injury is severe and seems to reflect the liver status of patients with liver surgery, such as transplants. This is corroborated by a tissue necrosis pattern and ALT levels greater than 20 times baseline, which validates our model. Our data showed that neutrophils progressively infiltrated the injured liver, which was directly correlated with damage severity and liver dysfunction (Fig 1, 9, 15). Strategies to prevent recruitment of neutrophils have been shown in different models of IR (Souza, Cassali et al. 2001, Souza, Bertini et al. 2004). Consistent with this, several studies of sterile inflammation have shown that depletion of neutrophils protects mice against liver injury (Liu, Han et al. 2006, Jaeschke and Liu 2007). It is suggested that strategies that limit neutrophil accumulation and/or activation may be a useful adjuvant in the treatment of ischemic disorders.

The occurrence of the IR injury certainly triggers an intense immune response. The role of neutrophils seems to be paramount since they are quickly recruited into the injured liver indicating their likely involvement in the pathogenesis of liver injury. ELR+ CXC chemokines are major mediators known to induce neutrophil activation and migration. Our data demonstrated an elevated production of CXCL1, CXCL2 and CXCL6 between 6 hours and 12 hours after reperfusion. Previous studies demonstrated that CXC chemokines mediate neutrophil infiltration during the acute inflammatory phase of hepatic IR injury (Lentsch, Yoshidome et al. 1998, Kataoka, Shimizu et al. 2002, Kuboki, Shin et al. 2008). Consistent with these observations, CXCL1 and CXCL2 have been shown to be expressed at the luminal surface of liver sinusoids around sites of necrosis. They form a chemokine gradient that guides neutrophil migration in the direction of the injury (McDonald, Pittman et al. 2010). In mice, hepatic expression of CXCL1 and CXCL2 increases in temporally distinct patterns after hepatic IR. The expression of CXCL2 increases in the early phase of reperfusion, before any detectable increase in neutrophil accumulation, suggesting that CXCL2 may be involved in the initial

recruitment of neutrophils to the ischemic lobe (Lentsch, Yoshidome et al. 1998). Neutralization of CXCL6 in an IR injury model resulted in reduced neutrophil sequestration in the liver and serum ALT levels (Colletti, Kunkel et al. 1996). Moreover, previous reports have shown that GCP-2/CXCL6 induces neutrophil influx (Wuyts, D'Haese et al. 1999, D'Haese, Wuyts et al. 2000). Indeed, when the chemokine is administered i.p. an increase in both circulating and peritoneal neutrophils is observed, consistent with the concept that these chemokines have a dual action, acting locally to stimulate recruitment and systemically to promote mobilization (Wengner, Pitchford et al. 2008). When these chemokines are produced locally, they are retained on the endothelial wall, creating a chemotactic gradient for neutrophils (McDonald, Pittman et al. 2010).

CXC chemokines act by activating G protein-coupled receptors on the surface of neutrophils. The murine receptor CXCR2 binds to and signals in response to murine CXCL1, CXCL2 and CXCL6. In addition, CXCL6 also interacts with murine CXCR1 (Fan, Patera et al. 2007). By recruiting and activating neutrophils, human CXCL1, CXCL2 and CXCL6 have been implicated in a wide range of diseases, including liver IR injury (Mosher, Dean et al. 2001, Li, Klintman et al. 2004). Because of their essential role for the migration of leukocytes, chemokines and their GPCRs can serve as potential targets for the development of new anti-inflammatory drugs. Thus, considering the role of CXCR1/2 in neutrophil recruitment, their blockade could protect the liver of mice subjected to IR. For this, we have studied the CXCR1/2 antagonist Reparixin (DF1681B) in this model. Reparixin is a compound that inhibits the effects of CXCL1 and CXCL2 by allosteric modulation of the CXCR1/2 receptors (Sousa, Coelho et al. 2013). Our data demonstrated that the administration of Reparixin was able to reduce neutrophil infiltration into the liver and lungs, which was associated with better histological outcome (Figure 5, 6). This reinforces the idea of direct contribution of neutrophils to the worsening of the hepatic injury by IR and is in agreement with preliminary findings in other IR models (Souza, Bertini et al. 2004, Cugini, Azzollini et al. 2005, Gorio, Madaschi et al. 2007). CXCR2 is one of the most important chemotactic receptors in neutrophils, and its blockade is already a target for the development of drugs for various diseases (Bertini, Barcelos et al. 2012). In addition, a favorable consequence of CXCR2 blockade is the likely inhibition of neutrophil egress from the bone marrow (Griffith, Sokol et al. 2014), providing a dual efficacy to prevent the arrival of neutrophils at the target site. Reparixin inhibited the expression of the cytokines TNF-α, IL-6 and the chemokine CCL3, whereas it was inefficient in reducing the levels of chemokines CXCL1 and CXCL6 (Figure 7). Our data corroborate with previous findings in a

different model of sterile liver injury, reporting no differences in chemokine levels in mice treated with a CXCR1/2 antagonist (Marques, Amaral et al. 2012). These data can be explained by the fact that although infiltrating neutrophils secrete chemokines, the primary source of chemokines in the liver are hepatocytes, Kupffer cells, stellate cells and sinusoidal endothelial cells. Together, these cells secrete an array of chemokines, such as human CXCL1, CXCL8, CXCL9 and CXCL10 or their murine counterparts that drive leukocyte infiltration, development of inflammation and liver injury (Karlmark, Wasmuth et al. 2008, Wasmuth, Lammert et al. 2009, Oo and Adams 2010, Wasmuth, Tacke et al. 2010). As Reparixin acts by blocking the CXCR1/2 receptors expressed on the neutrophil surface, it does not alter chemokine production by resident cells. On the other hand, CCL3/MIP1α is a chemokine primarily produced by leukocytes, especially macrophages. It can activate granulocytes, leading to acute neutrophilic inflammation. (Sherry, Tekamp-Olson et al. 1988).

Recent advances in intravital microscopy have enabled visualization and quantification of real-time biological processes *in situ*. To better define the status of neutrophils in liver IRI, we performed confocal intravital microscopy. Few studies have demonstrated real-time intravital neutrophil dynamics, leaving questions still open as how neutrophils behave during the sterile injury development. Beyond the intense neutrophil recruitment to the liver, our *in vivo* data show that IR caused cell death, as observed by high serum transaminase levels and deposits of extracellular DNA (Figure 3). Interestingly, DNA is spread through the tissue. This result corroborates with other models of sterile liver injury, showing that during liver injury induced by acetaminophen, deposits of DNA from damaged hepatocytes are found in almost all compartments of the liver (within the centrilobular veins and sinuses, around the hepatocytes and in the space of Disse), contributing to significantly increased systemic inflammation, neutrophil recruitment in the liver, and hepatotoxicity (Marques, Oliveira et al. 2015). In this way, we can consider that the lesion is generalized.

The observations on the movement of neutrophils helped us to understand their behavior within the injured liver. It is interesting to note that the arrival of neutrophils coincides perfectly with the presence of liver damage. We showed that IR induced polarization and activation of neutrophils during the liver injury development. During inflammation induced by IR, neutrophils were activated, as shown by their movement over longer distances, by their higher speed, formation of clusters and their larger size and polarity. (Figure 4). An interesting finding of our study is that the time-course of the production of CXCL1, CXCL2 and CXCL6 in serum showed a similar tendency to the time-course of neutrophil movement (track length), velocity,

size, formation of clusters and elongation of neutrophil shape. Honda and colleagues have shown that CXC chemokines modulate the behavior of neutrophils during sterile liver inflammation (Honda, Takeichi et al. 2013). As mentioned above, previous reports have shown that an intravascular gradient of chemokines and mitochondria-derived formyl peptides collaborate to guide neutrophils to site of liver necrosis (McDonald, Pittman et al. 2010). Thus, the recruitment of neutrophils to the site of sterile inflammation is controlled by a multistep cascade of molecular events. Our data demonstrated that pharmacological antagonism of CXCR1/2 was able to interfere with neutrophil polarization and activation in real-time, which collaborated to reduce liver injury and improve overall outcome.

The mechanism by which neutrophils damage the liver during IR injury is still poorly understood, however 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 (Borregaard, Sorensen et al. 2007). Among the different molecules within the granules of neutrophils, we investigated the functional significance of MMP-9 in our model. There is a growing body of evidence supporting key functions for MMPs expressed in the pathogenesis of liver diseases (Bissell 2010, Dechene, Sowa et al. 2010, Coito 2011). In this regard, the contribution of MMP-9 to the pathogenesis of liver IRI was clearly demonstrated when we used *Mmp-9*$^{-/-}$ animals. We have shown that *Mmp-9*$^{-/-}$ deficiency significantly reduced ALT levels, neutrophil recruitment, inflammation and significantly reduced liver damage by histological assessment, providing an indication that MMP-9 is indeed an active player in liver IRI (Figure 9, 10). The decrease of serum ALT activity and tissue necrosis are clear indications that hepatic damage has been reduced. MMP-9 has been implicated as an important enzyme during inflammation because of its ability to facilitate leukocyte trafficking through the degradation of ECM (Opdenakker, Van den Steen et al. 2001). We have shown MMP-9 expression in damaged livers. This is in accordance with previous reports showing detrimental roles of MMPs in liver preservation (Upadhya, Harvey et al. 1997). Others have established a correlation between disease severity and MMP-9 detection in the serum of patients with various types of liver injury, including IRI (Kuyvenhoven, Ringers et al. 2003), acute allograft rejection (Kuyvenhoven, Verspaget et al. 2004) and chronic viral hepatitis (Leroy, Monier et al. 2004). Our data are in agreement with others showing that in human orthotopic liver transplantation, MMP-9 has been detected in the serum of patients few minutes after reperfusion, and it remained elevated for days after transplantation (Kuyvenhoven, Verspaget et al. 2004, Duarte, Baber et al. 2015). Our observations are also in line with murine models of allergen-induced

airway inflammation and zymosan-induced peritonitis, in which *Mmp-9*-deficient mice showed significantly less leukocyte infiltration compared with that in their corresponding WT littermates (Cataldo, Tournoy et al. 2002, Kolaczkowska, Chadzinska et al. 2006).

MMP-9 is an inducible gelatinase expressed mostly by leukocytes. In our model, Ly6G neutrophils were strongly recruited to the livers of WT mice subjected to IR, whereas we showed a significant reduction in *Mmp-9*$^{-/-}$ animals (9). Indeed, previous reports have shown that Ly6G neutrophils and Mac-1 leukocytes were the major sources of MMP-9 in damaged WT liver (Hamada, Fondevila et al. 2008). In addition to showing reduced neutrophil migration, *Mmp-9*$^{-/-}$ mice were characterized by a profound reduction in the expression of proinflammatory cytokines after liver IR injury (Fig 10). In contrast, WT mice showed an elevated expression of TNF-α and IL-6, indicating that inflammation in these animals was still a continuous process. TNF-α, which is a potent proinflammatory cytokine, is often associated with neutrophil infiltration and liver damage (Shuh, Bohorquez et al. 2013). MMPs are secreted as inactive zymogens (proMMPs) requiring activation. However, the mechanisms of MMP activation in the injured livers is unclear. MMPs can be activated by proteolytic cleavage of the N-terminal propeptide, but under conditions of IR, the reactive oxygen peroxynitrite might activate proMMPs without propeptide removal (Okamoto, Akaike et al. 2001). Upon activation, MMPs might also decrease the blood flow, hence tissue viability, through their vasoconstrictive activities, leading to loss of cell viability and tissue integrity following IRI.

Interestingly, although *Mmp-9*$^{-/-}$ mice were found to be significantly protected against liver IR injury, the levels of major chemokines did not reduce (Figure 11). Chemokines and MMPs, in particular MMP-9 and MMP-8, play key roles in the migration of immune cells to sites of inflammation. However, they also have important regulatory roles, as they can regulate cytokine and chemokine activity by proteolytic processing. Upon chemokine stimulation, leukocytes produce and secrete proteolytic enzymes for innate immune defense mechanisms. Some of these proteases modify the biological activity of the chemokines. For instance, neutrophils secrete MMP-9 and MMP-8 after stimulation with IL-8. MMP8/9 processes IL-8 into a 10–30-fold more active chemokine. This results in an important positive feedback loop, as IL-8 induces the rapid release of MMP8/9 from the granules, indicating that gelatinases are not only effectors but also regulatory enzymes (McQuibban, Gong et al. 2000, Van den Steen, Proost et al. 2000). Moreover, as previously reported, the cleavage of mouse GCP-2 (CXCL6) by gelatinase B is highly efficient and results in potentiation of its biological activity, although to a lesser extent than with human IL-8 (Van Den Steen, Wuyts et al. 2003). So, although in

our hands, the levels of chemokines between WT and *Mmp-9*$^{-/-}$ mice are comparable, the chemokines produced by *Mmp-9*$^{-/-}$ mice may be less activated because of the lack of the enzyme.

MMPs and their specific inhibitors, the TIMPs, play an important role in inducing and preventing the degradation of the ECM, respectively (Kessenbrock, Plaks et al. 2010). Among the TIMPs, TIMP-1 is a widely expressed and secreted protein that plays a critical role in tissue remodeling via inhibiting members of the large family of MMPs (Chirco, Liu et al. 2006). In our model, we have shown that the expression of TIMP-1 was upregulated in WT mice, however is decreased in *Mmp-9*$^{-/-}$ mice (Figure 12). Moreover, expression of TIMP-1 occurred in parallel with MMP-9 (Figure 9, 12). This is in line with other reports showing that TIMP-1 has been found to be strongly upregulated in liver tissue during hepatic fibrogenesis in patients with liver disease and in animal models of hepatic fibrogenesis (Iredale, Benyon et al. 1996, Kluwe, Wongsiriroj et al. 2011). This result is also in accordance with reports, which have shown a time-dependent increase in MMP-9 and TIMP-1 gene expression relative to control, with peak expression occurring between 12 and 24 hours in murine models of post-operative ileus (Moore, Manthey et al. 2011). Moreover, Duarte and colleagues have shown that TIMP-1 deficiency is lethal in mice subjected to liver IRI (Duarte, Hamada et al. 2012).

TIMPs suppress protease activity by forming high-affinity, noncovalent complexes with the active MMPs (Bode, Fernandez-Catalan et al. 1999, Visse and Nagase 2003). Although in our model, the expression of TIMP-1 and MMP-9 occur together, TIMP-1 was not able to prevent MMP-9 expression. This is in accordance with Moore and colleagues. They have shown resistance of MMP-9 activity to TIMP-1 inhibition in a steatotic rat liver model of transplantation (Moore, Shen et al. 2007). A comparable situation has been observed in cancer patients, in which high levels of TIMP-1 were associated with poor patient prognosis (McCarthy, Maguire et al. 1999). This suggests that the function of TIMP-1 in liver IRI can be distinct from the classical concept that TIMP-1 regulates physiological or pathological processes through its ability to inhibit MMPs. Specific MMP inhibitors do not simply block protease activity but the role of TIMPs is to modulate MMP functioning. Different protease activation processes occur as a response to liver injury (Consolo, Amoroso et al. 2009). Usually, while injured cells release proteases, healthy cells release TIMPs, which are secreted by the cells surrounding these producing proteases. Thus, high levels of TIMPs occur simultaneously to an increase in proteases; in other words, both proteases and inhibitors could be produced by the same cell type at the same time (Iyer, Wei et al. 2007, Consolo, Amoroso et al. 2009). Keeping this in mind, several lines of evidence also indicate that TIMP-1 is a multifunctional

protein with functions distinct from protease inhibition. For instance, TIMP-1 can stimulate cell proliferation independently of its MMP inhibitory effect (Wurtz, Schrohl et al. 2005), raising the possibility that the pathophysiological role of TIMP-1 in liver IRI may be unrelated to MMP inhibition. Further experiments are required to test this hypothesis.

Another important factor in neutrophil-induced liver injury is MPO. This enzyme is crucial to generate cytotoxic oxidants, such as nitric oxide (NO)-derived inflammatory oxidants and reactive nitrogen species, which may be relevant in liver IRI, because livers express high levels of iNOS after IR, which generates large amounts of NO (Eiserich, Hristova et al. 1998). 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 MPO activation (Figure 9). We wondered whether the reduced MPO activity would be due to lower injury or whether MMP-9 could also control the production of enzymes including MPO by neutrophils. For this, 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 control neutrophil degranulation, since MPO activity, elastase and NGAL were reduced in stimulated *Mmp-9*$^{-/-}$ neutrophils (Figure 13). With regard to NGAL however, only *Mmp-9*$^{-/-}$ neutrophils stimulated with CXCL6 and fMLP showed a difference when compared with WT neutrophils. These data seem to show ligand-specific differences and also granule marker-specific differences. In general, the MMPs have for long been considered as secreted proteases with functions outside the cell. However, in the past few years, MMPs, originally discovered to function 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 others MMPs (Cauwe and Opdenakker 2010). This is a very new and relatively unexplored field. Historically, extracellular activation of pro-MMPs has been discovered mainly by *in vitro* studies with individual MMPs. The first examples were the activation of collagenase (MMP-1) by the serine protease plasmin (Eeckhout and Vaes 1977). Based on this, it is clear that different MMPs can also activate others enzymes as well as activate each other. For instance, Choi and colleagues demonstrated that serine proteases cleave and generate an activated form of MMP-3 inside stressed dopaminergic cells (Choi, Kim et al. 2008). Moreover, and interestingly, in MMP-26 the latency motif containing the conserved cysteine is inactive and MMP-26 is activated by autolytic cleavages (Marchenko, Marchenko et al. 2002). In addition, the major fraction of synthesized MMP-26 remains intracellularly and may activate pro-MMP-9 in the cytoplasm (Zhao, Xiao et al. 2003, Strongin

2006). Another example is the processing of antimicrobial α-defensins called cryptdins (crypt defensins) by the enzyme MMP-7. MMP-7 colocalizes with cryptdins in murine Paneth cells and mediates the processing and activation of various cryptdins *in vitro*. Moreover, MMP-7 knockout mice have a functional defect in clearance of intestinal infections, and they succumb more rapidly from and to lower doses of virulent *Salmonella typhimurium* compared with control mice (Wilson, Ouellette et al. 1999).

In our model, we also demonstrated a clear link between serine proteases and MMPs, since inhibition of elastase was able to decrease the gelatinolytic activity of neutrophils. This reinforced the concept of enzyme cascades. An activation network has evolved into a maze of dynamic protease interactions with other proteases, with inhibitors and with their substrates, which was termed the "protease web" (Overall and Kleifeld 2006) or the "proteolytic internet" (Kruger 2009). Hence, proteases may be the substrates of other MMPs, and these protease interaction network may also function inside cells. Then, MMPs may cleave various substrates within cells. Since many of the proteins are molecules with multiple functions, the modulation of these substrates by MMPs adds an additional layer of complexity to MMP inhibition in pathology (Cauwe and Opdenakker 2010). Altogether, our data demonstrated that MMP-9 seems to control neutrophil degranulation. Others experiments are required to test this hypothesis, such as evaluating the neutrophil migration in *Mmp9*$^{-/-}$ animals using intravital confocal microscopy (IVM). However, this is not possible at this time, since the *Mmp9*$^{-/-}$ animals are located in Leuven and our IVM device is here in UFMG.

As we showed, neutrophils play an essential role in the inflammatory response triggered by liver IRI. In this aspect, chemokines demonstrate a key role in the multistep process that contribute to sequential neutrophil tethering and rolling, activation, tight adhesion and transendothelial migration (Ley, Laudanna et al. 2007). All chemokines exert their biological activity by binding to GPCRs (CXCR1 and CXCR2). However, chemokines also interact with GAGs on endothelial cells and the ECM (Handel, Johnson et al. 2005). GAG-chemokine interaction is essential to inhibit the diffusion of chemokines away from their sites of production, enabling the formation of localized gradients that guide neutrophil recruitment (Wang, Fuster et al. 2005, Colditz, Schneider et al. 2007). Once leukocytes enter the tissue, they can migrate to the site of inflammation through the gradient of local GAG-bound chemokines. We wondered whether disruption of chemokine-GAG interactions would be protective against liver IRI. For this, the animals subjected to IR were treated with MIG 30 peptide, which is a COOH-terminal region from the chemokine CXCL9. Given the high number

of positive charges, one could presume a GAG binding function for the COOH-terminal region of CXCL9. Keeping this in mind, the following hypothesis was formulated. Do these COOH-terminal CXCL9 peptides (MIG 30), through competition with active chemokines for GAG binding, reduce chemokine immobilization and presentation, and do they thereby inhibit chemokine-induced leukocyte migration? Firstly, by evaluating heparan sulfate binding to a potent murine neutrophil chemoattractant CXCL6 in the presence of the chemically synthesized MIG 30 peptide, we showed clearly reduced CXCL6 binding to heparan sulfate, with a high efficacy (Figure 15). Previous reports of our group have shown that MIG 30 peptide could inhibit the *in vivo* chemotactic activity of CXCL8 by competing with CXCL8 for the binding to GAGs (Vanheule, Janssens et al. 2015).

The binding of chemokines to GAGs has been proven to be indispensable for chemokine activity. This importance was initially supported by studies that demonstrated binding of chemokines to purified GAGs *in vitro* (Witt and Lander 1994) and to endothelial cell surface GAGs *in vitro* and *in vivo* (Middleton, Neil et al. 1997, Kuschert, Coulin et al. 1999). Based on the necessity of chemokine binding to GAGs for exerting biological functions *in vivo*, an anti-inflammatory action for MIG 30 peptide through GAG binding competition was evaluated in our murine model of acute liver inflammation induced by IR. We showed, for the first time, that intravenous injection of the MIG 30 peptide efficiently inhibited liver injury induced by IR, as observed by reduced ALT levels in the serum, lower infiltration of neutrophils, better liver function and improved liver histology (Figure 16, 19). This result is in line with previous reports of our group showing that the peptide was able to inhibit neutrophil extravasation and inflammation in a murine model of monosodium urate (MSU) crystal-induced gout (Vanheule, Janssens et al. 2015, Vanheule, Boff et al. 2017). Previous reports have shown that mice lacking the GAG heparan sulfate showed impaired neutrophil infiltration. Decreased neutrophil infiltration was partially due to reduced chemokine presentation on the cell surface, resulting in decreased firm neutrophil adhesion and migration (Wang, Fuster et al. 2005). Moreover, this is supported by an approach showing that GAG-binding deficient chemokines were unable to promote cell migration in a simple intra-peritoneal animal model of cell recruitment (Proudfoot, Handel et al. 2003). This confirms the requirement for GAG-binding in maintaining high local concentrations of chemokines to induce cell migration.

Although chemokines are clearly beneficial in the battle against infectious organisms and during wound healing after tissue injury, excessive on-going expression of chemokines has been associated with inflammatory disorders, characterized by high levels of leukocyte

infiltration in tissues and consequently tissue damage (Proost, Struyf et al. 2006, Szekanecz, Vegvari et al. 2010). Thus, a reduction of chemokine activity at sites of excessive inflammation is needed. In our model, the major chemokines and cytokines were significantly expressed in mice subjected to liver IR. However, the reduction in inflammatory cell infiltrate was associated to a significant reduction in serum levels of chemokines and cytokines in mice treated with MIG 30 peptide (Figure 17, 18). Even this result is not expected, since the treatment with MIG 30 peptide doesn't block the production of chemokines, but its action. The decreased levels of chemokines can be explained by additional roles for chemokine–GAG interactions. For instance, binding to heparan sulfate can retain chemokines on the surface of cells and within the ECM and protect them from proteolytic cleavage (Webb, Ehrengruber et al. 1993, Metzemaekers, Mortier et al. 2017). This result indicates that chemokines may have been quickly removed or degraded, once they became displaced by MIG 30 peptide.

Recently, intact modified chemokines were developed that interfere with the binding of chemokines to GAGs (Rek, Krenn et al. 2009, Adage, Piccinini et al. 2012). These modified chemokines have, in comparison with their natural human chemokine counterparts, an enhanced affinity for GAGs but a decreased affinity for their GPCRs. In this way, the modified chemokines can compete with functional chemokines for GAG binding. Thereby, they reduce chemokine immobilization and presentation and enhance the inhibition of chemokine-induced leukocyte migration. It has been shown that PA401, a CXCL8-based decoy protein, exerts strong anti-inflammatory activity *in vivo* (Falsone, Wabitsch et al. 2013, Adage, del Bene et al. 2015, McElvaney, O'Reilly et al. 2015). Therefore, interfering with the chemokine-GAG system might constitute an attractive way of treating such diseases. In view of this, the MIG 30 peptide might be a lead molecule for the generation of therapeutic peptides that compete with functional chemokines for GAG binding, and its efficacy confirms the applicability of GAGs as a therapeutic target. This new approach may be used as a complementary option for chemokine receptors antagonism.

6. CONCLUSIONS

In summary, this study demonstrated that IR triggers an inflammatory process in the liver with recruitment of neutrophils into the parenchyma. Neutrophils use a multistep process to infiltrate the tissue, which includes participation of GPCRs, MMPs and GAG-chemokine interactions. The neutrophil migration correlated with liver injury and was dependent on CXCR1/2 and its ligands. Furthermore, we showed in real-time in this model that an antagonist of CXCR1/2 interfered with tissue damage, inflammatory cytokine production and the behavior of neutrophils in the tissue. 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. We also confirmed the pivotal role of GAGs in neutrophil-mediated liver injury, which further confirms that the interaction between GAGs and chemokines can be exploited therapeutically. 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.

In conclusion, we hope that this work will contribute to a better understanding of the mechanisms in the development of IRI and provides insights into improving the treatment regimen for IRI and an effective method for preventing or minimizing hepatic IRI during liver surgery.

7. SUPPLEMENTARY MATERIAL

7.1 Article accepted for publication on the Laboratory Investigation

Neutrophils: a cornerstone of liver ischemia and reperfusion injury

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Key words: Hepatic IRI, chemokines, glycosaminoglycans, metalloproteinases, neutrophil-mediated liver injury

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7.2 Article submitted for publication

Intravital microscopic evaluation of the effects of a CXCR2 antagonist in a model of Liver Ischemia Reperfusion Injury in Mice

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Short title: CXCR2 inhibition in murine liver inflammation

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7.3 Article in process for submission

Matrix Metalloproteinase-9 Gene Deletion Attenuates Liver Ischemia and Reperfusion Injury in Mice

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7.4 Article in process for submission

Disruption of Glycosaminoglycan-Chemokine Interaction Protects Against Liver Ischemia Reperfusion Injury in Mice

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Short title: GAG-chemokine disruption in murine liver inflammation

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