

Matrix-Leukocyte Interactions in Liver Ischemia-Reperfusion Injury

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WHAT HAPPENS WHEN “SELF” CHANGES...?

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SUMMARY

Orthotopic liver transplantation OLT is an effective therapeutic modality for end stage liver disease. The current scarcity of donor livers and high number of wait-listed patients has led to a greater use of marginal organs, normally discarded due to the higher risks of primary non-function or dysfunction after transplantation. Hepatic ischemia/reperfusion (I/R) injury is a complex inflammatory event that implicates the participation of a wide variety of chemical, molecular and cellular mediators and can greatly deteriorate the outcome of a liver transplant and lead to graft loss. It is a phenomenon whereby initial damage to the hypoxic liver is further accentuated by the return of blood flow and oxygen delivery. Hepatocellular damage caused by hepatic I/R injury is the result of an intricate network of inflammatory events, which include, intense oxidative stress, expression and release of pro-inflammatory cytokines and chemokines, and massive inflammatory leukocyte migration. Earlier studies from our laboratory have unveiled a critical role for fibronectin (FN), a key extracellular matrix (ECM) component, on leukocyte recruitment and subsequent tissue injury after organ transplantation. Fibronectin, up-regulated on the vascular endothelium after liver injury, interacts with $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins expressed by leukocytes. Additionally, matrix metalloproteinases, especially gelatinases (MMP-2 & MMP-9) and membrane-type MMPs, are essential for focal matrix degradation and the outcome of their activity in inflammation is greatly dependent on the endogenous regulation mediated by TIMPs. Therefore our aim was to further dissect the functions of fibronectin, and of relevant MMPs and their inhibitors in hepatic I/R injury. In the work contained in this PhD project, we report that FN- $\alpha 4\beta 1$ interactions regulated leukocyte expression of MMP-9 and MT1-MMP (MMP-14) via the p38 MAPK signaling pathway in a model of 24 hours cold liver I/R injury. Additionally, we show that CS-1 peptides, which block FN- $\alpha 4\beta 1$ integrin interactions, significantly depressed leukocyte infiltration, ameliorated liver injury and improved recipient survival from 50% to 100% after 14 days of transplantation. Furthermore, we show that FN- $\alpha 5\beta 1$ integrin interactions can also induce MMP-9 expression, and significantly contribute to cell injury and cell death in a model of steatotic liver I/R injury. Our laboratory has previously shown that MMP-9 is a critical mediator of leukocyte infiltration in hepatic I/R injury and that specifically targeting MMP-9 profoundly ameliorates tissue damage after liver I/R insult. Therefore, we next used TIMP-1^{-/-} mice in a model of 70% partial warm liver I/R injury to study the role of the endogenously expressed MMP-9 inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1). We show that TIMP-1 deficiency led to significant increase in MMP-9 activity and exacerbated MMP-9 mediated leukocyte infiltration in the liver, severely deteriorating liver

function and increasing hepatocellular death. Moreover, we provide novel findings on how the absence of TIMP-1 results in lethal I/R injury due to the inability of the liver to recover and regenerate after hepatic I/R injury. Finally, we and others have shown that elevated iNOS expression levels are correlated to liver damage post-IRI. We show here, using iNOS^{-/-} mice and specific iNOS inhibitor ONO-1714, that iNOS expression promotes MMP-9 mediated leukocyte infiltration and thus contributes to hepatocellular injury and impaired liver function after liver I/R injury.

RESUMO

Hoje em dia, o transplante hepático é uma modalidade terapêutica eficaz no tratamento de doenças hepáticas terminais. A actual escassez de fígados dadores associado ao elevado número de doentes em lista de espera tem levado ao maior uso de órgãos limítrofes, que são habitualmente descartados devido ao maior risco de provocar uma disfunção primária do órgão transplantado. A lesão de isquemia/reperfusão (I/R) hepática é um complexo evento inflamatório no qual participam um vasto número de mediadores químicos, moleculares e celulares que por sua vez provocam uma deterioração do órgão transplantado, e em muitos casos, a perda do mesmo órgão. Na lesão de I/R hepática o dano inicial no fígado em hipoxia é acentuado pelo excesso de oxigénio disponível durante o retorno do fluxo sanguíneo, o que envolve uma rede complexa de processos inflamatórios, tais como stress oxidativo excessivo, expressão e libertação de citocinas e quimiocinas inflamatórias e finalmente uma intensa infiltração e migração leucocitária. Estudos prévios do nosso laboratório revelaram um papel crítico da fibronectina (FN), uma componente chave da matriz extracelular, no recrutamento de leucócitos e consequente dano no tecido após o transplante. Induzida no endotélio vascular, a fibronectina interage com as integrinas $\alpha 4\beta 1$ e $\alpha 5\beta 1$ expressas na membrana celular dos leucócitos. As metaloproteínases de matriz (MMP), nomeadamente as gelatinases (MMP-2 e MMP-9) e as metaloproteínases de membrana (MT-MMP), são essenciais para a degradação focal da matriz e o resultado da sua atividade depende em grande parte da sua regulação endógena pelas TIMP. Assim, o objectivo deste estudo centrou-se em analisar as funções da fibronectina, bem como das mais relevantes MMPs e dos seus inibidores endógenos na lesão de I/R hepática. A investigação realizada neste projecto de doutoramento demonstra que, num modelo de lesão de I/R hepática fria de 24 horas, as interacções FN- $\alpha 4\beta 1$ regulam a expressão de MMP-9 e MT1-MMP (MMP-14) nos leucócitos através da via de sinalização p38 MAPK. Demonstra ainda que a administração de péptidos CS-1, cuja função é bloquear as interacções FN- $\alpha 4\beta 1$, reduziu significativamente a infiltração de leucócitos, bem como a lesão hepática, e melhorou a taxa de sobrevivência dos transplantados de 50 para 100% após 14 dias do transplante. Além disso, verificámos que, à semelhança das interacções FN- $\alpha 4\beta 1$, as interacções FN- $\alpha 5\beta 1$ também induzem a expressão de MMP-9 e contribuem significativamente para a lesão e morte celular num modelo de lesão de I/R em fígado esteatosico. A investigação desenvolvida no nosso laboratório mostrou que a MMP-9 é um mediador crítico de infiltração leucocitária durante a lesão de I/R hepática, e a sua inibição específica leva a uma profunda melhoria da lesão hepática provocada pelo insulto de I/R. Deste modo, o

passo seguinte consistiu em utilizar ratinhos TIMP-1^{-/-} num modelo de 70% lesão I/R hepática morna para estudar o papel do inibidor endógeno da MMP-9, TIMP-1. Nesta fase mostrámos que a ausência de TIMP-1 resultou num aumento significativo da actividade da MMP-9 e da infiltração de leucócitos mediado pela MMP-9. Consequentemente, verificámos uma severa deterioração da função hepática e um aumento de morte hepatocelular e pela primeira vez mostramos que a ausência da TIMP-1 torna o fígado incapaz de recuperar e regenerar depois da lesão de I/R hepática, resultando numa lesão letal. Finalmente, o nosso grupo, bem como outros, já demonstraram que uma elevada expressão de iNOS está associada a danos hepáticos durante a lesão de I/R hepática. Através da utilização de ratinhos iNOS^{-/-}, bem como de ratinhos tratados com um inibidor específico para iNOS, ONO-1714, demonstrámos que a expressão de iNOS promove a activação de MMP-9 e consequente infiltração de leucócitos que por sua vez se traduz num agravamento da lesão e numa deterioração da função hepática.

ABBREVIATION LIST

ALT - Alanine aminotransferase
AST - Aspartate aminotransferase
ATP - Adenosine triphosphate
BrdU - 5'-bromodeoxyuridine
CS-1 - Connecting segment-1
COX-2 - cyclooxygenase-2
cFN - Cellular fibronectin
DNA - Deoxyribonucleic acid
ECM - Extracellular matrix
EGF - Epidermal growth factor
FN - Fibronectin
GPCR - G protein-coupled receptor
HGF - Hepatocyte growth factor
HSC - Hepatic stellate cell
iNOS - Inducible nitric oxide synthase
ICAM - Intracellular adhesion molecule
IFN - Interferon
IL - Interleukin
I/R - Ischemia/Reperfusion
IRI - Ischemia reperfusion injury
KC - Kupffer cell
KO - Knockout
LFA-1 - Lymphocyte function-associated antigen 1
LPS - Lipopolysaccharide
NADPH - Nicotinamide adenine dinucleotide phosphate
NK - Natural Killer
MAPK - mitogen-activated protein kinase
mRNA - messenger ribonucleic acid
MIP - Macrophage inflammatory protein
MMP - Matrix metalloproteinase
MPO - myeloperoxidase
MT-MMP - membrane type matrix metalloproteinase
NO - Nitric Oxide
PCNA - proliferating cell nuclear antigen
pFN - Plasma fibronectin
RANTES - Regulated upon Activation, Normal T-cell Expressed, and Secreted
RNA - Ribonucleic acid
RNS - Reactive nitrogen species
ROS - Reactive oxygen species
TIMP - Tissue inhibitor of metalloproteinases
TNF - Tumor necrosis factor
UNOS - United network of organ sharing
VCAM - Vascular cell adhesion molecule
VLA-4 - Very late antigen-4
WT - Wild type

CHAPTER I

GENERAL INTRODUCTION

Over 40 years have passed since Dr. Thomas E. Starzl and his team of surgeons in Denver, Colorado, USA, performed the first liver transplant on a 3-year-old pediatric patient in 1963 [1]. This first case and a few more over the ensuing years were unsuccessful and, it was not until 1967 that Dr. Starzl's team performed the first successful liver transplants with recipient survival of one year [2, 3]. Despite the establishment of a few more liver transplant programs and the development of improved surgical techniques over the next decade, the recipient one-year survival rate endured at approximately 25% and liver transplantation remained as an experimental procedure [4]. Finally, in the 1980's, two landmark advancements in the field of transplantation aided the establishment of liver transplantation as a standard clinical treatment [5, 6]. In the early 1980's, Sir Roy Calne from Cambridge, UK, introduced the immunosuppressive drug cyclosporine that contributed to a significant improvement in prolonged recipient survival. In 1987 Folkert Belzer of the University of Wisconsin introduced a new organ preservation solution, named the UW solution, that improved ex vivo organ preservation for longer periods of times and consequently recipient outcomes in liver transplantation [7]. Since then, liver transplantation has progressed into a thriving clinical field and stands as the only effective treatment for patients with end stage liver disease or rare genetic disorders originating in hepatocytes [8]. Currently, in the United States of America alone, and according to data published by UNOS, there are about 5000 liver transplants performed every year [9]. Established in 1984 by Dr Ronald Busuttil, the Dumont-UCLA liver transplant center, where the work of this PhD thesis was carried out, is currently the most active liver transplant program in the world, having reached in September of 2010 the milestone 5000th liver transplant. As for liver transplantation in Portugal, according to the 2010 activity report of the Autoridade para os Serviços de Sangue e da Transplantação, a total of 3,074 liver transplants have been performed since the first transplant in 1988, with an average of 253 a year in the recent 5 year period spanning between 2006 and 2010 [10]. Despite the remarkable advancements, liver transplantation continues to have multiple obstacles that continue to contribute to graft dysfunction and patient death. In the initial period after transplantation, infections, acute rejection and ischemia reperfusion injury lead to primary graft dysfunction and contribute to the persisting one-year recipient mortality rates of 10-15% [11, 12]. In the long term, liver transplant recipients face issues like chronic rejection, chronic renal failure due to life-long use of immunosuppressors, and disease recurrence [12].

The optimization of liver transplantation and its outcomes has driven an increase in referrals for liver transplantation and demand for donor livers that is far higher than the number of available donor organs [13]. This sharp disparity results in the death of thousands of patients every year while on the waiting list [14]. Therefore, the need to

expand the donor population has impelled clinicians to increase the use of marginal donor livers that are normally discarded [15]. Prolonged ischemia, elevated steatosis and livers from non-heart-beating or elderly donors are a few background marginal donor conditions that contribute significantly to the etiology of liver ischemia reperfusion injury and therefore, correlate with a higher incidence of early liver dysfunction [16, 17].

Ischemia reperfusion (I/R) injury is a pathophysiological event characterized by the initial restriction of blood flow to an organ followed by the restoration of blood flow and reoxygenation of the tissue [18]. In addition to the hypoxic insult triggered by ischemia, reperfusion is associated with the exacerbation of tissue injury and an intense inflammatory response (reperfusion injury) [19].

Pathophysiology of Hepatic I/R injury

Liver I/R injury occurs frequently in a number of clinical settings that span from surgical procedures to hepatic pathologies where blood flow to the liver is partially or completely impeded. Importantly, liver I/R injury is inherent to liver transplantation due to the complete deprivation of blood flow during organ procurement. It is considered to be one of the major problems post liver transplantation where it contributes significantly to poorer graft outcomes and is the cause of up to 20% of primary graft dysfunctions [20-22]. According to the nature of the event, I/R injury can be classified as warm or cold I/R injury in a reference to the temperature at which ischemia occurs [18, 23]. While warm I/R injury occurs when ischemia takes place at regular body temperature, cold ischemia prevails during organ preservation in the transplantation setting where the liver is stored *ex vivo* in preservation solution at 4°C for an extended period of time. Both categories of I/R injury share a multitude of mechanisms but also display some noteworthy differences between them, such as more prominent endothelium injury in cold I/R injury and intense hepatocyte injury in warm I/R injury [18, 24]. Hepatic I/R injury is an antigen-independent event characterized by interplay between many complex pathways, involving numerous cell types, multiple injury response mechanisms and a wide range of inflammatory mediators that ultimately lead to significant cellular death, tissue damage and decline of liver function [25] [26].

The cellular mechanisms mediating hepatic I/R injury can be divided into two distinct, however strongly interconnected, phases; the ischemic phase and the reperfusion phase [18]. During the ischemic phase, hypoxic insult of the parenchymal and non-parenchymal O₂-dependent cells results in a shortage of nutrients and oxygen, anaerobic glycogen consumption, decreased oxidative phosphorylation, and finally a severe depletion of the cellular ATP [27, 28]. Subsequently, there are major disruptions of the cellular ion

homeostasis, H^+ is released from damaged lysosomes and hydrolases are activated while there is a loss of mitochondrial membrane potential, an osmotic cell swelling and finally cell membrane disruptions that increase cell membrane permeability. This interconnected network of events leads to an enhanced cellular injury and Kupffer cell activation [18, 29].

The restoration of blood flow to the liver initiates the reperfusion phase of injury. In the initial stages of this phase, there is a burst in the production of reactive oxygen species (ROS) by injured hepatocytes, endothelial cells and activated Kupffer cells. ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), superoxide radical ($O_2^\cdot$), and hypochlorous (HClO), are generated in these cells by the xanthine oxidase, NADPH oxidase and mitochondrial respiratory chain systems [30-34]. Another important component of reperfusion injury is the nitric oxide (NO) system [35]. NO is the product of the conversion of the L-arginine amino acid to L-citrulline by the intracellular nitric oxide synthase enzymes, eNOS, nNOS and iNOS. While the first two are constitutively expressed, iNOS is the inducible form that is normally expressed in inflammation. In hepatic I/R injury, the pro-inflammatory induction of iNOS expression leads to elevated levels of NO. NO can react with molecular oxygen or superoxide to generate potent damage inflicting reactive nitrogen species (RNS) such as, nitrogen dioxide (NO_2), peroxynitrate ($ONOO^\cdot$) and dinitrogen trioxide (N_2O_3) [36]. Altogether, the excessive ROS and RNS oxidize cell membrane lipids, damage respiratory chain enzyme complexes, oxidize proteases and their inhibitors, and damage DNA and RNA, further contributing to overall cellular damage resulting in apoptotic or necrotic cell death that leads to intense tissue injury [33, 34, 37].

With the reestablishment of the blood flow, kupffer cells are further activated and undergo morphological changes that allow them to extend in the sinusoidal lumen and contribute to the reduction of hepatic microcirculation during reperfusion [27, 38]. They also fuel the propagation of cellular injury and trigger the inflammatory response, by producing pro-inflammatory cytokines, orchestrating hepatocytes and endothelial cells to produce chemokines, and activating the endothelium expression of adhesion molecules [37][39, 40]. Indeed, cytokines like $TNF-\alpha$, IL-1, IL-12, IL-6 and $IFN-\gamma$ act both locally and systemically to promote further production of chemokines and expression of adhesion molecules on both leukocytes and endothelial cells, which prompts leukocyte recruitment and infiltration to sites of injury [41]. Once recruited to the liver, infiltrating inflammatory leukocytes play a central role in the post-ischemic reperfusion injury, damaging the hepatic parenchymal cells via the production and release of proteases, ROS, RNS, cytokines and multiple other inflammatory mediators [42-44].

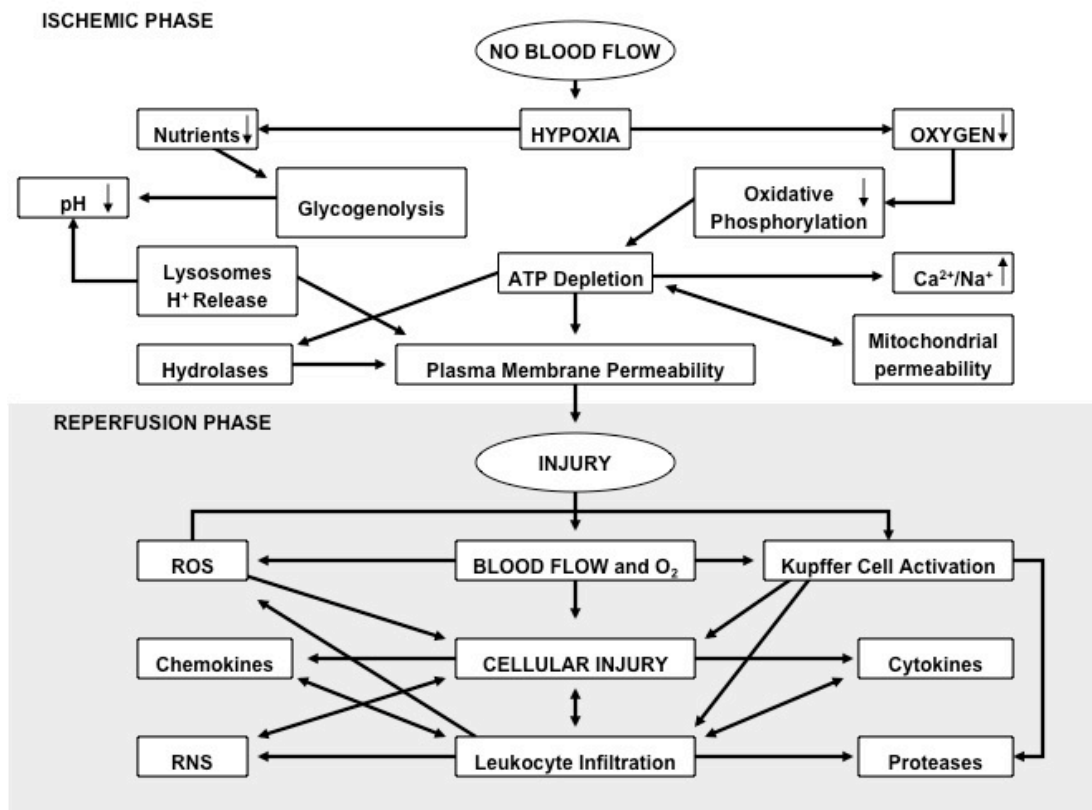


Figure 1 – Representation of some of the complex mechanisms of hepatic I/R injury in the ischemic and reperfusion phases. Cellular injury generated due to lack of oxygen and nutrients is aggravated with reperfusion and the onset of the inflammatory response, where leukocytes are critical players. ROS, reactive oxygen species; RNS, reactive nitrogen species.

Despite extensive research achievements and a broader understanding of the underlying mechanisms of hepatic I/R injury, a void remains for potential therapies that are successful in the clinical setting. Therefore, it is essential to maintain efforts to further unveil the mechanisms of hepatic I/R injury. Due to their broad role and significant impact in the pathogenesis of hepatic I/R injury, we are particularly interested in further understanding the specific mechanisms involving leukocytes during this inflammatory process.

Leukocytes

Soon after the onset of reperfusion, cells of the innate immune system begin to be recruited from the bloodstream to the sites of tissue inflammation in the liver [44]. During this process, leukocytes promote collateral tissue damage and contribute to further pathological activation of inflammation [40, 45]. Neutrophils are considered to be the first leukocytes to infiltrate and accumulate in the liver during hepatic I/R injury [46]. They

generate added tissue damage via proteolytic and oxidant-mediated mechanisms. Once activated, neutrophils release granules with a plethora of damage inflicting proteases, (matrix metalloproteinases, elastase, cathepsin G and heparanase) and hydrolytic enzymes [47]. These proteases can act individually or in a concerted effort. While matrix metalloproteinases promote neutrophil infiltration and migration by degradation of the basement membrane and extracellular matrix, elastase and cathepsin G attack and damage constituents of the hepatocyte and endothelial cell membrane [48][49]. Moreover, proteases can also activate many inflammatory mediators and their receptors, either by direct proteolytic cleavage or by release from their surrounding extracellular matrix [50-52]. Recently, a study by Uchida and colleagues demonstrated that inhibition of neutrophil elastase resulted in decreased hepatic I/R injury [53]. Activated neutrophils are also a major source of ROS throughout hepatic I/R injury [37]. Neutrophil-derived ROS are predominantly produced via the active NADPH oxidase pathway and released into the parenchyma where they are converted into multiple other forms [46]. Additionally, neutrophils also express and release high levels of myeloperoxidase, an enzyme that is stored in large amounts in azurophilic granules and catalyzes redox reactions that generate potent oxidants, such as hypochlorous acid (HOCl) [54]. Interestingly, there is a growing body of evidence that suggest that ROS have the particular ability to inactivate, via oxidation, endogenously expressed anti-proteases, which inhibit the activity of the leukocyte expressed proteases [42, 55, 56]. Thus, neutrophil production of ROS confers a degree of protection to the neutrophil derived proteases and promotes their activity in the vicinity of the cell.

There is growing evidence that T-lymphocytes play a significant role in liver I/R injury, although the mechanisms by which this occurs remain to be entirely understood. Current data appears to indicate that resident T-cell subsets in the liver, activated by hypoxia, can modulate the inflammatory response by expressing cytokines, such as IFN- γ , IL-4 and IL-17, and chemokines, such as RANTES and MIP-2. These mediators may then contribute to the rapid recruitment and accumulation of additional T-lymphocytes within 1h after reperfusion as shown in a study by Zwacka and coworkers [57]. This study and others that followed also showed that depletion of T-cells was able to reduce neutrophil recruitment and decrease liver injury [57]. Moreover, CD8 depletion had absolutely no effect on the outcome of hepatic I/R injury [57]. In contrast, CD4^{-/-} mice, when compared to WT mice, had significantly less liver injury and improved sinusoidal perfusion, suggesting a role for CD4⁺ T-cells in the pathogenesis of hepatic I/R injury [58]. This was supported by a second study proposing that CD4⁺ cells are rapidly recruited to the liver and facilitate neutrophil recruitment by expressing the cytokine IL-17. However, questions remain, since

they also report that these same CD4⁺ T-cells are important in reducing neutrophil activation [59].

Leukocyte migration in hepatic I/R injury

Inflammatory leukocyte migration from the vasculature to sites of injury is a key multi-step process that is the hallmark of inflammation. In hepatic I/R injury, the accumulation of leukocytes in the liver significantly enhances tissue damage. A remarkable part of the leukocyte-mediated injury in the liver occurs during leukocyte migration to and at the sites of inflammatory stimulus. Despite the significance of this phenomenon, to date, little is known about the mechanisms of leukocyte recruitment to inflammatory sites in liver. Continued efforts to better understand these processes could provide us with useful targets for the development of therapies to minimize their detrimental effects. Thus, in our group we are greatly invested in further understanding how leukocyte migration is regulated in hepatic I/R injury.

In the general inflammatory setting, leukocytes, in response to proinflammatory mediators, tether and roll on inflamed endothelial cells [60]. Subsequently, leukocytes express integrins that promote their firm adhesion to the vasculature by interacting with multiple adhesion molecules expressed on the cytokine activated endothelial cells [60]. Finally, leukocyte migration across the endothelial and extracellular matrix barriers involves complex cascades of adhesive and focal matrix degradation events [61]. This process is referred to as transmigration and is tightly regulated by matrix metalloproteinases [60, 61]. However, the liver has a remarkably complex vascular bed that is dominated by minute sinusoidal vessels with slow flow rates [62, 63]. Therefore, it is becoming increasingly apparent that leukocyte recruitment and migration in the liver may require some distinctive adhesive and de-adhesive mechanisms as compared with other organs [61]. Next, we refer to some of the current knowledge and debate on the mechanisms involved in the promotion of leukocyte migration during hepatic I/R injury.

Selectins

The initial step of leukocyte recruitment in inflammation is leukocyte tethering and rolling along the vessel wall [64]. This step is normally mediated by selectins, a family of adhesion molecules, composed of three calcium-dependent type I transmembrane glycoproteins [65]. Members of the family are similar in structure, with an N-terminal lectin like domain, and EGF-like domain, a variable number of consensus repeats (CRs), a

transmembrane domain, and a short cytoplasmic tail [66]. P-selectin and E-selectin are expressed by endothelial cells that have been stimulated by cytokines, ROS stress, activated complement products and shear stress [61, 67]. L-selectin is expressed activated leukocytes [66, 67]. It is well established that selectins initiate leukocyte-endothelial contact by interacting with P-selectin glycoprotein ligand (PSGL-1) and several other carbohydrate ligands expressed on leukocytes [65, 68]. However, the role of selectins in leukocyte recruitment in liver I/R injury is currently one of much debate and remains to be fully understood. Multiple studies have shown that selectin blockade was beneficial to liver I/R injury outcomes [69-71]. On the other hand, several other studies, including studies applying intravital microscopy, P-selectin and E-selectin deficient mice, have shown that selectin mediated tethering and rolling is not essential to sinusoidal leukocyte recruitment in the inflamed liver [63, 72, 73]. Instead these studies propose that leukocytes migrating in the tight sinusoidal vasculature at reduced speed simply undergo integrin-mediated adhesion to the endothelium [61, 62]. Furthermore, P-selectin blockade alone did not offer liver protection from hepatic I/R injury [74]. Hence, additional studies are necessary to better understand the actual contribution of selectins to leukocyte recruitment in hepatic I/R injury.

Chemokines

Chemokines are a large group of heparin-binding proteins with chemotactic properties that play a key role in the orchestration of the immune system by creating gradients that drive the migration and activation of leukocytes [60]. Leukocyte exposure to chemokines is important for the activation of leukocyte integrins that then mediate their firm adhesion to the inflamed endothelium [61]. Moreover, chemokines also regulate integrin avidity by modifying their affinity (conformational changes) and valency (integrin clustering) [75, 76]. Currently, they are grouped in to 4 different families that are characterized by the number of aminoacids (X) between the N-terminal cysteine residues: the CC, the CXC, the CX3C, and the C families [77]. They bind with high affinity to specific GPCRs expressed by different immune cells. Expressed mainly by activated kupffer cells and platelets, injured hepatocytes and endothelial cells, chemokines, have been implicated in a large number of different liver pathologies [77]. Multiple CXC chemokines have been implicated in hepatic I/R injury [44]. CXCL-1 (MIP-1), CXCL-2 (MIP-2) (murine homologues of human IL-8) and CINC are potent neutrophil chemoattractants expressed during the early stages of liver I/R injury [78, 79]. Studies performed by our group have shown that reduced expression of CXCL-2 was associated with a diminished neutrophil infiltration [80, 81]. Others have also shown that CXCR-3 and its ligands CXCL-9, CXCL-10 and CXCL-11 can mediate

activated T-lymphocyte and NK-cell recruitment in inflamed livers [82]. CC chemokines have also been implicated in hepatic inflammation. RANTES (CCL5) is a CC T-cell specific chemokine involved in regulating the swift T-cell recruitment observed in the first hours of reperfusion [83]. Kupffer cells, hepatocytes and hepatic stellate cells secrete CCL-2 (MCP-1) to promote monocyte and macrophage recruitment [84]. CCL-2's intense expression in liver I/R injury correlates with higher levels of liver injury (AST levels) in orthotopic liver transplant patients [84]. Interestingly, pro-forms of chemokines can be found bound to the liver extracellular matrix. Upon their release and activation by infiltrating leukocytes, they can engage these cells and establish additional chemotactic gradients [40]. The study of chemokines in hepatic I/R injury has grown over the years, however as we uncover some answers and mechanisms the roles of the multiple chemokines becomes increasingly complex and more questions arise leaving us with much to be discovered.

Integrins

Once a leukocyte has tethered, rolled, reduced its speed and been activated by chemokines and cytokines in the blood vessel, it must adhere to the endothelium before it can transmigrate through the vasculature and basement membrane [60]. Adhesion to the endothelium is a crucial step of leukocyte recruitment mediated by integrins. Integrins are $\alpha\beta$ transmembrane cell-surface receptors that mediate cell-cell and cell-extracellular matrix (ECM) adhesion [85]. Each integrin consists of non-covalently linked α and β subunits [86]. In total there are 18 α and 8 β subunits, giving rise to 24 distinct integrin molecules which all differ in substrate specificity [85]. Integrin subunits are composed of an extracellular domain, a single membrane-spanning domain, and a short cytoplasmic tail [86].

Due to their abundant expression on leukocytes, $\beta 1$ and $\beta 2$ integrins are the most relevant to leukocyte adhesion to the vasculature. Their importance in leukocyte migration has been well documented by the profound effects of their deficiency on the immune response [87-89]. Of these integrins, by far the most well studied have been the VLA-4 or $\alpha 4\beta 1$ integrin, the LFA-1 or $\alpha L\beta 2$ integrin and the Mac-1 or $\alpha M\beta 2$ integrin (CD11b/CD18) [90]. Leukocyte integrins interact with immunoglobulin superfamily members like intercellular adhesion molecule (ICAM 1-5), and vascular cell adhesion molecule (VCAM) expressed on the cytokine activated endothelial cells during inflammation. The $\beta 2$ integrin LFA-1, expressed on neutrophils, NK cells and T-cells, interacts with several ICAMs (1-4), especially ICAM-1, which is constitutively expressed on the hepatic vascular endothelium

[61]. Neutrophil expression of LFA-1 is up-regulated during the early stages of hepatic I/R injury, enabling neutrophils to firmly adhere and accumulate on the liver sinusoidal endothelium by binding to ICAM-1 [46, 47]. However, anti-ICAM-1 antibodies have proved to only moderately, or not at all, benefit the outcome of hepatic I/R injury [91]. $\alpha 4\beta 1$ integrin is the major $\beta 1$ integrin expressed on inflammatory leukocytes that mediates their adhesion and recruitment to sites of injury. The most widely studied ligand for $\alpha 4\beta 1$ in leukocyte recruitment is VCAM-1. Studies have shown that chemokine induced, high affinity $\alpha 4\beta 1$ integrin expression is required for VCAM-1 mediated firm adhesion on endothelial cells [92]. Other studies show that low affinity $\alpha 4\beta 1$ integrin can also mediate rolling on the endothelium by transient adhesive interactions with VCAM-1 [93, 94]. In addition, studies successfully applying anti- $\alpha 4$ antibody therapies have shown that there are multiple $\alpha 4\beta 1$ integrin dependent adhesion pathways in the pathogenesis of inflammatory pathologies [95-98]. Integrins also interact strongly with ECM molecules. Over recent years, evidence has accumulated of fibronectin (FN) as a key ECM molecule mediating leukocyte adhesion events in inflammatory processes [99]. FN promotes leukocyte firm adhesion through interactions with $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins, its 2 major receptors on leukocytes, which can bind to the connecting segment-1 (CS-1), located within the V region of FN, and the RGD sequence on the tenth type III repeats of FN, respectively [100][101]. Interestingly, studies that blocked both $\alpha 4\beta 1$ -FN(CS-1) and $\alpha 4\beta 1$ -VCAM-1 interactions, have shown that, cellular fibronectin (cFN) is the more relevant $\alpha 4\beta 1$ ligand on inflammatory stimulated endothelial cells [102]. Moreover, a number of studies by our group applying CS-1 and cyclic RGD blocking peptides have demonstrated that cFN- $\alpha 4\beta 1$ and cFN- $\alpha 5\beta 1$ interactions regulate leukocyte recruitment and migration during hepatic I/R injury in steatotic liver transplant recipients [103-106].

Extracellular matrix in liver I/R injury

Throughout their migration from blood stream to sites of inflammatory injury, leukocytes encounter and interact with multiple extracellular matrix (ECM) proteins that are expressed by the inflamed endothelium, compose the basement membrane and the interstitial matrix of intracellular spaces. Hence, it is not surprising that the individual components of ECM as well as its three-dimensional ultrastructure and biophysical properties can actively modulate the outcome of the inflammatory response at various levels [107]. Indeed, there is a growing field of evidence that leukocyte-ECM interactions can influence immune cell activation, migration, proliferation, and differentiation processes. Moreover, the immunological outcome of these interactions depends greatly

on the specific microenvironment in which they occur [107, 108]. The ECM is a complex network of elaborate carbohydrates and proteins that serves as a structural scaffold to support tissue integrity and cell adhesion [109]. It also serves as a lattice that enables cells to move [110]. The major components of the ECM are collagens (types I-V), fibronectin (FN), laminin, hyaluronan (HA), proteoglycans, and nidogen [109, 111]. Most ECM proteins are large proteins with distinct conserved structural domains that are glycosylated and frequently contain negatively charged sulphated glycosaminoglycan chains [109, 112]. The overall negative charge of the ECM, also enables it to serve as a reservoir for interactions with a multitude of chemokines, growth factors, and cytokines [113]. ECM proteins promote strong cell adhesion due to their potential to bind, via conserved sequences in their structural domains, directly with cell surface receptors such as integrins. At these sites of cell-matrix adhesions, signals transmitted through the integrins can promote cell survival, migration and proliferation [114]. In inflammatory responses, integrin mediated leukocyte firm adhesion to the ECM is critical to leukocyte migration and integrity. The mechanisms by which ECM proteins mediate leukocyte recruitment, extravasation/transmigration and migration through the basement membrane during inflammation are still poorly understood [107]. However, some ECM proteins, such as fibronectin and tenascin (TNC), have been implicated in leukocyte adhesion and migration through endothelial and ECM protein barriers.

Fibronectin

Of the many well-characterized ECM components, fibronectin is probably the most prominent and extensively studied protein in leukocyte-ECM interactions, specifically in organ transplantation where its expression in the vasculature is considered to be an adhesive factor that triggers leukocyte recruitment and an immune cascade that leads to allograft rejection [99, 115]. Functional fibronectin is a large dimeric glycoprotein with similar subunits of approximately 220-250 KDa, bound by 2 disulphide bonds near their carboxyl-termini. Each monomer is composed of a series of independently folding modular domains known as FN repeats I, II, III [116]. Contained in these repeating modular domains are the domains that control the protein assembly and mediate the cellular functions of FN [115]. Considerable FN structural diversity originates from the complex and regulated splicing of the FN primary transcript in three segments referred to as EIIIA, EIIB and V in rats (ED-A, ED-B and IIICS in Humans) [99, 117]. Multiple FN mRNA's and consequently multiple FN protein isoforms arise from this alternative splicing. In humans there are up to 20 possible isoforms (12 in mice and rats) [118]. Whereas the EIIIA and EIIB domains are either included or excluded in the protein, the V domain is completely

included or partially included or totally excluded. The totally included form contains an important integrin binding region/sequence termed the connecting segment – 1 (CS-1), essential to leukocyte adhesion [115].

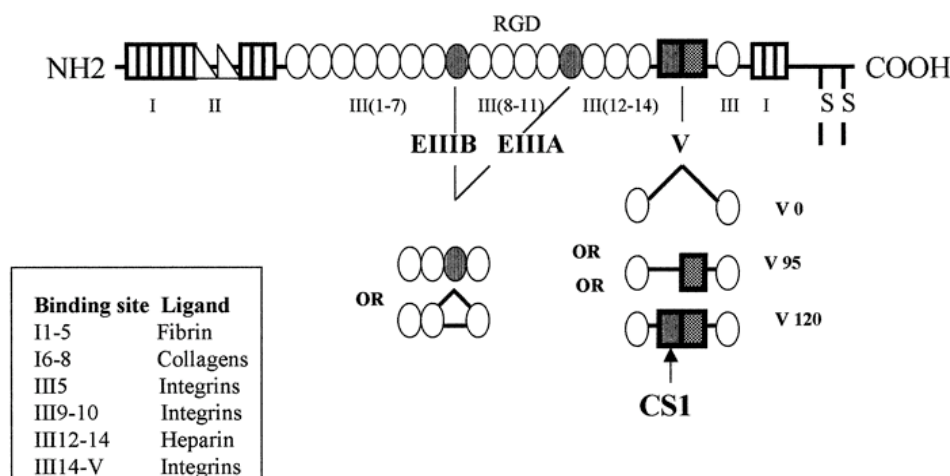


Figure 2 - Schematic diagram of the primary structure of monomeric fibronectin and its variants illustrating the 3 alternatively spliced domains designated EIIIA, EIIIB, and V and the independently folding modular domains known as FN repeats I (rectangles), II (triangles), and III (ovals). The diagram also illustrates the RGD cell binding domain and the cell adhesive segment CS1 that interact with the $\alpha 4\beta 1$ and $\alpha 4\beta 1$ integrins respectively. Figure adopted from Coito, A.J. *et al.* Extracellular matrix proteins in organ transplantation. *Transplantation* **69**, 2465-2473 (2000) [115].

The FN molecule has multiple sites for interactions with a wide array of other proteins, such as cell surface receptors, ECM proteins, growth factors, cytokines and complement components [118]. Integrins are the major cell surface receptors for FN [119]. Of the 12 different integrins that can bind to FN, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ are the main mediators of leukocyte adhesion to FN [120]. Leukocytes expressing these 2 members of the $\beta 1$ integrin family recognize and bind to specific sequences within the FN type III repeats [89, 121, 122]. The $\alpha 5\beta 1$ integrin can recognize the well-known RGD binding sequence, located on a flexible and exposed loop region connecting two β strands in the 10th FN type III repeat [123]. It can also recognize the PHSRN synergy sequence located in the adjacent 9th type III repeat, which contributes to more robust cell adhesion in humans [118, 124]. The preferred binding site for $\alpha 4\beta 1$ integrin is the CS1 sequence located within the V region of FN. However, $\alpha 4\beta 1$ also has the ability to interact with the RGD sequence, the KLDAPT sequence and the PEDGIHELFP sequence on the EIIIA domain [100, 125]. Therefore, these binding sites allow leukocytes to firmly adhere to the ECM, during an inflammatory response. *In vitro* experiments have determined that $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins mediate

macrophage adhesion and migration on FN while inducing the expression of inflammatory cytokines and matrix metalloproteinases [126]. The importance of these FN-leukocyte interactions in cell migration has been further emphasized *in vivo* where antibody blockade of the CS1- $\alpha 4\beta 1$ interactions in macrophages was able to ameliorate atherosclerosis [127]. Additionally, $\alpha 5\beta 1$ integrins can mediate neutrophil adhesion to FN and promote, along with $\alpha 4\beta 1$ integrins, neutrophil recruitment in LPS-induced lung inflammation [128, 129]. Similar studies have also implicated these interactions in lymphocyte adhesion and migration [130-132].

In general, there are two forms of fibronectin; 1) Plasma fibronectin (pFN) does not include EIIIA and EIIIB; 2) cellular fibronectin (cFN) protein includes both EIIIA and EIIIB domains [118]. While pFN is constitutively expressed by hepatocytes in adults and circulates in the blood, cFN expression is virtually absent in adults under normal conditions and solely detected during embryogenesis and upon the onset of certain pathological conditions [133]. Induction of cFN expression has been abundantly detected in psoriasis, rheumatoid arthritis, and liver fibrosis among many others [134-136]. In organ transplantation, cFN expression has been markedly detected in the vasculature of cardiac and hepatic grafts during the early post transplantation period [99, 103, 137]. These findings, associated with the importance of inflammatory leukocyte recruitment in ischemia reperfusion injury and graft rejection, suggested that FN play a crucial role in the leukocyte activation, adhesion and migration to the transplanted organ. Indeed, Coito and coworkers first showed this with elegant studies in cardiac allografts, where blockade of $\alpha 4\beta 1$ -cFN interactions, via a peptide sequence that mimics the role of the CS-1 sequence, was able to suppress mononuclear cell infiltration, endothelium activation and cytokine expression in cardiac allograft rejection [138-140]. In recent years, under the guidance of Dr. Coito, our group has extended this concept to the liver and shown that cFN has an essential role in leukocyte recruitment during ischemia reperfusion injury. In several published studies, CS-1 peptide therapy significantly improved the outcome of hepatic I/R injury in steatotic liver transplant recipients by reducing leukocyte recruitment, leukocyte MMP-9 expression and cytokine, iNOS and COX-2 expression [103-105]. Moreover, the overall outcome of rat steatotic liver transplant recipients was also improved by blockade of the FN- $\alpha 5\beta 1$ integrin interactions via treatment with cyclic RGD peptides [106].

Matrix Metalloproteinases in liver I/R injury

Upon integrin-mediated adhesion to the inflamed endothelium, leukocyte migration across the endothelium and ECM to sites of tissue injury requires a coordinated succession of adhesion release steps and focal matrix degradation events [61]. These events are tightly regulated by matrix metalloproteinases (MMPs) and result in profound ECM turnover and rearrangement, which can severely impact the outcome of the inflammatory process [49, 51].

Matrix metalloproteinases (MMPs) are a family of 24 zinc-dependent proteases renowned for their ability to cleave and degrade ECM proteins [141]. Since the description of the first MMP more than 40 years ago, it has become increasingly clear that MMPs are essential players in defining how a cell responds to its surrounding microenvironment, consequently participating in a wide variety of physiological and pathological processes such as tissue remodeling, embryogenesis and tumor metastasis [142, 143]. All members of the MMP family possess a conserved pro-domain, a catalytic domain in which a Zinc ion (Zn^{2+}) is ligated to 3 conserved histidine residues, a flexible proline rich hinge region, and a carboxy C-terminal hemopexin like domain that mediates substrate recognition [144]. Overall, the MMP family is subdivided into several different subclasses based mainly on substrate specificity or protein structure: (i) collagenases, which have the ability to degrade fibrillar collagens; (ii) gelatinases which degrade gelatin (denatured collagen) type IV and V collagen, fibronectin and elastin; (iii) stromelysins, which have a broad substrate specificity but don't degrade triple helical regions of interstitial collagens; (iv) membrane-type MMPs (MT-MMPs), which are anchored to the cell membrane due to extra their transmembrane and cytoplasmic domains; (v) matrilysins, which lack the C-terminal hemopexin like domain [145, 146]. MMPs are expressed as inactive proenzymes (Zymogen) in which a propeptide domain, located at the N-termini of the protein, blocks the enzyme's active site (pro-MMP) by establishing a non-covalent bond between a conserved cysteine residue in the propeptide and the Zn^{2+} ion located in the catalytic domain of the MMP [147]. Activation requires breaking of this bond and consequent removal of the pro-peptide.

One of the major conclusions retrieved from a collection of inflammatory pathologies and their characteristics, was that MMPs are involved in the great majority of inflammatory pathologies known to date [144, 148]. Moreover, a deregulation or up-regulation of MMP expression levels is a feature of all inflammatory or inflammatory-related diseases [144]. Therefore it is understood that despite being essential to physiological processes such as remodeling, repair and host defense, the uncontrolled, inappropriate or exacerbated expression of MMPs has detrimental and injurious consequences [51].

Among the many MMPs, the gelatinase subclass is one of special interest, due to the well-studied role its members have in the promotion of leukocyte migration [61]. MMP-2 (gelatinase A) 72KDa and MMP-9 (gelatinase B) 92KDa, the sole members of the gelatinase subclass, are characterized by the presence of gelatin-binding domains, which resemble fibronectin domains and allow them to bind and cleave a diverse array of substrates, especially gelatin, fibronectin and type IV collagen. These substrates compose the initial ECM barriers that leukocytes must overcome once they begin to migrate into the tissue. While MMP-2 is expressed constitutively by a variety of cells, MMP-9 expression is inducible in leukocytes upon activation. Indeed, elevated levels of MMP-9 have been associated with increased neutrophil infiltration and airway obstruction in acute asthma exacerbations [149]. MMP-9 can also promote the blood brain barrier breakdown and leukocyte mediated neuronal injury after focal transient ischemia [150]. Moreover, gelatinases are associated with leukocyte migration in lung and kidney I/R injury, among others [151, 152]. Several studies have reported elevated MMP-2 and MMP-9 levels in the serum of human liver transplant recipients during acute rejection, indicating a possible role for gelatinases in hepatic ischemia reperfusion injury. Indeed, our group has confirmed that MMP-9 expression is significantly elevated after 6 hours steatotic liver transplantation in rats [105]. Furthermore, in a follow-up study in our lab, specific MMP-9 inhibition and MMP-9 gene deletion improved the outcome of hepatic I/R injury by significantly reducing infiltration of inflammatory macrophages and neutrophils, clearly demonstrating a critical role for MMP-9 in liver I/R injury [48]. Recently, a similar effect was also shown by MMP-9 inhibition in a model of small-for-size liver graft injury [153]. On the other hand, the role of MMP-2 in hepatic I/R injury seems to be more complex. Administration of a broad gelatinase inhibitor to mice did not produce as much protection, suggesting that MMP-2 might actually have, in opposition to MMP-9, a beneficial role in hepatic I/R injury [48]. In fact, two studies have attributed a protective role to MMP-2 in acute colitis and brain and spinal cord inflammation, while another, by McQuibban and colleagues, has shown that MMP-2 can dampen inflammation by cleaving the potent macrophage pro-inflammatory chemokine, MCP-3, into a peptide with strong anti-inflammatory properties [154][155].

Membrane-type metalloproteinases are a distinct subclass of MMPs tethered to the cell membrane via an extra transmembrane domain, which confers to them the exceptional ability to perform pericellular proteolysis and influence an individual cell's interactions with its immediate surrounding microenvironment. This is specially featured in MT-MMPs impact on cell migration, where they play an important role in the processing of focal cell-matrix adhesion sites and therefore promote cell movement through the endothelial and basement membrane barriers. The 6 members of the MT-MMP family (MT1-MMP to MT6-MMP) present a broad range of substrate specificity degrading multiple ECM proteins, of

which the most noteworthy are native collagen and fibronectin [156]. Among the members of this family, MT1-MMP was the first to be identified and is by far the most studied due to the significance of its collagenolytic activity. While animals deficient in all MMP family members do not present any drastic phenotype, MT1-MMP^{-/-} null mice present severe deficiencies during embryogenesis and in bone formation. Since its discovery, multiple studies have identified MT1-MMP as a critical promoter of cell invasiveness and migration [157]. Moreover, MT1-MMP has been implicated in various inflammatory pathologies including heart and brain I/R injury [158-163]. *In vitro* studies have determined that FN can up-regulate the expression MT1-MMP in multiple cell types and that MT1-MMP is capable of mediating human monocyte migration *in vitro* [126,161]. Finally, MT1-MMP plays a vital role in the 2-step MMP-2 activation mechanism [162]. At the level of the cell membrane, TIMP-2 binds to one inactive MT1-MMP and one pro-MMP-2 protein, bridging their interaction and allowing for a second, neighboring MT1-MMP molecule to proteolytically activate the briefly anchored MMP-2 [163]. During cell invasion or migration, MT1-MMP promotes a coordinated and cooperative pericellular proteolysis by denaturing collagen into gelatin that MMP-2 and MMP-9 can subsequently further digest [162]. All together, these studies provide support to the idea that MT1-MMP, via specialized pericellular proteolysis, may have an important contribution to leukocyte recruitment mechanisms in inflammation, especially in hepatic I/R injury.

As a family, the MMPs are consistently attributed the main role in ECM destruction, turnover and rearrangement. However, many studies applying MMP inhibitors and MMP gene knockdowns have revealed surprisingly opposing results and most MMP inhibitors have failed in clinical trials, consequently forcing a reconsideration and deeper investigation of MMP function in inflammation [144]. There is extensive evidence that besides their role in the development of pathologies, individual MMPs have roles in the response mechanisms to these pathologies and in normal physiological processes. As a result we now understand that a broader MMP action contributes significantly to a tightly regulated inflammatory response. Proteolytic cleavage of inflammatory cytokines and chemokines can activate, inactivate or reverse their inflammatory function and as a result contribute to either the promotion or repression of inflammation [51]. Degradation of cell membrane receptors or their ligands can profoundly impact cell survival, proliferation and differentiation. Indeed, while TNF- α and IL-1 β can be activated by MMP-2, MMP-3 and MMP-9, IL-1 β can also be degraded by MMP-3, and MMP-2 can convert the inflammatory MCP-3 to an anti-inflammatory peptide [155].

Whether it is in a physiological or a pathological process, uncontrolled or excessive MMP activity is deleterious and contributes to severe injury. Therefore, in order to maintain homeostasis, it is critical that MMP activity be tightly regulated and fine-tuned.

Throughout evolution, the regulation of MMP activity has become a complex process that occurs at several different levels, including the transcriptional, post-transcriptional and protein levels (Figure 4) [61, 163]. A biologically active MMP requires the active site and Zn^{2+} ion to be accessible to the substrate, which is attained by interruption of the Zn^{2+} -propeptide sulphydryl bond and subsequent removal of the propeptide [147]. This mechanism is commonly referred to as the “cysteine-switch” and it can be achieved either by proteolytic cleavage of the propeptide or by a redox reaction between the thiol cysteine group of the propetide and ROS or RNS [164-167]. One exceptionally important mechanism of MMP activity regulation is the binding of a family of natural occurring endogenous MMP inhibitors, called tissue inhibitors of metalloproteinases (TIMPs), to the active site of the MMP [168].

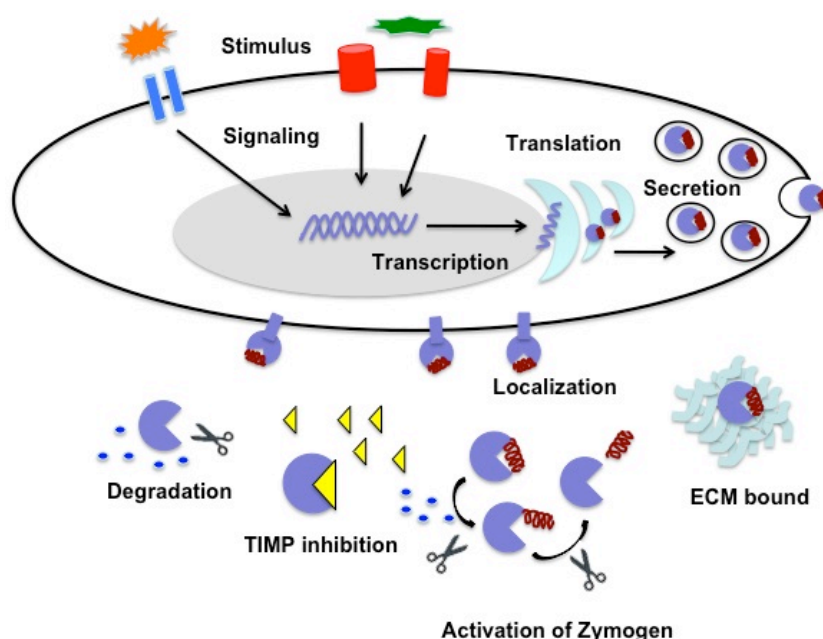


Figure 4 - MMP function can be regulated at many different levels as seen in the representation. MMP expression can be regulated by different stimulus via multiple signaling pathways and is then additionally regulated at the transcription and translational levels. Once the protein is formed, it can be regulated at the levels of: protein secretion; of cell membrane or extracellular localization; of zymogen activation (Cysteine Switch); of TIMP inhibition; and finally of protein degradation by multiple other proteases or oxidation. Figure adapted from figure in Page-McCaw A, *et al.* Matrix Metalloproteinases and the regulation of tissue remodeling. *Nature Reviews Molecular Cell Biology* 8, 221-233 (2007) [169].

Tissue Inhibitors of Metalloproteinases

The tissue inhibitor of metalloproteinase family consists of four distinct low molecular weight members, TIMP-1, -2, -3 and -4 [170]. TIMPs are composed of a N-terminal domain that forms high affinity bonds with the catalytic domain of the MMPs, inhibiting their activity, and a C-terminal domain that is responsible for multiple protein-protein

interactions [170]. Each TIMP has the ability to form tight binding, non-covalent inhibitory complexes with several members of the MMP family [168]. These stable 1:1 stoichiometric complexes are achieved through interactions between the Zn^{+2} of the MMP active site and the amino and carbonyl groups of the TIMP N-terminal cysteine residue [171]. However, the affinity that each TIMP binds with to a different MMP is variable [172]. TIMP-1, which can bind to the vast majority of MMPs, is most well known for its high affinity complex with MMP9 (both pro and active forms). TIMP-2 is well known for its ability to bind to and inhibit both MMP-2 and MT1-MMP. Moreover, TIMP-2 is a key component of the complex that promotes MT1-MMP mediated activation of MMP-2, by bringing them together at the cell membrane [173]. Although MMP expression is regulated at multiple levels, the ultimate control is achieved by means of this finely tuned MMP/TIMP balance. Therefore, TIMPs are endogenously expressed in an attempt to dampen processes such as leukocyte migration and control their deleterious tissue injury during inflammation. TIMP-1 is widely recognized for contributing significantly to the regulation of MMP-9-mediated leukocyte migration and subsequently inflammation [174]. Indeed, TIMP-1 has been shown to be an essential participant in several pathological settings like bleomycin induced acute lung injury, experimental autoimmune encephalomyelitis and focal cerebral ischemia [175-177]. In the liver, the majority of studies have focused on the role of TIMP-1 in several models of fibrosis and liver regeneration after hepatectomy [178-181]. In one recent study, the absence of TIMP-1 exacerbated carbon tetrachloride induced liver injury [182]. Interestingly, in liver transplantation, TIMP-1 has been detected in the serum of recipients [183]. It is also expressed in steatotic rat livers subject to cold liver I/R injury and mouse livers subject to warm liver I/R injury, in association with induced MMP-9 expression [105]. Furthermore, TIMP-1 expression is also present in mouse livers subject to warm liver I/R injury [48]. However, to date, studies on the role of TIMP-1 in liver I/R injury remain scarce or absent. Finally, TIMP-2 and TIMP-3 are expressed in the liver by kupffer cells and hepatocytes respectively [49, 184].

Just as with the MMPs, we now understand that individual TIMPs have other biological activities that extend their participation in physiological and pathological process beyond the initially characterized role of MMP inhibition. For example, TIMPs can regulate cellular division, survival (apoptosis), polarization and differentiation independently from their ability to inhibit MMPs [185, 186]. Notably, TIMP-1 has the ability to promote cell survival without direct participation of MMPs [187]. On the other hand TIMP-1 is also anti-apoptotic to hepatic stellate cells in an MMP-dependent mechanism [188]. It is unknown if TIMP-1 can play this sort of role in liver, influencing cell survival and proliferation, especially during and inflammatory response in the liver where there are elevated levels of cell death, and injury.

To fully understand the role of MMPs in leukocyte migration during hepatic I/R injury, it is essential to understand how their expression and activity is endogenously regulated. To that extent, a better understanding of their interactions with TIMPs in the ongoing inflammatory process is critical and could provide rationale for the development of more directed therapeutic approaches. Therefore, in this thesis we planned to focus some of our work on the role of TIMP-1 throughout the progression of hepatic I/R injury and understanding how its expression influences MMP-9 activity, leukocyte migration and the outcome of hepatic I/R injury.

Final Introductory Remarks

Hepatic Ischemia Reperfusion injury is a complex inflammatory event that implicates the participation of a wide variety of chemical, molecular and cellular mediators. Altogether it is an intricate network of mechanisms with many overlapping and synergistic effects. Of particular interest to us is the understanding of the mechanisms of leukocyte transmigration across the vascular endothelium and ECM barriers. Overall, activated leukocytes undergo a succession of firm adhesion and focal matrix degradation events to transmigrate through the endothelial and basement membrane towards the sites of injury. Fibronectin, expressed on the endothelium is likely a key extracellular matrix protein involved in the firm adhesion interactions with leukocyte $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins. Matrix metalloproteinases, especially gelatinases (MMP-2 & MMP-9) and membrane-type MMPs are important for focal matrix degradation. The outcome of MMP activity is greatly dependent on the endogenous regulation mediated by TIMPs. Therefore, with this in consideration, this PhD project was designed to further dissect the functions of fibronectin and relevant MMPs in hepatic I/R injury. The results presented here are divided into 4 different chapters. Earlier studies from our laboratory have demonstrated that cellular FN is up-regulated in the vascular endothelium after organ transplantation preceding leukocyte recruitment. Chapter II extends previous studies and shows that FN- $\alpha 4\beta 1$ interactions regulate leukocyte MMP-9 and MT1-MMP expressions, via the p38 MAPK signaling pathway in a 24-hour model of prolonged cold hepatic ischemia-reperfusion injury in rats. Moreover, we show that blockade of the FN- $\alpha 4\beta 1$ interactions with a CS-1 peptide, which mimics the CS-1 domain of FN and binds to the leukocyte $\alpha 4\beta 1$ integrin, significantly depressed MMP-9 and MMP-14 mediated leukocyte infiltration after transplantation. Consequently, it ameliorated hepatic liver injury and significantly improved the rat 14-day survival rate (50% vs. 100%). Chapter III presents a study on the role of FN- $\alpha 5\beta 1$ integrin blockade in a rat model of steatotic liver I/R injury. It demonstrates that FN- $\alpha 5\beta 1$ interactions can also induce MMP-9 expression, and significantly contribute to cell injury and cell death in this model. Our laboratory was the first to show that specifically targeting MMP-9 profoundly ameliorates tissue damage after liver I/R insult. Chapter IV provides a study on the role of tissue inhibitor of metalloproteinase-1 (TIMP-1), the endogenously expressed MMP-9 inhibitor, in a mouse model of 70% warm hepatic I/R injury. We show that TIMP-1 deficiency significantly increases MMP-9 activity and MMP-9 mediated leukocyte infiltration in the liver, severely deteriorating liver function and increasing hepatocellular death. Moreover, it provides novel findings on how the absence of TIMP-1 results in lethal I/R injury due to the inability of the liver to recover and

regenerate after hepatic I/R injury. We and others have shown that elevated iNOS expression levels are correlated to liver injury post-I/R. Chapter V dissects the functional significance of iNOS expression on MMP-9 activation in hepatic I/R injury. Finally Chapter VI will provide an integrated discussion of the results presented in the preceding chapters and their impact on the field of hepatic I/R injury and liver transplantation.

REFERENCES

1. Starzl, T.E., *et al.*, 1963. *Homotransplantation of the Liver in Humans*. Surg Gynecol Obstet. **117**: p. 659-76.
2. Starzl, T.E., *et al.*, 1968. *Orthotopic homotransplantation of the human liver*. Ann Surg. **168**(3): p. 392-415.
3. Starzl, T.E., *et al.*, 1968. *Extended survival in 3 cases of orthotopic homotransplantation of the human liver*. Surgery. **63**(4): p. 549-63.
4. Starzl, T.E., *et al.*, 1974. *Progress in and deterrents to orthotopic liver transplantation, with special reference to survival, resistance to hyperacute rejection, and biliary duct reconstruction*. Transplant Proc. **6**(4 Suppl 1): p. 129-39.
5. 1984. *National Institutes of Health Consensus Development Conference Statement: liver transplantation--June 20-23, 1983*. Hepatology. **4**(1 Suppl): p. 107S-110S.
6. 1984. *National Institutes of Health Consensus Development Conference on Liver Transplantation. Sponsored by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases and the National Institutes of Health Office of Medical Applications of Research*. Hepatology. **4**(1 Suppl): p. 1S-110S.
7. Southard, J.H. and F.O. Belzer, 1995. *Organ preservation*. Annu Rev Med. **46**: p. 235-47.
8. Mehrabi, A., *et al.*, 2008. *Current concepts in transplant surgery: liver transplantation today*. Langenbecks Arch Surg. **393**(3): p. 245-60.
9. UNOS and US Department of Health and Human Services. Organ Procurement and Transplantation Network Registry - <http://optn.transplant.hrsa.gov/latestData/rptData.asp>
10. Relatório Estatístico 2010 - Colheita e transplantação de órgãos, tecidos e células - Autoridade para os serviços de sangue e da transplantação.
11. Merion, R.M. *Current status and future of liver transplantation*. Semin Liver Dis. **30**(4): p. 411-21.
12. Adam, R. and E. Hoti, 2009. *Liver transplantation: the current situation*. Semin Liver Dis. **29**(1): p. 3-18.
13. Wertheim, J.A., *et al.* *Major challenges limiting liver transplantation in the United States*. Am J Transplant. **11**(9): p. 1773-84.
14. Selzner, M. and P.A. Clavien, 2001. *Fatty liver in liver transplantation and surgery*. Semin Liver Dis. **21**(1): p. 105-13.
15. Busuttil, R.W. and K. Tanaka, 2003. *The utility of marginal donors in liver transplantation*. Liver Transpl. **9**(7): p. 651-63.
16. Attia, M., M.A. Silva, and D.F. Mirza, 2008. *The marginal liver donor--an update*. Transpl Int. **21**(8): p. 713-24.
17. Briceno, J. and R. Ciria. *Early graft dysfunction after liver transplantation*. Transplant Proc. **42**(2): p. 631-3.
18. de Groot, H. and U. Rauen, 2007. *Ischemia-reperfusion injury: processes in pathogenetic networks: a review*. Transplant Proc. **39**(2): p. 481-4.
19. Ioannou, A., J. Dalle Lucca, and G.C. Tsokos. *Immunopathogenesis of ischemia/reperfusion-associated tissue damage*. Clin Immunol. **141**(1): p. 3-14.
20. de Rougemont, O., P. Dutkowski, and P.A. Clavien. *Biological modulation of liver ischemia-reperfusion injury*. Curr Opin Organ Transplant. **15**(2): p. 183-9.
21. Clavien, P.A., P.R. Harvey, and S.M. Strasberg, 1992. *Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies*. Transplantation. **53**(5): p. 957-78.
22. Nakano, H., *et al.*, 1997. *The effects of N-acetylcysteine and anti-intercellular adhesion molecule-1 monoclonal antibody against ischemia-reperfusion injury of*

- the rat steatotic liver produced by a choline-methionine-deficient diet.* Hepatology. **26**(3): p. 670-8.
23. Ikeda, T., et al., 1992. *Ischemic injury in liver transplantation: difference in injury sites between warm and cold ischemia in rats.* Hepatology. **16**(2): p. 454-61.
 24. Massip-Salcedo, M., et al., 2007. *The response of the hepatocyte to ischemia.* Liver Int. **27**(1): p. 6-16.
 25. Walsh, K.B., et al., 2009. *Inflammatory mediators of liver ischemia-reperfusion injury.* Exp Clin Transplant. **7**(2): p. 78-93.
 26. Fondevila, C., R.W. Busuttil, and J.W. Kupiec-Weglinski, 2003. *Hepatic ischemia/reperfusion injury--a fresh look.* Exp Mol Pathol. **74**(2): p. 86-93.
 27. Montalvo-Jave, E.E., et al., 2008. *Factors in the pathophysiology of the liver ischemia-reperfusion injury.* J Surg Res. **147**(1): p. 153-9.
 28. Rauen, U. and H. de Groot, 2004. *New insights into the cellular and molecular mechanisms of cold storage injury.* J Investig Med. **52**(5): p. 299-309.
 29. Jaeschke, H. and A. Farhood, 2002. *Kupffer cell activation after no-flow ischemia versus hemorrhagic shock.* Free Radic Biol Med. **33**(2): p. 210-9.
 30. Urakami, H., Y. Abe, and M.B. Grisham, 2007. *Role of reactive metabolites of oxygen and nitrogen in partial liver transplantation: lessons learned from reduced-size liver ischaemia and reperfusion injury.* Clin Exp Pharmacol Physiol. **34**(9): p. 912-9.
 31. Gonzalez-Flecha, B., J.C. Cutrin, and A. Boveris, 1993. *Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion.* J Clin Invest. **91**(2): p. 456-64.
 32. Jaeschke, H. and J.R. Mitchell, 1989. *Mitochondria and xanthine oxidase both generate reactive oxygen species in isolated perfused rat liver after hypoxic injury.* Biochem Biophys Res Commun. **160**(1): p. 140-7.
 33. Li, J.M. and A.M. Shah, 2001. *Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase.* Cardiovasc Res. **52**(3): p. 477-86.
 34. Ozaki, M., et al., 2000. *Inhibition of the Rac1 GTPase protects against nonlethal ischemia/reperfusion-induced necrosis and apoptosis in vivo.* FASEB J. **14**(2): p. 418-29.
 35. Chen, T., et al., 2003. *Role of nitric oxide in liver injury.* Curr Mol Med. **3**(6): p. 519-26.
 36. Abu-Amara, M., et al. *Liver ischemia/reperfusion injury: processes in inflammatory networks--a review.* Liver Transpl. **16**(9): p. 1016-32.
 37. Jaeschke, H., 2003. *Role of reactive oxygen species in hepatic ischemia-reperfusion injury and preconditioning.* J Invest Surg. **16**(3): p. 127-40.
 38. Shiratori, Y., et al., 1994. *Modulation of ischemia-reperfusion-induced hepatic injury by Kupffer cells.* Dig Dis Sci. **39**(6): p. 1265-72.
 39. Jaeschke, H., C.V. Smith, and J.R. Mitchell, 1988. *Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver.* J Clin Invest. **81**(4): p. 1240-6.
 40. Jaeschke, H. and A. Farhood, 1991. *Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver.* Am J Physiol. **260**(3 Pt 1): p. G355-62.
 41. Lentsch, A.B., et al., 1999. *Requirement for interleukin-12 in the pathogenesis of warm hepatic ischemia/reperfusion injury in mice.* Hepatology. **30**(6): p. 1448-53.
 42. Lentsch, A.B., et al., 2000. *Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury.* Hepatology. **32**(2): p. 169-73.
 43. Teoh, N.C. and G.C. Farrell, 2003. *Hepatic ischemia reperfusion injury: pathogenic mechanisms and basis for hepatoprotection.* J Gastroenterol Hepatol. **18**(8): p. 891-902.
 44. Teoh, N.C. *Hepatic ischemia reperfusion injury: Contemporary perspectives on pathogenic mechanisms and basis for hepatoprotection-the good, bad and deadly.* J Gastroenterol Hepatol. **26 Suppl 1**: p. 180-7.

45. Vardanian, A.J., R.W. Busuttil, and J.W. Kupiec-Weglinski, 2008. *Molecular mediators of liver ischemia and reperfusion injury: a brief review*. Mol Med. **14**(5-6): p. 337-45.
46. Jaeschke, H. and T. Hasegawa, 2006. *Role of neutrophils in acute inflammatory liver injury*. Liver Int. **26**(8): p. 912-9.
47. Ramaiah, S.K. and H. Jaeschke, 2007. *Role of neutrophils in the pathogenesis of acute inflammatory liver injury*. Toxicol Pathol. **35**(6): p. 757-66.
48. Hamada, T., et al., 2008. *Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury*. Hepatology. **47**(1): p. 186-98.
49. Viappiani, S., M. Sariahmetoglu, and R. Schulz, 2006. *The role of matrix metalloproteinase inhibitors in ischemia-reperfusion injury in the liver*. Curr Pharm Des. **12**(23): p. 2923-34.
50. Wiedow, O. and U. Meyer-Hoffert, 2005. *Neutrophil serine proteases: potential key regulators of cell signalling during inflammation*. J Intern Med. **257**(4): p. 319-28.
51. Manicone, A.M. and J.K. McGuire, 2008. *Matrix metalloproteinases as modulators of inflammation*. Semin Cell Dev Biol. **19**(1): p. 34-41.
52. Van Lint, P. and C. Libert, 2007. *Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation*. J Leukoc Biol. **82**(6): p. 1375-81.
53. Uchida, Y., et al. *The protective function of neutrophil elastase inhibitor in liver ischemia/reperfusion injury*. Transplantation. **89**(9): p. 1050-6.
54. Arnhold, J. and J. Flemmig. *Human myeloperoxidase in innate and acquired immunity*. Arch Biochem Biophys. **500**(1): p. 92-106.
55. Weiss, S.J., 1989. *Tissue destruction by neutrophils*. N Engl J Med. **320**(6): p. 365-76.
56. Jaeschke, H. *Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts*. J Gastroenterol Hepatol. **26 Suppl 1**: p. 173-9.
57. Zwacka, R.M., et al., 1997. *CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver*. J Clin Invest. **100**(2): p. 279-89.
58. Khandoga, A., et al., 2006. *CD4+ T cells contribute to postischemic liver injury in mice by interacting with sinusoidal endothelium and platelets*. Hepatology. **43**(2): p. 306-15.
59. Caldwell, C.C., et al., 2005. *Divergent functions of CD4+ T lymphocytes in acute liver inflammation and injury after ischemia-reperfusion*. Am J Physiol Gastrointest Liver Physiol. **289**(5): p. G969-76.
60. Ley, K., et al., 2007. *Getting to the site of inflammation: the leukocyte adhesion cascade updated*. Nat Rev Immunol. **7**(9): p. 678-89.
61. Coito, A.J. *Leukocyte transmigration across endothelial and extracellular matrix protein barriers in liver ischemia/reperfusion injury*. Curr Opin Organ Transplant.
62. Lee, W.Y. and P. Kubes, 2008. *Leukocyte adhesion in the liver: distinct adhesion paradigm from other organs*. J Hepatol. **48**(3): p. 504-12.
63. Wong, J., et al., 1997. *A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature*. J Clin Invest. **99**(11): p. 2782-90.
64. Petri, B., M. Phillipson, and P. Kubes, 2008. *The physiology of leukocyte recruitment: an in vivo perspective*. J Immunol. **180**(10): p. 6439-46.
65. Kelly, M., J.M. Hwang, and P. Kubes, 2007. *Modulating leukocyte recruitment in inflammation*. J Allergy Clin Immunol. **120**(1): p. 3-10.
66. Kansas, G.S., 1996. *Selectins and their ligands: current concepts and controversies*. Blood. **88**(9): p. 3259-87.
67. Ley, K., 2003. *The role of selectins in inflammation and disease*. Trends Mol Med. **9**(6): p. 263-8.
68. Muller, W.A., 2003. *Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response*. Trends Immunol. **24**(6): p. 327-34.

69. Garcia-Criado, F.J., et al., 1997. *Sulfo-Lewis(x) diminishes neutrophil infiltration and free radicals with minimal effect on serum cytokines after liver ischemia and reperfusion*. J Surg Res. **70**(2): p. 187-94.
70. Dulkanchainun, T.S., et al., 1998. *Reduction of hepatic ischemia/reperfusion injury by a soluble P-selectin glycoprotein ligand-1*. Ann Surg. **227**(6): p. 832-40.
71. Tsuchihashi, S., et al., 2006. *Molecular characterization of rat leukocyte P-selectin glycoprotein ligand-1 and effect of its blockade: protection from ischemia-reperfusion injury in liver transplantation*. J Immunol. **176**(1): p. 616-24.
72. Essani, N.A., et al., 1998. *Increased P-selectin gene expression in the liver vasculature and its role in the pathophysiology of neutrophil-induced liver injury in murine endotoxin shock*. J Leukoc Biol. **63**(3): p. 288-96.
73. Gaboury, J.P. and P. Kubes, 1994. *Reductions in physiologic shear rates lead to CD11/CD18-dependent, selectin-independent leukocyte rolling in vivo*. Blood. **83**(2): p. 345-50.
74. Wyllie, S., et al., 2008. *Failure of P-selectin blockade alone to protect the liver from ischemia-reperfusion injury in the isolated blood-perfused rat liver*. World J Gastroenterol. **14**(44): p. 6808-16.
75. Laudanna, C., et al., 2002. *Rapid leukocyte integrin activation by chemokines*. Immunol Rev. **186**: p. 37-46.
76. Shattil, S.J., C. Kim, and M.H. Ginsberg. *The final steps of integrin activation: the end game*. Nat Rev Mol Cell Biol. **11**(4): p. 288-300.
77. Wasmuth, H.E., F. Tacke, and C. Trautwein. *Chemokines in liver inflammation and fibrosis*. Semin Liver Dis. **30**(3): p. 215-25.
78. Lentsch, A.B., et al., 1998. *Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC*. Hepatology. **27**(4): p. 1172-7.
79. Mosher, B., et al., 2001. *Inhibition of Kupffer cells reduced CXC chemokine production and liver injury*. J Surg Res. **99**(2): p. 201-10.
80. Hamada, T., et al., 2008. *Cyclooxygenase-2 deficiency enhances Th2 immune responses and impairs neutrophil recruitment in hepatic ischemia/reperfusion injury*. J Immunol. **180**(3): p. 1843-53.
81. Kuriyama, N., et al. *Tenascin-C: A novel mediator of hepatic ischemia and reperfusion injury*. Hepatology. **54**(6): p. 2125-36.
82. Oo, Y.H., S. Shetty, and D.H. Adams. *The role of chemokines in the recruitment of lymphocytes to the liver*. Dig Dis. **28**(1): p. 31-44.
83. Day, Y.J., et al., 2004. *Protection from ischemic liver injury by activation of A2A adenosine receptors during reperfusion: inhibition of chemokine induction*. Am J Physiol Gastrointest Liver Physiol. **286**(2): p. G285-93.
84. Moench, C., et al., 2003. *The role of monocyte chemoattractant protein-1 in orthotopic liver transplantation*. Transplant Proc. **35**(4): p. 1452-5.
85. Hynes, R.O., 2002. *Integrins: bidirectional, allosteric signaling machines*. Cell. **110**(6): p. 673-87.
86. Janik, M.E., A. Litynska, and P. Vereecken. *Cell migration-the role of integrin glycosylation*. Biochim Biophys Acta. **1800**(6): p. 545-55.
87. Kakkar, A.K. and D.J. Lefer, 2004. *Leukocyte and endothelial adhesion molecule studies in knockout mice*. Curr Opin Pharmacol. **4**(2): p. 154-8.
88. Potocnik, A.J., C. Brakebusch, and R. Fassler, 2000. *Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow*. Immunity. **12**(6): p. 653-63.
89. Sixt, M., et al., 2006. *Beta1 integrins: zip codes and signaling relay for blood cells*. Curr Opin Cell Biol. **18**(5): p. 482-90.
90. Hogg, N., et al., 2003. *T-cell integrins: more than just sticking points*. J Cell Sci. **116**(Pt 23): p. 4695-705.

91. Casillas-Ramirez, A., et al., 2006. *Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation*. Life Sci. **79**(20): p. 1881-94.
92. Chan, J.R., S.J. Hyduk, and M.I. Cybulsky, 2001. *Chemoattractants induce a rapid and transient upregulation of monocyte alpha4 integrin affinity for vascular cell adhesion molecule 1 which mediates arrest: an early step in the process of emigration*. J Exp Med. **193**(10): p. 1149-58.
93. Berlin, C., et al., 1995. *alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow*. Cell. **80**(3): p. 413-22.
94. Chen, C., et al., 1999. *High affinity very late antigen-4 subsets expressed on T cells are mandatory for spontaneous adhesion strengthening but not for rolling on VCAM-1 in shear flow*. J Immunol. **162**(2): p. 1084-95.
95. Yednock, T.A., et al., 1992. *Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin*. Nature. **356**(6364): p. 63-6.
96. Lobb, R.R. and M.E. Hemler, 1994. *The pathophysiologic role of alpha 4 integrins in vivo*. J Clin Invest. **94**(5): p. 1722-8.
97. O'Connor, P., 2007. *Natalizumab and the role of alpha 4-integrin antagonism in the treatment of multiple sclerosis*. Expert Opin Biol Ther. **7**(1): p. 123-36.
98. Fiorino, G., et al. *Leukocyte traffic control: a novel therapeutic strategy for inflammatory bowel disease*. Expert Rev Clin Immunol. **6**(4): p. 567-72.
99. Coito, A.J., M. de Sousa, and J.W. Kupiec-Weglinski, 2000. *Fibronectin in immune responses in organ transplant recipients*. Dev Immunol. **7**(2-4): p. 239-48.
100. Liao, Y.F., et al., 2002. *The E11A segment of fibronectin is a ligand for integrins alpha 9beta 1 and alpha 4beta 1 providing a novel mechanism for regulating cell adhesion by alternative splicing*. J Biol Chem. **277**(17): p. 14467-74.
101. Hynes, R.O., 1992. *Integrins: versatility, modulation, and signaling in cell adhesion*. Cell. **69**(1): p. 11-25.
102. Man, S., et al., 2009. *alpha4 Integrin/FN-CS1 mediated leukocyte adhesion to brain microvascular endothelial cells under flow conditions*. J Neuroimmunol. **210**(1-2): p. 92-9.
103. Amersi, F., et al., 2003. *Fibronectin-alpha 4 beta 1 integrin-mediated blockade protects genetically fat Zucker rat livers from ischemia/reperfusion injury*. Am J Pathol. **162**(4): p. 1229-39.
104. Moore, C., et al., 2005. *Fibronectin-alpha4beta1 integrin interactions modulate p42/44 MAPK phosphorylation in steatotic liver cold ischemia-reperfusion injury*. Transplant Proc. **37**(1): p. 432-4.
105. Moore, C., et al., 2007. *Fibronectin-alpha4beta1 integrin interactions regulate metalloproteinase-9 expression in steatotic liver ischemia and reperfusion injury*. Am J Pathol. **170**(2): p. 567-77.
106. Fondevila, C., et al., 2005. *Cyclic RGD peptides with high affinity for alpha5beta1 integrin protect genetically fat Zucker rat livers from cold ischemia/reperfusion injury*. Transplant Proc. **37**(4): p. 1679-81.
107. Sorokin, L. *The impact of the extracellular matrix on inflammation*. Nat Rev Immunol. **10**(10): p. 712-23.
108. Vaday, G.G., et al., 2001. *Combinatorial signals by inflammatory cytokines and chemokines mediate leukocyte interactions with extracellular matrix*. J Leukoc Biol. **69**(6): p. 885-92.
109. Hynes, R.O., 2009. *The extracellular matrix: not just pretty fibrils*. Science. **326**(5957): p. 1216-9.
110. Morwood, S.R. and L.B. Nicholson, 2006. *Modulation of the immune response by extracellular matrix proteins*. Arch Immunol Ther Exp (Warsz). **54**(6): p. 367-74.
111. Marastoni, S., et al., 2008. *Extracellular matrix: a matter of life and death*. Connect Tissue Res. **49**(3): p. 203-6.

112. Hohenester, E. and J. Engel, 2002. *Domain structure and organisation in extracellular matrix proteins*. Matrix Biol. **21**(2): p. 115-28.
113. Korpos, E., C. Wu, and L. Sorokin, 2009. *Multiple roles of the extracellular matrix in inflammation*. Curr Pharm Des. **15**(12): p. 1349-57.
114. Berrier, A.L. and K.M. Yamada, 2007. *Cell-matrix adhesion*. J Cell Physiol. **213**(3): p. 565-73.
115. Coito, A.J. and J.W. Kupiec-Weglinski, 2000. *Extracellular matrix proteins in organ transplantation*. Transplantation. **69**(12): p. 2465-73.
116. Hynes, R.O., 1986. *Fibronectins*. Sci Am. **254**(6): p. 42-51.
117. Tamkun, J.W., J.E. Schwarzbauer, and R.O. Hynes, 1984. *A single rat fibronectin gene generates three different mRNAs by alternative splicing of a complex exon*. Proc Natl Acad Sci U S A. **81**(16): p. 5140-4.
118. White, E.S., F.E. Baralle, and A.F. Muro, 2008. *New insights into form and function of fibronectin splice variants*. J Pathol. **216**(1): p. 1-14.
119. Pankov, R. and K.M. Yamada, 2002. *Fibronectin at a glance*. J Cell Sci. **115**(Pt 20): p. 3861-3.
120. French-Constant, C., 1995. *Alternative splicing of fibronectin--many different proteins but few different functions*. Exp Cell Res. **221**(2): p. 261-71.
121. Guan, J.L. and R.O. Hynes, 1990. *Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1*. Cell. **60**(1): p. 53-61.
122. Davis, L.S., et al., 1990. *Fibronectin promotes proliferation of naive and memory T cells by signaling through both the VLA-4 and VLA-5 integrin molecules*. J Immunol. **145**(3): p. 785-93.
123. Barczyk, M., S. Carracedo, and D. Gullberg. *Integrins*. Cell Tissue Res. **339**(1): p. 269-80.
124. Aota, S., M. Nomizu, and K.M. Yamada, 1994. *The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function*. J Biol Chem. **269**(40): p. 24756-61.
125. Moyano, J.V., et al., 1997. *Fibronectin type III5 repeat contains a novel cell adhesion sequence, KLDAPT, which binds activated alpha4beta1 and alpha4beta7 integrins*. J Biol Chem. **272**(40): p. 24832-6.
126. Matias-Roman, S., et al., 2005. *Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium*. Blood. **105**(10): p. 3956-64.
127. Huo, Y., A. Hafezi-Moghadam, and K. Ley, 2000. *Role of vascular cell adhesion molecule-1 and fibronectin connecting segment-1 in monocyte rolling and adhesion on early atherosclerotic lesions*. Circ Res. **87**(2): p. 153-9.
128. Burns, J.A., et al., 2001. *The alpha 4 beta 1 (very late antigen (VLA)-4, CD49d/CD29) and alpha 5 beta 1 (VLA-5, CD49e/CD29) integrins mediate beta 2 (CD11/CD18) integrin-independent neutrophil recruitment to endotoxin-induced lung inflammation*. J Immunol. **166**(7): p. 4644-9.
129. Bohnsack, J.F. and X.N. Zhou, 1992. *Divalent cation substitution reveals CD18- and very late antigen-dependent pathways that mediate human neutrophil adherence to fibronectin*. J Immunol. **149**(4): p. 1340-7.
130. Redondo-Munoz, J., et al., 2006. *MMP-9 in B-cell chronic lymphocytic leukemia is up-regulated by alpha4beta1 integrin or CXCR4 engagement via distinct signaling pathways, localizes to podosomes, and is involved in cell invasion and migration*. Blood. **108**(9): p. 3143-51.
131. Yakubenko, V.P., et al., 2000. *Differential induction of gelatinase B (MMP-9) and gelatinase A (MMP-2) in T lymphocytes upon alpha(4)beta(1)-mediated adhesion to VCAM-1 and the CS-1 peptide of fibronectin*. Exp Cell Res. **260**(1): p. 73-84.
132. Olivier, P., et al., 1999. *Urokinase-type plasminogen activator inhibits alpha 4 beta 1 integrin-mediated T lymphocyte adhesion to fibronectin independently of its catalytic activity*. Eur J Immunol. **29**(10): p. 3196-209.

133. White, E.S. and A.F. Muro. *Fibronectin splice variants: understanding their multiple roles in health and disease using engineered mouse models*. IUBMB Life. **63**(7): p. 538-46.
134. McFadden, J.P., et al. *Psoriasis and extra domain A fibronectin loops*. Br J Dermatol. **163**(1): p. 5-11.
135. Elices, M.J., et al., 1994. *Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature*. J Clin Invest. **93**(1): p. 405-16.
136. Jarnagin, W.R., et al., 1994. *Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis*. J Cell Biol. **127**(6 Pt 2): p. 2037-48.
137. Coito, A.J., et al., 1997. *Expression of fibronectin splicing variants in organ transplantation: a differential pattern between rat cardiac allografts and isografts*. Am J Pathol. **150**(5): p. 1757-72.
138. Coito, A.J., et al., 1998. *Blockade of very late antigen-4 integrin binding to fibronectin in allograft recipients: I. Treatment with connecting segment-1 peptides prevents acute rejection by suppressing intragraft mononuclear cell accumulation, endothelial activation, and cytokine expression*. Transplantation. **65**(5): p. 699-706.
139. Coito, A.J., et al., 1998. *Blockade of alpha 4 beta 1-integrin-fibronectin adhesive interactions prevents chronic allograft rejection in sensitized recipients*. Transplant Proc. **30**(4): p. 939-40.
140. Korom, S., et al., 1998. *Blockade of very late antigen-4 integrin binding to fibronectin in allograft recipients. II. Treatment with connecting segment-1 peptides prevents chronic rejection by attenuating arteriosclerotic development and suppressing intragraft T cell and macrophage activation*. Transplantation. **65**(6): p. 854-9.
141. Nagase, H., R. Visse, and G. Murphy, 2006. *Structure and function of matrix metalloproteinases and TIMPs*. Cardiovasc Res. **69**(3): p. 562-73.
142. Brinckerhoff, C.E. and L.M. Matrisian, 2002. *Matrix metalloproteinases: a tail of a frog that became a prince*. Nat Rev Mol Cell Biol. **3**(3): p. 207-14.
143. Lee, M.H. and G. Murphy, 2004. *Matrix metalloproteinases at a glance*. J Cell Sci. **117**(Pt 18): p. 4015-6.
144. Parks, W.C., C.L. Wilson, and Y.S. Lopez-Boado, 2004. *Matrix metalloproteinases as modulators of inflammation and innate immunity*. Nat Rev Immunol. **4**(8): p. 617-29.
145. Klein, T. and R. Bischoff. *Physiology and pathophysiology of matrix metalloproteinases*. Amino Acids. **41**(2): p. 271-90.
146. Bourboulia, D. and W.G. Stetler-Stevenson. *Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion*. Semin Cancer Biol. **20**(3): p. 161-8.
147. Van Wart, H.E. and H. Birkedal-Hansen, 1990. *The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family*. Proc Natl Acad Sci U S A. **87**(14): p. 5578-82.
148. Nathan, C., 2002. *Points of control in inflammation*. Nature. **420**(6917): p. 846-52.
149. Chakrabarti, S. and K.D. Patel, 2005. *Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology*. Exp Lung Res. **31**(6): p. 599-621.
150. Gidday, J.M., et al., 2005. *Leukocyte-derived matrix metalloproteinase-9 mediates blood-brain barrier breakdown and is proinflammatory after transient focal cerebral ischemia*. Am J Physiol Heart Circ Physiol. **289**(2): p. H558-68.
151. Soccia, P.M., et al., 2004. *Matrix metalloproteinase inhibition decreases ischemia-reperfusion injury after lung transplantation*. Am J Transplant. **4**(1): p. 41-50.
152. Kunugi, S., et al. *Inhibition of matrix metalloproteinases reduces ischemia-reperfusion acute kidney injury*. Lab Invest. **91**(2): p. 170-80.

153. Ma, Z.Y., et al. *Inhibition of matrix metalloproteinase-9 attenuates acute small-for-size liver graft injury in rats*. Am J Transplant. **10**(4): p. 784-95.
154. Garg, P., et al., 2006. *Selective ablation of matrix metalloproteinase-2 exacerbates experimental colitis: contrasting role of gelatinases in the pathogenesis of colitis*. J Immunol. **177**(6): p. 4103-12.
155. McQuibban, G.A., et al., 2000. *Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3*. Science. **289**(5482): p. 1202-6.
156. Hernandez-Barrantes, S., et al., 2002. *Regulation of membrane type-matrix metalloproteinases*. Semin Cancer Biol. **12**(2): p. 131-8.
157. Itoh, Y., 2006. *MT1-MMP: a key regulator of cell migration in tissue*. IUBMB Life. **58**(10): p. 589-96.
158. Rajavashisth, T.B., et al., 1999. *Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques: evidence for activation by proinflammatory mediators*. Circulation. **99**(24): p. 3103-9.
159. Konttinen, Y.T., et al., 1998. *New collagenolytic enzymes/cascade identified at the pannus-hard tissue junction in rheumatoid arthritis: destruction from above*. Matrix Biol. **17**(8-9): p. 585-601.
160. Graesser, D., et al., 1998. *The interrelationship of alpha4 integrin and matrix metalloproteinase-2 in the pathogenesis of experimental autoimmune encephalomyelitis*. Lab Invest. **78**(11): p. 1445-58.
161. Esparza, J., et al., 1999. *Fibronectin upregulates gelatinase B (MMP-9) and induces coordinated expression of gelatinase A (MMP-2) and its activator MT1-MMP (MMP-14) by human T lymphocyte cell lines. A process repressed through RAS/MAP kinase signaling pathways*. Blood. **94**(8): p. 2754-66.
162. Sato, H. and T. Takino. *Coordinate action of membrane-type matrix metalloproteinase-1 (MT1-MMP) and MMP-2 enhances pericellular proteolysis and invasion*. Cancer Sci. **101**(4): p. 843-7.
163. Hadler-Olsen, E., et al. *Regulation of matrix metalloproteinase activity in health and disease*. FEBS J. **278**(1): p. 28-45.
164. Murphy, G., et al., 1994. *Regulation of matrix metalloproteinase activity*. Ann N Y Acad Sci. **732**: p. 31-41.
165. Tallant, C., A. Marrero, and F.X. Gomis-Ruth. *Matrix metalloproteinases: fold and function of their catalytic domains*. Biochim Biophys Acta. **1803**(1): p. 20-8.
166. Okamoto, T., et al., 1997. *Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide*. Arch Biochem Biophys. **342**(2): p. 261-74.
167. McCarthy, S.M., et al., 2008. *Nitric oxide regulation of MMP-9 activation and its relationship to modifications of the cysteine switch*. Biochemistry. **47**(21): p. 5832-40.
168. Brew, K., D. Dinakarpanian, and H. Nagase, 2000. *Tissue inhibitors of metalloproteinases: evolution, structure and function*. Biochim Biophys Acta. **1477**(1-2): p. 267-83.
169. Page-McCaw, A., A.J. Ewald, and Z. Werb, 2007. *Matrix metalloproteinases and the regulation of tissue remodelling*. Nat Rev Mol Cell Biol. **8**(3): p. 221-33.
170. Lambert, E., et al., 2009. *TIMP-1 binding to proMMP-9/CD44 complex localized at the cell surface promotes erythroid cell survival*. Int J Biochem Cell Biol. **41**(5): p. 1102-15.
171. Stetler-Stevenson, W.G., 2008. *Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities*. Sci Signal. **1**(27): p. re6.
172. Gomez, D.E., et al., 1997. *Tissue inhibitors of metalloproteinases: structure, regulation and biological functions*. Eur J Cell Biol. **74**(2): p. 111-22.
173. Bode, W., et al., 1999. *Insights into MMP-TIMP interactions*. Ann N Y Acad Sci. **878**: p. 73-91.

174. Clutterbuck, A.L., et al., 2009. *Targeting matrix metalloproteinases in inflammatory conditions*. *Curr Drug Targets*. **10**(12): p. 1245-54.
175. Kim, K.H., et al., 2005. *Tissue inhibitor of metalloproteinase-1 deficiency amplifies acute lung injury in bleomycin-exposed mice*. *Am J Respir Cell Mol Biol*. **33**(3): p. 271-9.
176. Crocker, S.J., et al., 2006. *Persistent macrophage/microglial activation and myelin disruption after experimental autoimmune encephalomyelitis in tissue inhibitor of metalloproteinase-1-deficient mice*. *Am J Pathol*. **169**(6): p. 2104-16.
177. Fujimoto, M., et al., 2008. *Tissue inhibitor of metalloproteinases protect blood-brain barrier disruption in focal cerebral ischemia*. *J Cereb Blood Flow Metab*. **28**(10): p. 1674-85.
178. Mohammed, F.F., et al., 2005. *Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell cycle via HGF activation in murine liver regeneration*. *Hepatology*. **41**(4): p. 857-67.
179. Iredale, J.P., 1997. *Tissue inhibitors of metalloproteinases in liver fibrosis*. *Int J Biochem Cell Biol*. **29**(1): p. 43-54.
180. Arthur, M.J., D.A. Mann, and J.P. Iredale, 1998. *Tissue inhibitors of metalloproteinases, hepatic stellate cells and liver fibrosis*. *J Gastroenterol Hepatol*. **13 Suppl**: p. S33-8.
181. Arthur, M.J., J.P. Iredale, and D.A. Mann, 1999. *Tissue inhibitors of metalloproteinases: role in liver fibrosis and alcoholic liver disease*. *Alcohol Clin Exp Res*. **23**(5): p. 940-3.
182. Wang, H., et al. *Tissue inhibitor of metalloproteinase 1 (TIMP-1) deficiency exacerbates carbon tetrachloride-induced liver injury and fibrosis in mice: involvement of hepatocyte STAT3 in TIMP-1 production*. *Cell Biosci*. **1**(1): p. 14.
183. Kuyvenhoven, J.P., et al., 2004. *Plasma MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 during human orthotopic liver transplantation. The effect of aprotinin and the relation to ischemia/reperfusion injury*. *Thromb Haemost*. **91**(3): p. 506-13.
184. Knittel, T., et al., 1999. *Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta1*. *J Hepatol*. **30**(1): p. 48-60.
185. Chirco, R., et al., 2006. *Novel functions of TIMPs in cell signaling*. *Cancer Metastasis Rev*. **25**(1): p. 99-113.
186. Cruz-Munoz, W. and R. Khokha, 2008. *The role of tissue inhibitors of metalloproteinases in tumorigenesis and metastasis*. *Crit Rev Clin Lab Sci*. **45**(3): p. 291-338.
187. Liu, X.W., et al., 2005. *Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells from extrinsic cell death: a potential oncogenic activity of tissue inhibitor of metalloproteinase-1*. *Cancer Res*. **65**(3): p. 898-906.
188. Murphy, F.R., et al., 2002. *Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis*. *J Biol Chem*. **277**(13): p. 11069-76.

CHAPTER II

FIBRONECTIN- α 4 β 1 INTERACTIONS IN HEPATIC COLD ISCHEMIA REPERFUSION INJURY: REGULATION OF MMP-9 AND MT1-MMP VIA THE p38 MAPK PATHWAY

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**FIBRONECTIN- α 4 β 1 INTERACTIONS IN HEPATIC COLD ISCHEMIA REPERFUSION
INJURY: REGULATION OF MMP-9 AND MT1-MMP VIA THE p38 MAPK PATHWAY**

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Running Title: CS-1 peptides in prolonged cold liver IRI

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FOOTNOTES

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Liver ischemia-reperfusion injury (IRI) remains a challenging problem in clinical settings. The expression of fibronectin (FN) by endothelial cells is a prominent feature of the hepatic response to injury. Here we investigate the effects of the connecting segment-1 (CS-1) peptide therapy, which blocks fibronectin (FN)- $\alpha4\beta1$ integrin leukocyte interactions, in a well-established model of 24-hour cold liver IRI. CS-1 peptides significantly inhibited leukocyte recruitment and local release of proinflammatory mediators (IFN- γ , COX-2 and iNOS), ameliorating liver IRI and improving recipient survival rate. CS1 therapy inhibited the phosphorylation of p38 MAPK, a kinase linked to inflammatory processes. Moreover, in addition to downregulating the expression of matrix metalloproteinase-9 (MMP-9) in hepatic IRI, as previously shown, CS-1 peptide therapy depressed the expression of membrane type 1-matrix metalloproteinase (MT1-MMP/MMP-14) by macrophages, a membrane-tethered MMP important for focal matrix proteolysis. Inhibition of p38 MAPK activity, with its pharmacological antagonist SB203580, downregulated MMP-9 and MT1-MMP/MMP-14 expressions by fibronectin-stimulated macrophages, suggesting that p38 MAPK kinase pathway controls fibronectin mediated inductions of MMP-9 and MT1-MMP/MMP-14. Hence, this study provides new insights on the role of fibronectin in liver injury, which can potentially be applied to the development of new pharmacological strategies for the successful protection against hepatic IRI.

INTRODUCTION

Ischemia-reperfusion injury (IRI) represents a major problem in orthotopic liver transplantation (OLT). IRI is a multifactorial antigen-independent inflammatory process that can lead to early graft failure and to a higher incidence of both acute and chronic organ dysfunction after transplantation ^{1,2}.

The migration of leukocytes into tissues is a central event in inflammatory processes ³, including in acute inflammatory liver injury ⁴. Leukocyte transmigration across endothelial and extracellular matrix (ECM) protein barriers is dependent on complex series of adhesion and focal matrix degradation events ^{5,6}. Fibronectin (FN) is a well characterized ECM glycoprotein implicated in a variety of pathological conditions that are associated with cell turnover and migration such as tumor metastasis ⁷, rheumatoid arthritis ⁸, multiple sclerosis ⁹, and organ transplantation ¹⁰. Moreover, clinical trials using humanized antibodies against the $\alpha 4$ integrin, a receptor for the connecting segment-1 (CS-1) region of fibronectin ¹¹, have been effective in controlling inflammatory conditions like multiple sclerosis (MS) ¹² and inflammatory bowel disease ¹³. The role of fibronectin in leukocyte adhesion, migration and activation has been extensively reported ¹⁴. Indeed, it was recently demonstrated that adhesion of leukocytes from MS patients to brain microvascular endothelial cells under flow conditions is preferentially mediated by the $\alpha 4$ integrin/FN-CS1 interactions ¹⁵.

We have previously shown that CS-1 peptides, which are FN-specific peptides that interact with the $\alpha 4\beta 1$ -integrin and inhibit its binding to FN ¹⁶, profoundly depressed leukocyte recruitment, and improved liver function and recipient survival rate of suboptimal steatotic liver transplants in a model of ex vivo 4-hour cold ischemia followed by isotransplantation ^{17, 18}. In the present study, we evaluated the effects of the CS-1 peptide therapy in a well-established rat liver model of prolonged cold hepatic IRI, in

which normal livers are cold stored for 24 hours prior to being transplanted in syngeneic recipients. Our results show a beneficial role for CS-1 peptides in ameliorating prolonged cold hepatic IRI. Moreover, they provide evidence that FN regulates the expression of both matrix metalloproteinase-9 (MMP-9) and membrane type 1-matrix metalloproteinase (MT1-MMP/MMP-14) through activation of the p38 MAP kinase cell signaling pathway.

Abbreviations: alanine aminotransferase (ALT); aspartate aminotransferase (AST); connecting segment-1 (CS-1); cyclooxygenase-2 (COX-2); fibronectin (FN); extracellular matrix (ECM); inducible nitric oxide synthase (iNOS); matrix metalloproteinase (MMP); membrane type 1-matrix metalloproteinase (MT1-MMP); mitogen-activated protein kinase (MAPK); myeloperoxidase (MPO);

MATERIALS AND METHODS

Animals, grafting techniques and CS-1 peptide therapy

Male Sprague Dawley rats (250-300 g) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Syngeneic OLTs were performed using livers harvested from Sprague Dawley donors stored for 24 hours at 4°C in University of Wisconsin (UW) solution before being transplanted in syngeneic recipients. The standard techniques of liver harvesting and orthotopic transplantation with revascularization without hepatic artery reconstruction were performed with an anhepatic phase of 16–20 min and according to the previously described Kamada's and Calne's cuff technique¹⁹. Cellular FN was significantly up-regulated in the liver vasculature after 24h of cold storage followed by OLT (Fig. 1). Based on these observations and in our previous studies^{17, 18}, CS-1 peptides (500 µg/rat), which block $\alpha 4\beta 1$ integrin-FN interactions^{20, 21}, were administered ex vivo via portal vein to livers before cold storage, and immediately prior to reperfusion; OLT recipients received an additional dose of CS-1 peptides post-transplantation. Control recipients received vehicle in a similar fashion as in the CS-1-treated group. Rat recipients of liver transplants were sacrificed at 6 h and 24 h after OLT or followed for survival studies. All animals were cared for humanly according to the criteria in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health.

Assessment of liver Damage

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in blood samples obtained at 6 and 24 hours after hepatic reperfusion. Measurements were made with an auto analyzer by ANTECH Diagnostics (Los Angeles, CA). Liver specimens were fixed in 10% buffered formalin solution and

embedded in paraffin. Paraffin sections were stained with H&E and the histological severity of IRI in the liver was assessed as previously described ¹⁷.

Immunohistochemistry

Liver specimens were snap-frozen in liquid nitrogen for peroxidase and immunofluorescence staining, as previously described ^{18, 22}. Appropriate primary antibodies against rat T-Cells (R73), NK-cells (CD161), monocyte/macrophages (ED1) (AbD Serotec, Indianapolis, IN), cellular FN (IST-9) (Accurate Chemical, Westbury, NY), MMP-9 (gelatinase-B) (EMDchemicals, Gibbstown, NJ) and MMP-14 (H-72) (Santa Cruz Biotech, Santa Cruz, CA) were added at optimal dilutions. Bound primary antibody was detected using biotinylated anti-mouse IgG or biotinylated anti-rabbit IgG and streptavidin peroxidase-conjugated complexes (Dako, Carpinteria, CA). The peroxidase reaction was developed with DAB Substrate Kit (Vector Laboratories). Negative and positive controls were included for each stain. Sections were evaluated blindly by counting the labeled cells in triplicates within 10 high-power fields per each section. Dual Staining was achieved by immunofluorescence with Alexa Fluor 594-red anti-rabbit IgG (H+L) and Alexa Fluor 488-green IgG (H+L) antibodies (Molecular probes, Carlsbad, CA). Slides were analyzed using a Nikon Eclipse 90i Fluorescent Microscope and a Leica Confocal Microscope (UCLA Brain Research Institute, Confocal Microscope Core Facility)

Myeloperoxidase (MPO) Assay

MPO is a naturally occurring constituent of neutrophils and is frequently used as a marker for neutrophil infiltration in rat livers. Snap-frozen liver tissue was immediately homogenized for 30 seconds in an iced solution of 0.5%hexadecyltrimethyl-ammonium (Sigma, St Louis, MO) and 50mM KPhos (Sigma, St Louis MO) pH 5. After samples

were homogenized and centrifuged, the supernatant was mixed with a solution of hydrogen peroxide-sodium acetate and tetramethyl benzidine (Sigma, St Louis, MO). One unit of myeloperoxidase activity was defined as the quantity of enzyme that degraded 1 μ M of peroxide per minute at 25°C. MPO activity was expressed as U/g of protein for specific activity.

Western Blot and Zymography Analyses

Snap-frozen liver tissue was immediately homogenized as previously described ¹⁸. Liver protein content was determined using a BCA Protein Assay Kit (Pierce Chemical, Rockford, IL). For western blots 40 μ g of protein in SDS-loading buffer were electrophoresed through 10%-12% SDS- polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Thermo Scientific, Rockford, IL). The membranes were blocked with 5% dry milk and 0.05% Tween 20 (USB, Cleveland, OH) in Tris-buffered saline (TBS) and incubated with specific primary antibodies against MMP-9 (Millipore, Billerica, MA), MT1-MMP (Sigma, St Louis), phospho-p38, phospho-p44/42, p38, and p44/42 (Cell Signaling Technology, Danvers, MA). The filters were washed and then incubated with horseradish peroxidase conjugated secondary antibodies, followed by detection with SuperSignal West Pico Chemiluminescent Substrate (Pierce). After development, membranes were striped and re-blotted with an antibody against β -actin (Abcam). Gelatinolytic activity was detected in liver extracts, at a final protein content of 100 μ g, or in 200 μ l of cell supernatant by 10% SDS-PAGE contained 1mg/ml of gelatin (Invitrogen, Carlsbad, CA) under non-reducing conditions ¹⁸. After SDS-page, the gels were soaked twice with Novex Zymogram Renaturing Buffer (Invitrogen) for 30 min each time, rinsed in water, and incubated overnight at 37°C in Novex Zymogram Developing Buffer (Invitrogen). The gels were then stained with coomassie brilliant blue

R-250 (Bio-rad, Hercules, CA), and destained with methanol/acetic acid/water (20:10:70). A clear zone indicated the presence of enzymatic activity. Positive controls for MMP-9 (BIOMOL International, Plymouth, PA), and prestained molecular weight markers (Fermentas) served as standards. Relative quantities of protein were determined using a densitometer (Image J, NIH free software).

RNA Extraction and Reverse Transcriptase PCR

For evaluation of the gene expressions, RNA was extracted from livers with Trizol (Life Technologies Inc., New York, NY) using a Polytron RT-3000 (Kinematica AG, Littau-luzmen, Switzerland) as described ¹⁸. Reverse transcription was performed using 5µg of total RNA in a first-strand cDNA synthesis reaction with SuperScript II RNaseH Reverse Transcriptase (Life Technologies, Inc.) as recommended by the manufacturer. One µl of the resulting reverse transcriptase product was used for polymerase chain reaction amplification. PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide. Each sample was normalized to β-actin gene expression.

Cell Culture

Murine macrophages were isolated as previously described ²³. Briefly, 1 ml of 3% thioglycollate medium was injected into the peritoneal cavity 72 hours before collecting macrophages. The peritoneal cavities were lavaged with Hanks' balanced salt solution (HBSS), and the aspirate was placed on ice and centrifuged at 1200 rpm for 5 minutes at 4°C. The pellets were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cell viability was determined by trypan blue exclusion. Isolated macrophages were cultured in medium without fetal bovine serum overnight and

pretreated for 30 min with the p38 inhibitor SB203580 (10 μ M) prior to being plated on FN-coated plates (Biocoat, BD Biosciences, San Jose, CA). Controls included combination of cells cultured on polylysine-coated plates, cells stimulated with lipopolysaccharide (10 ng/ml, LPS, Sigma), and cells treated with a specific LPS inhibitor, Polymixin B (10 ng/ml). Cells were cultured on 24-well plates at a concentration of 5×10^5 cells/well and incubated at 37°C, 5% CO₂ for 12 hours. After, incubation, cells and supernatants were collected for RT-PCR and zymography, respectively.

Data Analysis. Statistical Analysis

Data are shown as means $\pm$ SD. Statistical comparisons between groups were performed by Student's t-test using the statistical package SPSS (SPSS Inc., Chicago, IL, USA) when data had a normal distribution. P values of <0.05 were considered statistically significant.

RESULTS

CS-1 peptide therapy ameliorates hepatocellular damage and increases recipient survival and in 24h cold liver IRI.

We examined the effects of the CS-1 peptide therapy on the development of IRI in a well-established model of 24-hour liver cold ischemia followed by OLT. Recipients of livers that had been treated with CS-1 peptides were characterized by improved liver function, as shown by the decreased serum transaminase levels (IU/L) at 6h (sAST: 1412 ± 420 vs. 2866 ± 864 , $p < 0.008$; sALT: 728 ± 428 vs. 1690 ± 211 , $p < 0.004$) and 24h (sAST: 1350 ± 142 vs. 4000 ± 1358 , $p < 0.006$; sALT: 1261 ± 233 vs. 3051 ± 958 , $p < 0.005$) post-OLT (Fig. 2A). CS-1 treated OLTs showed mild vascular congestion, reduced necrosis and good preservation of the lobular architecture, contrasting with high vascular congestion, extensive necrosis, and significant disruption of lobular architecture observed in control livers (Fig. 2B). The improved liver function/histological preservation observed in the CS-1 peptide treated OLTs correlated with a significantly increased 14-day survival rate (100% vs. 50%, $p < 0.005$; $n=8/\text{group}$) in these animals (Fig 2C). Therefore, our results are in line with previous observations in a 4h model of steatotic liver IRI¹⁷ and provide further support for a broadly beneficial role of FN- $\alpha 4\beta 1$ integrin blockade in cold hepatic IRI.

CS-1 peptide therapy disrupts leukocyte infiltration in 24h cold liver IRI.

We evaluated the role of CS-1 peptide therapy on leukocyte infiltration in prolonged cold liver IRI. CS-1 peptide therapy significantly depressed T lymphocyte (31 ± 8 vs. 64 ± 3 , $p < 0.002$), NK cell (19 ± 2 vs. 41 ± 3 , $p < 0.003$) and ED1 monocyte/macrophage (21 ± 6 vs. 32 ± 1 , $p < 0.008$) infiltration at 6h post-OLT (Fig. 3). The decrease in leukocyte numbers by CS-1 peptide therapy was a sustained effect, as

T-cells (30 ± 3 vs. 83 ± 15 , $p < 0.002$), NK cells (16 ± 3 vs. 30 ± 8 , $p < 0.003$) and ED1 macrophages (35 ± 7 vs. 57 ± 16 , $p < 0.003$) were also depressed in the CS-1 peptide treated recipients 24h post-OLT (Fig. 3). MPO activity (U), which is an index of neutrophil infiltration, was significantly reduced at 6h (1.22 ± 0.48 vs. 2.93 ± 0.57 , $p < 0.005$) and 24h (0.48 ± 0.02 vs. 3.18 ± 0.94 , $p < 0.02$) post-transplantation, as compared with the respective controls (Fig. 3E).

CS-1 peptide therapy decreases iNOS, COX-2 and proinflammatory cytokine expression in prolonged cold liver IRI.

Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are two of the most prominent inflammatory mediators²⁴ and their expressions have been linked to liver IRI^{23, 25}. Therefore, we analyzed the expression of iNOS and COX-2 in liver grafts treated with the CS-1 peptides. As shown in figure 4, CS1 peptide therapy reduced the intragraft mRNA expression of both iNOS (0.06 ± 0.02 vs. 1.40 ± 0.41 , $p < 0.01$) and COX-2 (0.80 ± 0.11 vs. 2.20 ± 0.61 , $p < 0.01$) at 6h post-OLT. IFN- γ mRNA expression, which is an initiator of liver reperfusion injury²⁶, was also significantly depressed in the CS-1 treated livers (0.41 ± 0.11 vs. 0.58 ± 0.06 , $p < 0.05$), at 6h post-OLT (Fig. 4). Furthermore, other pro-inflammatory mediators, such as IL1 β (0.35 ± 0.13 vs. 0.74 ± 0.30 , $p < 0.05$), IL-6 (0.45 ± 0.17 vs. 1.10 ± 0.32 , $p < 0.02$), and TNF- α (0.03 ± 0.04 vs. 0.83 ± 0.26 , $p < 0.005$), were markedly depressed in the CS-1 treated OLTs, as compared to respective controls, (Fig. 4).

CS-1 peptide therapy downregulates MMP-9 and MT1-MMP/MMP-14 expressions in 24h cold liver IRI.

Our earlier studies have shown that MMP-9 is induced upon leukocyte attachment to fibronectin in damaged steatotic livers¹⁸ and that MMP-9 mediates leukocyte migration in liver IRI²². Others have shown that MT1-MMP/MMP-14 is capable of mediating human monocyte migration in vitro²⁷. Therefore, we evaluated whether CS-1 peptide mediated therapy affected the expressions of MMP-9 and MT1-MMP/MMP-14 in our liver model of ex vivo 24h cold storage followed by OLT. As shown in figure 5, CS-1 peptide therapy reduced the intragraft MMP-9 expression at mRNA (0.10 ± 0.12 vs. 0.50 ± 0.35 , $p < 0.03$) and protein (0.03 ± 0.01 vs. 0.23 ± 0.14 , $p < 0.03$) levels at 6h post-OLT. MMP-9 expression was also depressed at mRNA (0.33 ± 0.19 vs. 1.13 ± 0.22 , $p < 0.005$) and protein (0.15 ± 0.07 vs. 0.70 ± 0.14 , $p < 0.03$) levels in CS-1 peptide treated OLTs at 24h post-IRI, as compared to controls. Moreover, while control OLTs were characterized by significant MMP-9+ leukocyte infiltration, CS-1 peptide treated livers showed only very few intragraft MMP-9+ leukocytes, (Fig. 5C). MT1-MMP/MMP-14 was virtually absent from naïve livers and upregulated in livers post-OLT; however, its expression was significantly depressed in CS-1 peptide treated OLTs at mRNA (0.27 ± 0.20 vs. 0.76 ± 0.09 ; $p < 0.02$) and protein (0.32 ± 0.07 vs. 0.61 ± 0.04 ; $p < 0.003$) levels at 6h post-OLT, as compared to respective controls (Fig. 5 D and E). MT1-MMP/MMP-14 expression was similar in CS-1 peptide treated and control OLTs at 24h post-transplantation (not shown), raising the possibility that the MT1-MMP/MMP-14 expression could perhaps be more relevant during the initial phase of liver IRI. To identify the sources of MT1-MMP/MMP-14 in OLTs, we performed double immunofluorescence staining in serial sections of CS1 peptide-treated and control OLTs. MT1-MMP/MMP-14 staining was predominantly detected in infiltrating monocyte/macrophages of liver grafts 6h post-cold IRI, (Fig. 5F).

CS-1 peptide therapy inhibits the phosphorylation of p38 mitogen-activated protein kinase in 24h cold liver IRI.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases implied in the regulation of cellular responses to the environment ²⁸. In an attempt to elucidate the significance of CS-1 peptide therapy upon MAPK cell signaling, we evaluated the activation of p38 MAPK and p44/42 MAPK signaling pathways in our rat liver model of ex vivo 24h cold ischemia followed by transplantation. The phosphorylation of the p38 MAPK threonine and tyrosine residues (Thr180/Tyr182), which was virtually undetected in naïve livers, was slightly upregulated in CS-1 treated livers (0.06 ± 0.01 vs. 0.27 ± 0.13 ; $p < 0.03$ $n=6/\text{group}$) at 6h post-reperfusion, contrasting with the strong p38 phosphorylation levels detected in the respective control OLTs, (Fig. 6). On the other hand, significant differences in p44/42 MAPK threonine and tyrosine phosphorylation (Thr202/Tyr204) were not detected between CS-1 peptide treated and control OLTs (0.60 ± 0.28 vs. 0.57 ± 0.27 ; $n=6/\text{group}$) at 6h post-reperfusion, (Fig. 6). Thus, our data suggest that CS-1 peptide therapy preferentially inhibits the p38 MAPK signaling pathway in cold liver IRI.

Induction of MMP-9 and MT1-MMP/MMP-14 by fibronectin is mediated by p38 mitogen-activated protein kinase.

We have previously shown that fibronectin-leukocyte interactions regulate MMP-9 expression in a macrophage cell line ¹⁸. Here, we tested whether fibronectin was able to upregulate the expressions of both MMP-9 and MT1-MMP/MMP-14 in isolated macrophages. Indeed, in addition to MMP-9 upregulation (0.80 ± 0.24 vs. 0.36 ± 0.11 ; $p < 0.009$), we observed that FN is also capable of upregulating MT1-MMP/MMP-14 expression in cultured macrophages (0.97 ± 0.16 vs. 0.48 ± 0.11 ; $p < 0.001$) (Fig 7A). It

has been shown that human monocytes stimulated with LPS express MT1-MMP/MMP-14²⁹; polymyxin B was added to some cultures to eliminate the effect of potential endotoxin contamination. We further determined the role of the p38 MAP kinase pathway in fibronectin-stimulated MMP-9 and MT1-MMP/MMP-14 expressions by culturing isolated macrophages in fibronectin in the absence or presence of the pharmacological inhibitor of p38 MAPK SB203580. As shown in figure 7, SB203580 significantly inhibited fibronectin-stimulated MMP-9 (0.36 ± 0.07 vs. 0.80 ± 0.24 ; $p < 0.02$) and MT1-MMP/MMP-14 (0.62 ± 0.04 vs. 0.97 ± 0.16 ; $p < 0.002$) mRNA expressions. Moreover, gelatin zymography carried out on protein extracts from the cultures of fibronectin-stimulated macrophages confirmed a significant decrease in MMP-9 activity ($p < 0.005$) in the presence of SB203580. Thus, our data suggest that fibronectin-stimulated MMP-9 and MT1-MMP/MMP-14 expressions are p38 MAPK signaling pathway-dependent.

DISCUSSION

It is generally accepted that hepatic IRI associated with leukocyte recruitment and release of cytokines and free radicals plays a major role in liver dysfunction after OLT³⁰; nevertheless, the goal of improving therapies for liver IRI has been hindered by the need to develop a thorough understanding about which factors drive leukocyte recruitment and production of inflammatory cytokines. This study provides new insights on the role of fibronectin in the pathophysiology of hepatic IRI. FN can exist in two forms, plasma and cellular FN; plasma FN circulates in the blood in a closed (allegedly) non-active form, while cellular FN, which exists as part of the extracellular matrix, has been linked to most of the FN activities in the body^{31, 32}. Fibronectin structural diversity occurs by regulated alternative splicing of a single gene transcript in three segments termed EIIIA, EIIIB and V (or IIICS) domains; the latter form contains the CS-1 region that binds the $\alpha 4\beta 1$ integrin^{15, 33}. Cellular FN, which is virtually absent in normal adult tissues, is abundantly present in the matrices of tissues under several pathological conditions, such as tumor metastasis⁷, rheumatoid arthritis⁸, and organ transplantation¹⁴. In liver, fibronectin is expressed very early by sinusoidal endothelial cells as a response to injury³⁴, including to hepatic IRI¹⁷.

We have previously demonstrated that CS-1 peptide facilitate blockade of $\alpha 4\beta 1$ -FN interactions disrupted leukocyte infiltration and ameliorated steatotic liver IRI in a model of ex vivo 4-hour cold ischemia followed by isotransplantation¹⁷. Comparable beneficial effects of both reducing leukocyte infiltration and increasing recipient survival were observed with the CS-1 peptide therapy in prolonged cold liver IRI. In this well-established rat liver model of ex vivo 24-hour cold ischemia followed by isotransplantation, CS-1 peptides significantly improved liver histological preservation and increased recipient survival. CS-1 peptide therapy disrupted the recruitment of T cells, NK cells, macrophages, and neutrophils, which are leukocytes associated with the

development of liver IRI^{35, 36}. Moreover, the CS-1 peptide therapy suppressed the release of several pro-inflammatory mediators, such as TNF- α , IFN- γ , COX-2 and iNOS, in the prolonged cold liver IRI model. TNF- α as well as IFN- γ are critical mediators of liver IRI³⁵. COX-2 and iNOS have been shown by us^{23, 25} and by others^{11, 37} to have deleterious effects on liver IRI.

Leukocyte transmigration across vascular barriers is dependent on both adhesive and matrix degradation mechanisms. Whereas adhesion molecules are important to leukocyte transmigration by providing leukocyte attachment to the vascular endothelium, matrix metalloproteinases are critical for facilitating leukocyte movement across vascular barriers. Among different MMPs, MMP-9, an inducible gelatinase expressed by leukocytes during hepatic IRI, is emerging as an important mediator of leukocyte traffic to inflamed liver⁵. Fibronectin- α 4 β 1 interactions are capable of upregulating MMP-9 expression by infiltrating leukocytes¹⁸, which is a critical mediator of leukocyte recruitment in liver IRI²². There is a growing body of evidence supporting that a complex spatiotemporal regulation of the proteolytic activity is involved in focal matrix degradation during extravasation²⁷. In addition to downregulating the expression of MMP-9, CS-1 peptide therapy also depressed the expression of MT1-MMP/MMP-14 in hepatic IRI. The membrane-anchored MT1-MMP/MMP-14 is a MMP involved in the breakdown of several adhesion molecules, including fibronectin³⁸. Unlike soluble MMPs, MT1-MMP/MMP-14 has a stretch of hydrophobic amino acids that anchors the enzyme to the plasma membrane and restricts its activity to the cell surface^{35, 39}. We show for the first time that MT1-MMP/MMP-14 expression, which was undetectable in naïve livers, was upregulated by infiltrating monocyte/macrophages after prolonged liver IRI, suggesting a potential role for MT1-MMP/MMP-14 in liver IRI, particularly on leukocyte recruitment. MT1-MMP/MMP-14 has been associated to focalized ECM degradation and to migration

of a variety of cell types, including endothelial and tumor cells ⁴⁰. Indeed, it has been recently demonstrated that MT1-MMP/MMP-14 is needed to increase the migration of cancer cells in mammary tumors ³⁵. Moreover, MT1-MMP/MMP-14 inhibition impairs the *in vitro* migration of stimulated human monocytes on fibronectin ²⁷. All together, these observations support the view that MT1-MMP/MMP-14 may act as an amplifier of leukocyte recruitment in liver IRI; additional experimentation is warranted to further unveil the role of MT1-MMP/MMP-14 in inflamed livers.

Integrins transmit information from the ECM to the cell resulting in activation of cell signaling pathways important for regulating different cell functions, including adhesion, migration, and proliferation ⁴¹. The p38 MAPK signaling transduction pathway, which is activated through extracellular stimuli, plays an essential role in regulating inflammatory processes ⁴², including hepatic IRI ⁴³. Moreover, it has been suggested that the main biological response of p38 MAPK activation has been linked to initiation of leukocyte recruitment and activation ⁴³. Indeed, the results of this study show that CS-1 mediated blockade of the FN- α 4 β 1 interactions, which disrupted leukocyte recruitment, markedly depressed the phosphorylation of p38 MAPK. A better understanding of the intracellular signaling pathways linked to MMP expression may lead to improved therapies in liver IRI. We next investigated whether treatment with the pharmacological inhibitor of p38 MAPK SB203580 would affect the expressions of MMP-9 and MT1-MMP/MMP-14 in fibronectin-stimulated macrophages. Macrophages are major sources of MMP-9 in cold liver IRI ¹⁸ and, as we report here, of MT1-MMP/MMP-14 as well. Indeed, inhibition of p38 MAPK activity with the antagonist SB203580 depressed the expressions of MMP-9 and MT1-MMP/MMP-14 by fibronectin-stimulated macrophages in culture, suggesting that the p38 MAPK kinase pathway controls fibronectin MMP-9 and MT1-MMP/MMP-14 inductions. In this regard, activation of p38 MAPK has been

implied in $\text{TNF}\alpha$ mediated MMP-9 induction ⁴⁴ and in promoting cancer cell invasion via regulation of MMP mRNA stability ⁴⁵.

In summary, the findings we report here further emphasize an important role for the FN- $\alpha 4\beta 1$ integrin interactions in cold hepatic IRI. Our results show that CS-1 peptide therapy down-regulated the expressions of MMP-9 and MT1-MMP/MMP-14, disrupted leukocyte recruitment, and decreased the release of pro-inflammatory mediators, resulting in protection against prolonged cold liver IRI and increased OLT recipient survival. Additionally, the CS-1 peptide facilitated blockade of the FN- $\alpha 4\beta 1$ integrin interactions depressed the phosphorylation of p38 MAPK, which is considered to be an attractive target for pharmacologic intervention ⁴⁶, and therefore implying a regulatory role for fibronectin on the activation of p38 MAPK in hepatic IRI. Furthermore, we provide evidence that p38 MAPK kinase pathway controls the fibronectin mediated induction of both MMP-9 and MT1-MMP/MMP-14 by macrophages. Thus, this work provides new insights on the role of fibronectin that can potentially be applied to the development of new pharmacological strategies to ameliorate hepatic IRI.

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REFERENCES:

1. Selzner N, Rudiger H, Graf R, Clavien PA: Protective strategies against ischemic injury of the liver, *Gastroenterology* 2003, 125:917-936
2. Busuttil RW, Tanaka K: The utility of marginal donors in liver transplantation, *Liver Transpl* 2003, 9:651-663
3. Carman CV, Springer TA: Trans-cellular migration: cell-cell contacts get intimate, *Curr Opin Cell Biol* 2008, 20:533-540
4. Jaeschke H, Hasegawa T: Role of neutrophils in acute inflammatory liver injury, *Liver Int* 2006, 26:912-919
5. Coito AJ: Leukocyte transmigration across endothelial and extracellular matrix protein barriers in liver ischemia/reperfusion injury, *Curr Opin Organ Transplant* 2011, 16:34-40
6. Bianchi E, Bender JR, Blasi F, Pardi R: Through and beyond the wall: late steps in leukocyte transendothelial migration, *Immunol Today* 1997, 18:586-591
7. Garmy-Susini B, Avraamides CJ, Schmid MC, Foubert P, Ellies LG, Barnes L, Feral C, Papayannopoulou T, Lowy A, Blair SL, Cheresh D, Ginsberg M, Varner JA: Integrin $\alpha 4 \beta 1$ signaling is required for lymphangiogenesis and tumor metastasis, *Cancer Res* 2010, 70:3042-3051
8. Lefebvre JS, Levesque T, Picard S, Pare G, Gravel A, Flamand L, Borgeat P: Extra domain A of fibronectin primes leukotriene biosynthesis and stimulates neutrophil migration through activation of Toll-like receptor 4, *Arthritis Rheum* 2011, 63:1527-1533

9. van Horssen J, Dijkstra CD, de Vries HE: The extracellular matrix in multiple sclerosis pathology, *J Neurochem* 2007, 103:1293-1301
10. Coito AJ, Brown LF, Peters JH, Kupiec-Weglinski JW, van de Water L: Expression of fibronectin splicing variants in organ transplantation: a differential pattern between rat cardiac allografts and isografts, *Am J Pathol* 1997, 150:1757-1772
11. Moon KH, Hood BL, Mukhopadhyay P, Rajesh M, Abdelmegeed MA, Kwon YI, Conrads TP, Veenstra TD, Song BJ, Pacher P: Oxidative inactivation of key mitochondrial proteins leads to dysfunction and injury in hepatic ischemia reperfusion, *Gastroenterology* 2008, 135:1344-1357
12. O'Connor P: Natalizumab and the role of alpha 4-integrin antagonism in the treatment of multiple sclerosis, *Expert Opin Biol Ther* 2007, 7:123-136
13. Fiorino G, Correale C, Fries W, Repici A, Malesci A, Danese S: Leukocyte traffic control: a novel therapeutic strategy for inflammatory bowel disease, *Expert Rev Clin Immunol* 2010, 6:567-572
14. Coito AJ, de Sousa M, Kupiec-Weglinski JW: Fibronectin in immune responses in organ transplant recipients, *Dev Immunol* 2000, 7:239-248
15. Guan JL, Hynes RO: Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1, *Cell* 1990, 60:53-61
16. Perez VA, Ali Z, Alastalo TP, Ikeno F, Sawada H, Lai YJ, Kleisli T, Spiekerkoetter E, Qu X, Rubinos LH, Ashley E, Amieva M, Dedhar S,

Rabinovitch M: BMP promotes motility and represses growth of smooth muscle cells by activation of tandem Wnt pathways, *J Cell Biol* 2011, 192:171-188

17. Amersi F, Shen XD, Moore C, Melinek J, Busuttil RW, Kupiec-Weglinski JW, Coito AJ: Fibronectin-alpha 4 beta 1 integrin-mediated blockade protects genetically fat Zucker rat livers from ischemia/reperfusion injury, *Am J Pathol* 2003, 162:1229-1239

18. Moore C, Shen XD, Gao F, Busuttil RW, Coito AJ: Fibronectin-alpha4beta1 integrin interactions regulate metalloproteinase-9 expression in steatotic liver ischemia and reperfusion injury, *Am J Pathol* 2007, 170:567-577

19. Kamada N, Davies HS, Wight D, Culank L, Roser B: Liver transplantation in the rat. Biochemical and histological evidence of complete tolerance induction in non-rejector strains, *Transplantation* 1983, 35:304-311

20. Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR: VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site, *Cell* 1990, 60:577-584

21. Elices MJ, Tsai V, Strahl D, Goel AS, Tollefson V, Arrhenius T, Wayner EA, Gaeta FC, Fikes JD, Firestein GS: Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature, *J Clin Invest* 1994, 93:405-416

22. Hamada T, Fondevila C, Busuttil RW, Coito AJ: Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury, *Hepatology* 2008, 47:186-198

23. Hamada T, Duarte S, Tsuchihashi S, Busuttil RW, Coito AJ: Inducible nitric oxide synthase deficiency impairs matrix metalloproteinase-9 activity and disrupts leukocyte migration in hepatic ischemia/reperfusion injury, *Am J Pathol* 2009, 174:2265-2277
24. Kim SF, Huri DA, Snyder SH: Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2, *Science* 2005, 310:1966-1970
25. Hamada T, Tsuchihashi S, Avanesyan A, Duarte S, Moore C, Busuttil RW, Coito AJ: Cyclooxygenase-2 deficiency enhances Th2 immune responses and impairs neutrophil recruitment in hepatic ischemia/reperfusion injury, *J Immunol* 2008, 180:1843-1853
26. Lappas CM, Rieger JM, Linden J: A2A adenosine receptor induction inhibits IFN-gamma production in murine CD4+ T cells, *J Immunol* 2005, 174:1073-1080
27. Matias-Roman S, Galvez BG, Genis L, Yanez-Mo M, de la Rosa G, Sanchez-Mateos P, Sanchez-Madrid F, Arroyo AG: Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium, *Blood* 2005, 105:3956-3964
28. Johnson GL, Lapadat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases, *Science* 2002, 298:1911-1912
29. Shankavaram UT, Lai WC, Netzel-Arnett S, Mangan PR, Ardans JA, Caterina N, Stetler-Stevenson WG, Birkedal-Hansen H, Wahl LM: Monocyte

membrane type 1-matrix metalloproteinase. Prostaglandin-dependent regulation and role in metalloproteinase-2 activation, *J Biol Chem* 2001, 276:19027-19032

30. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, Pina E, Geller DA: Factors in the pathophysiology of the liver ischemia-reperfusion injury, *J Surg Res* 2008, 147:153-159

31. Pankov R, Yamada KM: Fibronectin at a glance, *J Cell Sci* 2002, 115:3861-3863

32. Wierzbicka-Patynowski I, Schwarzbauer JE: The ins and outs of fibronectin matrix assembly, *J Cell Sci* 2003, 116:3269-3276

33. Schwarzbauer JE, Tamkun JW, Lemischka IR, Hynes RO: Three different fibronectin mRNAs arise by alternative splicing within the coding region, *Cell* 1983, 35:421-431

34. Jarnagin WR, Rockey DC, Koteliensky VE, Wang SS, Bissell DM: Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis, *J Cell Biol* 1994, 127:2037-2048

35. Le Moine O, Louis H, Demols A, Desalle F, Demoor F, Quertinmont E, Goldman M, Deviere J: Cold liver ischemia-reperfusion injury critically depends on liver T cells and is improved by donor pretreatment with interleukin 10 in mice, *Hepatology* 2000, 31:1266-1274

36. Gao B, Jeong WI, Tian Z: Liver: An organ with predominant innate immunity, *Hepatology* 2008, 47:729-736

37. Ozturk H, Gezici A: The effect of celecoxib, a selective COX-2 inhibitor, on liver ischemia/reperfusion-induced oxidative stress in rats, *Hepatol Res* 2006, 34:76-83
38. Sternlicht MD, Werb Z: How matrix metalloproteinases regulate cell behavior, *Annu Rev Cell Dev Biol* 2001, 17:463-516
39. Seiki M: Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion, *Cancer Lett* 2003, 194:1-11
40. Moss NM, Wu YI, Liu Y, Munshi HG, Stack MS: Modulation of the membrane type 1 matrix metalloproteinase cytoplasmic tail enhances tumor cell invasion and proliferation in three-dimensional collagen matrices, *J Biol Chem* 2009, 284:19791-19799
41. Mainiero F, Gismondi A, Soriani A, Cippitelli M, Palmieri G, Jacobelli J, Piccoli M, Frati L, Santoni A: Integrin-mediated ras-extracellular regulated kinase (ERK) signaling regulates interferon gamma production in human natural killer cells, *J Exp Med* 1998, 188:1267-1275
42. Ono K, Han J: The p38 signal transduction pathway: activation and function, *Cell Signal* 2000, 12:1-13
43. King LA, Toledo AH, Rivera-Chavez FA, Toledo-Pereyra LH: Role of p38 and JNK in liver ischemia and reperfusion, *J Hepatobiliary Pancreat Surg* 2009, 16:763-770
44. Li H, Mittal A, Paul PK, Kumar M, Srivastava DS, Tyagi SC, Kumar A: Tumor necrosis factor-related weak inducer of apoptosis augments matrix metalloproteinase 9 (MMP-9) production in skeletal muscle through the activation

of nuclear factor-kappaB-inducing kinase and p38 mitogen-activated protein kinase: a potential role of MMP-9 in myopathy, J Biol Chem 2009, 284:4439-4450

45. Kumar B, Koul S, Petersen J, Khandrika L, Hwa JS, Meacham RB, Wilson S, Koul HK: p38 mitogen-activated protein kinase-driven MAPKAPK2 regulates invasion of bladder cancer by modulation of MMP-2 and MMP-9 activity, Cancer Res 2010, 70:832-841

46. Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S, Braunwart J, Glascock BJ, Klevitsky R, Kimball TF, Hewett TE, Molkentin JD: Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling, J Clin Invest 2003, 111:1475-1486

DISCLOSURE: The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Figure Legends

Figure 1. Representative cellular fibronectin staining in 24h cold liver IRI. Cellular fibronectin was virtually absent in naïve livers (A) and it was abundantly upregulated in livers after 24h of cold storage followed by 6h (B) and 24h (C) of reperfusion (n=4/group).

Figure 2. Transaminase levels, histology, and recipient survival in 24h cold liver IRI. CS-1 peptide therapy significantly improved liver function as evidenced by the lower AST and ALT levels (panel A) in the CS-1 peptide treated recipients at 6h and 24h post-cold liver IRI. Hematoxylin and eosin staining of liver grafts (panel, B) indicated a better histological preservation in the CS-1 peptide treated liver OLTs (c, and d) as compared with respective controls (a, and b), at 6h (a, and c) and 24h (b, and d) post-OLT. Moreover, CS-1 peptide treated OLTs had a significantly prolonged survival rate (panel C) as compared to respective controls at 14-day post-OLT (*p<0.008, **p<0.006, &p<0.004, and &&p<0.005; x100 H&E; panels A and B n=5-7 rats/group; panel C n=8 rats/group).

Figure 3. Leukocyte infiltration in 24h cold liver IRI. The infiltration of T lymphocytes (panel A), NK cells (panel B), and monocyte/ macrophages (panel C) was significantly depressed in CS-1 peptide treated OLTs at 6h and 24h post-reperfusion. Panel D illustrates immunoperoxidase staining of T cells (a, and b), NK lymphocytes (c, and d), and macrophages (e, and f) in CS-1 (b, d, and f) and control (a, c, and e) OLTs at 6h post-transplantation. MPO activity (panel E), an index of neutrophil infiltration, was significantly depressed in CS-1 peptide treated OLTs at 6h and 24h post-transplantation

as compared to respective controls (* $p < 0.002$, ** $p < 0.003$, & $p < 0.008$, && $p < 0.005$, $\tau < 0.02$; x200; n=5-6 rats/group).

Figure 4. COX-2, iNOS and proinflammatory cytokine gene expression in cold liver IRI. The mRNA expressions of COX-2 and iNOS, two major tissue injury mediators, as well as of various proinflammatory cytokines were profoundly depressed in the CS-1 treated grafts at 6h post-IRI (* $p < 0.005$, ** $p < 0.05$, *** $p < 0.02$, & $p < 0.01$; n=4 rats/group).

Figure 5. MMP-9 and MT1-MMP expressions in 24h cold liver IRI. The expressions of MMP-9 (panels A-C) and MT1-MMP (panels D-F) at mRNA (panel A and D) and protein (panel B and E) levels were readily detected in control OLTs (lanes 1, and 2) and only slightly detected in CS-1 peptide treated livers at 6h post-IRI (lanes 3, and 4); MMP-9 and MT1-MMP expressions were nearly undetectable in naïve livers (lane 5). Panel C shows MMP-9 + leukocyte infiltration in control (a) and CS-1 peptide treated (b) OLTs at 6h post-IRI. Panel F displays MT1-MMP and Mac-1 immunofluorescence staining in control (a, c, and e) and CS-1 peptide treated (b, d, and f) livers at 6h post-OLT. Mac-1 (a, and b) is stained in green (Alexa Fluor 488) and MT1-MMP (c, and d) is labeled in red (Alexa Fluor 594); cell colocalization of Mac-1/MT1-MMP markers is shown in yellow-orange (e, and f) (* $p < 0.03$, ** $p < 0.02$, and & $p < 0.003$; arrows denote positive labeling; x 200; n=5-6 rats/group).

Figure 6. Mitogen-activated protein kinase pathways in 24h cold liver IRI.

Levels of p38 MAPK and p44/42 MAPK phosphorylation (panel A) in control (lanes 1, and 2) and in CS-1 peptide (lanes 3, and 4) treated livers at 6h post-IRI. Densitometric analysis (panel B) revealed a marked decrease in p38 MAPK activation in CS-1 peptide treated OLTs as compared to controls; there were no significant

differences on p44/42 MAPK phosphorylation between CS-1 peptide treated and control OLTs (* $p < 0.03$; $n = 6/\text{group}$).

Figure 7. Fibronectin-mediated MMP-9 and MT1-MMP expressions are dependent on p38 MAPK activation. Attenuation of FN-mediated MMP-9 and MT1-MMP mRNA expressions (panel A) by inhibition of p38 MAPK in isolated macrophages treated with SB-203580 (10 $\mu\text{g/ml}$). While polymyxin B (10 ng/ml) profoundly depressed the expression of MMP-9 and MT1-MMP in LPS-stimulated macrophages, it didn't significantly affect the production of MMP-9 or MT1-MMP induced by fibronectin in isolated macrophages. Conditioned media obtained from cultured macrophages was subjected to a gelatin zymography assay (panel B); inhibition of p38 MAPK with SB-203580 significantly depressed the MMP-9 activity in fibronectin-stimulated macrophages (graph represents fold increases in enzymatic activity over unstimulated macrophages). MMP levels expressed as mean $\pm$ SD of four experiments (* $p < 0.009$, ** $p < 0.001$, *** $p < 0.002$ and $\&p < 0.005$, relative to unstimulated controls; $\delta p < 0.02$, $\delta\delta p < 0.002$, and $\delta\delta\delta p < 0.005$, relative to fibronectin stimulated controls; $\tau p < 0.004$, and $\tau\tau p < 0.001$ relative to LPS stimulated controls).

FIGURE 1

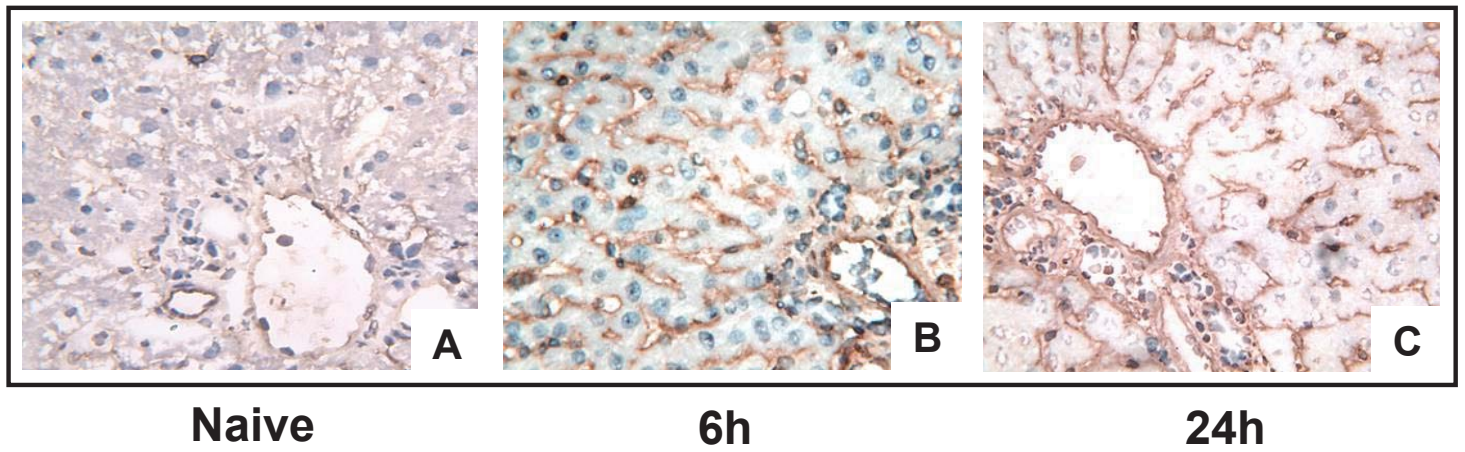


FIGURE 2

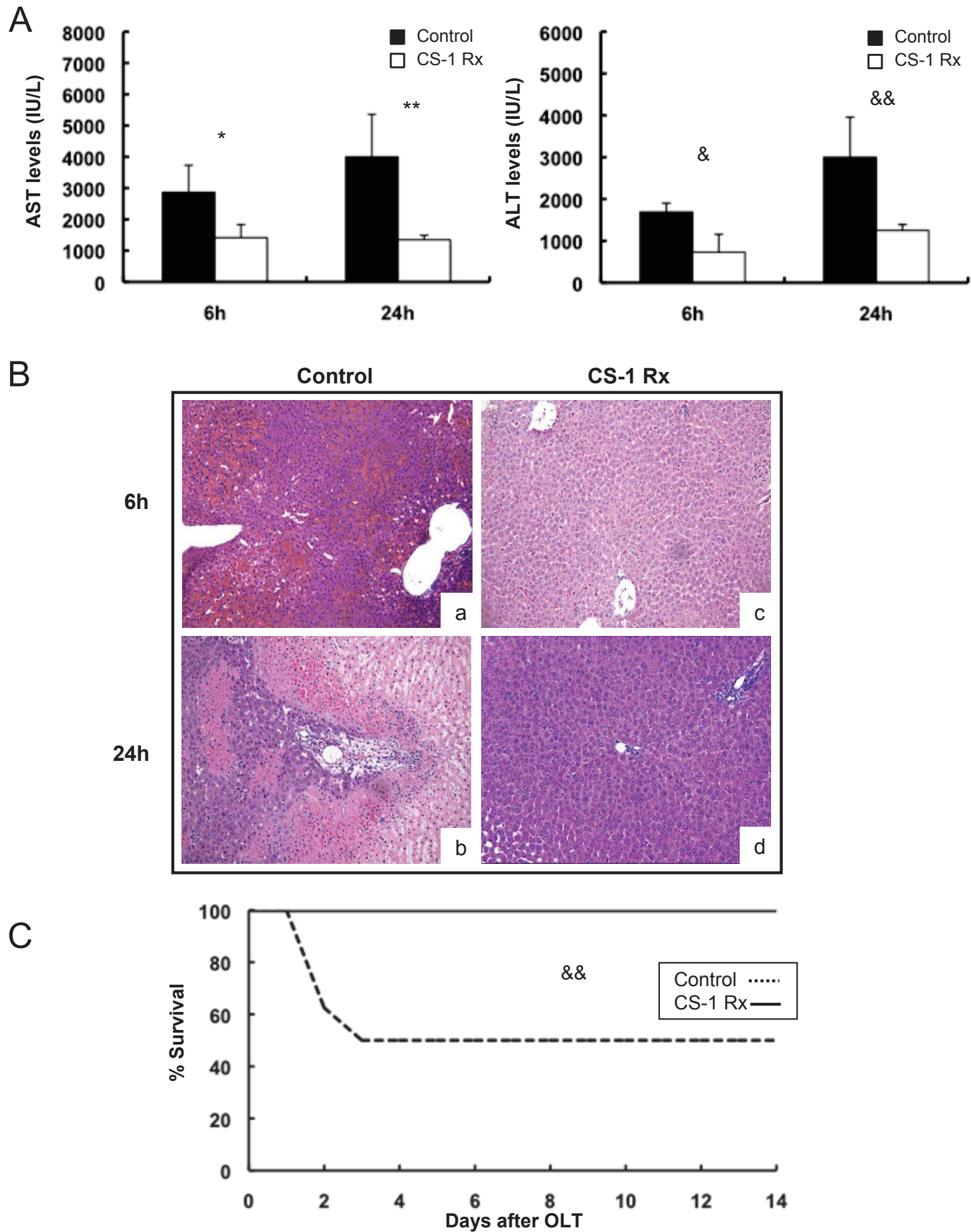


FIGURE 3

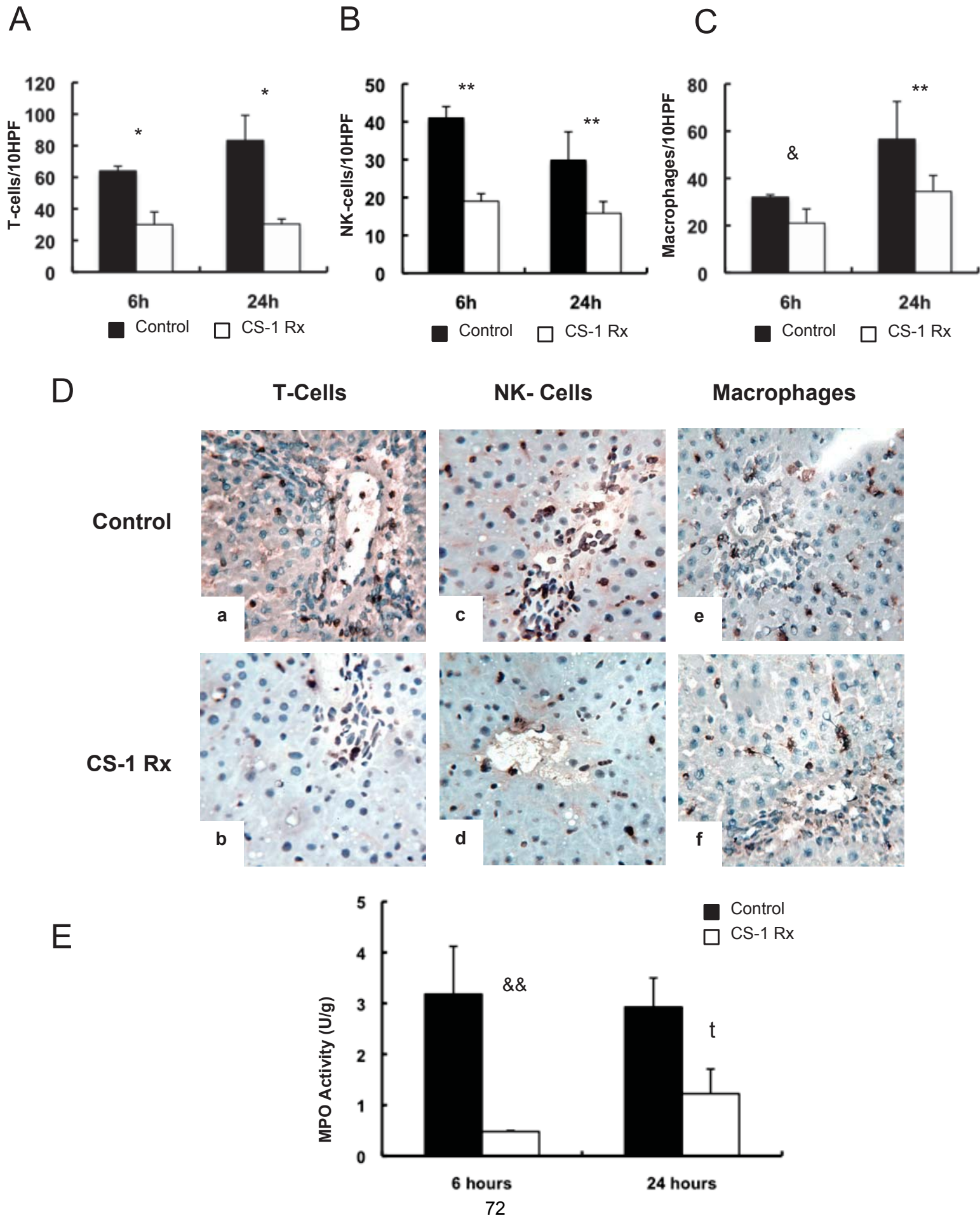


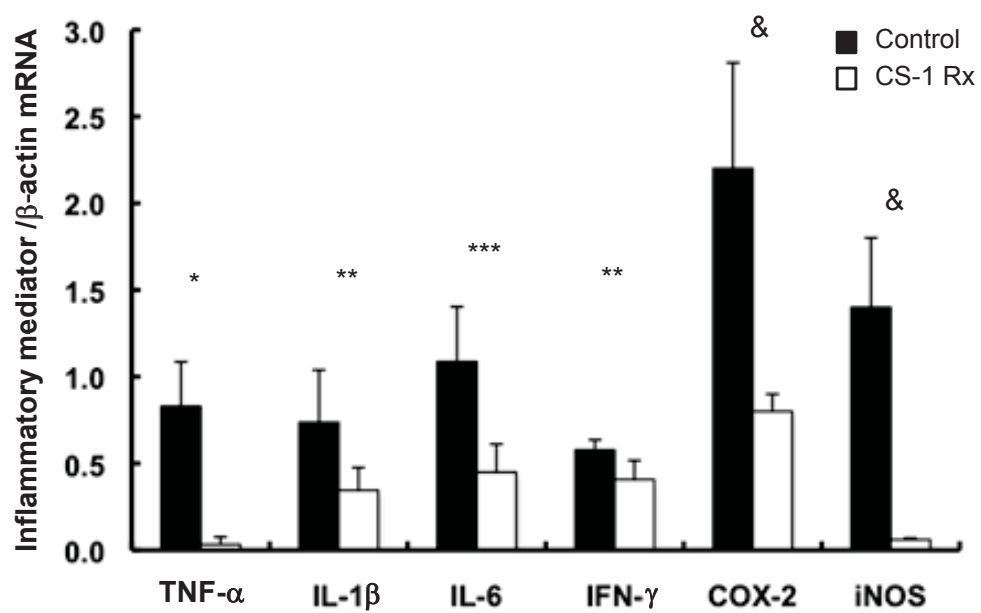
FIGURE 4**A**

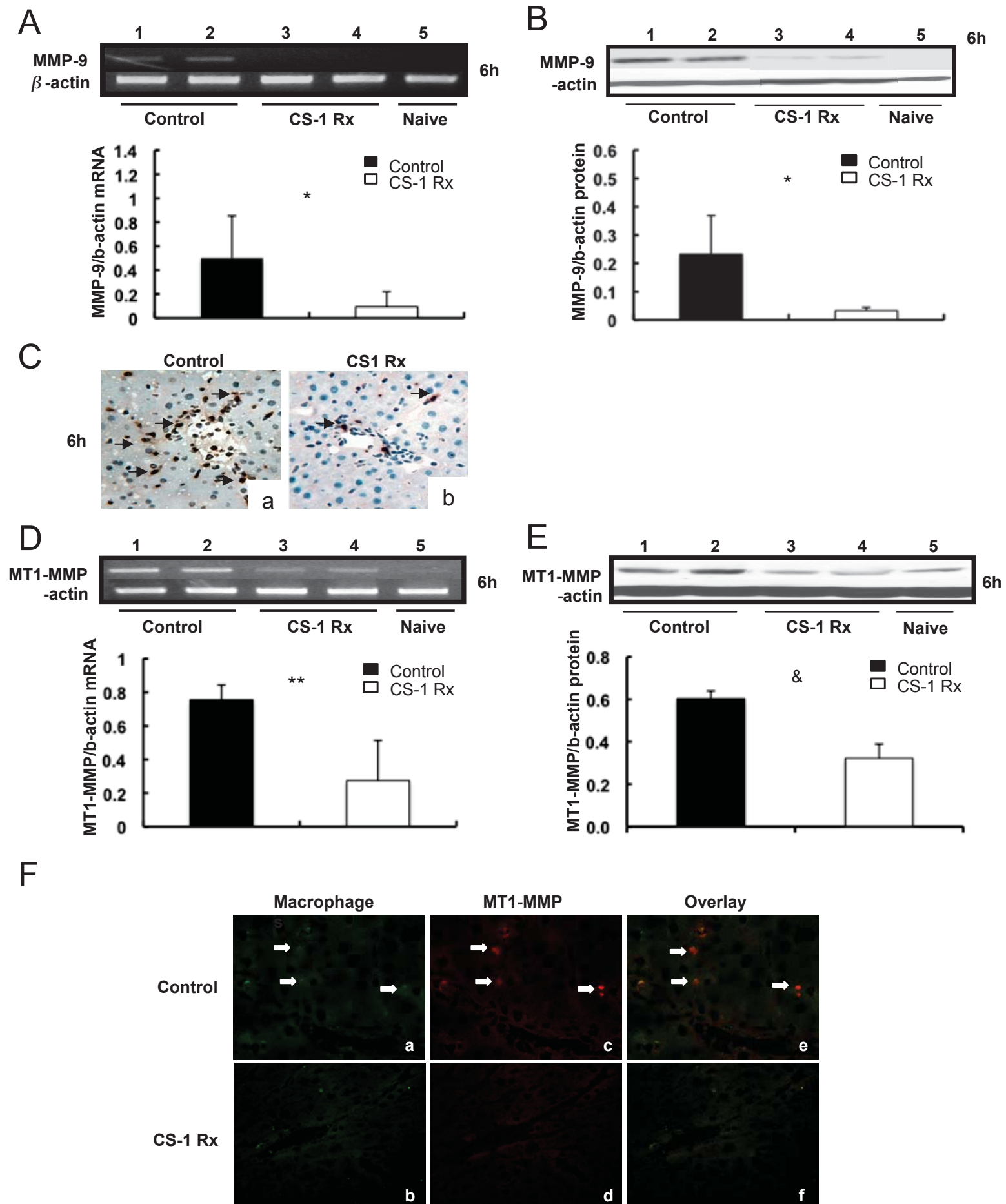
FIGURE 5

FIGURE 6

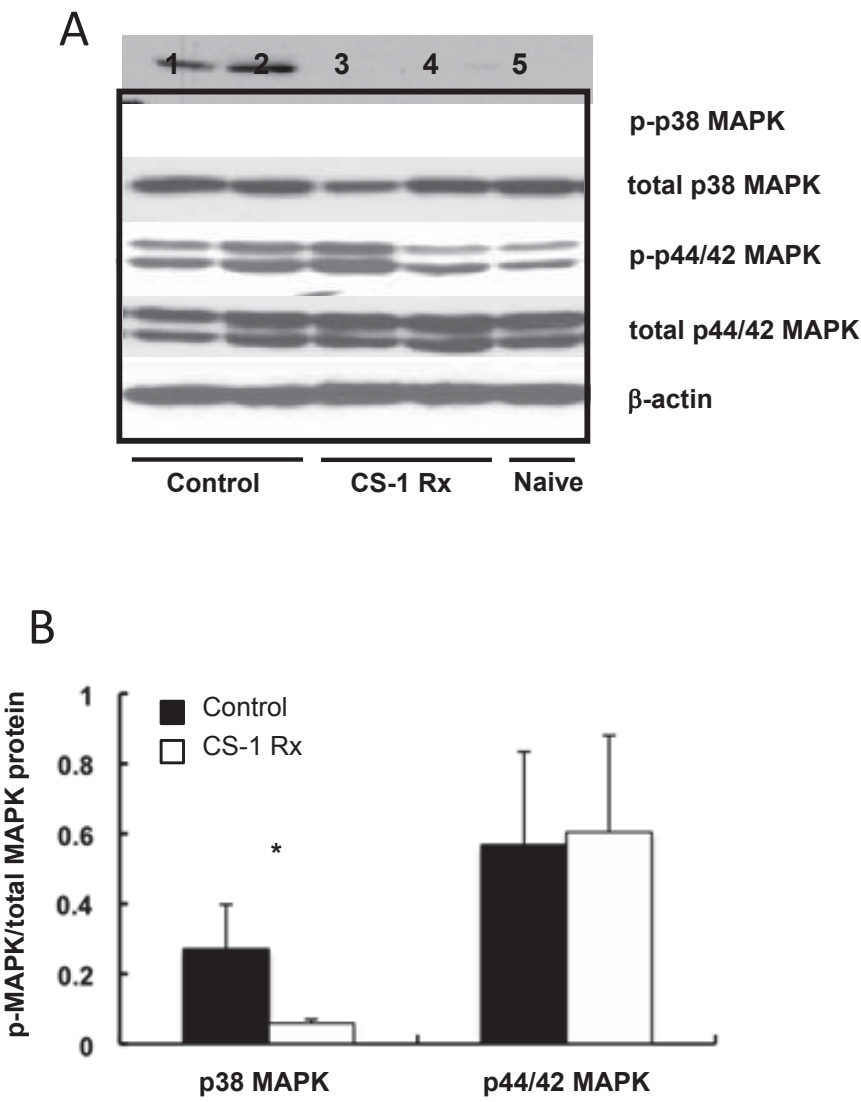
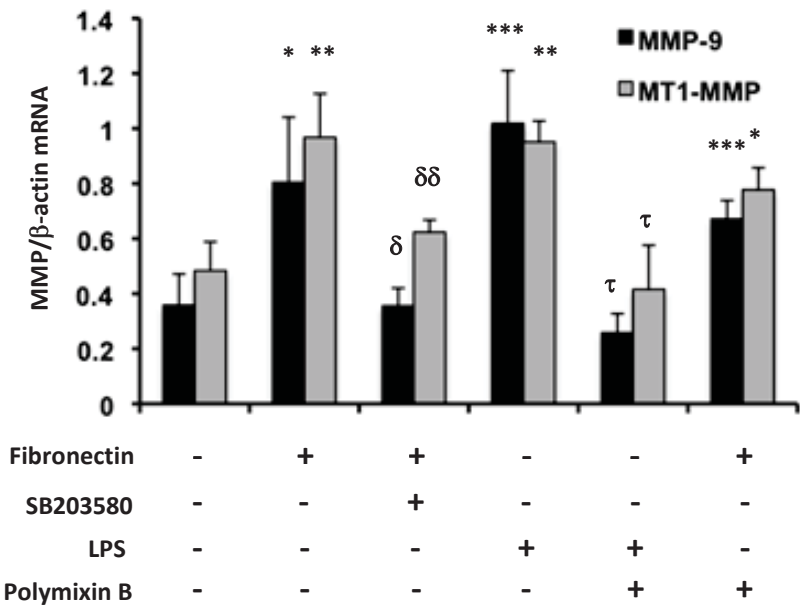
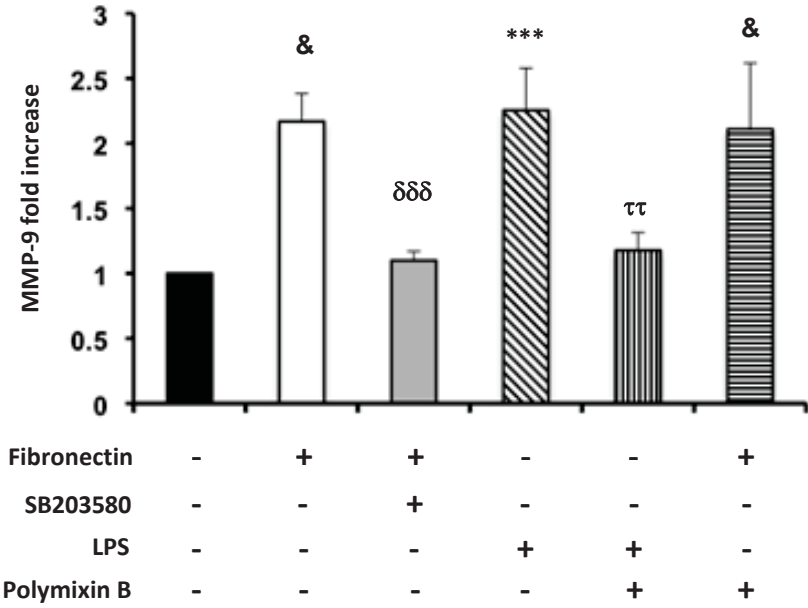


FIGURE 7

A



B



CHAPTER III

CYTOPROTECTIVE EFFECTS OF A CYCLIC RGD PEPTIDE IN STEATOTIC LIVER COLD ISCHEMIA AND REPERFUSION INJURY

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and Ana J. Coito

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Cytoprotective Effects of a Cyclic RGD Peptide in Steatotic Liver Cold Ischemia and Reperfusion Injury

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The serious need for expanding the donor population has attracted attention to the use of steatotic donor livers in orthotopic liver transplantation (OLT). However, steatotic livers are highly susceptible to hepatic ischemia–reperfusion injury (IRI). Expression of fibronectin (FN) by endothelial cells is an important feature of hepatic response to injury. We report the effect of a cyclic RGD peptide with high affinity for the $\alpha 5\beta 1$, the FN integrin receptor, in a rat model of steatotic liver cold ischemia, followed by transplantation. RGD peptide therapy ameliorated steatotic IRI and improved the recipient survival rate. It significantly inhibited the recruitment of monocyte/macrophages and neutrophils, and depressed the expression of pro-inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and interferon (IFN)- γ . Moreover, it resulted in profound inhibition of metalloproteinase-9 (MMP-9) expression, a gelatinase implied in leukocyte migration in damaged livers. Finally, we show that RGD peptide therapy reduced the expression of the 17-kDa active caspase-3 and the number of apoptotic cells in steatotic OLTs. The observed protection against steatotic liver IRI by the cyclic RGD peptides with high affinity for the $\alpha 5\beta 1$ integrin suggests that this integrin is a potential therapeutic target to allow the successful utilization of marginal steatotic livers in transplantation.

Key words: Fibronectin, hepatic steatosis, inflammation, integrin, ischemia/reperfusion injury, liver transplantation

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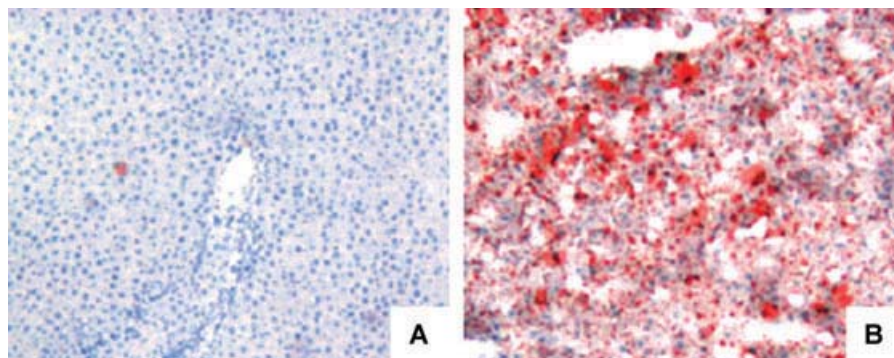
OLT is an effective therapeutic modality for end-stage liver disease. However, due to the shortage of organ donors, many patients die every year while on the waiting list (1).

The serious need in expanding the donor population has attracted attention to the possible use of steatotic donor livers, which are frequently discarded because of the fear of primary nonfunction, or dysfunction, after transplantation (2). IRI is a multifactorial antigen-independent inflammatory process that can lead to graft loss, particularly with marginal donor organs (2,3). Indeed, a growing body of evidence shows that IRI is poorly tolerated in fatty livers (4–6).

The migration of leukocytes is a key event in acute inflammatory liver injury (7). The transmigration of these cells across endothelial and extracellular matrix (ECM) protein barriers is dependent on a cascade of adhesion and focal matrix degradation events (8). FN is a large glycoprotein with a central role in cellular adhesion and migration. The very early expression of the ‘so-called’ cellular FN by sinusoidal endothelial cells is a prominent feature of hepatic response to injury (9), including IRI in steatotic livers (10). The role of FN in leukocyte adhesion, migration and activation has been extensively reported (11). FN has been implicated in multiple pathological conditions, including tumor metastasis (12), rheumatoid arthritis (13), cardiac allograft rejection (14), liver fibrosis (9) and liver IRI (10). The effects of FN are primarily mediated by integrins, a superfamily of cell surface receptors (15). $\alpha 4\beta 1$ (VLA-4) and $\alpha 5\beta 1$ (VLA-5) integrins are the two major FN receptors expressed on leukocytes; of these, the $\alpha 5\beta 1$ integrin is highly selective for FN and requires the RGD sequence on the tenth type III repeats of FN for ligand recognition (16). This integrin is expressed on T lymphocytes, monocyte/macrophages and polymorphonuclear cells (17–19).

In the present study, we have examined the effects in steatotic liver IRI of a cyclic RGD peptide, which avidly binds the $\alpha 5\beta 1$ integrin and particularly inhibits cell attachment to FN (20). We show that the cyclic RGD peptide therapy (1) ameliorated hepatocellular injury in steatotic OLTs and prolonged recipient survival, (2) disrupted monocyte/macrophage and neutrophil infiltration, (3) downregulated metalloproteinase-9 (MMP-9) expression, (4) depressed proinflammatory mediators, and (5) resulted in Akt upregulation and decreased apoptosis in steatotic OLTs. These data support the concept of the $\alpha 5\beta 1$ integrin as a potential therapeutic target in steatotic liver I/R injury.

Figure 1: Representative Oil Red O staining in naïve livers of normal (fa/-) and genetically obese (fa/fa) Zucker rats. Steatotic naïve livers (B) were characterized by >30% steatosis, whereas normal lean naïve livers (A) showed virtually no signs of steatosis.



Materials and Methods

Animals, grafting techniques and cyclic RGD peptide therapy

Genetically obese (fa/fa-) male Zucker (230–275 g), and lean (fa/-) Zucker (260–300 g) rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Syngenic OLTs were performed using fatty livers that were recovered from obese Zucker rats. Steatotic livers were stored at 4°C in University of Wisconsin (UW) solution for 4 h before being transplanted into lean Zucker recipients. The standard techniques of liver harvesting and orthotopic transplantation without hepatic artery reconstruction were performed according to the previously described Kamada's and Calne's cuff technique (21) and an anhepatic phase of 16–20 min. Cyclic CRGDGWC (RGD) peptides (500 µg/rat) that avidly bind the $\alpha 5\beta 1$ integrin (20) were administered *ex vivo* via portal vein to steatotic livers before cold storage, and immediately prior to reperfusion. In addition, OLT recipients received a 3-day course of cyclic RGD peptides (1 mg/rat per day, ip) posttransplantation. Cyclization of RGD peptides increases their affinity and inhibitory properties (22), and these cyclic RGD peptides are potent inhibitors of cell attachment to FN (20). Control recipients received vehicle or a scrambled peptide in a similar fashion as in the RGD-treated group. Rat recipients of steatotic OLTs were followed for survival. Separate groups of rats were sacrificed at 6 h, 24 h and day 7 after OLT, and liver samples were collected for further analysis. Animals were fed a standard rodent diet and water *ad libitum* and cared for according to guidelines approved by the American Association of Laboratory Animal Care. Oil Red-O staining confirmed the high content of fat in the steatotic donor livers. As shown in Figure 1, while naïve livers recovered from normal Zucker rats (fa/-) (recipients) showed virtually no steatosis, naïve livers recovered from fatty Zucker rat (fa/fa) (donors) showed over 30% steatosis. Indeed, Fatty Zucker rats of 230–275 g body weight have >30% liver steatosis, which sets them as marginal donors (10).

Assessment of hepatocellular damage

Serum glutamic-oxaloacetic transaminase (sGOT), an indicator of hepatocellular injury, was measured in blood samples obtained at 6 and 24 h, and day 7 after hepatic reperfusion. Measurements were made with an auto analyzer by ANTECH Diagnostics (Los Angeles, CA).

Histology

Liver specimens were fixed in 10% buffered formalin solution and embedded in paraffin. Sections were made at 4 µm and stained with H&E. The histological severity of IRI in the liver was graded using modified Suzuki's criteria (23). In this classification, sinusoidal congestion, hepatocyte necrosis and ballooning degeneration are graded from 0 to 4. The absence of necrosis, congestion or centrilobular ballooning is given a score of 0, while severe congestion and ballooning degeneration, as well as >60% lobular necrosis is given a value of 4.

Immunohistochemistry

Steatotic OLTs were also examined for leukocyte infiltration and FN deposition, as previously described (10). Briefly, cryostat sections were incubated with primary mouse antibody (Ab) against rat T cells (R73), monocytes/macrophages (ED1) (Abd Serotec, Indianapolis, IN), MMP-9 (gelatinase B) (NeoMarkers, Fremont, CA) and cellular FN (IST-9) (Accurate Chemical, Westbury, NY) at optimal dilutions. Bound primary antibody (Ab) was detected using biotinylated anti-mouse IgG and streptavidin peroxidase-conjugated complexes (Dako, Carpinteria, CA). Negative controls included sections in which the primary Ab was replaced with either dilution buffer or normal mouse serum. Control sections from inflammatory tissues known to be positive for each stain were included as positive controls. Sections were evaluated by counting the number of labeled cells within 20 high-power fields (HPF) per section. The relative abundance of some antigens was judged as (–) negative, (+) little, (++) moderately abundant and (+++, >200 cells/20 HPF) highly abundant.

Myeloperoxidase (MPO) assay

MPO is a naturally occurring constituent of neutrophils and is frequently used as a marker for neutrophil infiltration in rat livers (24). Frozen tissue was thawed and suspended in iced 0.5% hexadecyltrimethylammonium and 50 mmol potassium phosphate buffer solution (Sigma, St Louis, MO, USA), of pH 5. After samples were homogenized and centrifuged, 0.1 mL of the supernatant was mixed in the solution of hydrogen peroxide–sodium acetate and tetramethyl benzidine (Sigma). One unit of myeloperoxidase activity was defined as the quantity of enzyme that degraded 1 µmol peroxide per minute at 25°C per gram of tissue.

RNA extraction and reverse transcriptase-PCR

For evaluation of the gene expressions of the pro-inflammatory cytokines, iNOS and MMP-9, RNA was extracted from livers with Trizol (Life Technologies, Inc., Grand Island, NY) using a Polytron RT-3000 (Kinematica AG, Cincinnati, OH), as described (25). Reverse transcription was performed using 4 µg of total RNA in a first-strand cDNA synthesis reaction with SuperScript II RNaseH Reverse Transcriptase (Life Technologies, Inc.). One µL of the resulting reverse transcriptase product was used for polymerase chain reaction amplification.

Western blots

Snap-frozen liver tissue was immediately homogenized as previously described (10). The protein content was determined by a colorimetric assay (Bio-Rad, Hercules, CA). Proteins (40 µg/sample) in the sodium dodecyl sulfate (SDS)-loading buffer were electrophoresed through 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad). The gels were then stained with Coomassie blue to document equal protein loading. The membranes were blocked with 5% dry milk and 0.1% Tween 20 (USB, Cleveland, OH) in PBS and incubated with specific primary antibodies against Caspase-3 (Santa Cruz

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Biotechnology, Santa Cruz, CA), Akt and p-AKT Thr 308 (Cell Signaling Technology, Beverly, MA). The filters were washed and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Amersham, Arlington Heights, IL). After development, membranes were stripped and reblotted with an antibody against actin (Santa Cruz Biotechnology). Relative quantities of protein were determined using a densitometer (Kodak Digital Science 1D Analysis Software, Rochester, NY).

TUNEL assay

The TUNEL assay was performed on 5- μ m cryostat sections using the *In Situ* Cell Death detection kit (Roche) according to the manufacturer's protocol. TUNEL-positive cells were detected under light microscopy. Terminal transferase was omitted as a negative control. Positive controls were generated by treatment with DNase 1 (30 U/mL in 40 mmol/L Tris-Cl (pH 7.6), 6 mmol/L MgCl₂, and 2 mmol/L CaCl₂ for 30 min).

Data and statistical analysis

All values are expressed as the mean $\pm$ the standard deviations. Differences between groups were compared using the Mann-Whitney test for continuous variables, and a two-tailed p-value <0.05 was considered significant. Animal survival was analyzed according to the method of Kaplan-Meier, and the differences between the two groups were evaluated according to the log-rank test. Calculations were made using SPSS software (SPSS Inc., Chicago, IL).

Results**Cyclic RGD peptide therapy prolongs recipient survival, improves hepatic function and ameliorates hepatocellular injury in steatotic OLTs**

We examined the effects of a cyclic RGD peptide, which has high affinity for the FN receptor α 5 β 1 integrin (20), on the development of IRI in a well-established model of steatotic OLT. Steatotic OLTs treated with the cyclic RGD peptides had a significantly prolonged survival rate compared with respective controls at 14-days post-OLT ($p < 0.02$, $n = 10/\text{group}$) (Figure 2). The prolonged survival rate observed in the RGD-treated steatotic OLTs correlated with improved liver function in these rat recipients, as shown by the decreased sGOT levels (U/L) at 6 h (3470 ± 400 vs. 6900 ± 830 ; $n = 4/\text{gr}$ $p < 0.001$), day 1 (720 ± 80 vs. 2440 ± 900 ; $p < 0.002$, $n = 5/\text{gr}$ $p < 0.001$) and day 7 (170 ± 70 vs. 1330 ± 250 ; $p < 0.006$, $n = 5/\text{gr}$) post-OLT, (Figure 3A). RGD-treated steatotic OLTs showed mild signs of vascular congestion or necrosis, contrasting with severe hepatocyte necrosis and disruption of lobular architecture observed in respective control livers at day 1 post-OLT (Figure 2B). Indeed, the modified Suzuki score was significantly decreased in the cyclic RGD-treated OLTs as compared with respective controls (0.5 ± 0.4 vs. 2.5 ± 0.5 respectively, $p < 0.003$; day 1 after ischemic insult, $n = 5/\text{gr}$). Moreover, RGD therapy showed a long-lasting histological improvement; RGD-treated steatotic livers were characterized by a significant better histological preservation when compared with respective controls at day 7 post-OLT (Figure 3B).

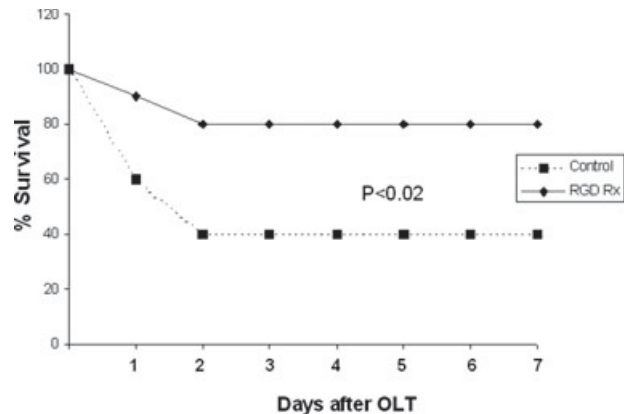


Figure 2: Survival of steatotic OLT recipients. Lean Zucker rats were transplanted with livers recovered from obese Zucker donors. Steatotic OLTs treated with the cyclic RGD peptides, which bind avidly to the α 5 β 1 integrin, had a significantly prolonged survival rate as compared with respective controls at 14-day post-OLT ($p < 0.02$, $n = 10/\text{group}$).

Cyclic RGD peptide therapy disrupts monocyte/macrophage and neutrophil infiltration in steatotic OLTs

We evaluated the role of cyclic RGD peptide therapy on leukocyte infiltration in steatotic OLTs. T lymphocytes (17 ± 5) and ED1+ monocytes/macrophages (29 ± 9) were detected in modest numbers in naïve steatotic livers ($n = 3$). Our earlier studies have shown that the blockade of FN- α 4 β 1 interactions significantly depressed T-cell infiltration, while monocyte/macrophage infiltration was little affected in steatotic livers at day 1 post-OLT (10). Interestingly, in contrast with the blockade of the α 4 β 1 integrin in steatotic OLTs, which preferentially affected T-cell recruitment (10), the cyclic RGD peptide therapy was less effective in reducing T-lymphocyte infiltration at 6 h (66 ± 20 vs. 95 ± 19) and at day 1 post-OLT (70 ± 8 vs. 110 ± 28) (Table 1 and Figure 4; $n = 4\text{--}5/\text{gr}$); it significantly inhibited the intra-graft infiltration of monocytes/macrophages at 6 h (66 ± 18 vs. 105 ± 16 ; $p < 0.02$) and at day 1 (74 ± 5 vs. 173 ± 34 ; $p < 0.001$) post-OLT (Table 1 and Figure 4). On the other hand, both T cells (81 ± 12 vs. 192 ± 26 ; $p < 0.001$) and monocytes/macrophages (47 ± 12 vs. 129 ± 42 ; $p < 0.001$) were detected in the RGD-treated steatotic OLTs in significantly reduced numbers as compared with respective controls, at day 7 posttransplantation (Table 1 and Figure 4). MPO activity (U/g), which is an index of neutrophil infiltration, was significantly reduced at day 1 (0.96 ± 0.12 vs. 1.89 ± 0.35 ; $p < 0.03$) and at day 7 (0.27 ± 0.17 vs. 1.88 ± 0.16 ; $p < 0.01$) posttransplantation as compared with the respective controls (Figure 5). Moreover, cellular FN, which is virtually undetectable in steatotic naïve livers (10), was upregulated in both RGD-treated and control OLTs at 6 h and at day 1 posttransplantation. However, while controls OLTs still showed high levels of FN deposition at day 7 posttransplantation, RGD-treated livers at day 7

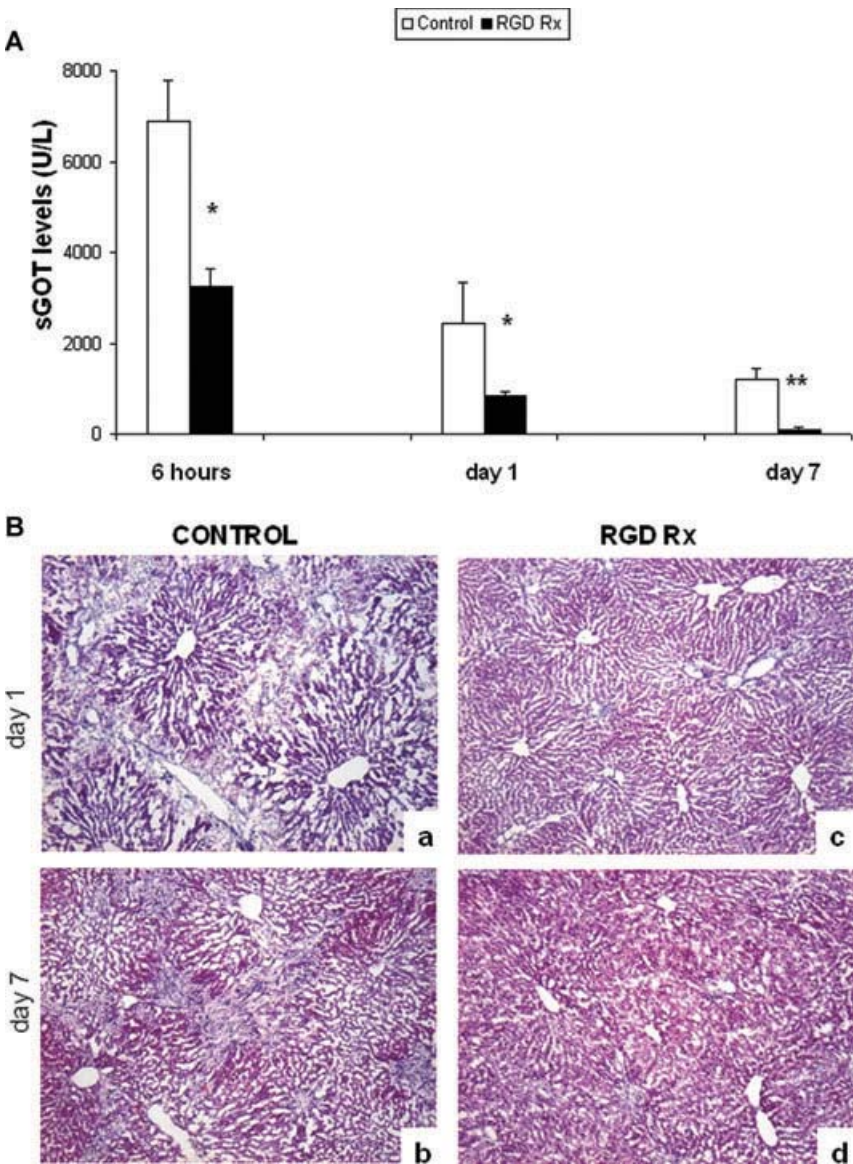


Figure 3: sGOT levels and histology in steatotic OLTs. Cyclic RGD peptide therapy significantly improved liver function as evidenced by sGOT levels (upper panel, A). Levels of sGOT were significantly lower at 6 h, at day 1 and at day 7 in the cyclic RGD peptide-treated recipients as compared with respective controls. Moreover, hematoxylin and eosin staining of steatotic liver grafts (lower panel, B) indicated a better histological preservation in the cyclic RGD peptide-treated steatotic OLTs (c and d) as compared with respective controls (a and b), at day 1 (a and c) and at day 7 (b and d) post-OLT (*p < 0.001 and **p < 0.006; $\times 100$ H&E; n = 4–5 rats/group).

were characterized by reduced deposition of this adhesion molecule in the vascular endothelium (Figure 4). Therefore, our data suggest that cyclic RGD peptide therapy preferentially affected the initial monocyte/macrophage and neutrophil infiltration in steatotic OLTs, and protected against the ongoing inflammatory process observed in the control recipients.

Cyclic RGD peptide therapy downregulates MMP-9 expression in steatotic OLTs

Leukocyte migration requires a coordinated series of adhesion and focal matrix degradation events. We have recently shown that MMP-9, a gelatinase implied in FN breakdown, is a critical mediator of leukocyte migration in liver IRI (26,27) and that $\alpha 4\beta 1$ -FN interactions regulate MMP-9 expression by leukocytes in damaged livers (25). Others have

shown that MMP-9 expression, which is associated with lung cancer invasion, is upregulated in lung cancer cells by the $\alpha 5\beta 1$ -FN interactions (28). We evaluated here whether

Table 1: Sequential immunohistochemical analysis of infiltrating cells

Time after OLT	Immunostaining (mean $\pm$ SD)			
	R73 + lymphocytes		ED + macrophages	
	Control	RGD Rx	Control	RGD Rx
6 h	95 $\pm$ 19	66 $\pm$ 20	105 $\pm$ 16	66 $\pm$ 18
Day 1	110 $\pm$ 28	70 $\pm$ 8	173 $\pm$ 34	74 $\pm$ 5
Day 7	192 $\pm$ 26	81 $\pm$ 12	129 $\pm$ 42	47 $\pm$ 12

Immunoperoxidase staining analysis of steatotic liver grafts at 6 h, at day 1 and at day 7 post-OLT. The results are expressed as mean $\pm$ SD of stained cells in 20 HPF/section; n = 4–5 rats/group.

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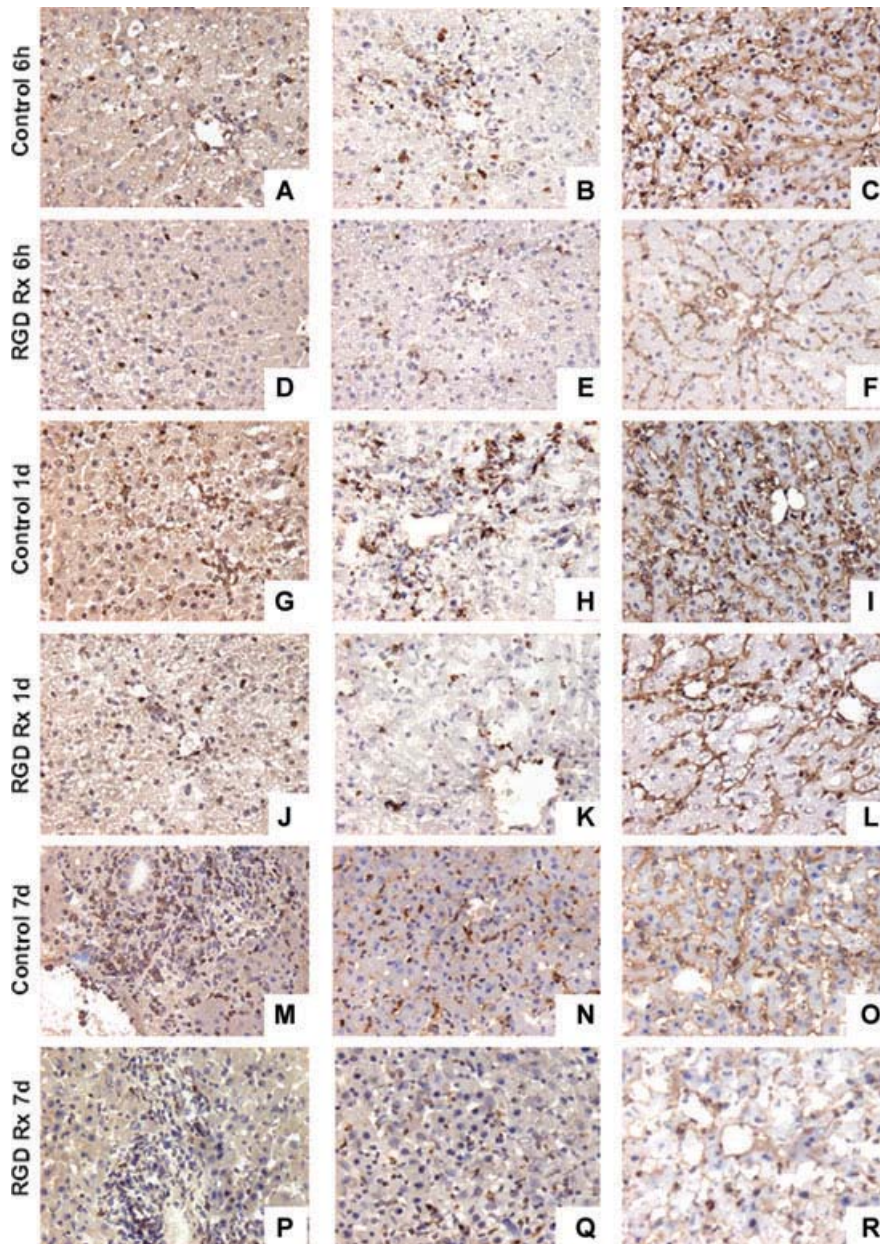


Figure 4: Mononuclear cell infiltration and fibronectin deposition in steatotic OLTs. Immunoperoxidase staining of T lymphocytes (A, D, G, J, M and P), monocyte/macrophages (B, E, H, K, N and Q) and cellular FN (C, F, I, L, O and R) in cyclic RGD peptide-treated fatty liver grafts (D–F, J–L and P–R) and respective controls (A–C, G–I and M–O), at 6 h (A–F), at day 1 (G–L) and day 7 (M–R) post-OLT. Steatotic livers at 6 h and at day 1 post-OLT showed signs of mononuclear cell infiltration and cellular FN deposition; however, the initial monocyte/macrophage infiltration was significantly depressed in the cyclic RGD peptide-treated group as compared with respective controls. Cyclic RGD peptide therapy in steatotic livers at day 7 post-OLT was associated with markedly reduced mononuclear cell infiltration and decreased cellular fibronectin deposition as compared with respective controls ($\times 200$; $n = 4$ –5 rats/group).

the cyclic RGD peptide therapy affected MMP-9 expression in steatotic OLTs. Indeed, MMP-9 gene expression was profoundly depressed in RGD peptide-treated livers at 6 h (0.5 ± 0.1 vs. 1.4 ± 0.5 , $p < 0.003$), day 1 (0.4 ± 0.1 vs. 2 ± 0.2 , $p < 0.01$) and day 7 (0.35 ± 0.2 vs. 2.1 ± 0.1 , $p < 0.0001$) after OLT, and contrasting with high MMP-9 expression levels detected in respective controls (Figure 6A and B). Moreover, the numbers of MMP-9+ leukocytes were also profoundly depressed in the RGD-treated OLTs as compared to controls at 6 h (34 ± 21 vs. 97 ± 19 , $p < 0.01$), day 1 (46 ± 12 vs. 167 ± 43 , $p < 0.01$) and at day 7 (54 ± 19 vs. 152 ± 32 , $p < 0.01$) post-OLT (Figure 6C). Naïve steatotic livers were virtually negative for MMP-9+ leukocytes.

Cyclic RGD peptides therapy decreases iNOS and proinflammatory cytokine expression in steatotic OLTs

iNOS generates NO in a sustained manner for prolonged periods of time, leading to large amounts of NO (29) which have been associated with liver pathologic conditions (10,30). Therefore, we analyzed the expression of iNOS in steatotic OLTs treated with the cyclic RGD peptides. As shown in Figure 7, cyclic RGD peptide therapy reduced the intragraft mRNA expression of iNOS at 6 h (0.6 ± 0.5 vs. 2.2 ± 0.9 , $p < 0.01$), at day 1 (1 ± 0.5 vs. 3.2 ± 0.5 , $p < 0.001$) and at day 7 (0.1 ± 0.1 vs. 2.7 ± 0.1 , $p < 0.0001$) post-OLT. IFN- γ mRNA expression, which is an initiator of liver reperfusion injury (31), was also

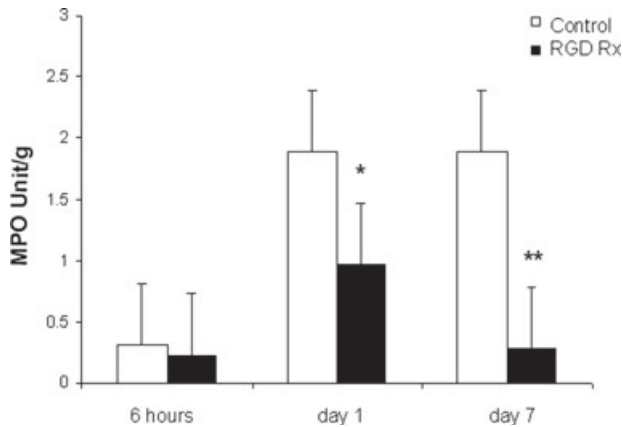
$\alpha 5\beta 1$ Integrin in Steatotic Liver Injury

Figure 5: MPO activity in steatotic OLTs. MPO activity, an index of neutrophil infiltration, was significantly depressed in cyclic RGD peptide-treated OLTs at day 1 and at day 7 posttransplantation as compared with respective controls (* $p < 0.03$ and ** $p < 0.01$; $n = 4$ –5 rats/group).

significantly depressed in the RGD-treated steatotic livers at 6 h (0.1 ± 0.1 vs. 0.4 ± 0.1 , $p < 0.002$), at day 1 (0.2 ± 0.1 vs. 0.5 ± 0.2 , $p < 0.003$) and at day 7 (0.1 ± 0.1 vs. 1 ± 0.3 , $p < 0.0001$) post-OLT (Figure 7). Furthermore, other pro-inflammatory mediators such as IL1 β (5.1 ± 0.05 vs. 3.2 ± 0.2 , $p < 0.001$), IL-2 (3.5 ± 0.4 vs. 2.1 ± 0.6 , $p < 0.05$) and TNF- α (3.1 ± 0.2 vs. 1.2 ± 0.2 , $p < 0.0001$) were markedly depressed in the RGD-treated OLTs at day 7, as compared with respective control OLTs (Figure 7).

Cyclic RGD therapy results in Akt upregulation and less apoptosis in steatotic OLTs

Apoptosis is considered an important mechanism in liver IRI (32). To determine whether apoptosis played a role in our settings, we evaluated the expression of Caspase-3, a pro-apoptotic marker associated with liver IRI (27). Caspase 3 is expressed in tissues as an inactive 32-kDa precursor. During apoptosis, the 32-kDa caspase-3 is cleaved and generates a 17-kDa mature active form, which is associated with caspase-3 activity (33). While 17-kDa caspase-3

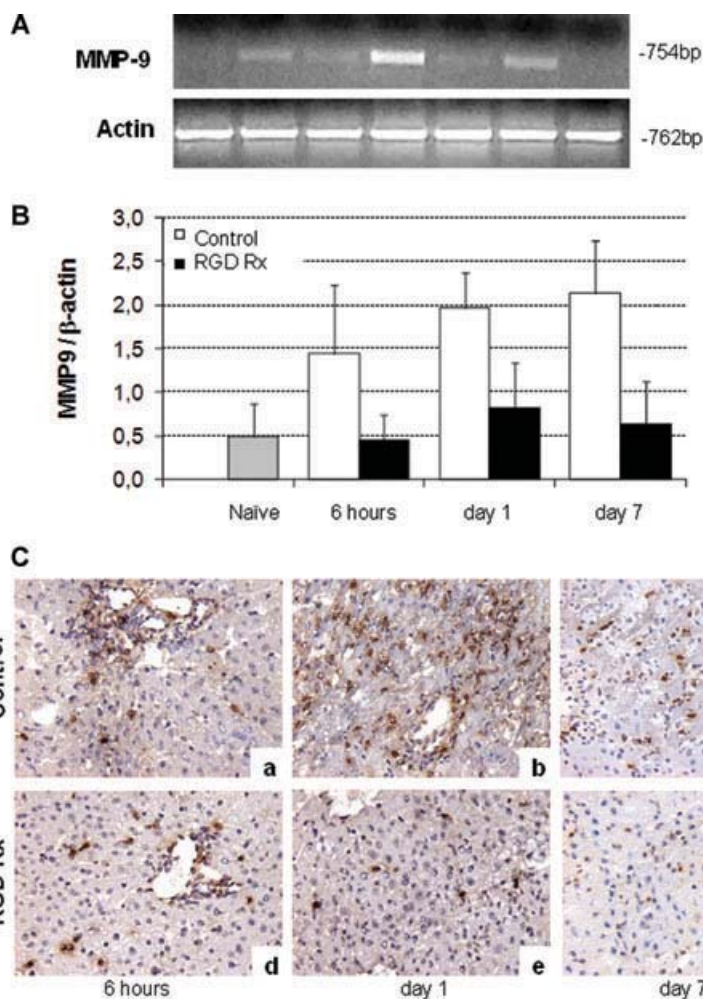


Figure 6: MMP-9 expression in steatotic OLTs. MMP-9 mRNA expression (panel A), which was almost undetectable in naïve steatotic livers (lane 1), was found readily expressed in control steatotic OLTs (lanes 2, 4 and 6) and slightly detected in cyclic RGD-treated steatotic liver grafts (lanes 3, 4 and 6) at 6 h (lanes 2 and 3), at day 1 (lanes 4 and 5) and at day 7 (lanes 6 and 7) post-IRI. Densitometric analysis (panel B) showed that MMP-9 mRNA expression was significantly decreased in the cyclic RGD peptide-treated OLTs as compared with respective controls. Moreover, MMP-9+ leukocytes (panel C) were detected in significantly lower numbers in the cyclic RGD peptide-treated livers (d, e and f), as compared with respective control OLTs (a, b and c) at 6 h (a and d), day 1 (b and e) and day 7 (c and f) posttransplantation (* $p < 0.003$, ** $p < 0.01$, and *** $p < 0.0001$; $\times 200$; $n = 4$ –5 rats/group).

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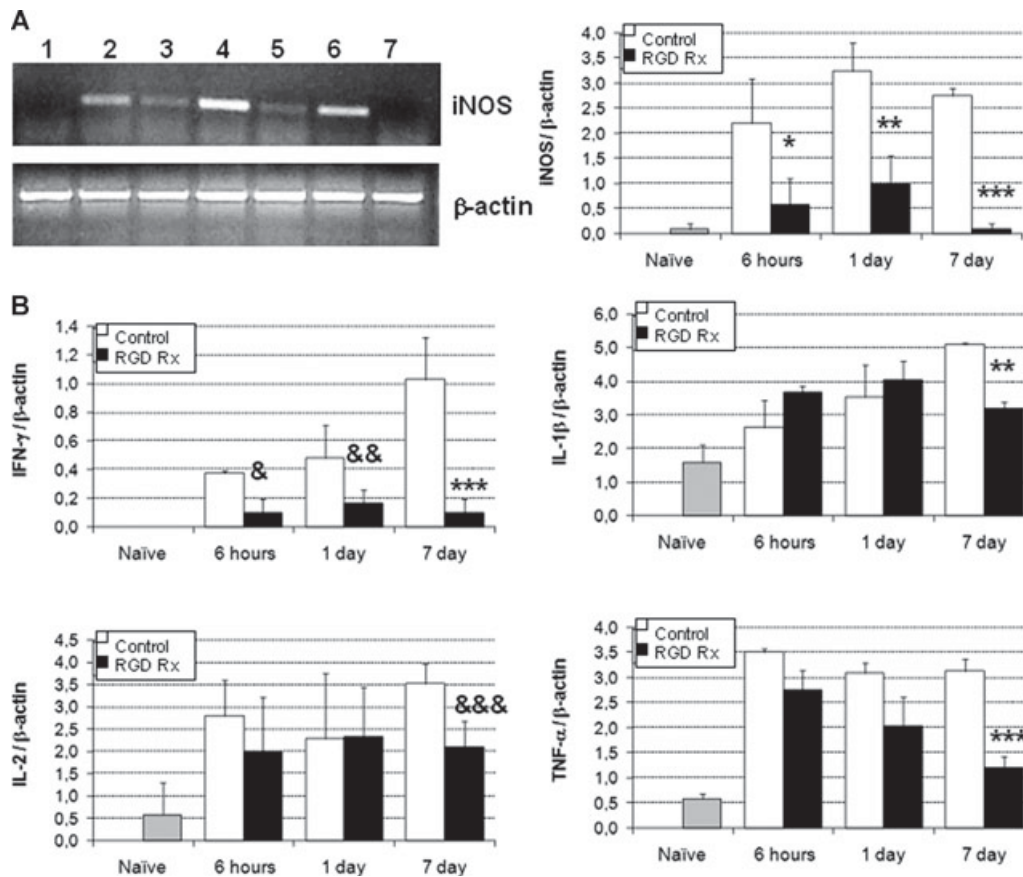


Figure 7: iNOS and proinflammatory cytokine gene expression in steatotic OLTs. Panel A illustrates iNOS expression and densitometric analysis in steatotic naïve livers (lane 1) and OLTs at 6 h (lanes 2 and 3), day 1 (lanes 4 and 5) and day 7 (lanes 6 and 7) posttransplantation. Control livers (lanes 2, 4 and 6) showed high levels of iNOS mRNA expression in particular at day 1 post-OLT (lane 4). In contrast, iNOS mRNA expression was virtually absent in naïve (lane 7), and significantly depressed in cyclic RGD peptide-treated OLTs at 6 h (lane 3), at day 1 (lane 5) and at day 7 (lane 7) as compared to respective controls. Panel B illustrates cytokine expression in steatotic liver OLTs. IFN- γ expression was significantly decreased in cyclic RGD peptide-treated OLTs as compared with respective controls at 6 h, day 1 and day 7 post-IRI. Moreover, IL-1 β , IL-2 and TNF- α expressions were significantly reduced in steatotic liver grafts as compared with controls at day 7 post-OLT (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, & $p < 0.002$, && $p < 0.003$ and &&& $p < 0.05$; $n = 4$ –5 rats/group).

was readily detected in control livers, it was virtually undetectable in RGD-treated livers post-OLT (Figure 8A). Moreover, TUNEL-positive cells were also less detected in the RGD-treated livers when compared with controls, in particular at 24 h post-OLT (2 ± 0.5 vs. 13 ± 10 , $p < 0.04$; $n = 4$ /group) (Figure 8B and C). Interestingly, Akt which is a 57-kD protein-serine/threonine kinase with functions mainly associated with pro-survival (anti-apoptotic) (34) was preferentially phosphorylated in the RGD-treated livers (6 h: 1.01 ± 0.26 vs. 0.03 ± 0.05 , $p < 0.01$; day 1: 0.25 ± 0.02 vs. 0.05 ± 0.02 , $p < 0.004$; $n = 4$ /group) (Figure 9).

Discussion

Ischemic damage in the liver associated with leukocyte recruitment, release of cytokines and free radicals, plays

a major role in post-IRI, leading to a decline of liver function and potentially resulting in graft loss (4). In general, leukocyte recruitment to target tissues is dependent on weak rolling adhesions, which are mostly mediated by selectins, and on firm integrin-mediated adhesions (35). However, we should have in consideration that liver is a venous driven vascular bed with slow flow rates and may require a distinct cascade of adhesive events as compared with other organs with higher flow rates. In this regard, it has been demonstrated that selectins are not essential for leukocyte recruitment into inflamed liver microvasculature (36). Lower shear rates may lead to selectin-independent leukocyte weak rolling adhesion mechanisms (37). This concept is perhaps even more relevant for fatty livers in view of the observations that steatosis further decreases sinusoidal blood flow by approximately 50% in humans and rats (38,39). Together, these observations highlight the

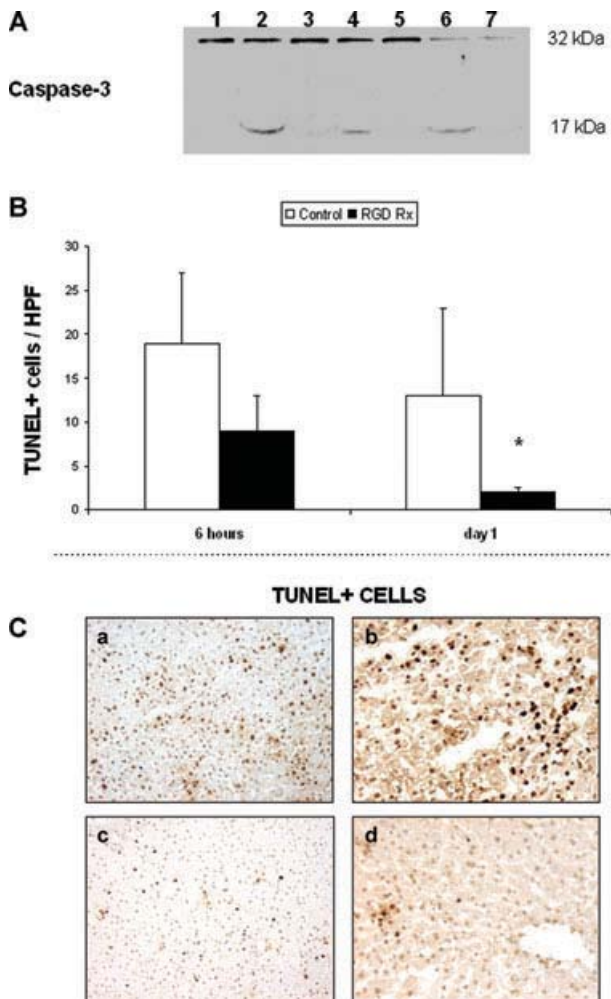
$\alpha 5\beta 1$ Integrin in Steatotic Liver Injury

Figure 8: Caspase-3 expression and TUNEL+ cell detection in steatotic OLTs. Panel A illustrates caspase-3 expression in steatotic naïve livers (lane 1), and in steatotic liver grafts at 6 h (lanes 2 and 3), at day 1 (lanes 4 and 5) and at day 7 (lanes 6 and 7) post-OLT. The 17-kDa caspase-3 form associated with caspase-3 activity was modestly detected in control OLTs (lanes 2, 4 and 6) and virtually absent in naïve livers (lane 1) and in cyclic RGD peptide-treated OLTs (lanes 3, 5 and 7). Moreover, TUNEL-positive cells (panel B) were also less detected in the RGD-treated livers when compared with controls, in particular at 24 h post-OLT. Panel C illustrates TUNEL+ labeling in control OLTs (a and b) and cyclic RGD peptide-treated OLTs (c and d) at 6 h (a and c) and 24 h (b and d) post-OLT (* $p < 0.04$; $\times 200$; $n = 4$ /group).

importance that firm adhesion mediated by integrins may have in steatotic OLTs.

We have shown that FN deposition in the vascular endothelium is a very early feature in response to IRI in steatotic liver grafts (10). The $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins are the two major FN receptors expressed on leukocytes. We have previously demonstrated that CS1 peptide facilitates the blockade of $\alpha 4\beta 1$ -FN interactions ameliorated steatotic

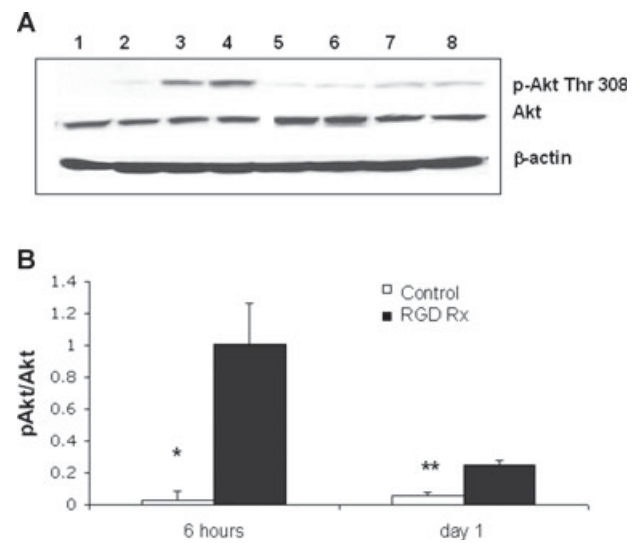


Figure 9: Akt protein expression in steatotic OLTs. Akt was preferentially phosphorylated in the RGD-treated livers (lanes 3, 4, 7 and 8) as compared with respective control OLTs (lanes 1, 2, 5 and 6) at 6 h (lanes 1–4) and at day 1 (lanes 5–8) post-OLT (* $p < 0.01$ and ** $p < 0.004$; $n = 4$ /group).

liver I/R injury (10). We report here the effect of cyclic RGD peptides, which are selective ligands for the $\alpha 5\beta 1$ integrin and inhibit cell attachment to FN (20). We found that administration of cyclic RGD peptides to steatotic OLT recipients significantly reduced liver damage and improved the recipient survival rate. Our observations are in line with early reports showing that RGD peptides are capable of ameliorating ischemic acute renal failure (40) and that RGD analogs protect against concanavalin A-induced liver damage in mice and against the development of liver cirrhosis in rats (41,42).

One of the most striking effects observed in the cyclic RGD peptide-treated steatotic OLT recipients was a marked decrease in monocyte/macrophage infiltration in the liver grafts. Monocyte/macrophages modulate inflammatory processes through the release of cytokines, growth factors and oxygen radicals (43), and therefore, their migration across ECM proteins during inflammation is an important event. Interestingly, while the blockade of $\alpha 4\beta 1$ -FN interactions was not very effective in decreasing the initial numbers of monocyte/macrophages infiltrating steatotic liver grafts (10), cyclic RGD peptide-treated steatotic OLTs were characterized by a marked reduction of these cells, suggesting an important role for the $\alpha 5\beta 1$ integrin in the recruitment of monocyte/macrophages in steatotic liver IRI. This is in agreement with previous observations indicating that $\alpha 5\beta 1$ is the predominant receptor for FN in monocytes (44) and that $\alpha 5\beta 1$ is implicated in monocyte influx into inflamed sites (18). Interestingly, it has been shown that cyclic RGD peptides were capable of decreasing macrophage infiltration in kidneys and in carotid artery

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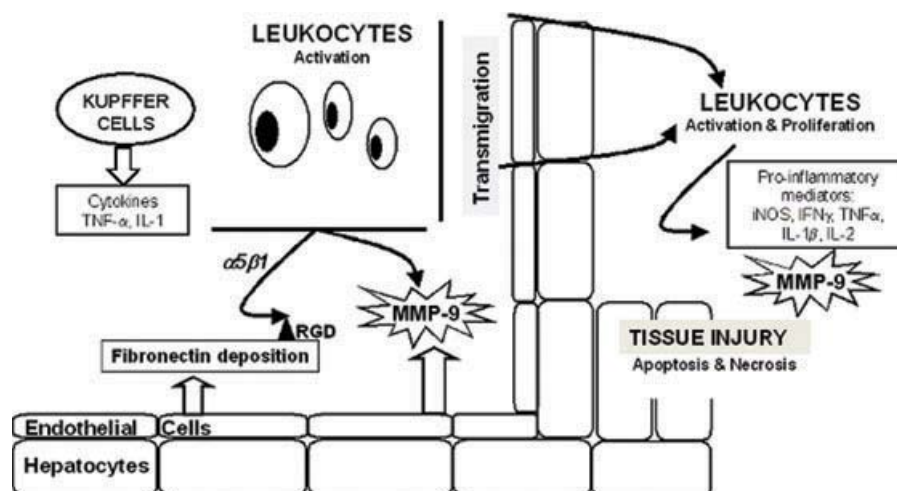


Figure 10: Schematic representation of cyclic RGD peptide-mediated protection against steatotic liver IRI. Major pathways in liver IRI include release of pro-inflammatory mediators by activated Kupffer cells such as TNF-α, which can contribute to upregulation of fibronectin by endothelial cells. Leukocyte transmigration across endothelial and ECM barriers depends upon adhesive interactions and focal matrix degradation mechanisms. Fibronectin binding to its α5β1 integrin receptor expressed on leukocytes stimulates the expression of MMP-9 and facilitates leukocyte transmigration. Cyclic RGD peptides, which block α5β1-FN adhesive interactions, disrupted MMP-9 expression and leukocyte infiltration in steatotic livers after IRI. Reduced leukocyte infiltration resulted in decreased levels of pro-inflammatory mediators, less tissue injury and improved liver function of steatotic OLTs.

lesions of apo-E-deficient mice (45). Neutrophils, which are considered to be critical mediators in acute inflammatory liver injury (7), were also depressed in the cyclic RGD peptide-treated steatotic OLTs. These observations are supported by others, showing that neutrophil adherence to FN is mediated by the α5β1 integrin (46) and that the α5β1 integrin has a role on neutrophil recruitment in lung injury (47). Indeed, it was reported that α4β1 and α5β1 integrins have a major function in mediating neutrophil recruitment into lung during acute LPS-induced inflammation (47).

Leukocyte transmigration across endothelial and ECM barriers is a complex process, which include cell activating chemokines and adhesive interactions, as well as focal matrix degradation mechanisms (8). While adhesion molecules are critical to successfully promote leukocyte transmigration by providing leukocyte attachment to the vascular endothelium, matrix proteases are important to facilitate leukocyte movement across vascular barriers. We have recently shown that MMP-9 is a critical mediator of leukocyte migration in liver IRI (26,48) and that α4β1-FN interactions are capable of regulating MMP-9 expression by leukocytes in damaged livers (25). The present study provides evidence that in addition to the α4β1 integrin, the α5β1 integrin is also capable of regulating the expression of MMP-9 in steatotic OLTs. Indeed, FN has been shown to affect MMP-9 expression in several systems (25,49–52). Moreover, it has also been demonstrated that MMP-9 gene expression in human HL-60 myeloid leukemia cells and in lung cancer cells is upregulated by α5β1-FN interactions (28,49).

MMP-9, in addition to facilitate leukocytes infiltration in livers after I/R injury, may also cause parenchyma cell detachment from ECM and, consequently, promote adhesion-related apoptosis/anoikis of these cells (48). In these experimental settings, the 17-kDa caspase-3, which is associated with the caspase-3 pro-apoptotic activity (33), was readily detected in control livers and virtually undetectable in RGD-treated livers post-OLT. Interestingly, the protein-serine/threonine kinase Akt, functions of which have been mainly associated with pro-survival (anti-apoptotic) (34), was preferentially phosphorylated in the cyclic RGD peptide-treated steatotic livers after IRI. While hepatocyte necrosis has been considered to be the predominant mode of cell death following IRI in steatotic livers (6), and in our settings, control steatotic OLTs were characterized by extensive necrosis, it has been shown that hepatocyte apoptosis also occurs in damaged steatotic livers (53). Indeed, as suggested by Lemasters (54), apoptosis and necrosis are not distinct forms of cell death, and they often coexist in tissue injury due to ischemia-reperfusion. Thus, independent of the form of cell death, cyclic RGD peptide-treated livers were characterized by much less injury as compared to respective controls after transplantation.

The expression of pro-inflammatory mediators, such as iNOS and IFN-γ, has been linked to tissue injury, including hepatic injury (10,31,55–57). We show here that both iNOS and IFN-γ were significantly depressed in the cyclic RGD peptide-treated steatotic OLTs, which were correlated with the improved liver function observed in these treated animals. Moreover, TNF-α expression, which was initially

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upregulated in both cyclic RGD peptide-treated and control OLTs, was significantly depressed in well-preserved long-term cyclic RGD peptide-treated liver grafts. However, it is important to note that the liver cytokine environment is complex and that cytokines, depending on the context, may be involved in both regenerative and injury processes. In this regard, TNF- α , whose inhibition has some detrimental effects in liver regeneration after hepatectomy (58), has been linked to liver injury in obesity (59).

In summary, this article is the first full report on the function of a cyclic RGD peptide, which avidly binds the $\alpha 5\beta 1$ integrin and particularly inhibits cell attachment to FN (20), in liver IRI. Our observations, which are outlined in Figure 10, show that cyclic RGD peptide therapy down-regulated MMP-9 expression, decreased leukocyte infiltration and depressed the release of pro-inflammatory mediators, leading to protection against steatotic liver IRI and increased OLT recipient survival. Therefore, this work provides the rationale to identify therapeutic approaches based in novel concepts that would allow the successful utilization of marginal steatotic livers in organ transplantation and, consequently, to expand the donor population.

Acknowledgments

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References

- Wight C, Cohen B. Shortage of organs for transplantation. *BMJ* 1996; 312: 989–990.
- Koneru B, Dikdan G. Hepatic steatosis and liver transplantation current clinical and experimental perspectives. *Transplantation* 2002; 73: 325–330.
- Strasberg SM, Howard TK, Molmenti EP, Hertl M. Selecting the donor liver: Risk factors for poor function after orthotopic liver transplantation. *Hepatology* 1994; 20: 829–838.
- Taneja C, Prescott L, Koneru B. Critical preservation injury in rat fatty liver is to hepatocytes, not sinusoidal lining cells. *Transplantation* 1998; 65: 167–172.
- Caraceni P, Nardo B, Domenicali M et al. Ischemia–reperfusion injury in rat fatty liver: Role of nutritional status. *Hepatology* 1999; 29: 1139–1146.
- Selzner M, Clavien PA. Fatty liver in liver transplantation and surgery. *Semin Liver Dis* 2001; 21: 105–113.
- Jaeschke H, Hasegawa T. Role of neutrophils in acute inflammatory liver injury. *Liver Int* 2006; 26: 912–919.
- Bianchi E, Bender JR, Blasi F, Pardi R. Through and beyond the wall: Late steps in leukocyte transendothelial migration. *Immunol Today* 1997; 18: 586–591.
- Jarnagin WR, Rockey DC, Kotliansky VE, Wang SS, Bissell DM. Expression of variant fibronectins in wound healing: Cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. *J Cell Biol* 1994; 127: 2037–2048.
- Amersi F, Shen XD, Moore C et al. Fibronectin- $\alpha 4\beta 1$ integrin-mediated blockade protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *Am J Pathol* 2003; 162: 1229–1239.
- Coito AJ, de Sousa M, Kupiec-Weglinski JW. Fibronectin in immune responses in organ transplant recipients. *Dev Immunol* 2000; 7: 239–248.
- Yi M, Ruoslahti E. A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis. *Proc Natl Acad Sci USA* 2001; 98: 620–624.
- Elices MJ, Tsai V, Strahl D et al. Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature. *J Clin Invest* 1994; 93: 405–416.
- Coito AJ, Brown LF, Peters JH, Kupiec-Weglinski JW, Van De WL. Expression of fibronectin splicing variants in organ transplantation: A differential pattern between rat cardiac allografts and isografts. *Am J Pathol* 1997; 150: 1757–1772.
- Hynes RO. Cell-matrix adhesion in vascular development. *J Thromb Haemost* 2007; 5(Suppl 1): 32–40.
- Hynes RO. Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 1992; 69: 11–25.
- Matsuyama T, Yamada A, Kay J et al. Activation of CD4 cells by fibronectin and anti-CD3 antibody. A synergistic effect mediated by the VLA-5 fibronectin receptor complex. *J Exp Med* 1989; 170: 1133–1148.
- Shang XZ, Lang BJ, Issekutz AC. Adhesion molecule mechanisms mediating monocyte migration through synovial fibroblast and endothelium barriers: Role for CD11/CD18, very late antigen-4 (CD49d/CD29), very late antigen-5 (CD49e/CD29), and vascular cell adhesion molecule-1 (CD106). *J Immunol* 1998; 160: 467–474.
- Bohnsack JF, Chang J, Zhou X, Yednock TA. Mechanisms of beta 1 integrin-dependent adherence of granulocytic HL60 to fibronectin. *J Leukoc Biol* 1995; 57: 592–599.
- Koivunen E, Wang B, Ruoslahti E. Phage libraries displaying cyclic peptides with different ring sizes: Ligand specificities of the RGD-directed integrins. *Biotechnology (NY)* 1995; 13: 265–270.
- Kamada N, Calne RY. A surgical experience with five hundred thirty liver transplants in the rat. *Surgery* 1983; 93: 64–69.
- Pierschbacher MD, Ruoslahti E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *J Biol Chem* 1987; 262: 17294–17298.
- Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. *Transplantation* 1993; 55: 1265–1272.
- Kato A, Yoshidome H, Edwards MJ, Lentsch AB. Regulation of liver inflammatory injury by signal transducer and activator of transcription-6. *Am J Pathol* 2000; 157: 297–302.
- Moore C, Shen XD, Gao F, Busuttill RW, Coito AJ. Fibronectin- $\{\alpha\}4\{\beta\}1$ integrin interactions regulate metalloproteinase-9 expression in steatotic liver ischemia and reperfusion injury. *Am J Pathol* 2007; 170: 567–577.
- Hamada T, Fondevila C, Busuttill RW, Coito AJ. Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury. *Hepatology* 2008; 47: 186–198.
- Hamada T, Tsuchihashi S, Avanesyan A et al. Cyclooxygenase-2 deficiency enhances Th2 immune responses and impairs neutrophil recruitment in hepatic ischemia/reperfusion injury. *J Immunol* 2008; 180: 1843–1853.
- Han S, Ritzenthaler JD, Sitaraman SV, Roman J. Fibronectin increases matrix metalloproteinase 9 expression through activation

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- of c-Fos via extracellular-regulated kinase and phosphatidylinositol 3-kinase pathways in human lung carcinoma cells. *J Biol Chem* 2006; 281: 29614–29624.
29. Nathan C. Inducible nitric oxide synthase: What difference does it make? *J Clin Invest* 1997; 100: 2417–2423.
 30. Isobe M, Katsuramaki T, Hirata K, Kimura H, Nagayama M, Matsuno T. Beneficial effects of inducible nitric oxide synthase inhibitor on reperfusion injury in the pig liver. *Transplantation* 1999; 68: 803–813.
 31. Lappas CM, Rieger JM, Linden J. A2A adenosine receptor induction inhibits IFN-gamma production in murine CD4+ T cells. *J Immunol* 2005; 174: 1073–1080.
 32. Rudiger HA, Graf R, Clavien PA. Liver ischemia: Apoptosis as a central mechanism of injury. *J Invest Surg* 2003; 16: 149–159.
 33. Han Z, Hendrickson EA, Bremner TA, Wyche JH. A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 in vitro. *J Biol Chem* 1997; 272: 13432–13436.
 34. Mullonkal CJ, Toledo-Pereyra LH. Akt in ischemia and reperfusion. *J Invest Surg* 2007; 20: 195–203.
 35. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell* 1991; 65: 859–873.
 36. Wong J, Johnston B, Lee SS et al. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. *J Clin Invest* 1997; 99: 2782–2790.
 37. Gaboury JP, Kubes P. Reductions in physiologic shear rates lead to CD11/CD18-dependent, selectin-independent leukocyte rolling in vivo. *Blood* 1994; 83: 345–350.
 38. Hayashi M, Tokunaga Y, Fujita T, Tanaka K, Yamaoka Y, Ozawa K. The effects of cold preservation on steatotic graft viability in rat liver transplantation. *Transplantation* 1993; 56: 282–287.
 39. Gao W, Connor HD, Lemasters JJ, Mason RP, Thurman RG. Primary nonfunction of fatty livers produced by alcohol is associated with a new, antioxidant-insensitive free radical species. *Transplantation* 1995; 59: 674–679.
 40. Noiri E, Gailit J, Sheth D et al. Cyclic RGD peptides ameliorate ischemic acute renal failure in rats. *Kidney Int* 1994; 46: 1050–1058.
 41. Bruck R, Herschkoviz R, Lider O et al. Inhibition of experimentally-induced liver cirrhosis in rats by a nonpeptidic mimetic of the extracellular matrix-associated Arg-Gly-Asp epitope. *J Hepatol* 1996; 24: 731–738.
 42. Bruck R, Herschkoviz R, Lider O, Shirin H, Aeed H, Halpern Z. The use of synthetic analogues of Arg-Gly-Asp (RGD) and soluble receptor of tumor necrosis factor to prevent acute and chronic experimental liver injury. *Yale J Biol Med* 1997; 70: 391–402.
 43. Laskin DL, Pendino KJ. Macrophages and inflammatory mediators in tissue injury. *Annu Rev Pharmacol Toxicol* 1995; 35: 655–677.
 44. Weber C, Alon R, Moser B, Springer TA. Sequential regulation of alpha 4 beta 1 and alpha 5 beta 1 integrin avidity by CC chemokines in monocytes: Implications for transendothelial chemotaxis. *J Cell Biol* 1996; 134: 1063–1073.
 45. Elitok S, Brodsky SV, Patschan D et al. Cyclic arginine-glycine-aspartic acid peptide inhibits macrophage infiltration of the kidney and carotid artery lesions in apo-E-deficient mice. *Am J Physiol Renal Physiol* 2006; 290: F159–F166.
 46. Bohnsack JF, Zhou XN. Divalent cation substitution reveals. *J Immunol* 1992; 149: 1340–1347.
 47. Burns JA, Issekutz TB, Yagita H, Issekutz AC. The alpha 4 beta 1 (very late antigen (VLA)-4, CD49d/CD29) and alpha 5 beta 1 (VLA-5, CD49e/CD29) integrins mediate beta 2 (CD11/CD18) integrin-independent neutrophil recruitment to endotoxin-induced lung inflammation. *J Immunol* 2001; 166: 4644–4649.
 48. Hamada T, Duarte S, Tsuchihashi S et al. Inducible nitric oxide synthase deficiency impairs matrix metalloproteinase-9 activity and disrupts leukocyte migration in hepatic ischemia/reperfusion injury. *Am J Pathol* 2009; 174: 2265–2277.
 49. Xie B, Laouar A, Huberman E. Fibronectin-mediated cell adhesion is required for induction of 92-kDa type IV collagenase/gelatinase (MMP-9) gene expression during macrophage differentiation. The signaling role of protein kinase C-beta. *J Biol Chem* 1998; 273: 11576–11582.
 50. Chana RS, Martin J, Rahman EU, Wheeler DC. Monocyte adhesion to mesangial matrix modulates cytokine and metalloproteinase production. *Kidney Int* 2003; 63: 889–898.
 51. Anderson DG, Li X, Balian G. A fibronectin fragment alters the metabolism by rabbit intervertebral disc cells in vitro. *Spine* 2005; 30: 1242–1246.
 52. Redondo-Munoz J, Escobar-Diaz E, Samaniego R, Terol MJ, Garcia-Marco JA, Garcia-Pardo A. MMP-9 in B-cell chronic lymphocytic leukemia is up-regulated by alpha4beta1 integrin or CXCR4 engagement via distinct signaling pathways, localizes to podosomes, and is involved in cell invasion and migration. *Blood* 2006; 108: 3143–3151.
 53. Baskin-Bey ES, Canbay A, Bronk SF et al. Cathepsin B inactivation attenuates hepatocyte apoptosis and liver damage in steatotic livers after cold ischemia-warm reperfusion injury. *Am J Physiol Gastrointest Liver Physiol* 2005; 288: G396–G402.
 54. Lemasters JJ. V. Necroptosis and the mitochondrial permeability transition: Shared pathways to necrosis and apoptosis. *Am J Physiol* 1999; 276: G1–G6.
 55. Hierholzer C, Harbrecht B, Menezes JM et al. Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. *J Exp Med* 1998; 187: 917–928.
 56. Sass G, Koerber K, Bang R, Guehring H, Tiegs G. Inducible nitric oxide synthase is critical for immune-mediated liver injury in mice. *J Clin Invest* 2001; 107: 439–447.
 57. Nathan C, Calingasan N, Nezezon J et al. Protection from Alzheimer's-like disease in the mouse by genetic ablation of inducible nitric oxide synthase. *J Exp Med* 2005; 202: 1163–1169.
 58. Serandour AL, Loyer P, Garnier D et al. TNFalpha-mediated extracellular matrix remodeling is required for multiple division cycles in rat hepatocytes. *Hepatology* 2005; 41: 478–486.
 59. Yang SQ, Lin HZ, Lane MD, Clemens M, Diehl AM. Obesity increases sensitivity to endotoxin liver injury: Implications for the pathogenesis of steatohepatitis. *Proc Natl Acad Sci USA* 1997; 94: 2557–2562.

CHAPTER IV

TISSUE INHIBITOR OF METALLOPROTEINASES-1 (TIMP-1) DEFICIENCY LEADS TO LETHAL PARTIAL HEPATIC ISCHEMIA AND REPERFUSION INJURY

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TISSUE INHIBITOR OF METALLOPROTEINASES-1 (TIMP-1) DEFICIENCY LEADS TO LETHAL PARTIAL HEPATIC ISCHEMIA AND REPERFUSION INJURY

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Running Title: TIMP-1 deficiency exacerbates liver ischemia and reperfusion injury

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ABSTRACT

Hepatic ischemia and reperfusion injury (IRI) remains an important challenge in clinical orthotopic liver transplantation (OLT). Tissue inhibitor of metalloproteinase-1 (TIMP-1) is the major endogenous regulator of matrix metalloproteinase-9 (MMP-9). In this study, we investigated the functional significance of TIMP-1 expression in a well-established mouse model of partial liver IRI. Compared to wild-type mice, TIMP-1^{-/-} mice showed further impaired liver function and histological preservation after IRI. Notably, TIMP-1 deficiency led to lethal liver IRI, as over 60% of the TIMP-1^{-/-} mice died post-reperfusion, whereas all TIMP-1^{+/+} mice recovered and survived surgery. Lack of TIMP-1 expression was accompanied by markedly high levels of MMP-9 activity, which facilitates leukocyte transmigration across vascular barriers in hepatic IRI. Indeed, TIMP-1^{-/-} livers were characterized by massive leukocyte infiltration and by upregulation of proinflammatory mediators, including TNF- α , IFN- γ , and iNOS, post-IRI. The inability of TIMP-1^{-/-} mice to express TIMP-1 increased the levels of active caspase-3 and depressed the expression of Bcl-2 and the phosphorylation of Akt, emphasizing an important role for TIMP-1 expression on hepatocyte survival. Using independent parameters of regeneration, 5-bromodeoxyuridine (BrdU) incorporation, proliferating cell nuclear antigen (PCNA) expression, and histone H3 phosphorylation, we provide evidence that hepatocyte progression into S phase and mitosis was impaired in TIMP-1 deficient livers after IRI. Inhibition of the cell cycle progression by TIMP-1 deficiency was linked to depressed levels of cyclins-D1 and -E and to disrupted c-Met signaling pathway, as evidenced by reduced phosphorylated c-Met expression and elevated c-Met ectodomain shedding post-liver IRI. In conclusion, these results support a critical protective function for TIMP-1 expression on promoting survival and proliferation of liver cells and on regulating leukocyte recruitment and activation in liver IRI.

Hepatic ischemia/reperfusion injury (IRI) occurs during trauma, shock, orthotopic liver transplantation (OLT), and other surgical procedures where blood supply to liver is temporarily interrupted ¹. Hepatic IR-related damage is the result of various factors that include leukocyte migration, release of cytokines and free radicals ^{1,2}.

Leukocytes migration across endothelial and extracellular matrix (ECM) barriers is dependent on cellular adhesion-release and focal matrix degradation mechanisms ³. While adhesion molecules are important for the successful leukocyte transmigration by providing leukocyte attachment to the endothelium, there is a growing body of evidence suggesting that matrix metalloproteinases (MMP) are critical for facilitating leukocyte movement across vascular barriers ³. In this regard, our previous studies showed an important role for leukocyte-expressed MMP-9, or gelatinase B, as a key mediator of leukocyte transmigration leading to liver injury ⁴.

Tissue inhibitors of metalloproteinases (TIMPs) are a family of naturally occurring inhibitors 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 ⁵. There are at least four identified members (TIMP 1-4) in the TIMP family, varying in tissue specific expression and in their ability to inhibit various MMPs ⁶. Among the different TIMPs, TIMP-1 is of particular interest; TIMP-1 is a 28.5 kDa soluble glycoprotein known to inhibit MMP-9 with high affinity, without interacting with MMP-2, or gelatinase A (the other member of the gelatinase family), as it lacks the required C-terminal MMP-2 interacting residues ^{7,8}.

In addition to its ability to inhibit MMP activity, TIMP-1 possesses other biological activities, such as cell growth regulation, that are just beginning to be recognized and characterized ⁹. The specific effects of the TIMPs are likely depending on the cell context and on the pathological condition. While TIMP-1 has been detected in the plasma of patients after liver transplantation ¹⁰, and in rat liver grafts after IRI ¹¹, its role in liver IRI,

or in OLT, remains to be established. Therefore, in the present study, we used mice lacking TIMP-1 to examine the significance of TIMP-1 expression in hepatic IRI.

MATERIALS AND METHODS

Mice and Model of Hepatic IRI

Male TIMP-1^{-/-} Knockout (KO) mice in the C57BL/6 background (B6.129S4-Timp1^{tm1Pd/J}) and respective TIMP-1^{+/+} wild-type C57BL-6 controls were obtained from the Jackson Laboratory. Hepatic IRI was performed as previously described ⁴. Briefly, arterial and portal venous blood supplies were interrupted to the cephalad lobes of the liver for 90 minutes using an atraumatic clip and mice were sacrificed after reperfusion. The animal studies were performed according to approved guidelines by the American Association of Laboratory Animal Care.

Assessment of Liver Damage

Serum alanine transaminase (ALT) and serum aspartate transaminase (AST) levels were measured with an auto analyzer by ANTECH Diagnostics (Los Angeles, CA), as described ⁴. Liver specimens were fixed with a 10% buffered formalin solution, embedded in paraffin and processed for H&E staining; to determine the percentage of necrotic area, ten random sections per slide were evaluated in duplicate using NIH IMAGE (Image-J).

Immunohistochemistry

Immunostaining was performed in cryostat sections, as described ^{4, 11}. Mac-1 (M1/70), and Ly-6G (1A8), from BD Biosciences, TIMP-1 (Ab86482; Abcam), MMP-9 (AF909; R&D Systems), and cleaved-caspase-3 (ASP175; Cell Signaling) antibodies were used at optimal dilutions. Sections were blindly evaluated by counting ten HPFs/section in triplicates. Dual/triple staining was detected by immunofluorescence with Alexa Fluor 594-red anti-goat IgG (H+L) (Molecular Probes), and Texas Red anti-rat IgG (H+L) antibodies (Vector Laboratories). Alexa Fluor 488 phalloidin (Molecular Probes)

and Vectashield mounting media with DAPI (Vector Laboratories) were used for F-actin and nuclear staining, respectively. Slides were analyzed using a Leica Confocal Microscope (UCLA Brain Research Institute).

Parameters of regeneration

Mice were injected i.p. with 50mg/kg of BrdU (Sigma) 2h prior to liver harvest, as described ¹². BrdU incorporation, PCNA, and phosphorylated histone H3_were detected by immunohistochemistry in paraffin sections using anti-BrdU (Bu20a; Neomarkers), anti-PCNA (PC-10; Neomarkers), and anti-pH3 (Ser10; Cell Signaling) antibodies. Proliferation indexes were determined in triplicate and quantified under light microscopy by counting ten, randomly chosen, HPFs/section. Data were expressed as the percentage of BrdU, PCNA, or pH3 stained hepatocytes per total number of hepatocytes.

Myeloperoxidase (MPO) Assay

Myeloperoxidase activity was evaluated in frozen tissue homogenized in an iced solution of 0.5% hexadecyltrimethyl-ammonium and 50 mmol/L of potassium phosphate buffer solution ⁴. After centrifugation, the supernatants were mixed in a solution of hydrogen peroxide-sodium acetate and tetramethyl benzidine (Sigma). The quantity of enzyme degrading 1 μ mol/L of peroxide/minute at 25°C per gram of tissue was defined as 1U of MPO activity.

Western blot and Zymography Analysis

Western blots and Zymography were performed as described ^{4, 11}. Proteins (40 μ g/sample) in sodium dodecyl sulfate (SDS)-loading buffer were electrophoresed through 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF

membranes. Membranes were incubated with specific antibodies against cleaved caspase-3 (ASP175), phospho-AKT (D9E; C31E5E), AKT (C67E7), phospho-c-Met (D26 and 130H2), c-Met (25H2) (Cell Signaling), Bcl-2 (Abcam) and cyclin D1 (BD Biosciences). After development, membranes were stripped and reblotted with anti-actin antibody (Santa Cruz).

Gelatinolytic activity was detected in liver extracts (100µg) by 10% SDS-PAGE contained 1mg/ml of gelatin (Invitrogen), under non-reducing conditions. After incubation in development buffer (50 mmol/L Tris-HCl, 5 mmol/L CaCl₂, and 0.02% NaN₃, pH 7.5), gels were stained with Coomassie brilliant blue R-250 (Bio-Rad), and destained with methanol/acetic acid/water (20:10:70). Prestained molecular weight markers (Bio-Rad) and MMP-9 (BIOMOL International) served as standards. Relative quantities of protein were determined using a densitometer (NIH Image J software)

RNA Extraction and Reverse Transcriptase PCR

RNA was extracted from livers with Trizol (Life Technologies) as described ⁴. Reverse transcription was performed using 5 µg of total RNA in a first-strand cDNA synthesis reaction with SuperScript III RNaseH Reverse Transcriptase (Life Technologies), as recommended by the manufacturer. The cDNA product was amplified by PCR using primers specific for each target cDNA.

Data Analysis

Data in the text and figures are expressed as mean $\pm$ standard deviation. Statistical comparisons between groups of normally distributed data were performed with the Student t-test using statistical package SPSS (SPSS Inc., Chicago, IL). Kaplan-

Meier analysis was used to determine statistical significance of the differences in mouse survival. P values of less than 0.05 were considered statistically significant.

RESULTS

Time Course of TIMP-1 Expression in Wild-Type Livers Post-IRI

TIMP-1 mRNA was almost undetectable in naïve livers and it was significantly upregulated in TIMP-1^{+/+} livers from 3h to 7d post-reperfusion, (Fig. 1A). TIMP-1 protein expression was mildly detected in TIMP-1^{+/+} naïve livers and it was markedly increase in livers after 6h of reperfusion, particularly at 24h and 48h post-IRI (Fig. 1B). Immunofluorescence analysis showed TIMP-1 staining in the surviving parenchyma predominantly around the portal triads of wild-type livers (Fig. 1C); TIMP-1+ staining was mostly detected in cells along hepatic sinusoids, likely HSC, and in scattered hepatocytes. *In vitro* studies have linked TIMP-1 production to hepatic stellate cells (HSC) and to hepatocytes¹³. Conversely, TIMP-1 staining was absent in TIMP-1^{-/-} livers after IRI (Fig. 1C).

TIMP-1 Deficient Mice Had Reduced Survival Rate after Hepatic IRI

To test the significance of TIMP-1 expression in liver IRI, our experiments included TIMP-1 deficient and respective wild-type (TIMP-1^{+/+}) control mice. The model of partial liver IRI is nonlethal¹⁴; regardless of the significant liver damage detected during the first few days of hepatic IRI, virtually every animal survives after reperfusion. Notably, TIMP-1 deficiency resulted in an unanticipated reduced survival rate post-IRI (37% vs. 100%; $p < 0.05$). Only 3 out of the 8 TIMP-1^{-/-} mice survived after reperfusion, while all 8 TIMP-1^{+/+} (WT) animals recovered from injury and survived up to 7 days post-IRI (Fig 2). TIMP-1^{-/-} mice failed to recover from the injury and succumbed between the second and fourth day post-IRI. Therefore, these results indicate an important role for TIMP-1 expression in hepatic IRI.

Liver Damage Was Increased in TIMP-1 Deficient Mice After IRI.

There were no detectable differences in liver histology and transaminase levels between naive TIMP-1^{-/-} and naive WT mice. Wild-type livers were characterized by significant sinusoidal congestion and extensive necrosis after reperfusion; however, TIMP-1 deficiency was associated with further lobular architecture disruption at 6h, 48h, and 7d post-IRI (Fig. 3A). Indeed, TIMP-1^{-/-} mice demonstrated 2-3-fold higher levels of hepatocellular necrosis ($p < 0.05$) when compared with TIMP-1^{+/+} mice at 48h post-IRI (Fig. 3B). TIMP-1^{-/-} mice that survived surgery showed improved liver histology at 7d post-IRI; however, levels of liver necrosis were still higher in these mice when compared to respective WT controls (Fig. 3A and B). The serum transaminase levels (U/L) were significantly increased in TIMP-1 mice at 6h (sAST: $30,040 \pm 12,104$ vs. $16,033 \pm 6,598$, $p < 0.05$; sALT: $40,660 \pm 21,970$ vs. $18,148 \pm 8,727$, $p < 0.05$), 48h (sAST: $3,290 \pm 2,170$ vs. 197.75 ± 82.44 , $p < 0.05$; sALT: $6,720 \pm 5,298$ vs. 571.25 ± 348.9 , $p < 0.05$), and 7d (sAST: $1,909 \pm 155$ vs. $1,472 \pm 62$, $p < 0.05$; sALT: 254 ± 88 vs. 119 ± 42 , $p < 0.05$) post-IRI, (Fig. 3C). All together, these data emphasize the concept that TIMP-1 has a protective function in hepatic IRI.

MMP-9 Expression and Activity Was Upregulated in TIMP-1 Deficient Livers After IRI.

TIMP-1^{-/-} mice showed significantly up-regulated MMP-9/ β -actin mRNA expression at 6h (0.44 ± 0.17 vs. 0.20 ± 0.11 ; $p < 0.05$), 48h (0.53 ± 0.15 vs. 0.29 ± 0.07 ; $p < 0.05$), and 7d (0.48 ± 0.13 vs. 0.19 ± 0.14 ; $p < 0.05$) after IRI, (Fig. 4A). Moreover, zymography analysis showed that MMP-9 activity was almost undetected in naïve livers and highly expressed in TIMP-1^{-/-} and WT livers post-IRI; however, MMP-9 activity was markedly upregulated in the livers of TIMP-1^{-/-} mice after 6h ($p < 0.05$) and 48h ($p < 0.05$) of reperfusion, as compared to controls (Fig. 4B). Indeed, the MMP-9 activity increase

observed in the TIMP-1^{-/-} mice was over 4-folds of that obtained in the control animals at 48h post-IRI (Fig. 4C). Finally, MMP-9⁺ leukocytes were present in significantly higher numbers in TIMP-1^{-/-} livers at 6h (52 ± 3 vs. 35 ± 14 ; $p < 0.05$), 48h (123 ± 13 vs. 87 ± 12 ; $p < 0.05$), and 7d (32 ± 4 vs. 15 ± 3 ; $p < 0.05$) post-IRI, (Fig. 4D and E). Thus, TIMP-1 deficiency is correlated to increased levels of MMP-9 expression/activity in hepatic IRI.

Deficiency in TIMP-1 Enhanced Myeloperoxidase Activity and Leukocyte Accumulation/Activation in Hepatic IRI.

MPO activity (U/g) was increased in TIMP-1^{-/-} livers (9.5 ± 2.1 vs. 4.7 ± 0.07 ; $p < 0.05$) at 6h post-reperfusion, as compared with TIMP-1^{+/+} controls (Fig. 5A). MPO activity was comparable in both TIMP-1^{-/-} and control livers at 24h post-IRI. However, MPO activity in TIMP-1^{-/-} livers increased again over controls at 48h (12.8 ± 4.9 vs. 5.1 ± 2.6 ; $p < 0.05$) and 7d (5.4 ± 2.0 vs. 1.8 ± 0.8 ; $p < 0.05$) post-IRI (Fig. 5A). MPO activity correlated with Ly-6G⁺ cell numbers; Ly-6G neutrophils were increased in the absence of TIMP-1 at 6h (73 ± 2 vs. 39 ± 10 ; $p < 0.05$), 48h (123 ± 13 vs. 88 ± 12 ; $p < 0.05$), and 7d (37 ± 9 vs. 20 ± 8 ; $p < 0.05$) post-IRI (Fig. 5B, and D). Moreover, TIMP-1 deficiency also caused a substantial increase of infiltrating Mac-1 macrophages at 6h (67 ± 3 vs. 37 ± 10 ; $p < 0.05$), 24h (73 ± 2 vs. 41 ± 8 ; $p < 0.05$), 48h (154 ± 34 vs. 101 ± 15 ; $p < 0.05$), and 7d (64 ± 19 vs. 30 ± 5 ; $p < 0.05$) post-IRI (Fig. 5C and D). The extent of leukocyte infiltration correlated with proinflammatory cytokine expression. TNF- α (0.66 ± 0.15 vs. 0.37 ± 0.28 ; $p < 0.05$), IL-1 β (1.08 ± 0.29 vs. 0.75 ± 0.24 ; $p < 0.05$), and IFN- γ (1.08 ± 0.29 vs. 0.75 ± 0.24 ; $p < 0.05$) were significantly upregulated in TIMP-1^{-/-} livers at 6h post-IRI (Fig 5E). TIMP-1^{-/-} livers at 48h (IL-1 β : 0.21 ± 0.04 vs. 0.10 ± 0.02 ; $p < 0.05$) and 7d (IL-1 β : 0.20 ± 0.04 vs. 0.14 ± 0.03 and TNF- α : 0.32 ± 0.07 vs. 0.21 ± 0.04 ; $p < 0.05$) post-IRI were also characterized by significantly increased proinflammatory cytokine expression. Further, iNOS expression,

which associates to liver injury ¹⁵, showed a ≈ 2.5 -fold increase ($p < 0.05$) in 6h TIMP-1^{-/-} livers. In contrast, IL-10, well-known for its protective role in hepatic IRI ¹⁶, was downregulated in TIMP-1^{-/-} livers at 48h (0.26 ± 0.13 vs. 0.65 ± 0.14 ; $p < 0.05$) and 7d (0.43 ± 0.21 vs. 0.82 ± 0.14 ; $p < 0.05$) post-IRI.

TIMP-1 Deficiency Did Not Alter the Expression of Major Chemokines in Hepatic IRI.

To determine whether TIMP-1 deficiency affects chemokine expression, we assessed major cell activating chemokines linked to liver IRI (Fig. 5F). CXCL-1 (1.16 ± 0.19 vs. 1.02 ± 0.03) and CXCL-2 (0.24 ± 0.18 vs. 0.24 ± 0.06), were comparably expressed in both TIMP-1^{-/-} and wild-type livers at 6h post-IRI. Moreover, TIMP-1^{-/-} and WT livers also expressed similar levels of MCP-1 (0.86 ± 0.11 vs. 0.66 ± 0.20) and SDF-1 (0.45 ± 0.13 vs. 0.45 ± 0.02) 6h post-reperfusion. The expression levels of these chemokines were also comparable in TIMP-1^{-/-} and WT livers at 24h, 48h, and 7d post-IRI (data not shown).

Deficiency in TIMP-1 Impaired Liver Regeneration after IRI.

To determine whether TIMP-1 deficiency interferes with cell proliferation, the percentage of cells in S phase, the BrdU and PCNA labeling indexes, and the percentage of phosphorylated histone H3 (P-H3)-positive cells, the mitotic index (MI), were evaluated after liver IRI. BrdU (0.53 ± 0.11 vs. 1.70 ± 0.13 ; $p < 0.05$), PCNA (0.51 ± 0.46 vs. 5.02 ± 2.98 ; $p < 0.05$) and MI (0.50 ± 0.46 vs. 2.96 ± 1.67) indexes were modestly detected at 24h post-IRI, with decreased proliferation indexes in the TIMP-1^{-/-} livers when compared to controls. While BrdU (0.92 ± 0.11 vs. 6.46 ± 0.24 ; $p < 0.05$), PCNA (2.65 ± 0.33 vs. 26.96 ± 2.74 ; $p < 0.05$), and MI (1.87 ± 1.71 vs. 10.74 ± 1.82 ; $p < 0.05$) indexes

were still almost negligible in TIMP-1^{-/-} livers at 48h post-IRI, they were significantly increased in TIMP-1^{+/+} controls (Fig 6A-C). Several TIMP-1^{-/-} animals died between the second and fourth day post-IRI; nonetheless, TIMP-1^{-/-} mice that survived surgery exhibited some evidence of delayed liver regeneration, as the mitotic index (7.16 ± 2.47 vs. 3.39 ± 1.17) was enhanced in these animals at 7d post-IRI. Moreover, cyclin D1, a regulator of the G1-to-S phase transition¹⁷, and cyclin E, also necessary for entry into S phase¹⁸, were downregulated at mRNA level in TIMP-1^{-/-} livers (cyclin D1: 0.21 ± 0.04 vs. 0.53 ± 0.11 ; $p < 0.05$; cyclin E: 0.44 ± 0.32 vs. 1.18 ± 0.42 ; $p < 0.05$) at 48h post-reperfusion (Fig 6D). Cyclin D1 was almost absent in TIMP-1^{-/-} livers at protein level (0.20 ± 0.26 vs. 1.19 ± 0.25 ; $p < 0.05$), contrasting with an approximate 6-fold increased expression detected in the wild-type livers at 48h post-IRI (Fig. 6E). c-Met-HGF interactions result in c-Met phosphorylation, which is the central stimulus for the G1–S progression of hepatocytes¹⁹. The inability of TIMP-1^{-/-} mice to express TIMP-1 led to markedly decreased HGF/c-Met signaling, as evidenced by the markedly reduced levels of phosphorylated c-Met (0.05 ± 0.07 vs. 0.35 ± 0.20 ; $p < 0.05$) in their livers at 48h post-IRI, (Fig. 7A). Further, c-Met ectodomain shedding, a process by which proteins are proteolytically released from the cell surface, negatively regulates c-Met signaling²⁰. In our settings, absence of TIMP-1 resulted in significantly enhanced c-Met ectodomain shedding in liver IRI, (Fig. 7B). Therefore, these results evidence that loss of TIMP-1 interferes with liver regeneration after IRI.

Lack of TIMP-1 Exacerbates Caspase-3 Activation in Liver IRI.

Caspase-3 is expressed in tissues as an inactive 32-kDa precursor, which is cleaved to generate a 17-kDa mature active form during apoptosis²¹. The active caspase-3 was absent in naïve livers and increased in TIMP-1^{-/-} and wild-type livers at

6h post-reperfusion; however, 17KDa caspase-3 expression was significantly higher (0.55 ± 0.22 vs. 0.12 ± 0.08 ; $p < 0.05$) in the livers of TIMP-1^{-/-} mice, as compared to controls. Notably, the active 17KDa caspase-3 was particularly increased in livers of mice deficient in TIMP-1 (1.79 ± 0.24 vs. 0.27 ± 0.16 ; $p < 0.05$) at 48h, preceding TIMP-1^{-/-} mouse death post-IRI, (Fig. 8A). Immunofluorescence analysis of cleaved-17KDa caspase-3 indicated that while the active form of caspase-3 was minimally expressed in scattered cells in wild-type livers at 48h post-IRI, it was readily detected in the TIMP-1 deficient livers in the still surviving tissue areas (Fig. 8B and C). Moreover, Bcl-2, a known inhibitor of cell death, was almost absent in the TIMP-1^{-/-} livers at 48h post-IRI (0.13 ± 0.08 vs. 0.69 ± 0.19 ; $p < 0.05$) (Fig. 8A). Finally, phosphorylation of Akt, a 57-kD protein-serine/threonine kinase with pro-survival associated functions²², was depressed in TIMP-1^{-/-} livers (0.10 ± 0.07 vs. 0.44 ± 0.30 ; $p < 0.05$) at 48h post-IRI (Fig. 8A). At 7d post-IRI, Bcl-2 was still reduced (≈ 0.6 -fold; $p < 0.05$) in TIMP-1^{-/-} livers, compared to controls. Hence, these results support a major protective role for TIMP-1 expression in hepatic IRI.

DISCUSSION

The understanding of the functions of TIMPs in liver IRI has the potential to contribute to the development of novel therapeutic approaches to prevent hepatic IRI, and consequently, to improve the outcome of liver transplantation. In this study, we investigated the functional significance of TIMP-1 expression in a well-established 90 min mouse model of partial liver warm IRI ⁴.

Interactions between ECM components and cell adhesion receptors regulate leukocyte functions; therefore, it is not unanticipated that enzymatic degradation of ECM can alter leukocyte behaviors ²³. Indeed, cells employ proteolytic enzymes, particularly MMPs, to control the ECM turnover, to release growth factors, and to migrate across ECM ²⁴. There is a growing body of evidence supporting key functions for MMP expression in the pathogenesis of liver diseases ^{3, 25, 26}. In this regard, we have previously shown that MMP-9 regulates leukocyte recruitment and contributes to hepatic IRI ⁴. While TIMP-1 can inhibit a broad range of MMPs, it is particularly potent for MMP-9 ²⁷. However, compared to MMP-9, the role of its natural inhibitor, TIMP-1, is virtually unknown in liver IRI. TIMP-1 expression is very low in naïve livers and it is induced after liver IRI; though, it is still insufficient to prevent an elevated MMP activity in liver IRI ¹¹. In the present study, we show that TIMP-1 deficiency resulted in further exaggerated upregulation of MMP-9 activity and, more strikingly, it led to a poor survival rate after reperfusion. This is particularly interesting having in consideration that the model of partial liver IRI is nonlethal ¹⁴. Indeed, all TIMP-1^{+/+} mice survived hepatic IRI despite the significant liver damage detected in the livers after reperfusion; in contrast, only 3 out of 8 TIMP-1^{-/-} mice survived more than 4 days after liver IRI. In general, TIMP-1^{-/-} mice showed additional impairment of liver function and more severe lesions, which likely led to their death between the second and fourth day post-reperfusion.

While infiltrating leukocytes are recognized as mediators of hepatic IRI ^{3, 28}, the mechanisms involved in their recruitment to sites of inflammatory stimulation in liver are still far from being understood. TIMP-1^{-/-} livers showed massive leukocyte accumulation post-IRI. This latter feature, together with the findings that MMP-9 enzymatic activity was significantly increased in the TIMP-1^{-/-} livers, strongly support an important regulatory role for TIMP-1 on leukocyte recruitment in hepatic IRI. Local concentrations of TIMP-1 are important for regulating MMP-9 activity in vivo ²⁹, and TIMP-1 has also been implicated in leukocyte infiltration into the damaged brain ³⁰. In addition to amplified leukocyte migration, TIMP-1 deficient mice showed significantly increased levels of proinflammatory mediators after liver injury. IFN- γ and iNOS, which have been linked to tissue injury, including hepatic injury ^{15, 31}, were markedly upregulated in the TIMP-1^{-/-} post-IRI. Moreover, TNF- α , whose expression is often associated with neutrophil infiltration and liver damage ³², was also significantly increased in the TIMP-1 livers after reperfusion.

Impaired liver regeneration/repair is one of the most frequent features in acute liver failure. Adult hepatocytes, which make up to 80% of hepatic cells, are long lived and normally do not undergo cell division; however, they maintain the ability to proliferate in response to injury ³³. Using three independent parameters of regeneration (BrdU, PCNA, and mitotic indexes), we provide evidence that hepatocyte progression into S phase and mitosis was disrupted in TIMP-1 deficient mice during the first 48h post-IRI. Cyclins D1 and E, which are necessary for entry into S phase ^{17, 18}, were profoundly depressed in the TIMP-1 deficient livers post-IRI. It is known that inhibition of cyclin D1 leads to growth arrest and to impaired hepatic regeneration ³⁴. It is perhaps important to stress that the role of TIMP-1 in liver regeneration may depend on the type of injury as TIMP-1 can negatively affect regeneration after substantial hepatic resection

³⁵.

Our results agree with previous findings indicating that TIMP-1 has a growth-promoting activity in a broad variety of cells^{9, 36, 37}, including in hepatocytes³⁸, and that TIMP-1 can stimulate the HGF/cMet pathway by inhibiting MMP-mediated c-Met shedding³⁹. Activation of the HGF/cMet signaling pathway requires phosphorylation of c-Met, which is needed for efficient liver regeneration⁴⁰. In our settings, the inability of TIMP-1^{-/-} mice to express TIMP-1 led to virtually undetectable phosphorylated c-Met levels after liver reperfusion. Further, TIMP-1 deficiency resulted in increased proteolytic cMet ectodomain shedding, which may account in part for the reduced levels of phosphorylated c-Met post-liver IRI; soluble c-Met shed ectodomains act as decoy receptors by interfering with HGF binding to c-Met²⁰. Therefore, our work strongly supports the view that TIMP-1^{-/-} livers have an impaired capability to regenerate after IRI.

In addition to impaired liver regeneration, cell death by necrosis, apoptosis, or necroapoptosis, is a prominent feature of liver IRI^{14, 41}. The expression of TIMP-1 was detected in the surviving parenchyma of wild-type mice after the ischemic insult, suggesting a potential role for TIMP-1 in conferring resistance to cell death. Indeed, TIMP-1^{-/-} deficient livers exhibited increased liver necrosis, particularly at 48h post-IRI. Moreover, caspase-3 activation, the executor of apoptosis⁴², was significantly increased in TIMP-1^{-/-} livers as compared with control littermates after IRI, and it was accompanied by a decrease in Bcl-2 expression. While morphologic alterations of apoptosis are mostly mediated by caspases⁴², Bcl-2 is an integral membrane anti-apoptotic protein expressed even in healthy cells⁴³. In this regard, it has been reported that TIMP-1 can inhibit apoptosis in a wide variety of cell types, including stellate cells⁴⁴, B cells⁴⁵, epithelial cells,⁴⁶ and mesangial cells⁴⁷, through MMP-dependent and -independent mechanisms. Moreover, it has also been shown that exogenous TIMP-1 confers resistance against apoptosis in isolated endothelial cells via activation of the PI-3/Akt

signaling pathway⁴⁸. Akt is a 57-kD protein-serine/threonine kinase with pro-survival functions²². In our settings, lack of TIMP-1 expression resulted in almost completely depleted Akt phosphorylation, without changing total Akt protein levels, suggesting that TIMP-1 activates the Akt signaling pathway in hepatic IRI. TIMP-1 inhibition of cell death can also be mediated via its regulatory role on MMP enzymatic activity. The extracellular matrix proteolysis mediated by MMPs can lead to detachment of liver cells resulting in apoptosis, by a phenomenon called “anoikis”⁴⁹. Indeed, we have previously shown that MMP-9, in addition to facilitate leukocytes infiltration in livers after IRI, induces hepatocyte apoptosis after IRI¹⁵.

In summary, these studies demonstrate an important protective role for TIMP-1 expression in liver IRI. Overall, we show that TIMP-1 has relevant functions on promoting cell survival and proliferation of liver cells and on regulating leukocyte recruitment and activation in liver IRI. The inability of TIMP-1^{-/-} mice to express TIMP-1 resulted in enhanced liver damage and in lethal hepatic IRI. Moreover, our data provide the rationale for studies, currently under development in our laboratory, aimed at efficiently overexpressing TIMP-1 *in vivo* as a potential therapeutic approach to improve hepatic IRI.

REFERENCES

1. Selzner N, Rudiger H, Graf R, Clavien PA. Protective strategies against ischemic injury of the liver. *Gastroenterology* 2003;125:917-36.
2. Busuttil RW, Tanaka K. The utility of marginal donors in liver transplantation. *Liver Transpl* 2003;9:651-63.
3. Coito AJ. Leukocyte transmigration across endothelial and extracellular matrix protein barriers in liver ischemia/reperfusion injury. *Curr Opin Organ Transplant* 2011; 16:34-40.
4. Hamada T, Fondevila C, Busuttil RW, Coito AJ. Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury. *Hepatology* 2008;47:186-98.
5. Chirco R, Liu XW, Jung KK, Kim HR. Novel functions of TIMPs in cell signaling. *Cancer Metastasis Rev* 2006;25:99-113.
6. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2:161-74.
7. Bjorklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta* 2005;1755:37-69.
8. Morgunova E, Tuuttila A, Bergmann U, Tryggvason K. Structural insight into the complex formation of latent matrix metalloproteinase 2 with tissue inhibitor of metalloproteinase 2. *Proc Natl Acad Sci U S A* 2002;99:7414-9.
9. Stetler-Stevenson WG. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. *Sci Signal* 2008;1:re6.
10. Kuyvenhoven JP, Molenaar IQ, Verspaget HW, Veldman MG, Palareti G, Legnani C, Moolenburgh SE, Terpstra OT, Lamers CB, van Hoek B, Porte RJ. Plasma MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 during

- human orthotopic liver transplantation. The effect of aprotinin and the relation to ischemia/reperfusion injury. *Thromb Haemost* 2004;91:506-13.
11. Moore C, Shen XD, Gao F, Busuttil RW, Coito AJ. Fibronectin-alpha4beta1 integrin interactions regulate metalloproteinase-9 expression in steatotic liver ischemia and reperfusion injury. *Am J Pathol* 2007;170:567-77.
 12. Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA. Platelet-derived serotonin mediates liver regeneration. *Science* 2006;312:104-7.
 13. Torres L, Garcia-Trevijano ER, Rodriguez JA, Carretero MV, Bustos M, Fernandez E, Eguinoa E, Mato JM, Avila MA. Induction of TIMP-1 expression in rat hepatic stellate cells and hepatocytes: a new role for homocysteine in liver fibrosis. *Biochim Biophys Acta* 1999;1455:12-22.
 14. Yadav SS, Sindram D, Perry DK, Clavien PA. Ischemic preconditioning protects the mouse liver by inhibition of apoptosis through a caspase-dependent pathway. *Hepatology* 1999;30:1223-31.
 15. Hamada T, Duarte S, Tsuchihashi S, Busuttil RW, Coito AJ. Inducible nitric oxide synthase deficiency impairs matrix metalloproteinase-9 activity and disrupts leukocyte migration in hepatic ischemia/reperfusion injury. *Am J Pathol* 2009;174:2265-77.
 16. Yoshidome H, Kato A, Edwards MJ, Lentsch AB. Interleukin-10 suppresses hepatic ischemia/reperfusion injury in mice: implications of a central role for nuclear factor kappaB. *Hepatology* 1999;30:203-8.
 17. Sherr CJ. Cancer cell cycles. *Science* 1996;274:1672-7.
 18. Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* 1995;15:2612-24.

19. Michalopoulos GK, DeFrances MC. Liver regeneration. *Science* 1997;276:60-6.
20. Petrelli A, Circosta P, Granziero L, Mazzone M, Pisacane A, Fenoglio S, Comoglio PM, Giordano S. Ab-induced ectodomain shedding mediates hepatocyte growth factor receptor down-regulation and hampers biological activity. *Proc Natl Acad Sci U S A* 2006;103:5090-5.
21. Han Z, Hendrickson EA, Bremner TA, Wyche JH. A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 in vitro. *J Biol Chem* 1997;272:13432-6.
22. Mullonkal CJ, Toledo-Pereyra LH. Akt in ischemia and reperfusion. *J Invest Surg* 2007;20:195-203.
23. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001;17:463-516.
24. Woessner JF, Jr. That impish TIMP: the tissue inhibitor of metalloproteinases-3. *J Clin Invest* 2001;108:799-800.
25. Dechene A, Sowa JP, Gieseler RK, Jochum C, Bechmann LP, El Fouly A, Schlattjan M, Saner F, Baba HA, Paul A, Dries V, Odenthal M, Gerken G, Friedman SL, Canbay A. Acute liver failure is associated with elevated liver stiffness and hepatic stellate cell activation. *Hepatology* 2010;52:1008-16.
26. Bissell DM. Inflammation and hepatic fibrosis. *Semin Liver Dis* 2010;30:211-4.
27. Gardner J, Ghorpade A. Tissue inhibitor of metalloproteinase (TIMP)-1: the TIMPed balance of matrix metalloproteinases in the central nervous system. *J Neurosci Res* 2003;74:801-6.
28. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. *FASEB J* 1990;4:3355-9.

29. Gough PJ, Gomez IG, Wille PT, Raines EW. Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. *J Clin Invest* 2006;116:59-69.
30. Crocker SJ, Whitmire JK, Frausto RF, Chertboonmuang P, Soloway PD, Whitton JL, Campbell IL. Persistent macrophage/microglial activation and myelin disruption after experimental autoimmune encephalomyelitis in tissue inhibitor of metalloproteinase-1-deficient mice. *Am J Pathol* 2006;169:2104-16.
31. Lappas CM, Rieger JM, Linden J. A2A adenosine receptor induction inhibits IFN-gamma production in murine CD4+ T cells. *J Immunol* 2005;174:1073-80.
32. Yang SQ, Lin HZ, Lane MD, Clemens M, Diehl AM. Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. *Proc Natl Acad Sci U S A* 1997;94:2557-62.
33. Taub R. Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol* 2004;5:836-47.
34. Natarajan A, Wagner B, Sibilio M. The EGF receptor is required for efficient liver regeneration. *Proc Natl Acad Sci U S A* 2007;104:17081-6.
35. Mohammed FF, Pennington CJ, Kassiri Z, Rubin JS, Soloway PD, Ruther U, Edwards DR, Khokha R. Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell cycle via HGF activation in murine liver regeneration. *Hepatology* 2005;41:857-67.
36. Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K. Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS Lett* 1992;298:29-32.
37. Fata JE, Leco KJ, Moorehead RA, Martin DC, Khokha R. Timp-1 is important for epithelial proliferation and branching morphogenesis during mouse mammary development. *Dev Biol* 1999;211:238-54.

38. Kopitz C, Gerg M, Bandapalli OR, Ister D, Pennington CJ, Hauser S, Flechsig C, Krell HW, Antolovic D, Brew K, Nagase H, Stangl M, von Weyhern CW, Brucher BL, Brand K, Coussens LM, Edwards DR, Kruger A. Tissue inhibitor of metalloproteinases-1 promotes liver metastasis by induction of hepatocyte growth factor signaling. *Cancer Res* 2007;67:8615-23.
39. Schelter F, Kobuch J, Moss ML, Becherer JD, Comoglio PM, Boccaccio C, Kruger A. A disintegrin and metalloproteinase-10 (ADAM-10) mediates DN30 antibody-induced shedding of the met surface receptor. *J Biol Chem* 2010;285:26335-40.
40. Huh CG, Factor VM, Sanchez A, Uchida K, Conner EA, Thorgeirsson SS. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proc Natl Acad Sci U S A* 2004;101:4477-82.
41. Malhi H, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology* 2006;43:S31-44.
42. Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 1997;326 (Pt 1):1-16.
43. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002;2:647-56.
44. Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, Benyon C, Iredale JP. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis. *J Biol Chem* 2002;277:11069-76.
45. Guedez L, Stetler-Stevenson WG, Wolff L, Wang J, Fukushima P, Mansoor A, Stetler-Stevenson M. In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1. *J Clin Invest* 1998;102:2002-10.

46. Liu XW, Taube ME, Jung KK, Dong Z, Lee YJ, Roshy S, Sloane BF, Fridman R, Kim HR. Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells from extrinsic cell death: a potential oncogenic activity of tissue inhibitor of metalloproteinase-1. *Cancer Res* 2005;65:898-906.
47. Lin H, Chen X, Wang J, Yu Z. Inhibition of apoptosis in rat mesangial cells by tissue inhibitor of metalloproteinase-1. *Kidney Int* 2002;62:60-9.
48. Boulday G, Fitau J, Coupel S, Soullillou JP, Charreau B. Exogenous tissue inhibitor of metalloproteinase-1 promotes endothelial cell survival through activation of the phosphatidylinositol 3-kinase/Akt pathway. *Ann N Y Acad Sci* 2004;1030:28-36.
49. Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 2001;13:555-62.

Figure Legends:

Figure 1. Time course of TIMP-1 expression in TIMP-1^{+/+} livers post-IRI. TIMP-1 mRNA expression (panel A) was almost absent in naïve wild-type livers (lane1) and it was significantly upregulated at 3h (lanes 2 and 3), 6h (lanes 4 and 5), 24h (lanes 6 and 7), 48h (lanes 8 and 9) and 7d (lanes 10 and 11) after liver IRI. TIMP-1 protein (panel B) was mildly expressed in naïve wild-type livers (lanes 1 and 2) and in livers at 3h (lanes 3 and 4) and 7h (lanes 10 and 11) post-IRI; however, it was markedly increased in livers at 6h (lanes 5 and 6), 24h (lanes 7 and 8) and 48h (lanes 9 and 10) after IRI. Panel C shows representative immunofluorescence staining in TIMP-1^{+/+} (a, c, e, and g) and TIMP-1^{-/-} (b, d, f, and h) livers at 6h post-IRI; TIMP-1 in red (a, b; Alexa Fluor 594), F-actin in green (c, d; Alexa Fluor 488 phalloidin), nuclear stain in blue (e,f; Dapi), and staining overlay (g,h); TIMP-1 positive staining was mostly detected in the surviving parenchyma surrounding the vasculature of wild-type livers post-IRI, whereas TIMP-1 staining was undetectable in the TIMP-1^{-/-} livers, (arrows denote TIMP-1 staining; n=4/group; *p<0.05 relative to naïve livers).

Figure 2. Mouse survival after liver IRI. TIMP-1^{-/-} deficient mice (dotted line) showed a significantly reduced survival rate at 7 days post-IRI, as compared with TIMP-1^{+/+} mice (solid line); TIMP-1^{-/-} animal survival was 37% versus 100% in the respective controls (n=8/group; p<0.05).

Figure 3. Liver histological preservation and serum transaminases in TIMP-1^{-/-} and TIMP-1^{+/+} mice. Representative H&E staining (panel A) of TIMP-1^{+/+} (a, c, e, and g) and TIMP-1^{-/-} (b, d, f, and h) livers at 6h (a, and b), 24h (c, and d), 48h (e, and f), and 7d (g, and h) post-I/R injury; TIMP-1 deficiency was associated with further disruption of

lobular architecture as compared with TIMP-1^{+/+} livers, particularly at 6h, 48h post-IRI, and 7d. The percentage of hepatocellular necrosis (panel B) was increased 2-3 fold in the TIMP-1^{-/-} livers at 48h after IRI. sAST and sALT levels (panel C) were measured in blood samples retrieved after IRI; transaminase levels were significantly increased in TIMP-1^{-/-} mice at 6h, 48h, and 7d post IRI, as compared with respective TIMP-1^{+/+} controls (n=4-6 mice/group *p<0.05).

Figure 4. MMP-9 expression and activity in TIMP-1^{-/-} and TIMP-1^{+/+} mice. MMP-9 mRNA expression (panel A), as detected by RT-PCR analysis, was significantly upregulated in TIMP-1^{-/-} mice at 6h, 48h, and 7d after IR injury, as compared to the respective wild-type controls. MMP-9 activity (panels B and C), analyzed by zymography in TIMP-1^{+/+} (lanes 1, 3, 4, 7, 8, 11, 12, 15, and 16) and TIMP-1^{-/-} (lanes 2, 5, 6, 9, 10, 13, 14, 17, and 18) livers; MMP-9 activity was almost absent in naïve livers of TIMP-1^{+/+} (lane 1) and TIMP-1^{-/-} (lane 2) mice and highly detectable in TIMP-1^{+/+} and TIMP-1^{-/-} livers at 6h (lanes 3-6), 24h (lanes 7-10), 48h (lanes 11-14), and 7d (lanes 15-18) post-IRI; however, compared to controls, MMP-9 activity was markedly upregulated in TIMP-1^{-/-} livers at 6h, 48h, and 7d after IRI. MMP-9+ cells (panel D and E) in wild-type controls (a, c, e) and TIMP-1^{-/-} livers (b, d and f) at 6h (a, and b), 24h (c, and d), and 48h (e, and f) post-IRI; MMP-9+ cells were detected in significantly higher numbers in TIMP-1^{-/-} livers, particularly at 6h, 48h, and 7d post-reperfusion, (n=4-5/group; *p<0.05).

Figure 5. Intrahepatic MPO enzyme activity and leukocyte infiltration/activation in TIMP-1^{-/-} and TIMP-1^{+/+} mice. MPO enzymatic activity (panel A), an index of neutrophil infiltration, was markedly upregulated in TIMP-1^{-/-} livers when compared to wild-type controls at 6h, 48h, and 7d after IRI. Ly-6G+ neutrophil infiltration (panel B) was significantly increased in livers of TIMP-1^{-/-} mice at 6h, 48h and 7d post-IRI. Mac-1+

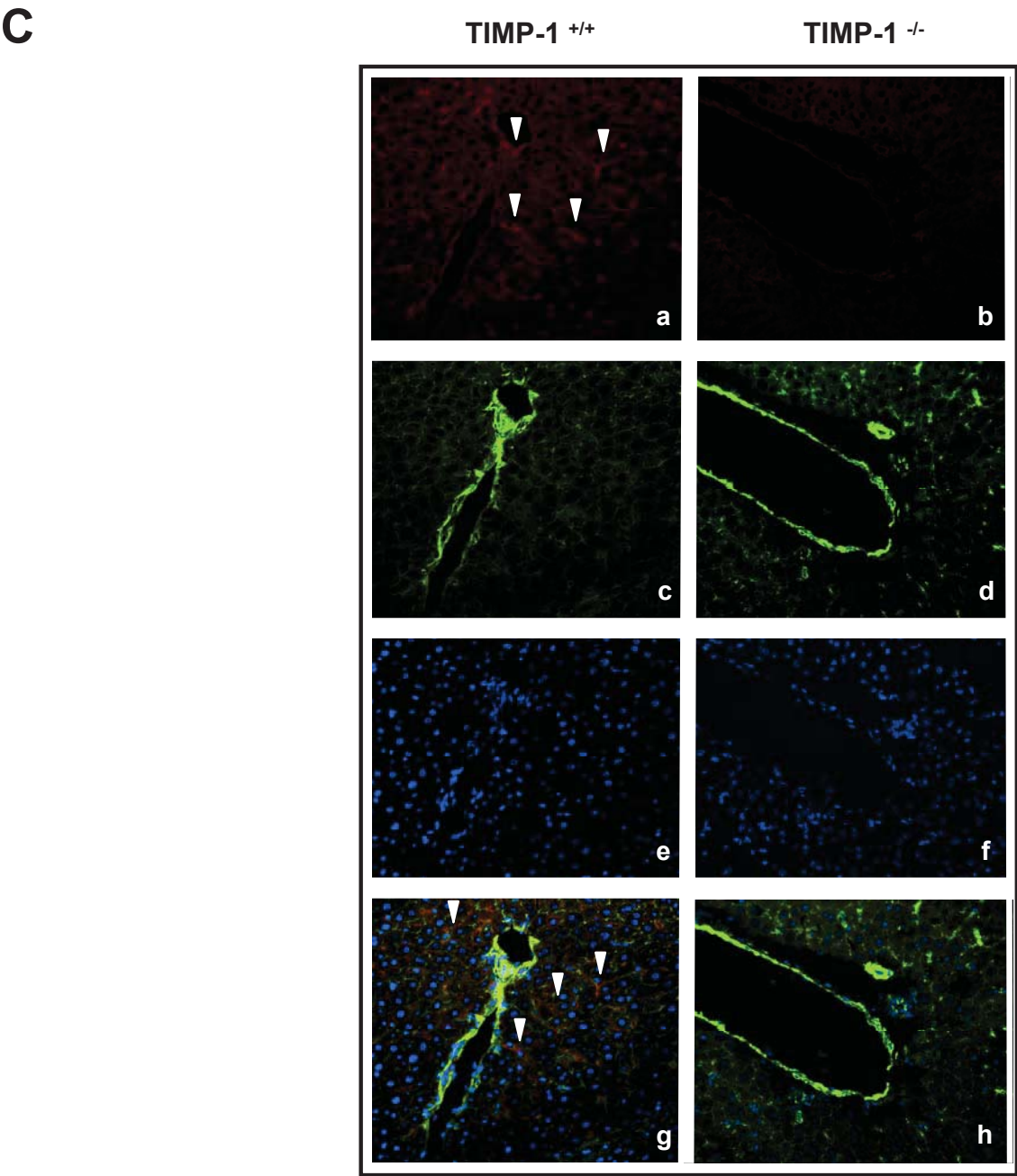
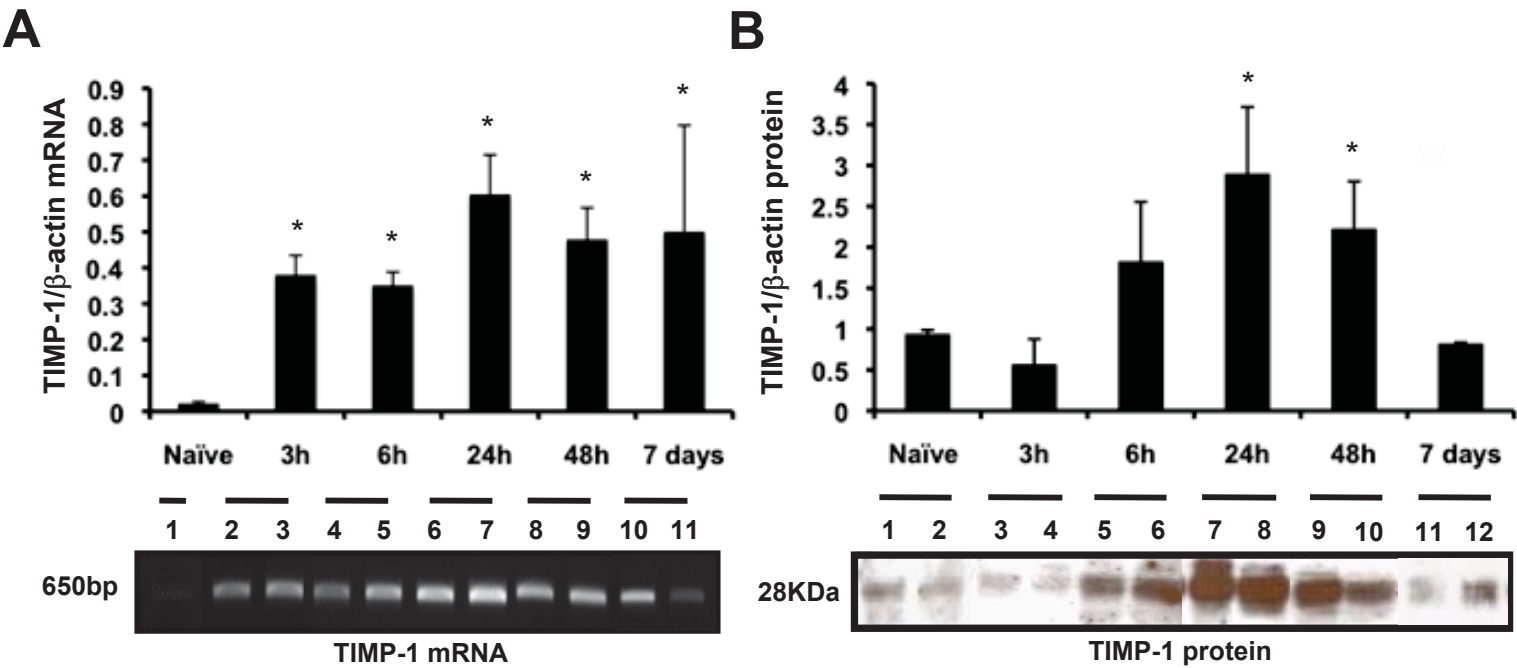
macrophages (panel C) were detected in higher numbers in livers of TIMP-1^{-/-} mice at 6h, 24h, 48h and 7d post-IRI. Panel D illustrates immunostaining of Ly-6G neutrophils (left) and Mac-1 macrophages (right) in TIMP-1^{+/+} livers (a) and TIMP-1^{-/-} (b) at 6h after IRI. Pro-inflammatory mediators (panel E) in TIMP-1^{+/+} and TIMP-1^{-/-} livers; TNF- α , IL-1 β , IFN- γ , and iNOS mRNA levels were significantly upregulated in TIMP-1^{-/-} deficient livers at 6h post-IRI, as compared to respective controls. Chemokine gene evaluation (panel F) showed comparable expressions of CXCL-1, CXCL-2, MCP-1 and SDF-1 in TIMP-1^{+/+} and TIMP-1^{-/-} livers after reperfusion (n=4-5/group; *p<0.05).

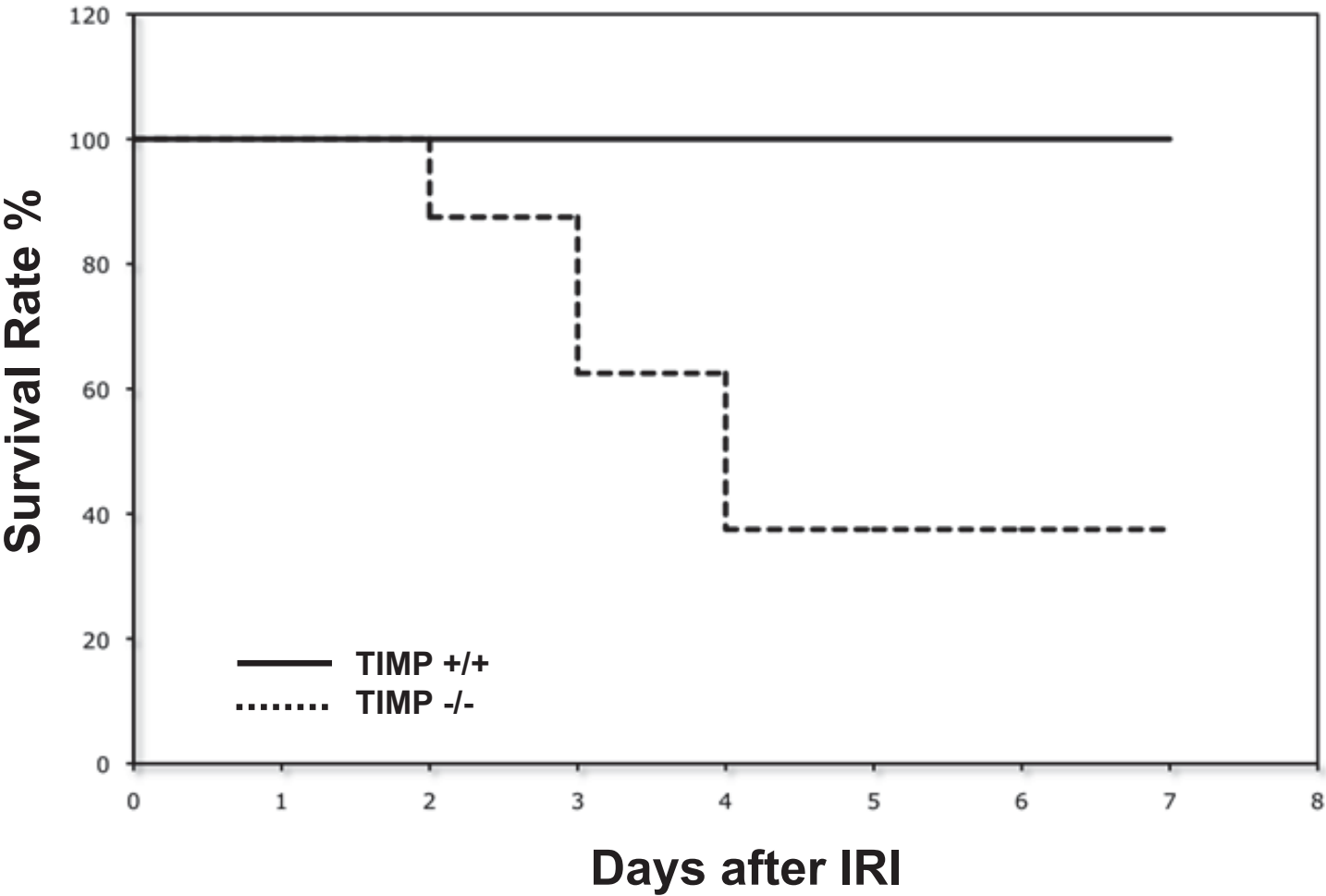
Figure 6. Expression of hepatic regenerative markers in TIMP-1^{-/-} and TIMP-1^{+/+} mice. Hepatocyte BrdU incorporation (panel A), PCNA labeling (panel B), and phosphorylated histone H3-positive cells (panel C) in TIMP-1^{+/+} (a, and c) and TIMP-1^{-/-} (b, and d) livers at 48h post-IRI; PCNA staining (c, and d) is shown in higher magnification to better illustrate positive (c) and virtually negative (d) PCNA hepatocyte-labeling in the surviving parenchyma of TIMP-1^{+/+} and TIMP-1^{-/-} livers, respectively. TIMP-1^{-/-} livers showed markedly diminished BrdU, PCNA, and mitotic labeling indexes, as compared to controls. The densitometric ratios of cyclin D1/ β -actin and cyclin E/ β -actin mRNA (panel D) were significantly depressed in TIMP-1^{-/-} livers at 48h post-IRI. Cyclin D1 at protein level (panel E) was also profoundly depressed in TIMP-1^{-/-} livers at 48h post-IRI, (n=4-5/group; *p<0.05).

Figure 7. cMet phosphorylation and c-Met ectodomain shedding in TIMP-1^{-/-} and TIMP-1^{+/+} livers. c-Met, the high affinity tyrosine kinase receptor for hepatocyte growth factor, was readily phosphorylated in TIMP-1^{+/+} wild-type livers (lanes 1, and 2), contrasting with the almost lack of c-Met phosphorylation detected in TIMP-1^{-/-} livers

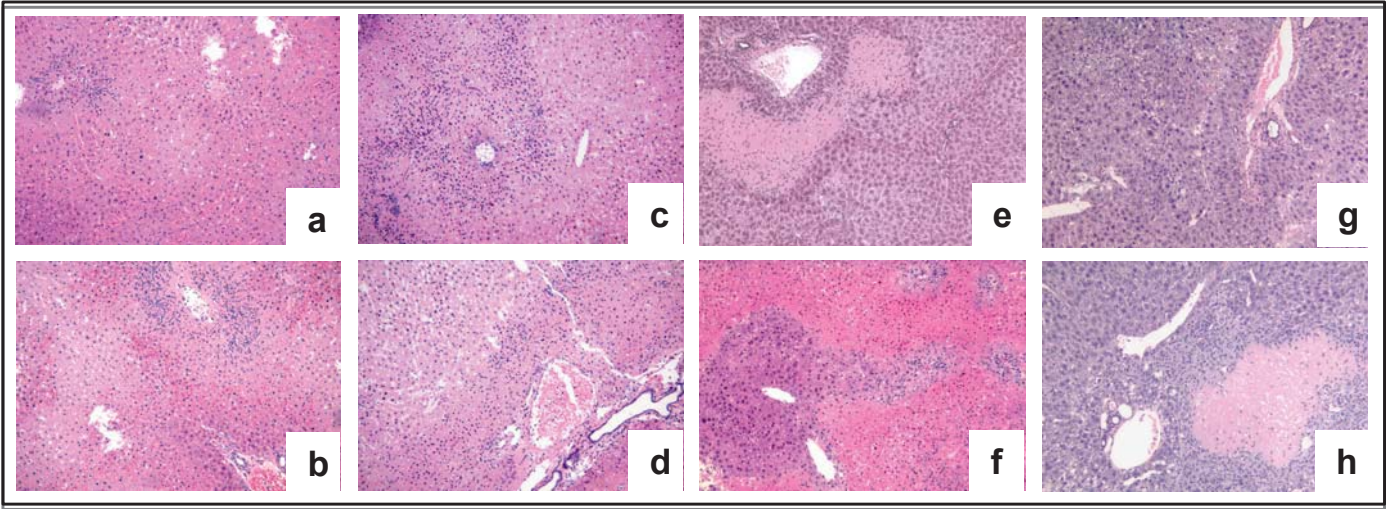
(lanes 3, and 4) post-IRI; the densitometric phospho-c-Met/c-Met ratio was decreased several-fold in the TIMP-1^{-/-} livers at 48h post-IRI, as compared to respective WT controls. Moreover, the c-Met ectodomain fragments 85 kDa and 75 kDa (panel B) were particularly elevated in TIMP-1^{-/-} livers (lanes 3, and 4) at 48h post-IRI when compared with respective matched wild-type controls (lanes 1, and 2), (n=4-5/group; *p<0.05).

Figure 8. Apoptotic and pro-survival markers in livers of TIMP-1^{-/-} and TIMP-1^{+/+} mice. Caspase-3, Bcl2, and Akt expressions (panel A) in wild-type (lanes 1 and 2) and TIMP-1^{-/-} (lanes 3 and 4) livers at 48h post-IRI; the densitometric ratio of active caspase-3/total caspase-3 was significantly increased in TIMP-1^{-/-} livers at 48h post-IRI, whereas the densitometric ratios of Bcl-2/ β -actin and pAkt/Akt were markedly reduced in these livers. Representative triple immunofluorescence in TIMP-1^{+/+} (panel B) and TIMP-1^{-/-} (C) livers of cleaved caspase-3 in red (a, Alexa Fluor 594), F-actin in green (d, Alexa Fluor 488 phalloidin), nuclear stain in blue (b, Dapi), and staining overlay (e, and f); H&E staining (c); active caspase-3 was predominantly detected in the still surviving tissue adjacent to the large vessels and surrounded by extensive areas of necrosis in TIMP-1^{-/-} livers at 48h post-IRI, (n=4-5/group; *p<0.05).

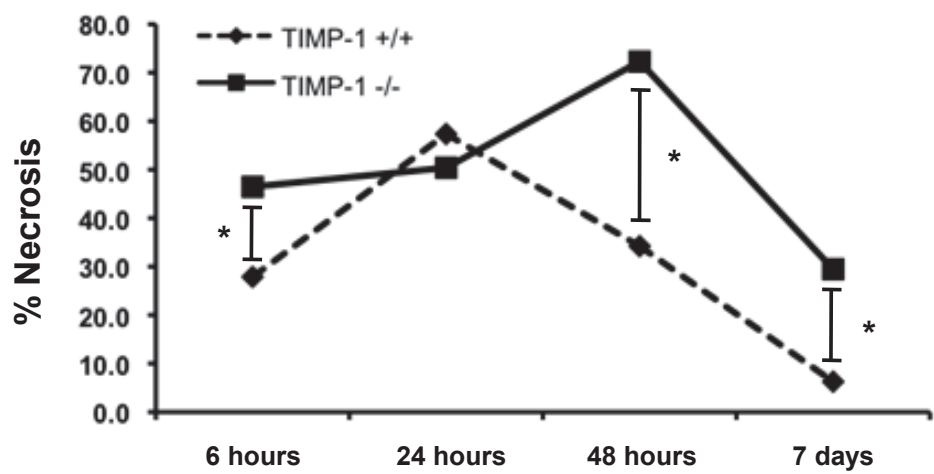




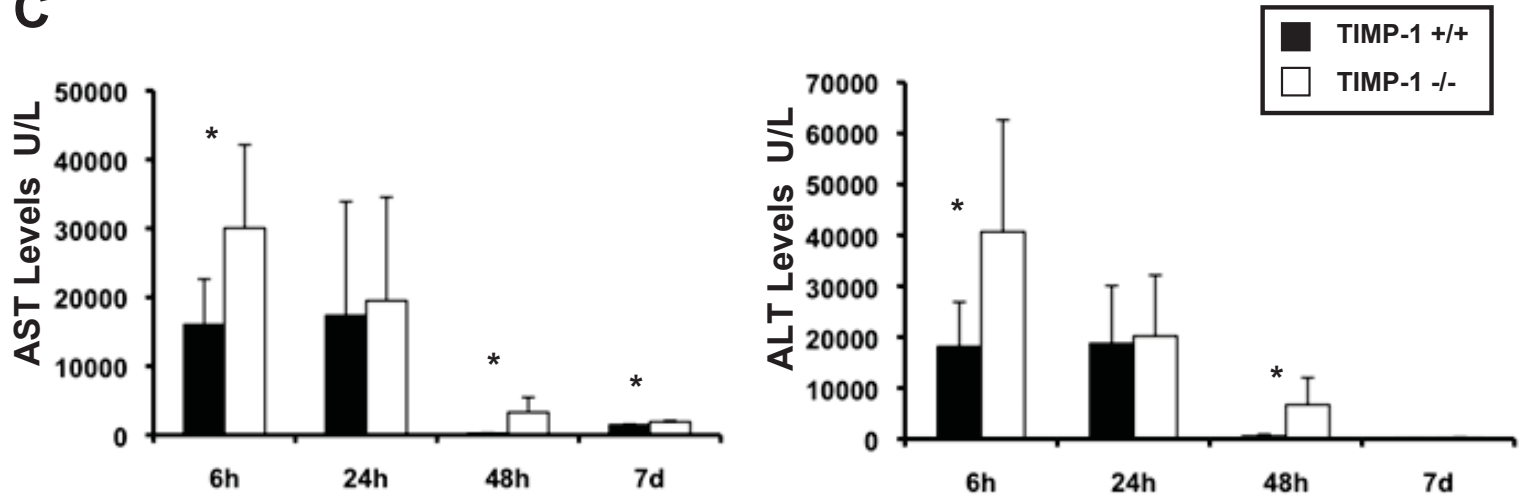
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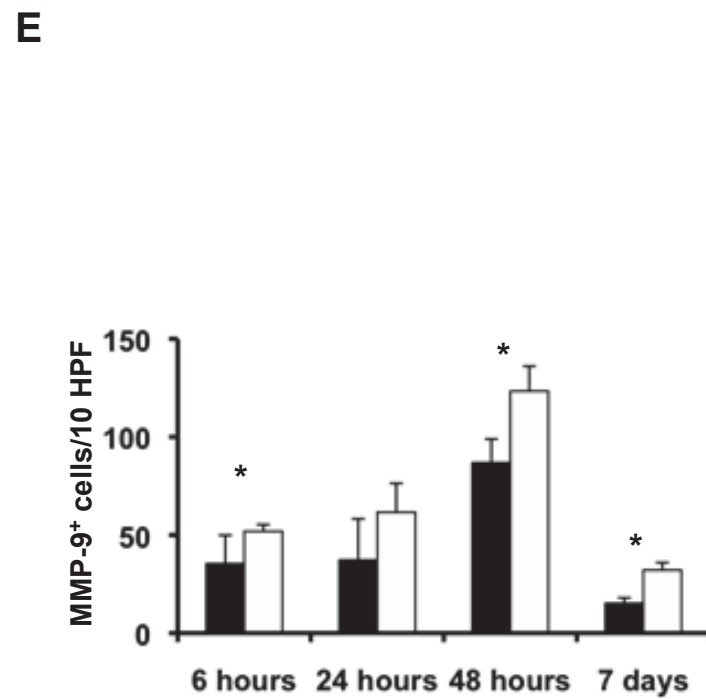
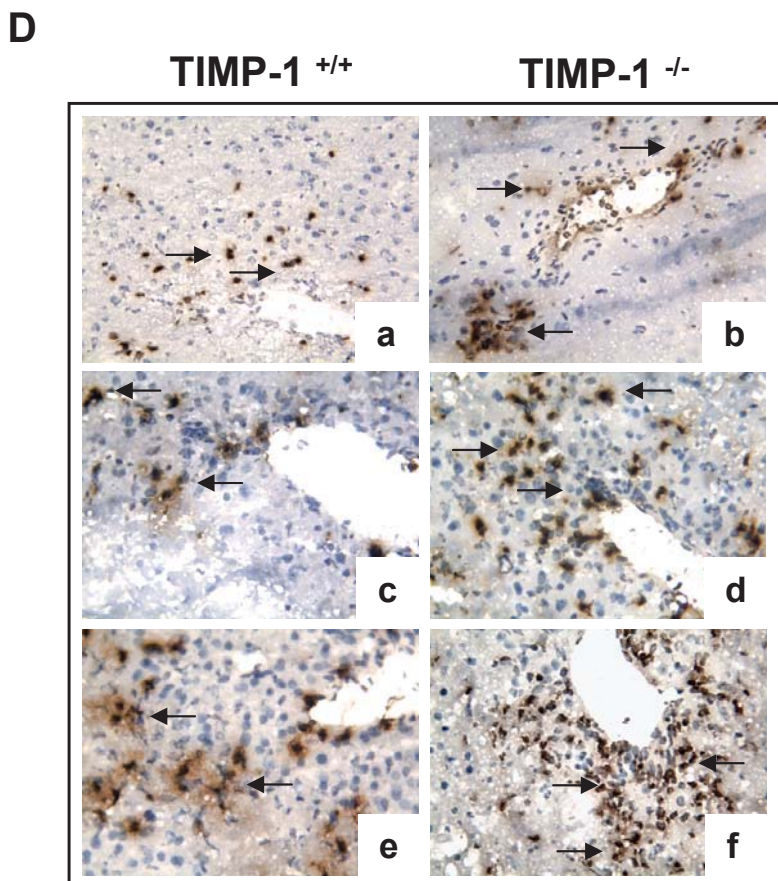
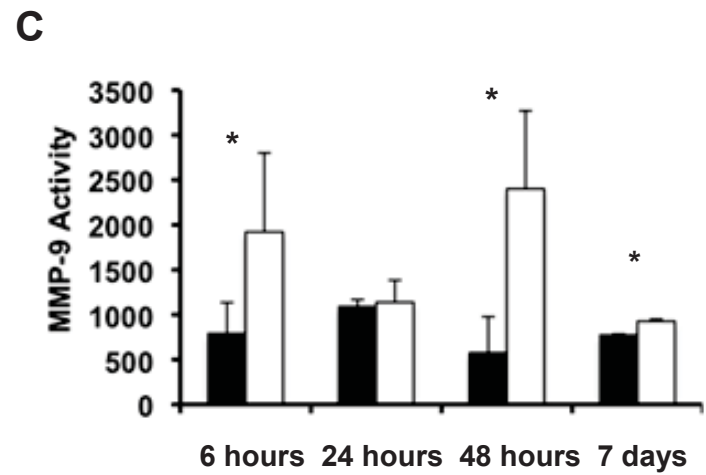
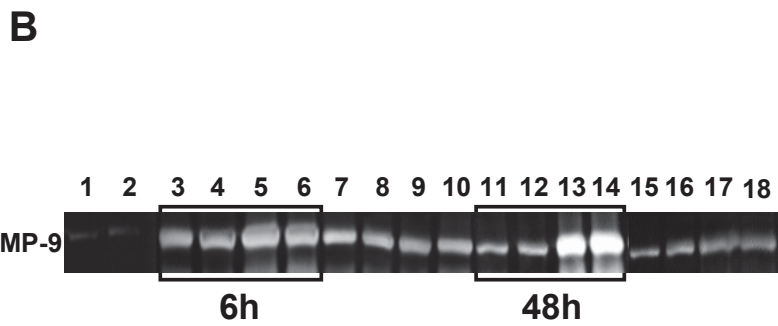
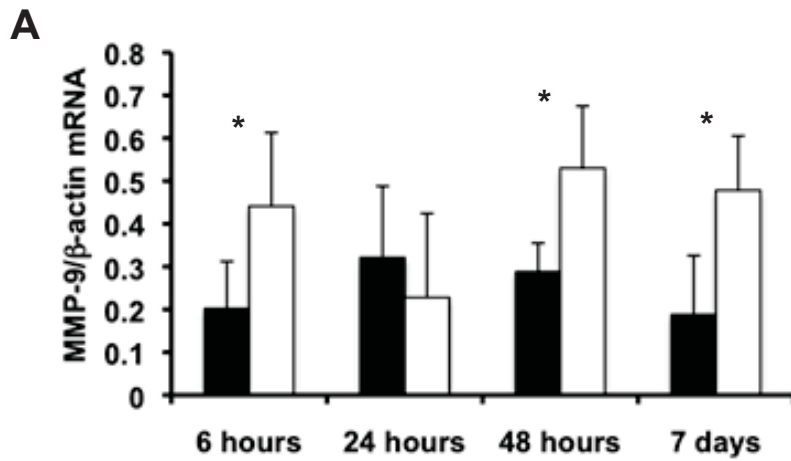


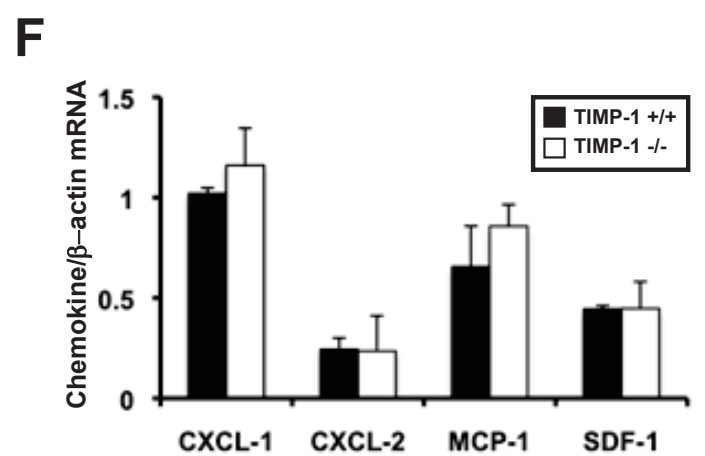
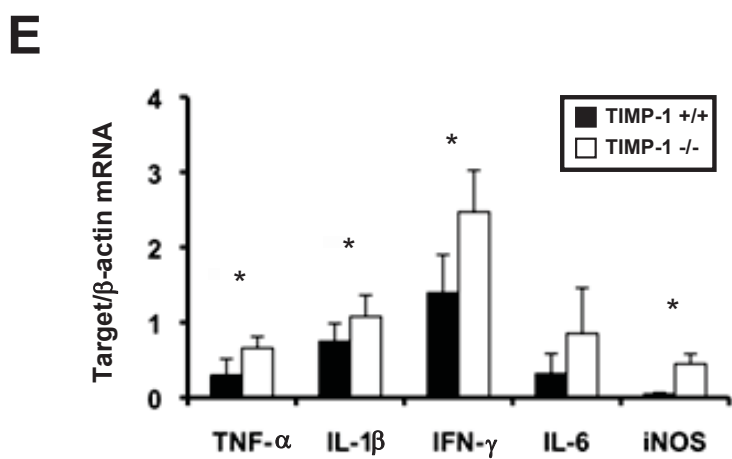
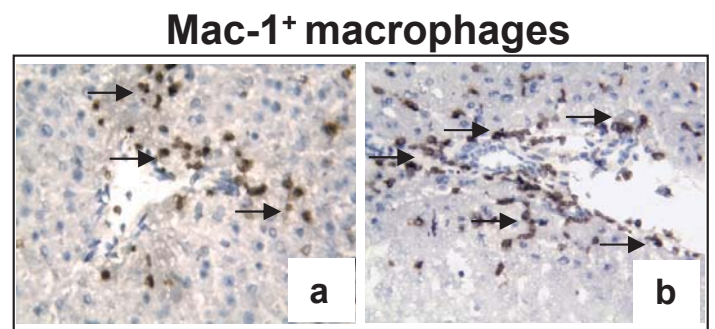
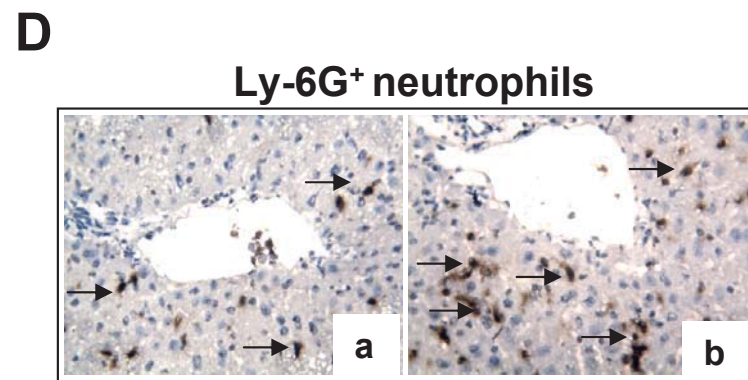
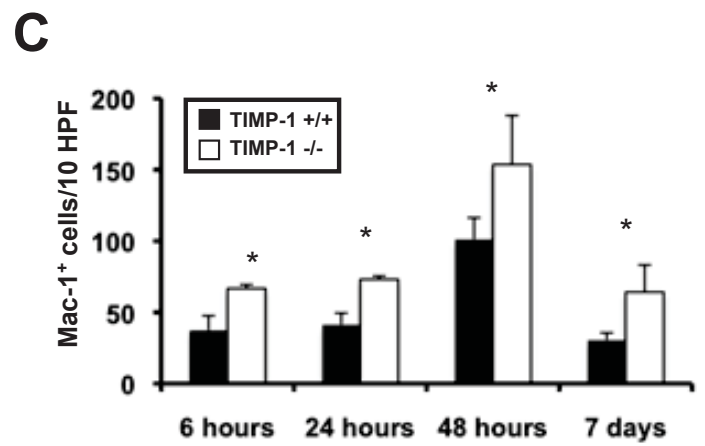
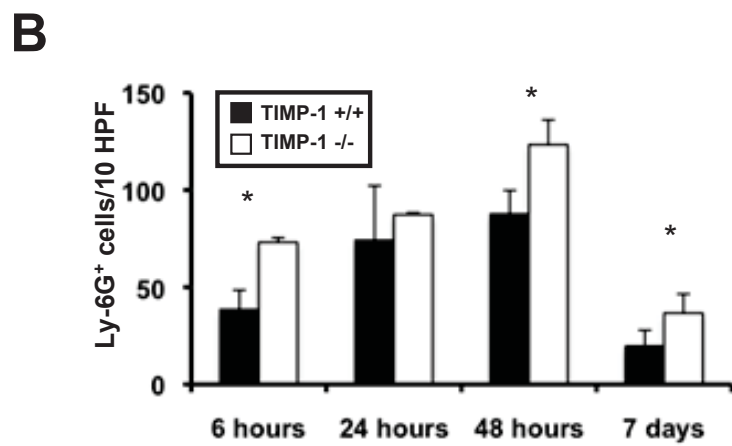
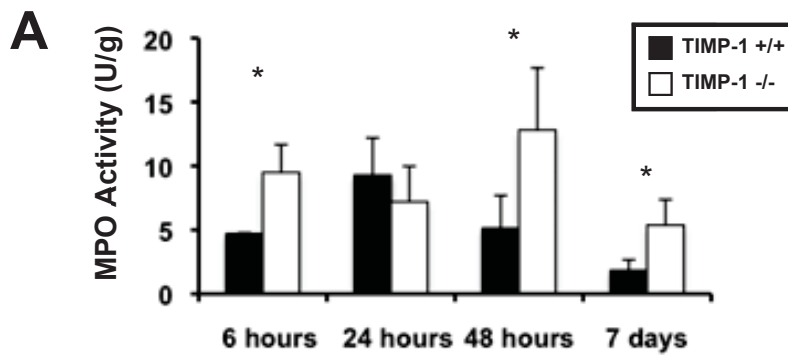
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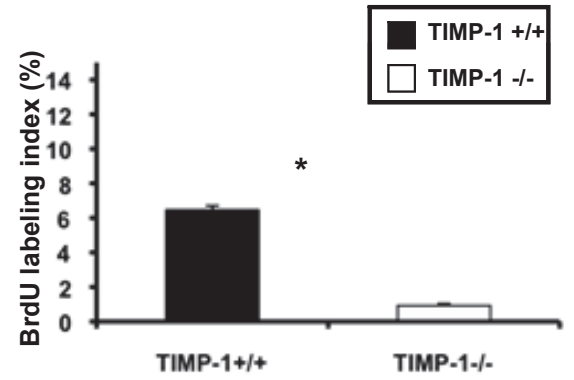
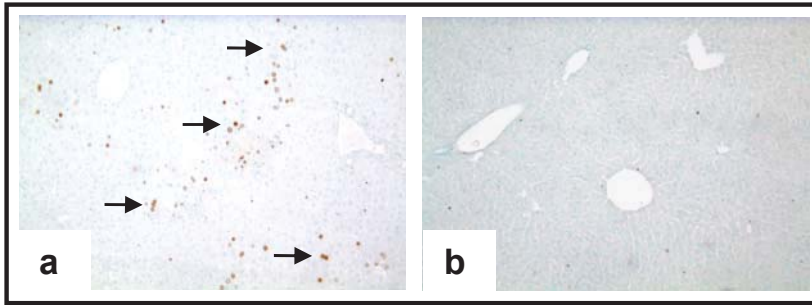
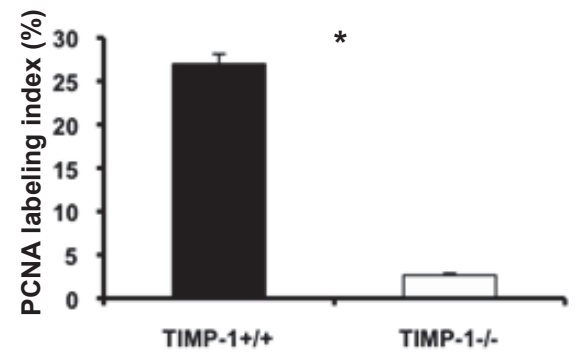
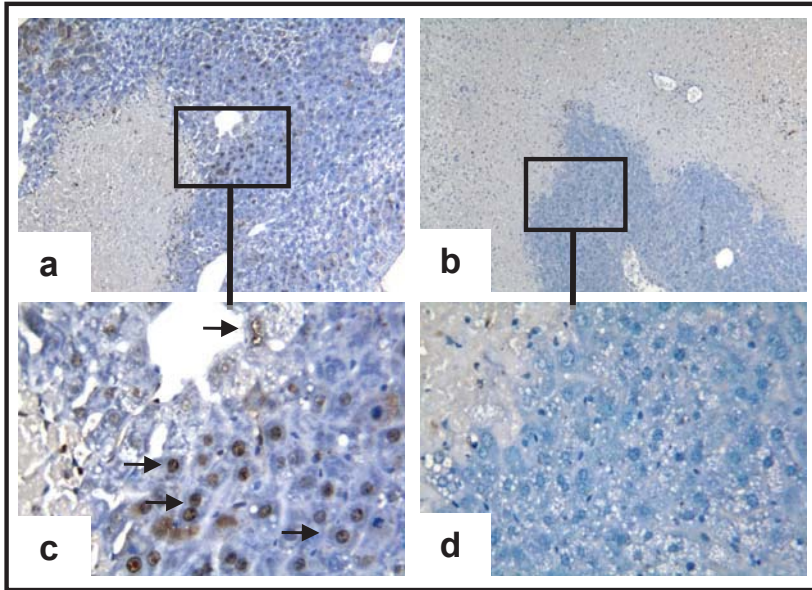
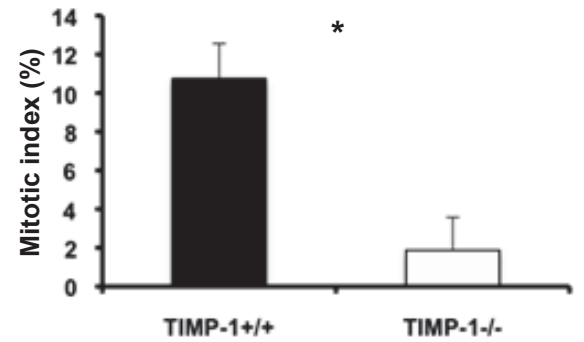
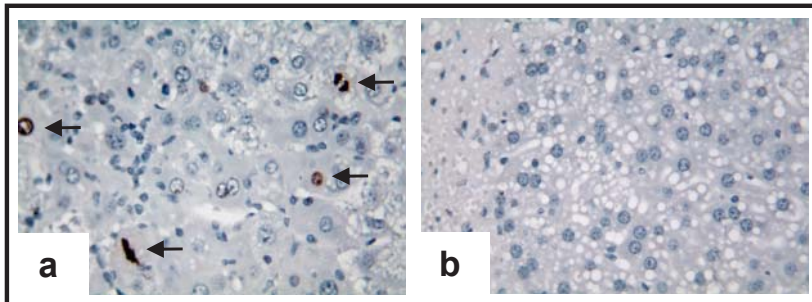
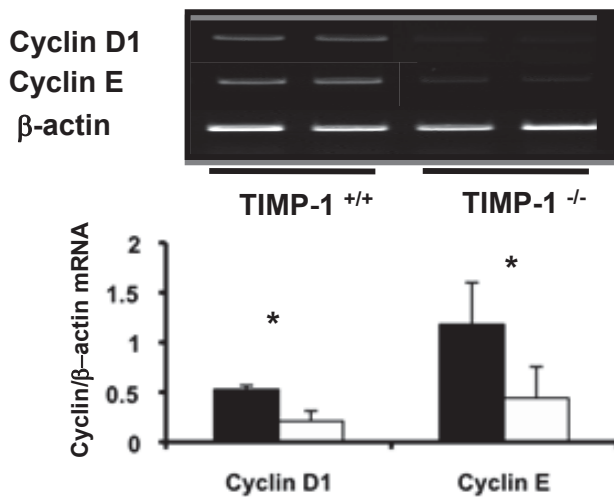
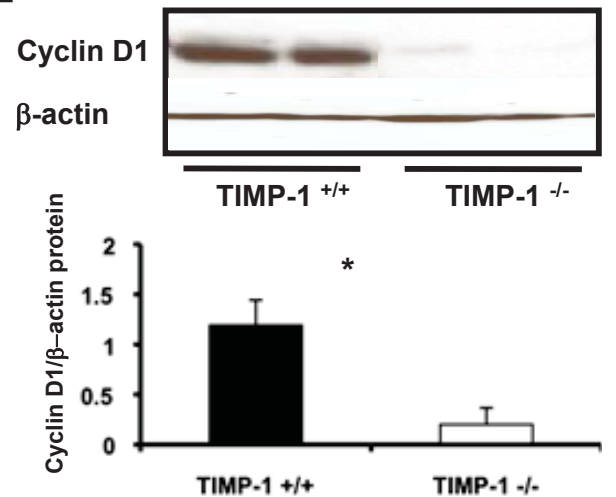


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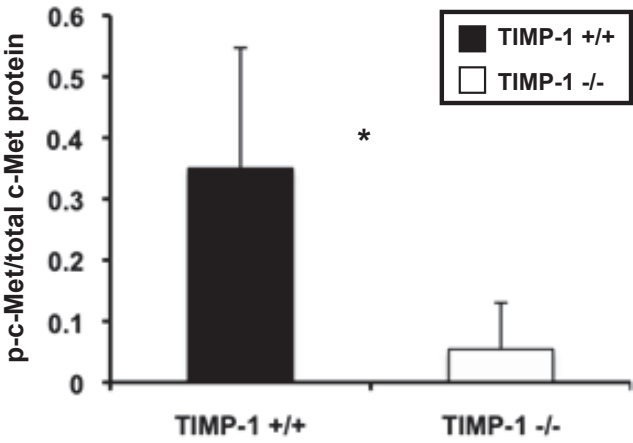
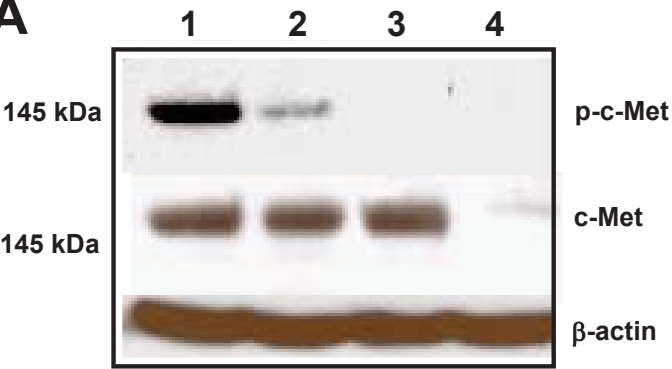




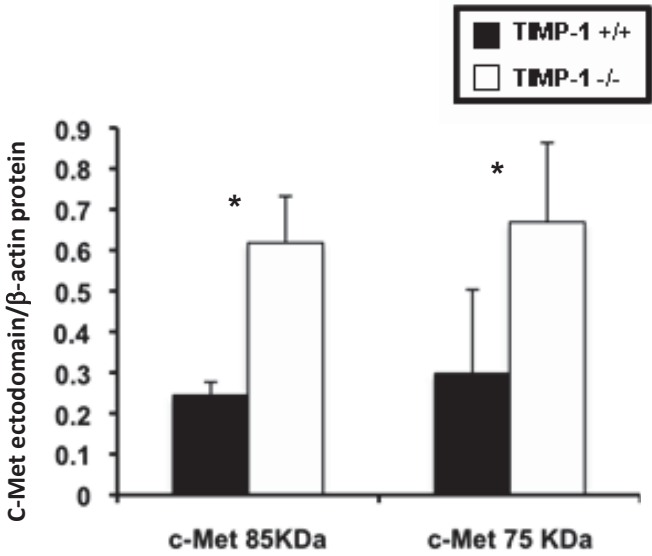
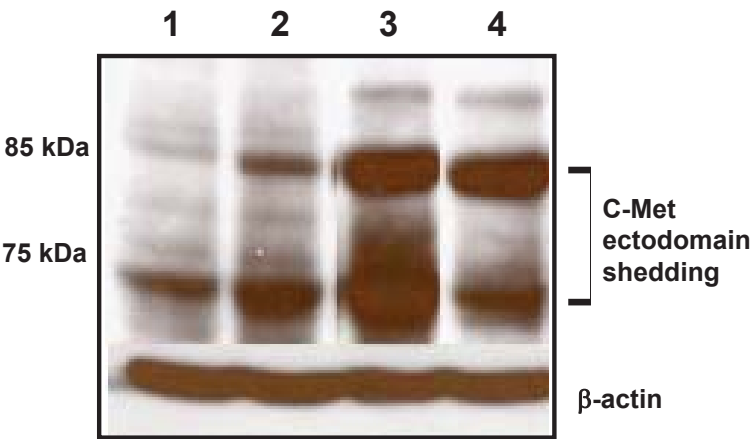


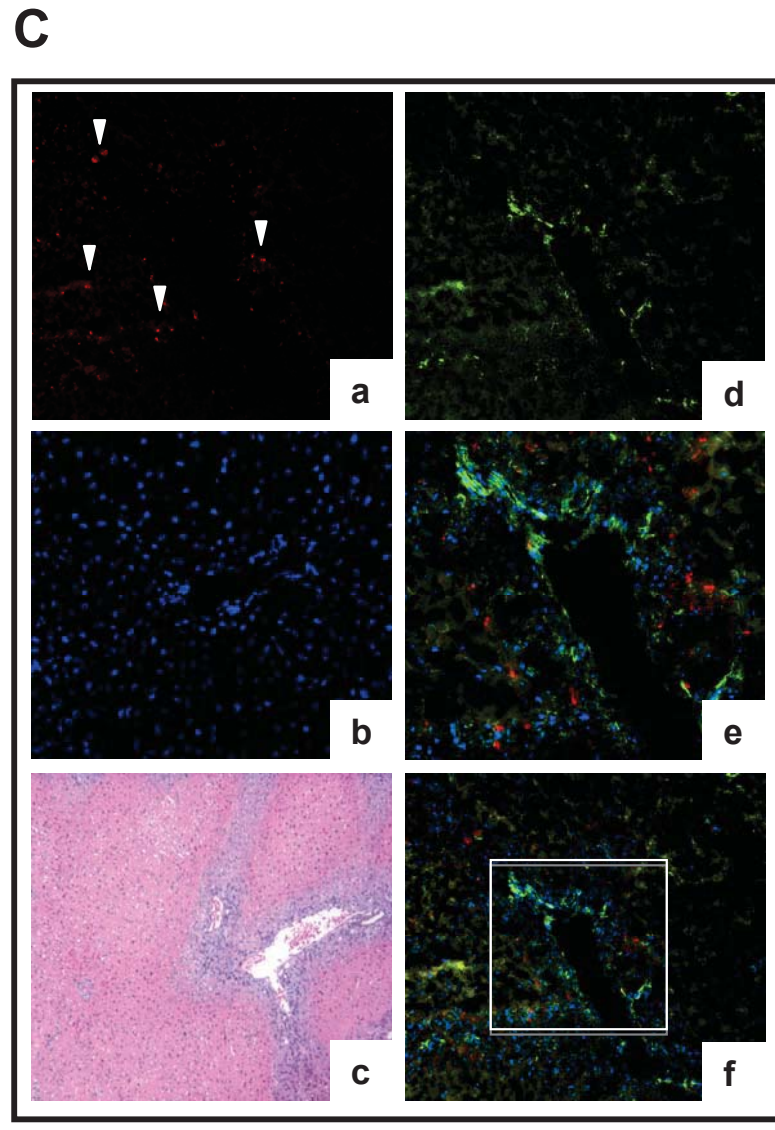
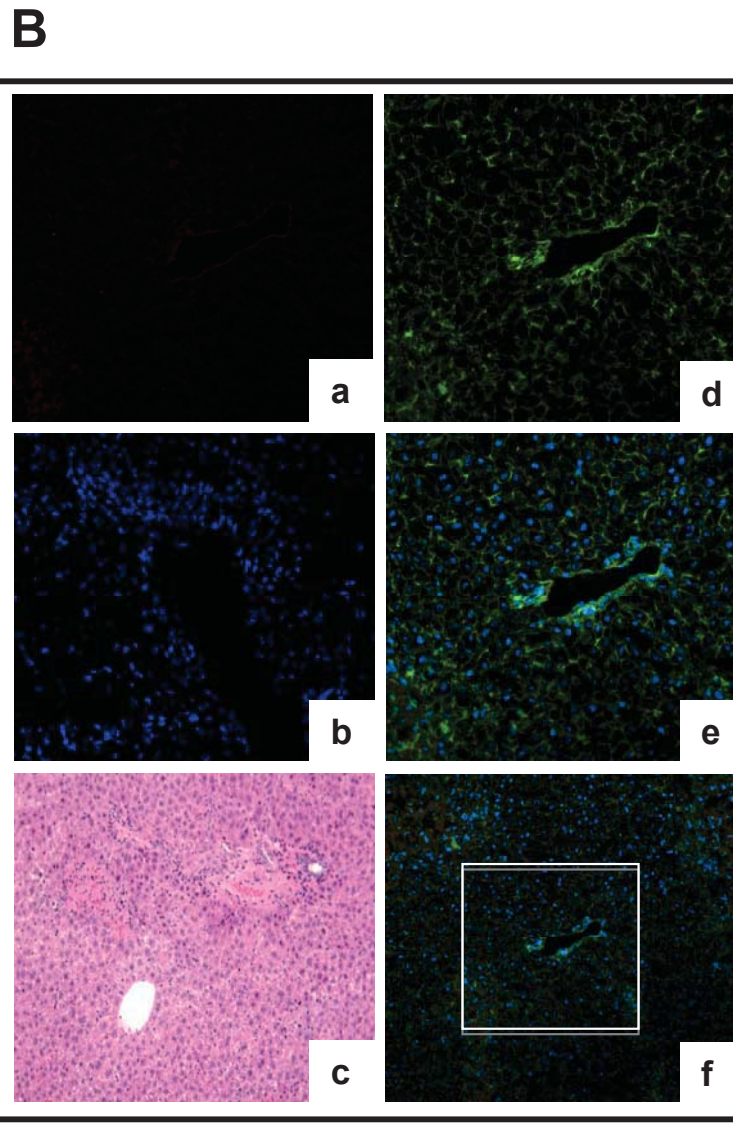
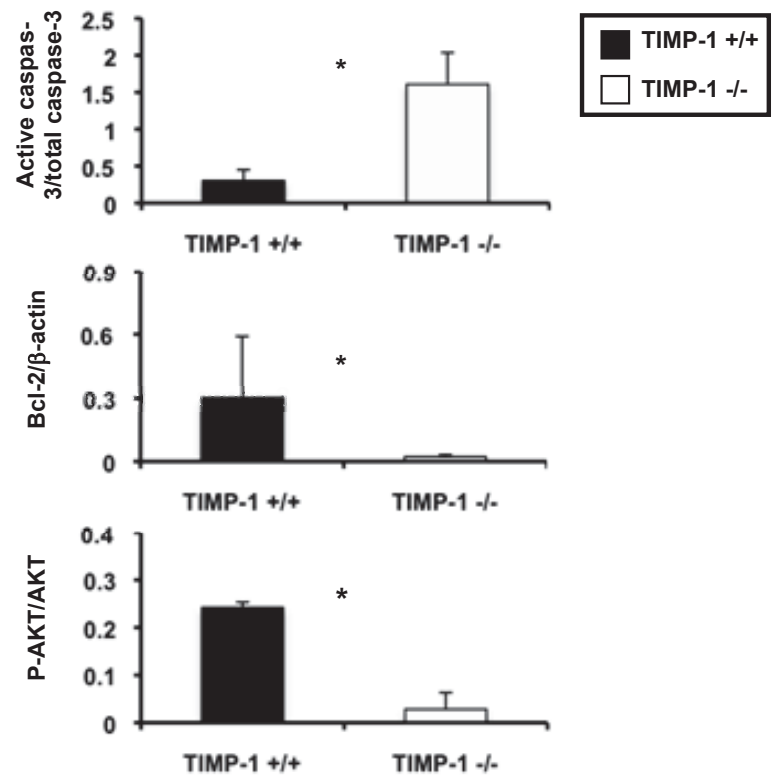
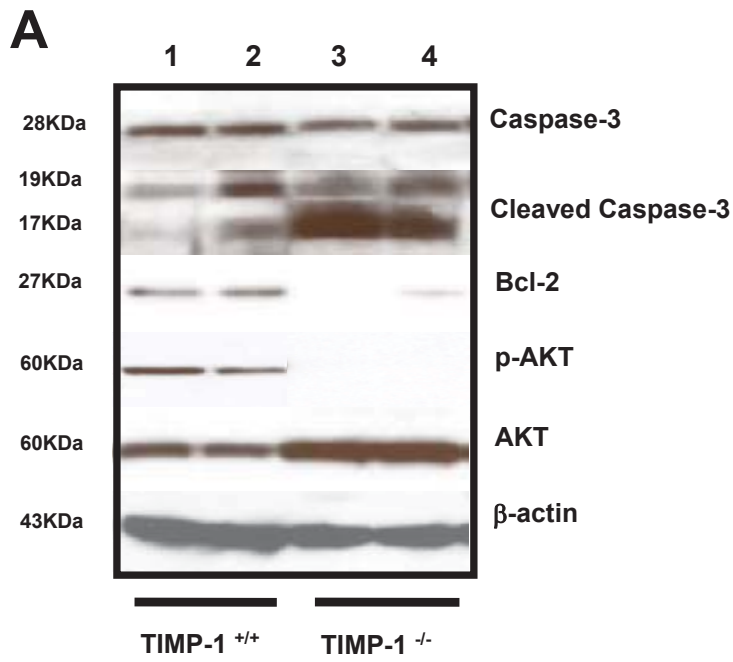
A**TIMP-1 ^{+/+}****TIMP-1 ^{-/-}****B****C****D****E**

A



B





CHAPTER V

INDUCIBLE NITRIC OXIDE SYNTHASE DEFICIENCY IMPAIRS MATRIX METALLOPROTEINASE-9 ACTIVITY AND DISRUPTS LEUKOCYTE MIGRATION IN HEPATIC ISCHEMIA/REPERFUSION INJURY

Takashi Hamada, **Sergio Duarte**, Seiichiro Tsuchihashi, Ronald W. Busuttil,
and Ana J. Coito

American Journal of Pathology 2009 Jun;174(6):2265-77

Matrix Pathobiology

Inducible Nitric Oxide Synthase Deficiency Impairs Matrix Metalloproteinase-9 Activity and Disrupts Leukocyte Migration in Hepatic Ischemia/Reperfusion Injury

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Matrix metalloproteinase 9 (MMP-9) is a critical mediator of leukocyte migration in hepatic ischemia/reperfusion (I/R) injury. To test the relevance of inducible nitric oxide synthase (iNOS) expression on the regulation of MMP-9 activity in liver I/R injury, our experiments included both iNOS-deficient mice and mice treated with ONO-1714, a specific iNOS inhibitor. The inability of iNOS-deficient mice to generate iNOS-derived nitric oxide (NO) profoundly inhibited MMP-9 activity and depressed leukocyte migration in livers after I/R injury. While macrophages expressed both iNOS and MMP-9 in damaged wild-type livers, neutrophils expressed MMP-9 and were virtually negative for iNOS; however, exposure of isolated murine neutrophils and macrophages to exogenous NO increased MMP-9 activity in both cell types, suggesting that NO may activate MMP-9 in leukocytes by either autocrine or paracrine mechanisms. Furthermore, macrophage NO production through the induction of iNOS was capable of promoting neutrophil transmigration across fibronectin in a MMP-9-dependent manner. iNOS expression in liver I/R injury was also linked to liver apoptosis, which was reduced in the absence of MMP-9. These results suggest that MMP-9 activity induced by iNOS-derived NO may also lead to detachment of hepatocytes from the extracellular matrix and cell death, in addition to regulating leukocyte migration across extracellular matrix barriers. These data provide evidence for a novel mechanism by which MMP-9 can mediate iNOS-induced liver

I/R injury. (*Am J Pathol* 2009, 174:2265–2277; DOI: 10.2353/ajpath.2009.080872)

Ischemia/reperfusion (I/R) injury is the pathophysiological process in which the hypoxic insult is further accentuated by restoration of blood flow to the compromised organ. This process causes up to 10% of early transplant failures and can lead to a significantly higher incidence of acute and chronic rejection.¹ Hepatic I/R injury is observed in many clinical situations other than transplantation, such as hepatectomy, shock, and cardiac arrest. Liver damage caused by I/R is the result of complex interactions between various inflammatory mediators, which include infiltrating leukocytes, reactive nitrogen species, reactive oxygen species, and cytokines.^{2–5} A better understanding of the molecular pathophysiology of I/R injury may eventually lead to advanced therapeutic strategies that could improve the success rate of organ transplantation.

Intracellular nitric oxide synthase (NOS) converts L-arginine to L-citrulline and to a free radical nitric oxide (NO).⁶ NO is a short-lived signaling molecule capable of regulating many physiological and pathological processes. There are at least three different isoforms of NOS able to generate NO; the neuronal NOS (nNOS or NOS1), the inducible NOS (iNOS or NOS2), and the endothelial NOS (eNOS or NOS3).⁶ While nNOS and eNOS are constitutively expressed, iNOS is triggered in many cell types by cytokines such as tumor necrosis factor- α or interferon (IFN- γ).⁷ Under normal conditions, only eNOS is present in the liver and low levels of NO regulate the hepatic perfusion.⁸ Alternatively, the excess production of nitric oxide, generated primarily by iNOS,⁹ has been implicated as a mediator of cellular injury at sites of inflamma-

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tion, including liver I/R injury.^{10–12} Under these circumstances, nitric oxide reacts with molecular oxygen or superoxide and generates reactive nitrogen species, which are capable of modifying bioorganic molecules¹³ and mediating many biological processes, including extracellular matrix (ECM) degradation.¹⁴

Leukocyte migration across ECM proteins is dependent on matrix degradation, not only for facilitating “matrix permeability” but also for generating ECM-derived fragments, which are biologically active, and can be highly chemotactic for leukocytes.^{15,16} Matrix metalloproteinase (MMP)-9 is one of two major gelatinases in the MMP family responsible for the turnover and degradation of several ECM proteins, including fibronectin,¹⁷ a key ECM protein expressed very early by liver endothelial cells in response to injury,¹⁸ including to I/R injury.¹⁹ The expression of MMP-9 has been linked to numerous pathological conditions, such as tumor invasion,²⁰ inflammation,¹⁷ arthritis,²¹ cerebral I/R injury²² liver I/R injury,^{15,23} and liver transplantation.²⁴

In general, MMPs have a large propeptide containing cysteine, a catalytic domain with zinc at the active center, and a hemopexin-like domain.²⁵ MMP activation typically requires dissociation of cysteine from the zinc ion, which is recognized as the switch that leads to enzymatic activation.²⁶ However, it has been recently shown that NO can interact with zinc ions and cysteine residues and activates MMP-9 in neuronal cells²² and in a macrophage cell line²⁷ *in vitro*. Similarly to iNOS, MMP-9 is virtually absent in naïve livers, and it is highly up-regulated in damaged livers after I/R injury.^{15,19,23}

In this study, we use iNOS deficient mice and mice treated with a specific iNOS inhibitor to test the hypothesis that iNOS expression has a regulatory function on MMP-9 activation in liver I/R injury. We demonstrate that specific iNOS inhibition markedly down-regulates MMP-9 activity, disrupts leukocyte migration, and reduces apoptosis in liver I/R injury. We present evidence that NO, possibly acting by paracrine mechanisms, regulates MMP-9 activity in neutrophils, which are critical mediators of acute inflammatory liver injury.²⁸ Moreover, we also show that macrophage-derived NO production through the induction of iNOS is capable of regulating neutrophil transmigration across fibronectin in a MMP-9 dependent manner.

Materials and Methods

Mice and Model of Hepatic I/R Injury

C57BL/6-NOS2^{-/-} (B6;129P2-Nos2^{tm1Lau}) and matched iNOS^{+/+} wild-type littermates (B6;129PF2/J), MMP-9^{-/-} (FVB.Cg-Mmp9^{tm1tvu}), and matched MMP-9^{+/+} wild-type littermates (FVB/NJ), and C57BL6 male mice 8 to 10 weeks old were purchased from the Jackson Laboratory. Mice were housed in the University of California at Los Angeles animal facility under specific pathogen-free conditions. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy

of Sciences and published by the National Institutes of Health. A warm hepatic I/R model was performed as previously described.¹⁵ Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) and injected with heparin (100 U/kg). Arterial and portal venous blood supplies were interrupted to the cephalad lobes of the liver for 90 minutes using an atraumatic clip. Mice were sacrificed at 6 hours and 24 hours after reperfusion and liver and blood samples were collected.

ONO-1714 Administration

ONO-1714 (0.05 mg/kg), a novel selective iNOS inhibitor, kindly provided by Drs. Naka and Maruyama from ONO Pharmaceutical Co. Ltd. (Osaka, Japan), was administered subcutaneously to C57BL6 mice 5 minutes before ischemia. Control mice were treated with vehicle in a similar fashion to ONO-1714 administration. ONO-1714 or vehicle administration had no effect in naïve animals.

Assessment of Liver Damage

Serum alanine transaminase (sALT), serum glutamate pyruvate transaminase, serum aspartate transaminase, and serum glutamic oxaloacetic transaminase, levels were measured with an autoanalyzer by ANTECH Diagnostics (Los Angeles, CA). Liver specimens were fixed with a 10% buffered formalin solution, embedded in paraffin, and processed for H&E staining.

Measurement of Nitrate and Nitrite Contents

Nitrite/nitrate levels in serum, liver homogenates, and cell supernatants were measured using Griess Reagent System (Promega, Madison, WI) according to manufacturer's instructions.

Myeloperoxidase Assay

Myeloperoxidase activity was evaluated as previously described.¹⁵ Frozen tissue was homogenized in an iced solution of 0.5% hexadecyltrimethyl-ammonium (Sigma, St. Louis, MO) and 50 mmol/L of potassium phosphate buffer solution (Sigma) with pH adjusted to 5. Samples were centrifuged at 15,000 rpm for 15 minutes at 4°C. Supernatants (100 μ l) were mixed in a solution of hydrogen peroxide-sodium acetate and tetramethyl benzidine (Sigma). The absorbance change at 655 nm in 1 minute was measured with PowerWave XS spectrophotometer (Bio-Tek, Winooski, VT). The quantity of enzyme degrading 1 μ mol/L of peroxide per minute at 25°C per g of tissue was defined as 1U of myeloperoxidase activity.

Immunohistochemistry

Liver specimens embedded in Tissue Tec OCT compound (Miles, Elkhart, IN) and snap frozen in liquid nitrogen were used for immunostaining, as previously described.¹⁹ Appropriate primary antibodies against mouse

CD3 (17A2; BioLegend San Diego, CA), CD4 (L3T4; BD Biosciences, San Jose, CA), macrophage antigen-1 (Mac-1, M1/70; BD Biosciences), Ly-6G (1A8; BD Biosciences), MMP-9 (AF909; R&D Systems, Minneapolis, MN), and vascular cell adhesion molecule1 (VCAM-1, MVCAM A 429; Serotec Inc., Raleigh, NC) were used at optimal dilutions. Bound primary antibody was detected using biotinylated anti-rat or anti-goat IgG, and then streptavidin peroxidase-conjugated complexes (Vector Laboratories, Burlingame, CA). Negative controls included sections in which the primary antibody was replaced with dilution buffer. Control sections from inflammatory tissues known to be positive for each stain were included as positive controls. The peroxidase reaction was developed with DAB Substrate Kit (Vector Laboratories). The sections were evaluated blindly by counting the labeled cells in triplicates within 40 high-power fields per section. Triple staining was detected by immunofluorescence with Alexa Fluor 488-green anti-rat IgG (H+L), Alexa Fluor 594-red anti-goat IgG (H+L), Alexa Fluor 647 anti-rabbit IgG (H+L) antibodies (Molecular Probes, Carlsbad, CA), and slides were analyzed using a Leica Confocal Microscope (University of California at Los Angeles Brain Research Institute, Confocal Microscope Core Facility).

RNA Extraction and Reverse Transcription-PCR

For evaluation of cytokine gene expression, livers were harvested and RNA was extracted with Trizol (Life Technologies Inc., Grand Island, New York) using a Polytron RT-3000 (Kinematica AG, Littau-Luzern, Switzerland), as previously described.²⁹ Reverse transcription was performed using 5 μ g of total RNA in a first-strand cDNA synthesis reaction with SuperScript II RNaseH Reverse Transcriptase (Life Technologies Inc), as recommended by the manufacturer. The cDNA product was amplified by PCR using primers specific for mouse cytokines and β -actin.

Western Blot and Zymography Analyses

Snap-frozen liver tissue was immediately homogenized as previously described.¹⁹ Protein content was determined using a BCA Protein Assay Kit (Pierce Chemical, Rockford, IL). For Western blots 40 μ g of protein in SDS-loading buffer were electrophoresed through 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The gels were then stained with Coomassie blue to document equal protein loading. The membranes were blocked with 5% dry milk and 0.05% Tween 20 (USB, Cleveland, OH) in Tris-buffered saline and incubated with specific primary antibodies against iNOS (Chemicon, Temecula, CA), and Bcl-xl (Cell Signaling Technology, Danvers, MA). The filters were washed and then incubated with horseradish peroxidase conjugated secondary antibodies, followed by detection with SuperSignal West Pico Chemiluminescent Substrate (Pierce). After development, membranes were striped and re-blotted with an antibody against β -actin

(Abcam). Relative quantities of protein were determined using a densitometer (Kodak Digital Science 1D Analysis Software, Rochester, NY).

Gelatinolytic activity was detected in liver extracts (100 μ g) or 200 μ l of cell supernatant by 10% SDS-polyacrylamide gel electrophoresis contained 1 mg/ml of gelatin (Invitrogen, Carlsbad, CA), under non-reducing conditions.²³ After SDS-polyacrylamide gel electrophoresis, the gels were soaked twice with Novex Zymogram Renaturing Buffer (Invitrogen) for 30 minutes each time, rinsed in water, and incubated overnight at 37°C in Novex Zymogram Developing Buffer (Invitrogen). The gels were then stained with Coomassie brilliant blue R-250 (Bio-rad, Hercules, CA), and destained with methanol/acetic acid/water (20:10:70). A clear zone indicates the presence of enzymatic activity. Positive controls for MMP-9 (BIOMOL International, Plymouth, PA), and prestained molecular weight markers (Kaleidoscope Prestained Standards; Bio-Rad) served as standards. Relative quantities of protein were determined using a densitometer (Kodak Digital Science 1D Analysis Software, Rochester, NY).

MMP-9 Protein Levels

Total MMP-9 protein levels were detected in cell supernatants using a Quantikine Mouse MMP-9 (total) Immunoassay Kit (RGD, Minneapolis, MN) according to the manufacturer's instructions.

MMP-9 Activity

MMP-9 activity was detected in liver homogenates (100 μ g of protein) and in cell supernatants using an Amersham Matrix Metalloproteinase-9 Biotrak Activity Assay System (GE Health care Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions.

Leukocyte Isolation

Isolation of adult murine neutrophils from bone marrow was performed as previously published.²⁹ Briefly, femurs and tibias were harvested and stripped of all muscle and sinew, and bone marrow was flushed with 2.5 ml of RPMI-1640 containing 5% fetal calf serum on ice. Cells were pelleted, and erythrocytes were removed by hypotonic lysis. The entire bone marrow preparation was resuspended at 5×10^7 cells/ml in Hanks' balanced saline solution. Cells were layered on a Percoll (Sigma-Aldrich) gradient (3 ml of 55%, top; 3 ml of 65%, middle; 4 ml of 80% Percoll) and centrifuged at 2000 rpm for 30 minutes at 10°C. Mature neutrophils were recovered at the interface of the 65% and 80% fractions and were >90% pure and >95% viable in the neutrophil-rich fraction as determined by Ly-6G immunostaining/morphology and trypan blue exclusion, respectively.

Murine macrophages were prepared using published methods. Briefly, 1 ml of 3% thioglycollate medium was injected into the peritoneal cavity 72 hours before collecting macrophages. The peritoneal cavities were lavaged with 5 ml of PBS, and the aspirate was placed on ice and

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centrifuged at 1200 rpm for 5 minutes at 4°C. The pellets are cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cell viability was determined by trypan blue exclusion.

iNOS Inhibition/NO Donor in Vitro Assays

Isolated leukocytes were cultured in medium without fetal bovine serum for 24 hours before being stimulated by lipopolysaccharide 1 to 100 ng/ml (LPS, Sigma) or by Formyl-Met-Leu-Phe-OH 5–50 nmol/L (fMLP, Calbiochem, San Diego, CA) for 24 hours in the presence or absence of ONO-1714. LPS and fMLP are commonly used to activate macrophages and neutrophils, respectively. After incubation, cell supernatants were collected for NO measurements. In addition, isolated leukocytes were also cultured for 6 hours with a NO donor with a long half-life of 27 hours, 2,2'-(hydroxynitrosohydrazono)-bis-ethanamine 5 to 500 μ mol/L (DETA NONOate; Sigma). Cell supernatants were collected for MMP-9 activity measurements by zymography.

Neutrophil Migration Assay

Macrophages previously stimulated with LPS (1 μ g/ml, Sigma) for 1 hour, and washed three times in Hanks' balanced saline solution (GIBCO BRL, Gaithersburg, MD) to remove LPS, were seeded (0.5×10^6 cells/250 μ l) in fresh LPS-free DMEM in 24-well tissue culture plates and incubated for 3 hours at 37°C and 5% CO₂ before neutrophil transmigration. Wells not seeded with macrophages had an equal volume of DMEM added to them. NO release by the adherent macrophages was significantly detected at 3 hours to 6 hours after LPS stimulation (not shown). Transmigration through fibronectin of isolated neutrophils, resuspended in DMEM without fetal bovine serum at a final concentration of 2.0×10^6 cells/ml, was performed using a commercially available *in vitro* cell migration assay kit (BD Bioscience, Bedford, MA), as previously described.¹⁵ Transwell inserts with 3- μ m pore size either coated with fibronectin or uncoated (control invasion chambers) were placed in the 24-well plates, and then neutrophils (4×10^5 cells/well) were added to the upper chambers. Where indicated, 10 nmol/L of MMP-9 inhibitor-I (C₂₇H₃₃N₃O₅S; Calbiochem, La Jolla, CA) or 20 nmol/L of iNOS inhibitor (ONO-1714) were included in the DMEM medium of the lower chambers. Cells were incubated at 37°C and 5% CO₂ for 4 hours, and the neutrophils that had migrated into the lower chambers were collected, stained and counted. NO contents and MMP-9 activity were also evaluated as previously described.

Cytokine-Mediated Neutrophil Stimulation

Isolated neutrophils were cultured in serum free medium for 24 hours before being treated with interleukin (IL)-6, 25 to 100 ng/ml, or IFN- γ , 25 to 100U/ml (eBioscience, San Diego, CA) for 24 hours. After incubation, cell supernatants were collected for MMP-9 activity measurements

by gelatin zymography. Gels were visualized using a Foto/Analyst FX (Fotodyne, Hartland, WI), and the bands were quantified by densitometry using Image J software (NIH, Bethesda, MA). Data are presented as fold increase over the unstimulated controls.

Caspase-3 Activity

Caspase-3 activity was determined in liver samples using a commercially available ApoAlert Caspase 3 Colorimetric Assay Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. Optical density measurements at 405 nm were performed using a microplate reader (Bio-Tek). Caspase activity was expressed in units with 1 unit being the amount of enzyme activity liberating 1 pmol of pNA per minute.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed on 5- μ m cryostat sections using the In Situ Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. TUNEL-positive(+) cells were detected under light microscopy. Terminal transferase was omitted as a negative control. Positive controls were generated by treatment with DNase 1 (30 U/ml in 40 mmol/L of Tris-Cl, pH 7.6, 6 mmol/L MgCl₂, and 2 mmol/L CaCl₂ for 30 minutes).

Data Analysis

Data in the text and figures are expressed as means $\pm$ SEM. Two-group comparisons were analyzed by the two-tailed Student's *t*-test for independent samples. Probability values of less than 0.05 were considered statistically significant.

Results

iNOS Expression in Hepatic I/R Injury

iNOS expression, as detected by Western blotting, was virtually undetectable and mildly detectable in naïve wild-type livers, and in livers after 90 minutes of warm ischemia (before reperfusion), respectively. However, iNOS expression was readily up-regulated at protein level at 3 hours, 6 hours, and 24 hours of I/R injury (Figure 1). These results were consistent with our previous observations in a rat model of liver transplantation, in which iNOS was highly expressed in damaged livers after I/R injury.¹⁹ The expression of iNOS in iNOS^{-/-} deficient mice was undetectable in naïve livers and in livers after 3 hours, 6 hours, and 24 hours of I/R injury.

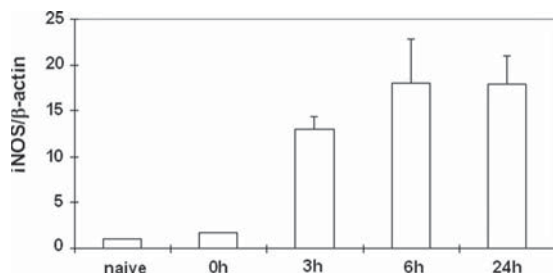


Figure 1. Western blot detection of iNOS in wild-type livers. iNOS expression was virtually absent or mildly expressed in naïve livers and in livers after 90 minutes of warm ischemia. In contrast, iNOS expression was highly detected at protein level in wild-type livers at 3 hours, 6 hours, and 24 hours of I/R injury.

Reduced I/R Injury Response in Livers from iNOS-Deficient Mice

There were no apparent differences either in transaminase levels or in liver histology between naïve iNOS^{-/-} and naïve wild-type mice. We then evaluated the liver injury produced by I/R in iNOS^{-/-} deficient mice; mice were sacrificed at 6 hours and 24 hours after liver I/R injury. iNOS^{-/-} mice showed significantly less liver damage, as evidenced by the reduced serum ALT levels (sALT, U/L: 1368 ± 1240 vs. 18,810 ± 4317; $P < 0.001$; and serum AST (sAST), U/L: 1587 ± 828 vs. 9293 ± 1166; $P < 0.001$, $n = 6/g$) at 6 hours after I/R injury (Figure 2A). A sustained protection was observed in iNOS^{-/-} mice, with sALT (U/L: 242 ± 98 vs. 1488 ± 306; $P < 0.001$, $n = 6/g$), and sAST (U/L: 329 ± 193 vs. 974 ± 193; $P < 0.003$, $n = 6/g$) levels depressed at 24 hours after I/R injury (Figure 2A). Moreover, improvement in liver function in iNOS^{-/-} mice was associated with significantly better histological preservation (Figure 2B). Elevated sinusoidal congestion and extensive areas of necrosis characterized livers from wild-type mice at 6 and 24 hours post-I/R injury, respectively. In contrast, iNOS knockout mice showed only mild signs of vascular changes and necrosis after liver I/R injury.

iNOS Deficiency Profoundly Disrupted Leukocyte Recruitment in Liver I/R Injury

We evaluated the contribution of iNOS expression on leukocyte infiltration in liver I/R injury. Myeloperoxidase activity (U/g), an index of neutrophil infiltration, was profoundly depressed in iNOS deficient livers at 6 hours (1.9 ± 1.5 vs. 13.7 ± 4.3 , $P < 0.004$; $n = 6/g$) and 24 hours (1.9 ± 0.4 vs. 3.6 ± 1.6 , $P < 0.04$; $n = 6/g$) of I/R injury, as compared with respective controls, (Figure 3A). Moreover, the myeloperoxidase activity results were correlated with the number of Ly-6G positive cells, a marker expressed primarily on granulocytes.³⁰ iNOS^{-/-} livers showed significantly lower numbers of Ly-6G neutrophils (2.3 ± 0.6 vs. 19.3 ± 1.5 , $P < 0.001$; $n = 6/g$), particularly at 6 hours after I/R, a time point that coincides with the highest serum transaminase levels, (Figure 3, B and C). Moreover, the numbers of CD3 lymphocytes (6 hours: 4.0 ± 1.0 vs. 8.3 ± 0.6 , $P < 0.003$; $n = 6/g$), CD4 T cells

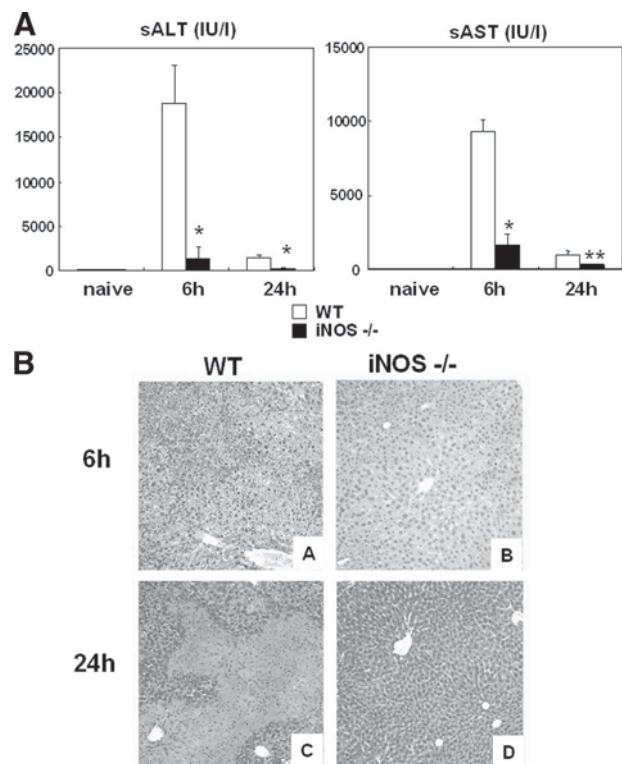


Figure 2. **A:** Liver transaminases and histological preservation in iNOS^{-/-} and wild-type (WT) mice. sALT and sAST levels (IU/L) were measured in the blood samples taken at 6 hours and 24 hours after I/R injury. sALT and sAST levels in the iNOS^{-/-} mice were significantly lower than those in the respective wild-type control littermates at both 6 hours, and 24 hours. Representative H&E staining of livers at 6 hours and 24 hours post-I/R injury. **B:** Control wild-type livers were mostly characterized by elevated sinusoidal congestion at 6 hours (A), and by large necrotic areas at 24 hours (C). In contrast, iNOS^{-/-} livers showed reduced sinusoidal congestion and rather good histological preservation at both 6 hours (B) and 24 hours (D) after liver I/R injury. H&E staining magnification = original $\times 100$; * $P < 0.001$, and ** $P < 0.003$.

(6 hours: 3.3 ± 1.5 vs. 8.0 ± 1.0 , $P < 0.01$; $n = 6/g$), and Mac-1 leukocytes (6 hours: 2.7 ± 0.6 vs. 21.7 ± 3.1 , $P < 0.001$; $n = 6/g$), a mouse macrophage antigen that is abundantly expressed on stimulated macrophages and, in lower amounts, on granulocytes,³¹ were profoundly depressed in iNOS^{-/-} livers as compared with respective wild-type controls after 6 hours of livers I/R injury (Figure 4, A–F). The extent of leukocyte infiltration was highly correlated with the degree of liver function and with the histological preservation observed in the different groups. Moreover, it was also correlated with the expression of pro-inflammatory cytokines (Figure 5). IL-6 expression, which is iNOS dependent in damaged livers and lungs after hemorrhagic shock,³² was profoundly depressed in iNOS^{-/-} livers ($P < 0.005$; $n = 4/g$) at 6 hours after I/R insult. IFN- γ expression, an initiator of liver reperfusion injury,³³ was also depressed in iNOS^{-/-} livers at 6 hours ($P < 0.01$; $n = 4/g$), and 24 hours ($P < 0.01$; $n = 4/g$) of reperfusion. However, the expression of tumor necrosis factor- α , a pro-inflammatory cytokine associated to iNOS-derived NO,³⁴ was up-regulated early (3 hours post-I/R) in both iNOS^{-/-} and wild-type livers after I/R, and it was virtually unchanged in both groups, suggesting that iNOS^{-/-} mice are capable of expressing

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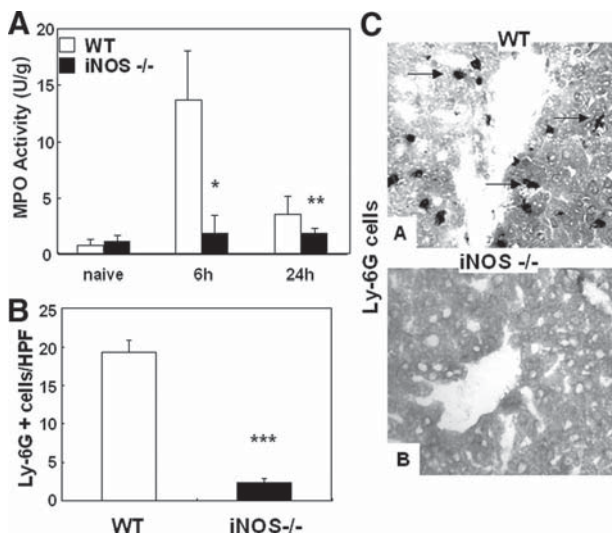


Figure 3. Intrahepatic myeloperoxidase enzyme activity and Ly-6G neutrophil infiltration in iNOS^{-/-} and wild-type (WT) mice. Myeloperoxidase enzymatic activity (A), an index of neutrophil infiltration, was markedly reduced in the iNOS^{-/-} mice at 6 hours and 24 hours of reperfusion following 90 minutes of warm ischemia. In addition, Ly-6G neutrophil infiltration (B) was predominantly detected in wild-type livers at 6 hours after I/R injury, contrasting with very little Ly-6G cell infiltration detected in iNOS^{-/-} livers. Representative immunostaining of Ly-6G neutrophils (C) in wild-type livers (A), and in iNOS^{-/-} livers (B) at 6 hours of I/R injury. Arrows indicate Ly-6G cell labeling in liver specimens. Immunostaining magnification = original $\times 200$; * $P < 0.004$, ** $P < 0.04$, and *** $P < 0.001$.

tumor necrosis factor- α by an iNOS independent pathway. In addition, CXCL-2, a neutrophil chemoattractant,³⁵ was down-regulated in the iNOS^{-/-} livers at both 6 hours ($P < 0.01$; $n = 4/g$), and 24 hours ($P < 0.005$; $n = 4/g$) after I/R injury (Figure 5).

MMP-9+ Leukocytes Were Detected in iNOS-Rich Areas of Damaged Livers

Leukocyte transmigration across endothelial and ECM barriers is a complex process, which is dependent on cell activating chemokines, and matrix degradation mechanisms. We have recently shown that MMP-9 is an important mediator in liver I/R injury.¹⁵ Others have shown that NO is capable of regulating MMP-9 activity in macrophages and neuronal cells^{22,27} *in vitro*. To evaluate whether iNOS and MMP-9 colocalize in damaged livers, we performed series of triple immunofluorescent assays in wild-type livers at 6 hours after I/R, a time point that coincides with high levels of iNOS expression, serum transaminases, and leukocyte infiltration in this experimental model. As shown in Figure 6, A–D, MMP-9+ leukocytes were detected in wild-type livers in the proximity of the vascular endothelium (stained for VCAM-1), either in the lumen of the vessels, before transmigration, or in the damaged liver tissues. Interestingly, MMP-9+ leukocytes were either positive for iNOS or were localized adjacent to iNOS-positive cells (Figure 6D). We have previously identified Mac-1+ macrophages and Ly-6G+ neutrophils as major sources of MMP-9 in this model of liver injury.¹⁵ To evaluate whether these cells were able to express iNOS, we stained wild-type-livers after 6 hours of

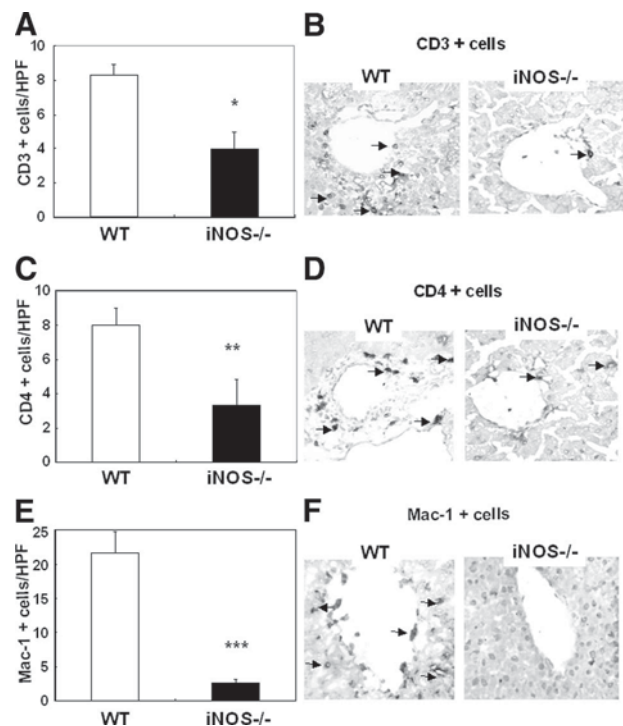


Figure 4. T and Mac-1 leukocyte infiltration in iNOS^{-/-} and wild-type (WT) mice. CD3 (A), CD4 (C), and Mac-1 (E) leukocyte infiltration was significantly reduced in both iNOS^{-/-} livers, as compared with respective controls at 6 hours post-I/R injury. Representative staining for CD3, CD4, and Mac-1 cells is illustrated in panels B, D, and F, respectively. Arrows indicate leukocyte labeling in liver specimens. Immunostaining magnification = original $\times 200$; * $P < 0.03$, ** $P < 0.01$, and *** $P < 0.001$.

I/R insult for simultaneous detection of leukocyte markers, iNOS, and MMP-9. While MMP-9+ Ly-6G+ neutrophils were virtually negative for iNOS (not shown), Mac-1 cells readily stained for both iNOS and MMP-9 (Figure 7, A–D). Therefore, these data show that Mac-1 macrophages co-expressed MMP-9 and iNOS, while other MMP-9+ leukocytes were localized adjacent to iNOS+ cells in damaged wild-type livers after I/R injury.

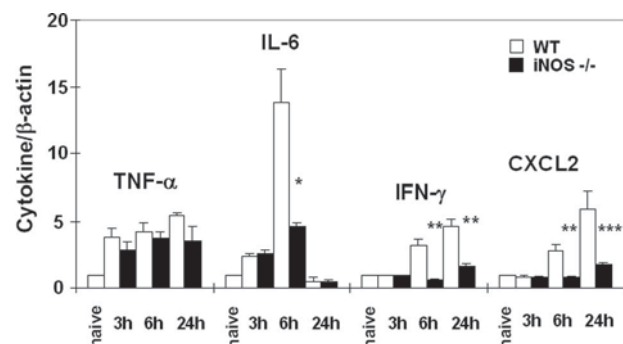


Figure 5. Cytokine and chemokine gene expression in iNOS^{-/-} and wild-type (WT) livers. Cytokine induction ratios were determined at 3 hours, 6 hours, and 24 hours of reperfusion following 90 minutes of warm ischemia. Pro-inflammatory IL-6, IFN- γ , and CXCL-2 expression was profoundly depressed in iNOS deficient livers as compared with respective controls. In contrast, tumor necrosis factor- α was comparably expressed in iNOS^{-/-} and wild-type livers after I/R injury, * $P < 0.006$, ** $P < 0.01$, and *** $P < 0.005$.

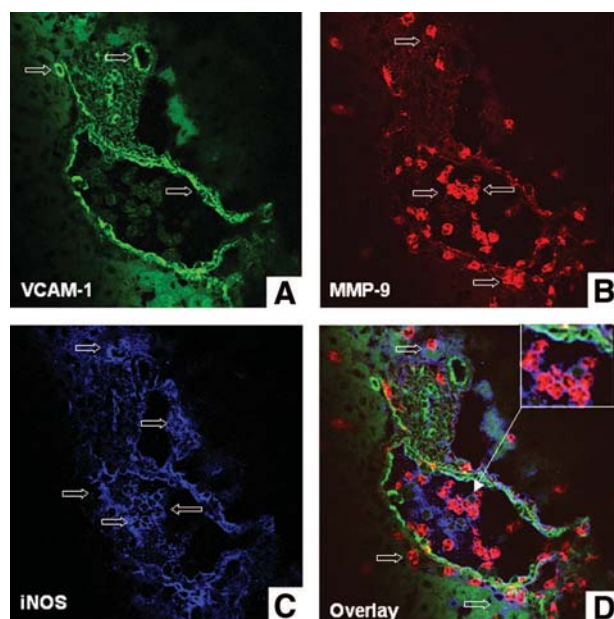


Figure 6. Confocal imaging of VCAM-1, MMP-9, and iNOS in wild-type livers. Triple immunofluorescence labeling of VCAM-1 (A, brilliant green), MMP-9 (B, brilliant red), iNOS (C, blue), and overlay image (D) of A, B, and C in wild-type livers at 6 hours post-I/R injury. MMP-9+ leukocytes were detected in damaged livers neighboring the vascular endothelium (VCAM-1 staining), before and after transmigration. MMP-9+ leukocytes either co-expressed iNOS (magenta) or were detected adjacent to iNOS+ cells (blue) in damaged livers. Open arrows indicate positive labeling; inset in D shows colocalization of MMP-9 and iNOS in leukocytes nearby the endothelium at higher magnification.

iNOS Deficiency Down-Regulated MMP-9 Activity after Liver I/R Injury

Gelatin zymography and a specific MMP-9 enzymatic activity kit were used to assess whether iNOS deficiency affected MMP-9 activity in liver I/R injury. MMP-9 activity, assessed by zymography, was markedly depressed in *iNOS*^{-/-} deficient livers (~sixfold decrease) at 6 hours after I/R injury (Figure 8A). In addition, *iNOS*^{-/-} deficient livers showed a significant decrease in the amount of active MMP-9 ($\mu\text{g/g}$) at both 6 hours (0.042 ± 0.009 vs. 1.289 ± 0.091 , $P < 0.0008$; $n = 6/\text{g}$) and 24 hours (0.098 ± 0.128 vs. 1.225 ± 0.352 , $P < 0.006$; $n = 6/\text{g}$), as compared with wild-type control livers after I/R injury (Figure 8B). The numbers of MMP-9+ leukocytes (6 hours: 3.3 ± 1.5 vs. 35.3 ± 5.1 , $P < 0.001$; $n = 6/\text{g}$) were also profoundly depressed in *iNOS*^{-/-} livers (Figure 8, C and D). Thus, these results show that MMP-9 activity was strongly reduced in the absence of iNOS in liver I/R injury.

ONO-1714-Mediated iNOS Inhibition Down-Regulated MMP-9 Activity and Ameliorated Liver I/R Injury

Knockout mice represent an important research tool; however, they often possess redundant mechanisms. Therefore, we performed additional experiments with ONO-1714, a powerful specific iNOS inhibitor.³⁶ The administration of the iNOS inhibitor to wild-type C56BL6 mice significantly decreased serum NO levels, transam-

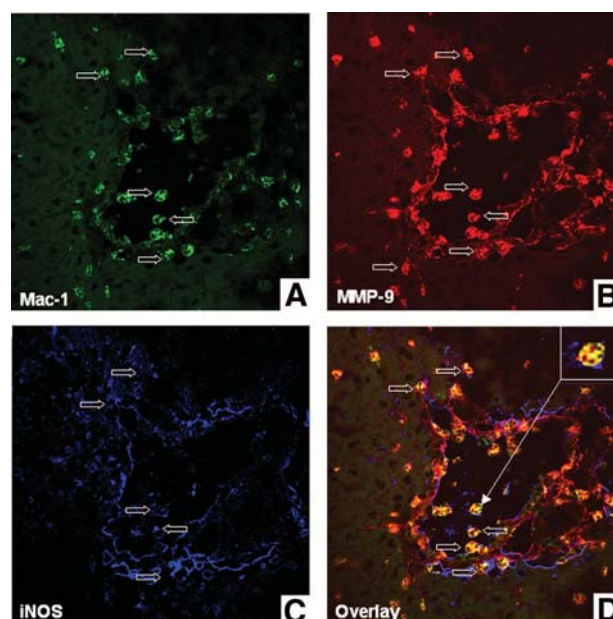


Figure 7. Confocal imaging of Mac-1, MMP-9, and iNOS in wild-type livers. Triple immunofluorescence labeling of Mac-1 (A, brilliant green), MMP-9 (B, brilliant red), iNOS (C, blue), and overlay image (D) of A, B, and C in wild-type livers at 6 hours post-I/R injury. Colocalization of Mac-1, MMP-9, and iNOS was detected in damaged livers. Open arrows indicate positive labeling; inset in D shows iNOS positive staining in MMP-9+ Mac-1 leukocytes at higher magnification.

inase levels (sAST: 3907 ± 1371 vs. $15,400 \pm 2107$ U/L, $P < 0.005$; $n = 5/\text{g}$), reduced liver vascular congestion, and improved liver preservation after 6 hours of I/R insult, (Figure 9, A–C). Moreover, ONO-1714 mediated iNOS inhibition significantly down-regulated MMP-9 activation (~threefold decrease), and profoundly decreased the number of infiltrating MMP-9+ leukocytes (3.2 ± 1.0 vs. 22.4 ± 2.5 , $P < 0.001$; $n = 4/\text{g}$), (Figure 9, D and E). Therefore, these results support our observations in *iNOS*-deficient mice, and are in agreement with previous studies in both pigs¹⁰ and rats,³⁷ which show that iNOS specific inhibition ameliorates liver I/R injury. The results also support the concept that MMP-9 is an important mediator of the effects of iNOS-derived NO in liver I/R injury.

NO Regulated MMP-9 Activity in Isolated Murine Neutrophils

Cultured isolated murine macrophages, in the absence of LPS stimulation, released low NO levels ($<5 \mu\text{mol/L}$). LPS mediated activation of macrophages significantly increased NO release levels (~15 to $25 \mu\text{mol/L}$); however, addition of ONO-1714 to LPS-activated macrophages returned NO release to almost unstimulated values (~4 to $6 \mu\text{mol/L}$), (Figure 10A). Alternatively, fMLP-activated neutrophils showed only a relatively modest increase in NO release levels (5 to $7 \mu\text{mol/L}$), which was not considerably affected by ONO-1714 mediated inhibition (Figure 10B). These results were somewhat correlated with our *in vivo* observations, in which iNOS expression was readily detectable in Mac-1 macrophages and virtually undetect-

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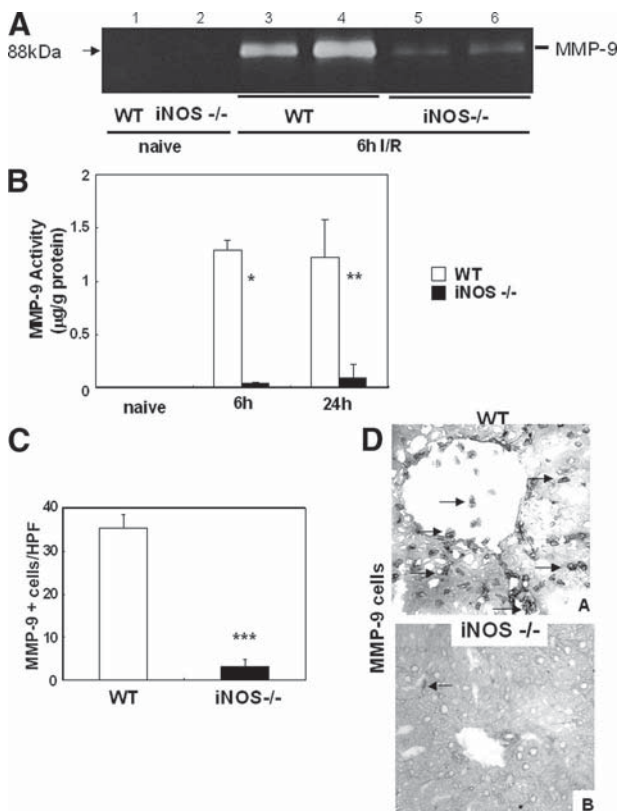


Figure 8. MMP-9 activity in *iNOS*^{-/-} and wild-type (WT) livers. MMP-9 activity detected by zymography (A) was virtually negative in wild-type (lane 1), and in *iNOS*^{-/-} (lane 2) naive livers. It was mildly detected in *iNOS*^{-/-} deficient livers at 6 hours of I/R (lanes 5, and 6) and highly up-regulated in the respective wild-type controls (lanes 3, and 4). Indeed, the amount of active MMP-9 (B) was several-fold decreased in *iNOS*^{-/-} livers as compared with controls at both 6 hours and 24 hours after I/R injury. In addition, MMP-9+ leukocyte infiltration was profoundly reduced in *iNOS*^{-/-} livers as compared with respective wild-type controls at 6 hours post-I/R injury (C). Representative staining for MMP-9 in wild-type livers (A) and in *iNOS*^{-/-} livers (B) is shown in D. Arrows indicate positive labeling in liver specimens. Immunostaining magnification = original $\times 200$; * $P < 0.0008$, ** $P < 0.006$, and *** $P < 0.001$.

able in Ly-6G neutrophils in the damaged wild-type livers after I/R injury. To test whether NO is capable of regulating MMP-9 expression and activity in isolated murine macrophages and neutrophils, we cultured these cells in the presence of a NO-generating agent, DETA-NONOate. We found that DETA-NONOate significantly up-regulated the expression of total MMP-9 protein levels in both macrophages and neutrophils, the latter being the cells that expressed higher levels of this gelatinase (Figure 11, A and B). For example, MMP-9 protein levels in macrophages and neutrophils were approximately 15 ng/ml and 50 ng/ml, respectively, at a DETA-NONOate concentration of 50 $\mu\text{mol/L}$. Moreover, it also up-regulated MMP-9 activity in both cell types (Figure 11, C and D). In macrophages, the higher levels of MMP-9 activity were predominantly detected in cells treated with DETA-NONOate at concentrations of 5 $\mu\text{mol/L}$ and 50 $\mu\text{mol/L}$ (Figure 11C). DETA-NONOate at a concentration of 500 $\mu\text{mol/L}$ seemed less effective in MMP-9 activation by macrophages. These results are supported by data obtained with a macrophage cell line in which NO up-regulates MMP-9 activity in this cell line; however, very

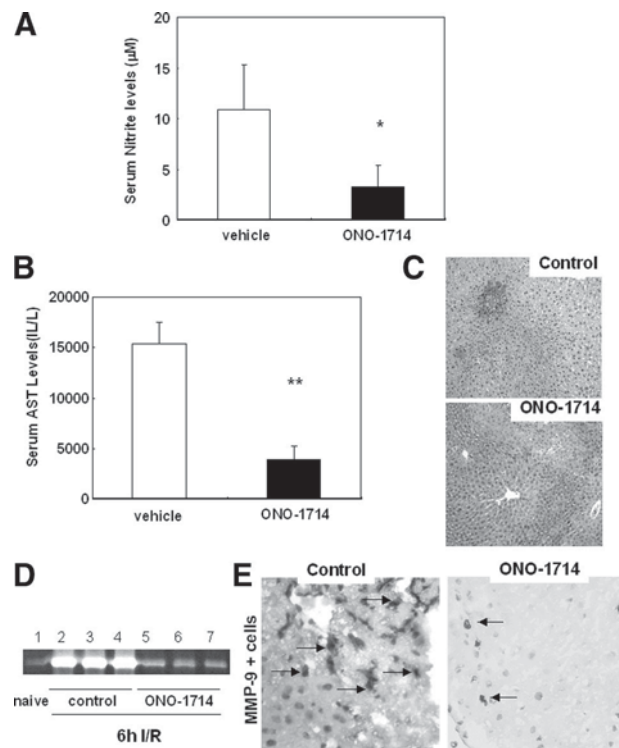


Figure 9. Liver function and MMP-9 activity in ONO-1714 treated livers at 6 hours post-I/R injury. ONO-1714 mediated iNOS selective inhibition in liver I/R injury profoundly depressed serum nitrite (A) and AST levels (B), and reduced sinusoidal congestion (C). Furthermore, amelioration of liver I/R injury by ONO-1714 was accompanied by a profound inhibition of MMP-9 activity (D). MMP-9 activity was highly detected in vehicle treated controls (lanes 2–4), and little expressed in ONO-1714 treated livers (lanes 5–7). In addition, MMP-9+ leukocyte infiltration (E) was depressed in ONO-1714 treated liver at 6 hours post-I/R injury. Arrows indicate positive labeling in liver specimens. H&E staining magnification = original $\times 100$; Immunostaining magnification = original $\times 200$; * $P < 0.05$; and ** $P < 0.005$.

high concentrations of the NO donor are less effective in increasing MMP activity by these cells.²⁷ On the other hand, MMP-9 enzymatic activity in neutrophils was increased at all studied concentrations of DETA-NONOate, with a more substantial increase observed at high concentrations of the NO donor (500 $\mu\text{mol/L}$), (Figure 11D). Therefore, these data provide evidence that NO is capable of regulating MMP-9 activity in neutrophils in addition to macrophages, and support our *in vivo* results of a regulatory function for iNOS-derived NO on activation of MMP-9 in liver I/R injury.

Macrophage-Derived NO Up-Regulated MMP-9 Activation and Promoted Neutrophil Migration across Fibronectin

Neutrophils are considered to be critical mediators in acute liver injury.²⁸ Having in consideration that neutrophils were nearly negative for iNOS in damaged livers and, that fMLP-activated neutrophils were only capable of releasing very low levels of NO, MMP-9 activity induced by iNOS-derived NO in neutrophils is likely mediated by NO produced by neighboring cells. Transwell experiments were performed to test whether macro-

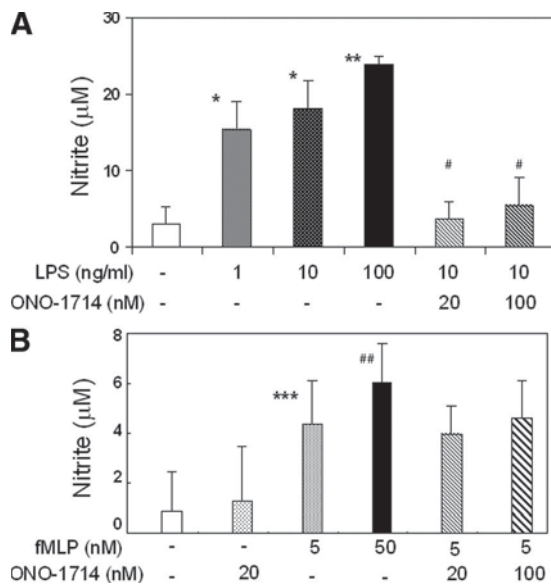


Figure 10. Nitrite levels in isolated murine macrophages and neutrophils. Nitrite levels, expressed as mean $\pm$ SD of three experiments, in macrophages (A) and in neutrophils (B). Nitrite levels in macrophages were significantly increased on LPS stimulation, and addition of ONO-1714 to LPS-activated macrophages returned nitrite release to almost unstimulated values. In contrast, compared with LPS-activated macrophages, release of nitrite by neutrophils was mildly detected on fMLP stimulation, and virtually unchanged on iNOS specific inhibition * $P < 0.001$, ** $P < 0.0001$, *** $P < 0.03$, and # $P < 0.01$, relative to unstimulated cells-white bars; ** $P < 0.001$, relative to stimulated cells-black dotted bars.

phage-derived NO is capable of regulating neutrophil migration across fibronectin, (Figure 12). MMP-9 activity and neutrophil migration across fibronectin-coated transwell membranes were modestly detected in the absence of activated macrophages plated in the lower chambers. In contrast, MMP-9 activity and neutrophil migration were increased by approximately sixfold and threefold, respectively, in the presence of activated macrophages; however, specific iNOS inhibition significantly depressed MMP-9 activity (0.61 ± 0.09 vs. 1.27 ± 0.26 , ng/ml, $P < 0.01$; $n = 4/g$) and neutrophil migration (37.22 ± 2.36 vs. 67.98 ± 11.04 , $P < 0.02$; $n = 4/g$) in a similar fashion to MMP-9 inhibition, (Figure 12, A and B). In fact, MMP-9 activity (0.35 ± 0.05 vs. 1.27 ± 0.26 , ng/ml, $P < 0.01$; $n = 4/g$) and neutrophil migration (32.09 ± 2.09 vs. 67.98 ± 11.04 , $P < 0.02$; $n = 4/g$) were markedly depressed in the MMP-9 inhibitor treated group as compared with controls, (Figure 12, A and B). While iNOS inhibition was highly effective in depressing NO release (3.5 ± 3.1 vs. 33.8 ± 4.4 , μM , $P < 0.003$; $n = 4/g$) and MMP-9 activity/neutrophil migration, MMP-9 inhibition depressed MMP-9 activity and neutrophil migration without disturbing the release of NO (30.4 ± 5.1 vs. 33.8 ± 4.4 , μM ; $n = 4/g$), (Figure 12, B and C), evidencing that MMP-9 is required for NO mediated neutrophil migration. Therefore, these results support our *in vivo* observations in which macrophage NO production, through the induction of iNOS, increases MMP-9 activity and promotes neutrophil migration. Overall, they support the concept that iNOS mediates leukocyte migration in a MMP-9-dependent manner.

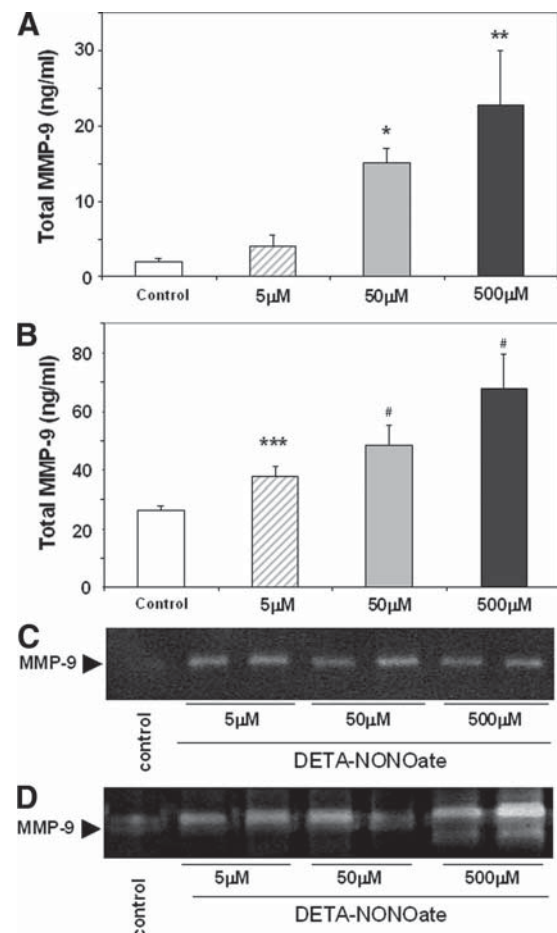


Figure 11. Regulation of MMP-9 expression and activity in isolated murine macrophages and neutrophils. Exposure of macrophages (A and C), and neutrophils (B and D), to exogenous NO increased MMP-9 expression/activity by both cell types with higher expression and activation levels detected in neutrophils. Total MMP-9 protein levels expressed as mean $\pm$ SD of three experiments * $P < 0.003$, ** $P < 0.02$, *** $P < 0.007$, and # $P < 0.01$, relative to controls.

Effects of IFN- γ and IL-6 on MMP-9 Activity in Isolated Murine Neutrophils

The regulation of MMP activity is a complex process and it can be done at transcriptional, post-transcriptional, and at protein levels.³⁸ It is important to consider that in addition to a possible NO-mediated metalloproteinase S-nitrosylation,²² NO may also contribute to MMP-9 activity via induction of cytokine or growth factor expression.³⁸ Indeed, IFN- γ and IL-6 were both found significantly depressed by iNOS deficiency in livers after I/R injury. In an attempt to evaluate whether these pro-inflammatory cytokines are capable of regulating MMP-9 activity, we performed additional experiments in isolated neutrophils. As shown in Figure 13, A–B, IFN- γ (~1.5- to 1.8-fold increase; $n = 3/g$) and IL-6 (~1.3- to 1.7-fold increase; $n = 3/g$) were capable of significantly up-regulating the levels of MMP-9 activity in cultured neutrophils, suggesting that these pro-inflammatory cytokines may contribute to NO-mediated MMP-9 activity in liver I/R injury.

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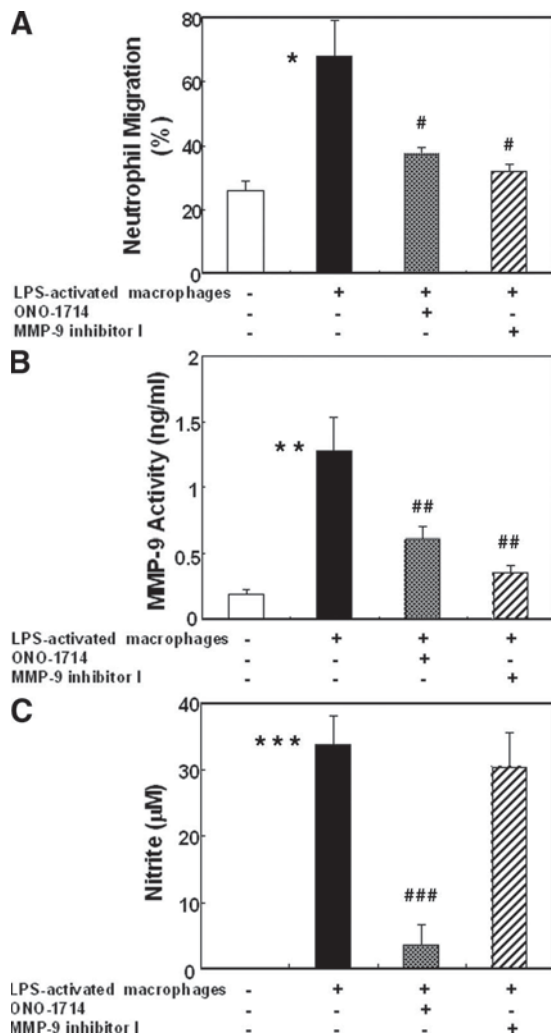


Figure 12. Regulation of neutrophil migration by macrophage NO produced through the induction of iNOS. Migration of neutrophils across fibronectin (A) was markedly increased in the presence of macrophages previously activated with LPS; however, selective iNOS inhibition as well as MMP-9 inhibition significantly reduced neutrophil migration to levels comparable with those observed in the absence of LPS-activated macrophages. MMP-9 activity (B) was profoundly depressed by iNOS and by MMP-9 inhibition. In contrast, nitrite release (C) was clearly reduced on iNOS inhibition, and remained unchanged on selective MMP-9 inhibition, suggesting that NO promoted neutrophil migration through MMP-9 activation, * $P < 0.02$, ** $P < 0.006$, and *** $P < 0.003$, relative to unstimulated controls-white bars; # $P < 0.02$, ## $P < 0.01$, and ### $P < 0.003$, relatively to stimulated controls-back bars.

iNOS^{-/-} Deficiency Decreased Caspase-3 Activity and TUNEL Staining in Liver I/R Injury

NO has been associated to adhesion-related apoptosis.³⁹ MMPs may not only facilitate leukocyte migration, but they may also lead to detachment of liver cells resulting in apoptosis. Activation of caspase-3 causes DNA fragmentation,⁴⁶ and caspase-3 activity is linked to I/R-induced liver apoptosis and damage.^{29,41} NO appears to have the dual capability of increasing⁴² and inhibiting caspase-3 activation.⁴³ Here, we show that caspase-3 activity was decreased in *iNOS^{-/-}* deficient livers at 6 hours (29.9 ± 3.7 vs. 45.8 ± 8.4 U/g, $P < 0.03$; $n = 6$ /g) of reperfusion as compared with the respective wild-type controls, (Figure 14A). Moreover, a decrease in caspase-3

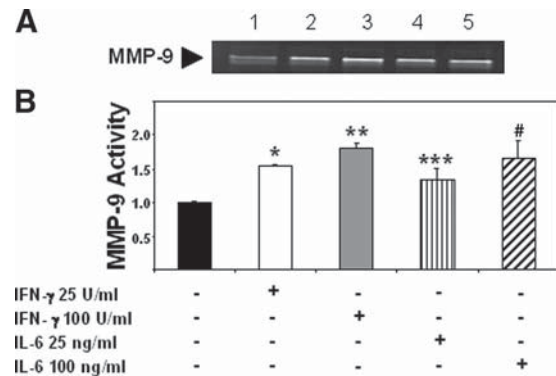


Figure 13. Regulation of MMP-9 activity by IFN- γ and IL-6. Conditioned media obtained from neutrophils stimulated with IFN- γ or IL-6 was subjected to a gelatin zymography assay (A); IFN- γ 25 and 100U/ml (lanes 2 and 3, respectively), and IL-6 25 and 100 ng/ml (lanes 4 and 5, respectively) were capable of increasing MMP-9 activity in cultured neutrophils relative to unstimulated cells (lane 1). Graph (B) represents fold increases in enzymatic activity over unstimulated neutrophils, * $P < 0.0003$, ** $P < 0.0002$, *** $P < 0.05$, and # $P < 0.03$, relative to unstimulated controls.

activity in the *iNOS^{-/-}* livers was accompanied by an approximately twofold increase in Bcl-XL, an important anti-apoptotic factor, and by a significant reduction in TUNEL + cells, with hepatocyte morphology, at 6 hours (4.3 ± 2.1 vs. 73.4 ± 7.7, $P < 0.001$; $n = 6$ /g), as compared with respective controls after I/R injury, (Figure 14, B-E). Indeed, liver TUNEL+ cells were negative for the pan-leukocyte marker CD45 (not shown). We also used MMP-9^{-/-} deficient mice to evaluate a possible contribution of MMP-9 on apoptosis. Indeed, TUNEL+ cells in the MMP-9^{-/-} deficient livers (32.8 ± 2.3 vs. 76.4 ± 6.5, $P < 0.004$; $n = 3$ /g) were detected in significantly fewer numbers as compared with respective controls at 6 hours after I/R injury. These results support the concept that iNOS deficiency is associated with decreased liver apoptosis after I/R injury, and that iNOS-derived NO-induced liver apoptosis may, in part, be mediated by MMP-9.

Discussion

In the present study, we investigated the functional significance of iNOS expression on MMP-9 activation in a well-established 90 minutes mouse model of partial liver warm I/R injury.^{4,15,29,41,44} We show here that MMP-9+ leukocytes either co-expressed iNOS or were detected adjacent to iNOS+ cells in damaged wild-type livers after the I/R insult. iNOS deficient mice showed (a) profound improvement in liver transaminases and in histological outcomes, (b) markedly inhibition of MMP-9 activity, (c) reduced leukocyte infiltration, (d) inhibition of cytokine and chemokine expression, and (e) decreased caspase-3 activity and apoptotic cell labeling after liver I/R injury. Moreover, specific iNOS inhibition with ONO-1714 down-regulated MMP-9 activity and significantly ameliorated liver I/R injury. We also show that activated neutrophils produced relatively negligible levels of iNOS and NO in contrast to activated macrophages, which expressed/released high levels of iNOS and NO; however, exogenous NO up-regulated MMP-9 activity in both leukocyte

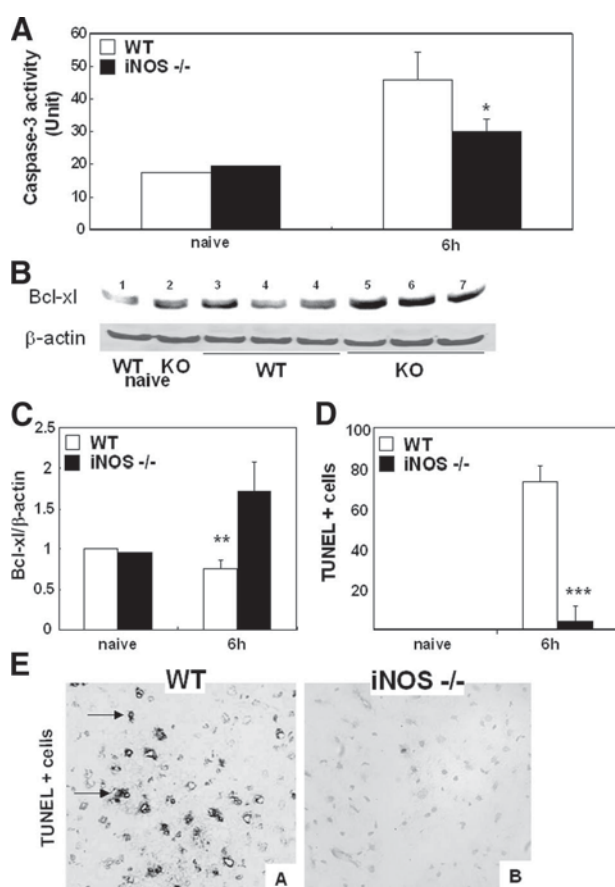


Figure 14. Apoptotic markers and TUNEL staining in iNOS^{-/-} and wild-type (WT) mice. Caspase-3 activity (**A**) was significantly depressed in iNOS^{-/-} livers at 6 hours post-I/R injury, when compared with respective controls. Alternatively, Bcl-XL expression (**B**) was up-regulated in iNOS^{-/-} livers at 6 hours after I/R injury (lanes 5–7), as compared with wild-type controls (lanes 3–4), to wild-type naive (lane 1), and to knockout naive (lane 2) livers. The densitometric ratios of Bcl-XL/β-actin are shown in (**C**). TUNEL+ cells (**D**) were readily detected in wild-type livers, and significantly depressed in iNOS^{-/-} livers at 6 hours of hepatic I/R injury. Representative TUNEL staining (**E**) in wild-type livers (**A**) and iNOS^{-/-} livers (**B**) at 6 hours post-I/R injury. Arrows denote TUNEL+ cells. TUNEL staining magnification = original ×200; **P* < 0.03, ***P* < 0.02, and ****P* < 0.001.

types. Furthermore, macrophage NO production through the induction of iNOS was capable of regulating neutrophil transmigration across fibronectin in a MMP-9-dependent manner.

While it is generally accepted that eNOS is beneficial to liver I/R injury, iNOS has generated more controversy. Our data shows that iNOS is highly expressed in wild-type livers after the I/R insult, and that iNOS-deficient mice, as compared with their wild-type counterparts, were significantly less susceptible to liver I/R reperfusion injury. iNOS-deficient mice showed reduced sALT and sAST levels and significantly improved histological preservation after the I/R insult, which indicates that liver damage was reduced in these mice, as compared with wild-type controls. However, studies performed by others in iNOS-deficient mice using a model of 45-minute partial warm liver ischemia followed by reperfusion, have indicated that iNOS has neither detrimental nor beneficial effects in liver during the acute phase of I/R injury,⁴⁵ or that iNOS deficiency renders these mice more sensitive

to liver damage.⁴⁶ This apparent contradiction may in part be explained by substantial differences between experimental models of liver I/R injury. Indeed, wild-type livers submitted to the 45-minute partial liver ischemia have undetectable iNOS expression after reperfusion, and absence of infiltrating neutrophils,^{45,46} which are critical mediators in inflammatory liver injury.²⁸ Therefore, as previously suggested, results obtained with the of 45-minute partial liver ischemia model may be explained by factors independent of liver iNOS.⁴⁷ Other reports of a protective role for NO in liver I/R injury have been mostly based in studies using non-selective NOS inhibitors, such as *N* ω-nitro-L-arginine methyl ester hydrochloride, which inhibit both iNOS and eNOS.^{48,49} There is a growing body of evidence that the toxic effects of NO vary according to the source of NO, concentration of NO, redox conditions, and the tissue environment.^{11,32,50} Reactive oxygen species/reactive nitrogen species are important mediators of I/R injury, and for example, peroxynitrite, which is a superoxide derivative of NO, has been shown to destroy proteins, lipids, and DNA.¹³ Our observations that lack of iNOS confers a protective role in our model of liver I/R injury are in line with several other studies in models of 60-minute partial liver I/R injury, ConA-induced liver injury, and hemorrhagic shock, in which liver damage is significantly ameliorated in iNOS^{-/-} mice.^{7,32,34,51} Furthermore, they are also supported by our own ONO-1714 studies, in which selective iNOS inhibition ameliorated mouse liver I/R injury, and by other publications showing that iNOS-specific inhibition is beneficial in pig and in rat liver I/R injury.^{10,37}

Infiltrating leukocytes have been implicated as major mediators of I/R injury in several organs, including liver.^{2,4} Infiltration of CD3, CD4, Mac-1, and Ly-6G leukocytes was markedly reduced in the iNOS-deficient livers after I/R injury. CXCL-2, a cytokine-induced neutrophil chemoattractant, was down-regulated in the iNOS^{-/-} livers after I/R, providing an indication that this chemokine may participate in neutrophil activation and recruitment in this model.²⁹ We have previously shown that MMP-9 facilitates leukocyte migration in liver I/R injury.¹⁵ We report here that iNOS deficiency, and ONO-1714-mediated iNOS selective inhibition, profoundly depressed MMP-9 activity and significantly reduced leukocyte recruitment to livers after I/R injury. In contrast to control livers, in which MMP-9+ leukocytes were detected in elevated numbers after I/R injury, iNOS-deficient livers, and ONO-1714 treated livers showed very little MMP-9+ leukocyte infiltration. MMP-9+ leukocytes either co-expressed iNOS or were detected adjacent to iNOS+ cells in damaged wild-type control livers after the I/R insult. Moreover, in addition to mediating MMP-9 activation in isolated macrophages *in vitro*, which is in line with a previous publication using a macrophage cell line,²⁷ we show here that NO is also capable of regulating MMP-9 expression and activity in neutrophils. In our experimental settings, cultured LPS-activated murine macrophages released relatively high levels of NO, which were profoundly depressed on selective iNOS inhibition. In contrast, fMLP-activated neutrophils released almost negligible NO, which was unchanged by iNOS inhibition. Furthermore,

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Mac-1 macrophages expressed both iNOS and MMP-9, while Ly-6G neutrophils expressed MMP-9, but were virtually negative for iNOS in damaged wild-type livers; thus, suggesting that NO-dependent MMP-9 activity in neutrophils may primarily be mediated by NO produced by adjacent cells. Fibronectin is a key ECM protein, which is expressed very early by liver endothelial cells in response to injury,¹⁸ including I/R injury.¹⁹ Interestingly, macrophage NO production through the induction of iNOS was capable of markedly up-regulating MMP-9 activity and significantly promoting neutrophil transmigration across fibronectin. Moreover, the observations that MMP-9 selective inhibition disrupted neutrophil migration, in the presence of high levels of iNOS-derived NO, provide evidence that MMP-9 is required for NO mediated neutrophil migration. Therefore, iNOS-derived NO regulates MMP-9 activity in neutrophils, likely by paracrine mechanisms, and promotes MMP-9-dependent neutrophil migration.

The extracellular matrix proteolysis mediated by metalloproteinases may not only facilitate leukocyte migration, but it may also lead to detachment of liver cells resulting in apoptosis, by a phenomenon called "anoikis."⁵² The molecular mechanisms initiating anoikis are still incompletely understood. In our experimental settings, Bcl-xL, which inhibits apoptosis in response to many cytotoxic insults,⁵³ was up-regulated in the iNOS-deficient livers after I/R injury. Moreover, activation of caspase-3, which triggers apoptosis,³³ and it is linked to liver damage,^{29,41} was significantly reduced in iNOS^{-/-} livers as compared with wild-type controls after I/R injury. Inhibition of caspase-3 activation was accompanied by a markedly reduced number of TUNEL-positive parenchyma cells in iNOS deficient livers after the I/R insult. Moreover, specific iNOS inhibition with ONO-1714 was also associated with a significant decrease in TUNEL-positive cells in the livers after the I/R insult (not shown). There is a growing evidence that NO induces adhesion-related apoptosis/anoikis,³⁹ possibly by NO-mediated MMP activity via metalloproteinase S-nitrosylation,²² and/or via induction of cytokines, or growth factors.³⁸ The regulation of MMP activity is a complex process, and the mechanisms by which NO may regulate MMP-9 activity in liver I/R injury are perhaps multifaceted. For example, we show that IL-6 and IFN- γ , which were found down-regulated by iNOS deficiency in livers after I/R injury, were capable of up-regulating MMP-9 activity in isolated neutrophils. Others have reported that S-nitrosylation mediates activation of MMP-9 causing neuronal cell death/anoikis.²² Thus, it is reasonable to postulate that MMP-9+ leukocytes infiltrating livers after I/R injury can cause parenchyma cell detachment from ECM and, consequently to promote apoptosis/anoikis of these cells, perhaps by a similar mechanism involved in neuronal cell death. Indeed, we have observed that MMP-9-deficient livers showed considerably fewer cells undergoing apoptosis after I/R injury, and others have shown that MMP inhibition with BB-94 leads to significant protection against apoptosis and necrosis of hepatocytes.⁵⁴

In conclusion, our data support the novel view that the pathological functions of iNOS-derived NO are, at least in

part, mediated by MMP-9 in liver I/R injury. As compared with wild-type mice, iNOS deficient mice and mice treated with a selective iNOS inhibitor, showed significantly greater protection against liver I/R injury. This study shows, for the first time, that specifically targeting iNOS-disrupted MMP-9+ leukocyte infiltration in livers after the I/R insult. Furthermore, it also shows that NO was capable of up-regulating MMP-9 expression/activation in neutrophils *in vitro* and that iNOS-derived NO regulated neutrophil transmigration across fibronectin in a MMP-9-dependent manner.

References

- Howard TK, Klintmalm GB, Cofer JB, Husberg BS, Goldstein RM, Gonwa TA: The influence of preservation injury on rejection in the hepatic transplant recipient. *Transplantation* 1990, 49:103–107
- Jaeschke H, Farhood A, Smith CW: Neutrophils contribute to ischemia/reperfusion injury in rat liver *in vivo*. *FASEB J* 1990, 4:3355–3359
- Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ: Kupffer cell activation and endothelial cell damage after storage of rat livers: effects of reperfusion. *Hepatology* 1991, 13:83–95
- Zwacka RM, Zhang Y, Halldorson J, Schlossberg H, Dudus L, Engelhardt JF: CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver. *J Clin Invest* 1997, 100:279–289
- Jessup JM, Battle P, Waller H, Edmiston KH, Stolz DB, Watkins SC, Locker J, Skena K: Reactive nitrogen and oxygen radicals formed during hepatic ischemia-reperfusion kill weakly metastatic colorectal cancer cells. *Cancer Res* 1999, 59:1825–1829
- Marletta MA, Hurshman AR, Rusche KM: Catalysis by nitric oxide synthase. *Curr Opin Chem Biol* 1998, 2:656–663
- Koerber K, Sass G, Kierner AK, Vollmar AM, Tiegs G: In vivo regulation of inducible NO synthase in immune-mediated liver injury in mice. *Hepatology* 2002, 36:1061–1069
- Li J, Billiar TR: Nitric oxide. IV. Determinants of nitric oxide protection and toxicity in liver. *Am J Physiol* 1999, 276:G1069–G1073
- Nathan C: Inducible nitric oxide synthase: what difference does it make? *J Clin Invest* 1997, 100:2417–2423
- Isobe M, Katsuramaki T, Hirata K, Kimura H, Nagayama M, Matsuno T: Beneficial effects of inducible nitric oxide synthase inhibitor on reperfusion injury in the pig liver. *Transplantation* 1999, 68:803–813
- Nathan C, Calingasan N, Nezezon J, Ding A, Lucia MS, La Perle K, Fuortes M, Lin M, Eht S, Kwon NS, Chen J, Vodovotz Y, Kipiani K, Beal MF: Protection from Alzheimer's-like disease in the mouse by genetic ablation of inducible nitric oxide synthase. *J Exp Med* 2005, 202:1163–1169
- Numata M, Suzuki S, Miyazawa N, Miyashita A, Nagashima Y, Inoue S, Kaneko T, Okubo T: Inhibition of inducible nitric oxide synthase prevents LPS-induced acute lung injury in dogs. *J Immunol* 1998, 160:3031–3037
- Ridnour LA, Thomas DD, Mancardi D, Espey MG, Miranda KM, Paolocci N, Feelisch M, Fukuto J, Wink DA: The chemistry of nitrosative stress induced by nitric oxide and reactive nitrogen oxide species. Putting perspective on stressful biological situations. *Biol Chem* 2004, 385:1–10
- Okamoto T, Akuta T, Tamura F, van Der Vliet A, Akaike T: Molecular mechanism for activation and regulation of matrix metalloproteinases during bacterial infections and respiratory inflammation. *Biol Chem* 2004, 385:997–1006
- Hamada T, Fondevila C, Busuttill RW, Coito AJ: Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury. *Hepatology* 2008, 47:186–198
- Lohr KM, Kurth CA, Xie DL, Seyer JM, Homandberg GA: The amino-terminal 29- and 72-Kd fragments of fibronectin mediate selective monocyte recruitment. *Blood* 1990, 76:2117–2124
- Ram M, Sherer Y, Shoenfeld Y: Matrix metalloproteinase-9 and autoimmune diseases. *J Clin Immunol* 2006, 26:299–307
- Jarnagin WR, Rockey DC, Koteliensky VE, Wang SS, Bissell DM: Expression of variant fibronectins in wound healing: cellular source

- and biological activity of the EIIIA segment in rat hepatic fibrogenesis. *J Cell Biol* 1994, 127:2037–2048
19. Amersi F, Shen XD, Moore C, Melinek J, Busuttil RW, Kupiec-Weglinski JW, Coito AJ: Fibronectin-alpha 4 beta 1 integrin-mediated blockade protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *Am J Pathol* 2003, 162:1229–1239
 20. Egeblad M, Werb Z: New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002, 2:161–174
 21. Tchertverikov I, Lard LR, DeGroot J, Verzijl N, TeKoppele JM, Breedveld FC, Huizinga TW, Hanemaaijer R: Matrix metalloproteinases-3, -8, -9 as markers of disease activity and joint damage progression in early rheumatoid arthritis. *Ann Rheum Dis* 2003, 62:1094–1099
 22. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA: S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 2002, 297:1186–1190
 23. Moore C, Shen XD, Gao F, Busuttil RW, Coito AJ: Fibronectin-[alpha]4[beta]1 integrin interactions regulate metalloproteinase-9 expression in steatotic liver ischemia and reperfusion injury. *Am J Pathol* 2007, 170:567–577
 24. Kuyvenhoven JP, Verspaget HW, Gao Q, Ringers J, Smit VT, Lamers CB, van Hoek B: Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. *Transplantation* 2004, 77:1646–1652
 25. Nagase H, Woessner JF Jr: Matrix metalloproteinases. *J Biol Chem* 1999, 274:21491–21494
 26. Visse R, Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003, 92:827–839
 27. Ridnour LA, Windhausen AN, Isenberg JS, Yeung N, Thomas DD, Vitek MP, Roberts DD, Wink DA: Nitric oxide regulates matrix metalloproteinase-9 activity by guanylyl-cyclase-dependent and -independent pathways. *Proc Natl Acad Sci USA* 2007, 104:16898–16903
 28. Jaeschke H, Hasegawa T: Role of neutrophils in acute inflammatory liver injury. *Liver Int* 2006, 26:912–919
 29. Hamada T, Tsuchihashi S, Avanesyan A, Duarte S, Moore C, Busuttil RW, Coito AJ: Cyclooxygenase-2 deficiency enhances th2 immune responses and impairs neutrophil recruitment in hepatic ischemia/reperfusion injury. *J Immunol* 2008, 180:1843–1853
 30. Ellis TN, Beaman BL: Murine polymorphonuclear neutrophils produce interferon-gamma in response to pulmonary infection with *Nocardia asteroides*. *J Leukoc Biol* 2002, 72:373–381
 31. Ho MK, Springer TA: Mac-1 antigen: quantitative expression in macrophage populations and tissues, and immunofluorescent localization in spleen. *J Immunol* 1982, 128:2281–2286
 32. Hierholzer C, Harbrecht B, Menezes JM, Kane J, MacMicking J, Nathan CF, Peitzman AB, Billiar TR, Tweardy DJ: Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. *J Exp Med* 1998, 187:917–928
 33. Lappas CM, Rieger JM, Linden J: A2A adenosine receptor induction inhibits IFN-gamma production in murine CD4+ T cells. *J Immunol* 2005, 174:1073–1080
 34. Sass G, Koerber K, Bang R, Guehring H, Tiegs G: Inducible nitric oxide synthase is critical for immune-mediated liver injury in mice. *J Clin Invest* 2001, 107:439–447
 35. Baggiolini M: Chemokines and leukocyte traffic. *Nature* 1998, 392:565–568
 36. Naito Y, Takagi T, Ichikawa H, Tomatsuri N, Kuroda M, Isozaki Y, Katada K, Uchiyama K, Kokura S, Yoshida N, Okanoue T, Yoshikawa T: A novel potent inhibitor of inducible nitric oxide inhibitor. ONO-1714, reduces intestinal ischemia-reperfusion injury in rats. *Nitric Oxide* 2004, 10:170–177
 37. Tsuchihashi S, Kaldas F, Chida N, Sudo Y, Tamura K, Zhai Y, Qiao B, Busuttil RW, Kupiec-Weglinski JW: FK330, a novel inducible nitric oxide synthase inhibitor, prevents ischemia and reperfusion injury in rat liver transplantation. *Am J Transplant* 2006, 6:2013–2022
 38. Sternlicht MD, Werb Z: How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001, 17:463–516
 39. Monteiro HP, Silva EF, Stern A: Nitric oxide: a potential inducer of adhesion-related apoptosis-anoikis. *Nitric Oxide* 2004, 10:1–10
 40. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S: A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 1998, 391:43–50
 41. Llacuna L, Mari M, Garcia-Ruiz C, Fernandez-Checa JC, Morales A: Critical role of acidic sphingomyelinase in murine hepatic ischemia-reperfusion injury. *Hepatology* 2006, 44:561–572
 42. Tamatani M, Ogawa S, Niitsu Y, Tohyama M: Involvement of Bcl-2 family and caspase-3-like protease in NO-mediated neuronal apoptosis. *J Neurochem* 1998, 71:1588–1596
 43. Kim YM, Talanian RV, Billiar TR: Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J Biol Chem* 1997, 272:31138–31148
 44. Zwacka RM, Zhang Y, Zhou W, Halldorson J, Engelhardt JF: Ischemia/reperfusion injury in the liver of BALB/c mice activates AP-1 and nuclear factor kappaB independently of IkappaB degradation. *Hepatology* 1998, 28:1022–1030
 45. Kawachi S, Hines IN, Laroux FS, Hoffman J, Bharwani S, Gray L, Leffer D, Grisham MB: Nitric oxide synthase and postischemic liver injury. *Biochem Biophys Res Commun* 2000, 276:851–854
 46. Hines IN, Harada H, Bharwani S, Pavlick KP, Hoffman JM, Grisham MB: Enhanced post-ischemic liver injury in iNOS-deficient mice: a cautionary note. *Biochem Biophys Res Commun* 2001, 284:972–976
 47. Hines IN, Kawachi S, Harada H, Pavlick KP, Hoffman JM, Bharwani S, Wolf RE, Grisham MB: Role of nitric oxide in liver ischemia and reperfusion injury. *Mol Cell Biochem* 2002, 234–235:229–237
 48. Wang Y, Mathews WR, Guido DM, Farhood A, Jaeschke H: Inhibition of nitric oxide synthesis aggravates reperfusion injury after hepatic ischemia and endotoxemia. *Shock* 1995, 4:282–288
 49. Cottart CH, Do L, Blanc MC, Vaubourdolle M, Descamps G, Durand D, Galen FX, Clot JP: Hepatoprotective effect of endogenous nitric oxide during ischemia-reperfusion in the rat. *Hepatology* 1999, 29:809–813
 50. Beckman JS, Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996, 271:C1424–C1437
 51. Lee VG, Johnson ML, Baust J, Laubach VE, Watkins SC, Billiar TR: The roles of iNOS in liver ischemia-reperfusion injury. *Shock* 2001, 16:355–360
 52. Frisch SM, Screaton RA: Anoikis mechanisms. *Curr Opin Cell Biol* 2001, 13:555–562
 53. Cory S, Adams JM: The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002, 2:647–656
 54. Wielockx B, Lannoy K, Shapiro SD, Itoh T, Itohara S, Vandekerckhove J, Libert C: Inhibition of matrix metalloproteinases blocks lethal hepatitis and apoptosis induced by tumor necrosis factor and allows safe antitumor therapy. *Nat Med* 2001, 7:1202–1208

CHAPTER VI

FINAL CONSIDERATIONS

Final Considerations

The ECM is a complex composition of secreted proteins and carbohydrates that serves as a structural scaffolding, holding cells and tissues together by interacting with receptors on their membrane surface [1]. The emerging relevance of leukocyte-ECM interactions to cell adhesion and migration emphasizes the role ECM proteins may play in the functioning of the immune system in both physiological and pathological conditions [2]. One of the major components of the ECM is fibronectin (FN), a large glycoprotein that is mainly expressed in 2 isoforms: the constitutively expressed plasma fibronectin (pFN), and inducible cellular fibronectin (cFN) that is normally absent in adult tissue. FN can interact with a wide variety of molecules and regulate many cell functions such as migration, proliferation and differentiation [3]. Consequently, FN has been implicated in many inflammatory pathologies such as psoriasis, rheumatoid arthritis, intestinal inflammation and as shown by us in organ transplantation and in hepatic I/R injury [4-10]. Integrin $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ are the major ligands, expressed on leukocytes, for the CS-1 and RGD domains of fibronectin, respectively [11-20]. We have previously demonstrated that blockade of $\alpha 4 \beta 1$ -FN interactions disrupted leukocyte infiltration and ameliorated steatotic liver IRI in a model of *ex vivo* 4-hour cold ischemia followed by isotransplantation [9, 21]. Our studies in chapters II and III extend these earlier findings on the role of fibronectin in hepatic I/R injury.

In chapter II, we used CS-1 peptide therapy to assess the role of $\alpha 4 \beta 1$ -FN interactions in a well-established model of 24 hours cold ischemia followed by isotransplantation. CS-1 peptides mimic the CS-1 sequence of FN and bind to $\alpha 4 \beta 1$ [4, 22]. In our study, the CS-1 facilitated blockade of FN- $\alpha 4 \beta 1$ interactions significantly depressed the elevated macrophage, neutrophil, T and NK-cell infiltration observed in control animals. Infiltrating leukocytes further amplify the inflammatory response by expressing high levels of pro-inflammatory mediators such as TNF- α , IL-1 β , IFN- γ , iNOS and COX-2 [23]. The CS-1 mediated depressed leukocyte infiltration correlated with a reduced expression of pro-inflammatory mediators. Moreover, reduced leukocyte infiltration resulted in less liver injury and a striking increase in the 14 day-OLT recipient survival rate from 50% in control animals to 100% in the treated animals.

In addition to $\alpha 4 \beta 1$ integrin, leukocytes also express $\alpha 5 \beta 1$ integrin. Expression of this integrin has been identified in T-cells, macrophages and neutrophils [24-26]. $\alpha 5 \beta 1$ integrin binds selectively to the RGD sequence located on the tenth type III repeat of FN [27]. In

chapter III, we dissected the impact of FN- $\alpha 5\beta 1$ interactions on leukocyte recruitment in steatotic liver I/R injury by administration of a cyclic RGD peptide that binds to $\alpha 5\beta 1$ and blocks its interactions with FN [28]. Indeed, treatment with cyclic RGD peptides significantly impaired macrophage and neutrophil infiltration. Moreover, RGD peptide blockade of macrophage and neutrophil infiltration reduced the inflammatory response and led to a significant improvement of liver injury, histology and lean recipient 14 day post-OLT survival rates. Interestingly, RGD peptides were not as effective blocking T lymphocyte infiltration early after transplantation, suggesting that FN- $\alpha 5\beta 1$ interactions may not be essential to T-lymphocyte infiltration during steatotic liver I/R injury.

Altogether these observations show that FN- $\alpha 4\beta 1$ and FN- $\alpha 5\beta 1$ interactions significantly impact liver I/R injury by mediating the crucial step of inflammatory leukocyte recruitment. They confirm that the development of vigorous intrahepatic inflammation is greatly dependent on leukocyte recruitment and infiltration. By blocking leukocytes ability to infiltrate the liver, we were able to hinder an aggravation of hepatic injury, reduce the positive feedback loop of pro-inflammatory mediator production and significantly improve survival. Furthermore, our results confirm a critical role for FN on leukocyte recruitment and subsequent tissue injury during hepatic I/R injury. The liver is a setting where the vasculature is extremely narrow and the blood flow rates are slower than in other organs. Leukocytes migrating through the liver sinusoids, do so with very low velocity making the role of selectin mediated rolling less relevant to the recruitment process [29, 30]. Instead, greater emphasis is likely placed on alternative firm adhesion mechanisms to promote leukocyte recruitment. Interestingly, endothelial cells secretion of matrix components is dynamic and greatly dependent on the state of the cell [31]. Our results demonstrate that during hepatic I/R injury stimulated endothelial cells favor alternative splicing mechanisms that lead to the expression of cFN on their surface to facilitate a stronger leukocyte adhesion to the endothelium. Consequently, our results confirm the role of leukocyte $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins in this mechanism, where by binding with cFN's specific CS-1 and RGD sequences respectively, they form cell matrix firm adhesion complexes that are essential to the cell migratory machinery. This dynamic link established on the vascular endothelium, between an ECM component and leukocyte integrins, allows leukocytes to undergo structural rearrangements that facilitate their directed migration to the sites of inflammation [32]. Therefore, our data contributes to a better understanding on the importance of firm focal adhesion mechanisms during leukocyte recruitment in the inflamed liver after I/R injury. Although $\alpha 5\beta 1$ integrin is highly specific for FN via its interaction with the RGD sequence, $\alpha 4\beta 1$ integrin interacts with the CS-1 sequence on

fibronectin, and with VCAM-1 expressed on the endothelial cell surface. While this suggests that administration of CS-1 peptides might unspecifically interfere with the $\alpha 4\beta 1$ -VCAM-1 interactions in addition to FN- $\alpha 4\beta 1$ interactions, studies have shown that VCAM-1 and CS-1 interact with $\alpha 4\beta 1$ at distinct binding sites [22]. Hence, it is not likely that CS-1 peptide therapy will inhibit the VCAM-1- $\alpha 4\beta 1$ interactions in our studies. This is further supported by a study showing that the concentration of CS-1 peptides able to interfere with VCAM-1- $\alpha 4\beta 1$ interactions is multiple times higher than that required to block FN- $\alpha 4\beta 1$ interactions [33]. Additionally, the $\alpha 4\beta 1$ integrin can also interact with the PEDGIELF sequence on the EIIIA domain of FN [2, 3]. Future studies should address whether the EIIIA domain of FN has a role in leukocyte recruitment and the development of hepatic I/R injury. Moreover, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ are not the only integrins expressed by leukocytes that can interact with FN, and we should aim to better understand the importance of these interactions in hepatic I/R injury.

Leukocyte transmigration across endothelial and extracellular matrix barriers is dependent on adhesive events, as well as on focal matrix degradation mechanisms [29]. Matrix metalloproteinases (MMP), due to their broad ECM breakdown abilities, are increasingly recognized as key regulators of leukocyte migration in inflammatory settings [34]. In Chapter II we observed that CS-1 peptide mediated blockade of FN- $\alpha 4\beta 1$ interactions profoundly depressed the MMP-9 expression and activity levels observed in livers post hepatic I/R injury. Additionally, infiltrating leukocytes were identified as the major sources of MMP-9 expression in control livers and, MMP-9+ leukocyte infiltration was significantly reduced in CS-1 treated livers. Moreover, cyclic RGD blockade of FN- $\alpha 5\beta 1$ interactions (Chapter III) also reduced MMP-9+ leukocyte infiltration post transplantation. The results are in line with previous studies from our group showing that FN- $\alpha 4\beta 1$ interactions up-regulate the expression of MMP-9 in steatotic liver I/R injury [21]. Furthermore, our observation is in conformity with multiple *in vitro* studies showing that leukocyte adhesion to FN can induce MMP-9 expression [16, 21, 35, 36]. Hence, our results demonstrate that FN- $\alpha 4\beta 1$ and FN- $\alpha 5\beta 1$ interactions regulate leukocyte MMP-9 expression during hepatic I/R injury. Given MMP-9's ability to facilitate inflammatory leukocyte trafficking through the ECM, our data is consistent with MMP-9 leukocyte mediated transmigration during hepatic I/R injury, as we have previously shown using MMP-9^{-/-} mice [37]. Leukocyte infiltration was strikingly depressed in MMP-9^{-/-} mice, which led to a marked improvement of hepatic I/R injury [37]. These results contribute to a growing body of evidence that support an important active role for MMP-9 in inflammatory pathologies [38-43].

Anchored to the cell membrane, MT1-MMP has the ability to modulate focal adhesion and promote cell migration and invasion by engaging in pericellular proteolysis of various cell adhesion molecules, including FN [44-46]. In chapter II we show for the first time the expression of MT1-MMP in damaged livers post-IRI. We identify macrophages/monocytes as the sources of MT1-MMP during hepatic I/R injury. Furthermore, we demonstrate that leukocyte expression of MT1-MMP is depressed by CS-1 peptide therapy, suggesting that FN- $\alpha 4\beta 1$ integrin interactions can influence MT1-MMP expression. In support of these results, several in vitro studies have shown that monocytes and T-cells express MT1-MMP when interacting with FN and that its expression mediates their migration on FN and stimulated endothelial cells [15, 36]. These observations suggest that MT1-MMP may amplify leukocyte recruitment in hepatic I/R injury. Furthermore, MT1-MMP has also been implicated in inflammatory pathologies like rheumatoid arthritis, experimental allergic encephalomyelitis and especially in heart and brain I/R injury [47-54]. Despite these strong indications that MT1-MMP contributes to leukocyte migration during hepatic I/R injury, further research into the role of MT1-MMP is necessary. To date, MT1-MMP is the only MMP for which there is no viable KO mouse strain available for studies. Despite being viable at birth, MMP-14^{-/-} mice have an extremely high postnatal death rate as a result of multiple problems that they develop from ablation of the collagenolytic activity [55]. Thus, for futures studies involving MT1-MMP, siRNA or neutralizing antibodies may be useful alternatives. Additionally, a conditional KO mouse strain with myeloid specific MT1-MMP deletion occurring under the control of the LysM promoter has the potential to provide some interesting insight into the specific role of macrophage MT1-MMP expression.

Integrins are not only ligands on the cell surface promoting cell adhesion. Additionally, they are also highly specialized cell signaling machines, that have the ability to relay information from the outside to the inside of the cell, influencing important cell processes such as survival, migration, differentiation, and proliferation [27, 56]. A key part of the cell matrix adhesion complexes established during leukocyte recruitment, integrin generated signaling cascades transfer informational inputs from the ECM that will influence the outcome of the cell migration process. Therefore, in chapter II, we attempted to dissect the cell signaling mechanisms that mediate FN- $\alpha 4\beta 1$ induced MMP-9/MT1-MMP expression in infiltrating leukocytes. The p38 MAPK signaling transduction pathway has been shown to participate in integrin signaling during leukocyte activation and recruitment as well as in the activation of inflammatory processes in hepatic ischemia reperfusion injury [57-60]. Indeed, in our study the activation of the p38 MAPK signaling pathway was

depressed by CS-1 peptides in association to the decreased leukocyte migration. Moreover, we showed in vitro that the up-regulation of MMP-9 and MT1-MMP in macrophages adhering to FN was abrogated by the inhibition of p38 MAPK signaling with the specific inhibitor SB203580. Hence, these results suggest that interactions between cFN and leukocyte integrins may stimulate macrophage MMP-9 and MT1-MMP expression via the p38 MAPK signaling pathway. They provide a mechanism by which the novel environment encountered by leukocytes upon recruitment and adhesion to the endothelium can significantly influence the immediate fate of the inflammatory leukocyte. However, further research is required to confirm p38 as the major transduction pathway leading to MMP expression for FN- $\alpha 4\beta 1$ interactions. Moreover, it is essential to better identify and dissect the role of intracellular adaptor and signaling proteins normally associated to focal adhesion and integrin signaling.

Increased expression of MMPs is considered to be a feature of several inflammatory pathologies [34]. MMPs contribute to the inflammatory process by regulating ECM degradation and rearrangement in the physical barriers through which leukocytes must migrate as well as processing inflammatory chemokines to establish gradients that guide that same migration [34]. The uncontrolled expression of many MMPs can likely cause the persistence of inflammation and consequently the aggravation of serious injury resulting from this process. Moreover, MMPs such as MMP-9 can contribute to cell death by degrading the ECM that supports parenchymal cells in the tissue, a process termed “anoikis” [61, 62]. In hepatic I/R injury, MMP-9 is now well established as a critical mediator leukocytes infiltration and injury [21, 37, 42]. Hence, unregulated and excessive MMP activity, contributes to excessive ongoing cell death, tissue injury, and organ dysfunction. However, MMPs are also essential to our defense and are key mediators of tissue repair through the inflammatory process. Therefore, it is essential that MMP's be subject to a tight spatiotemporal regulation and that their role in inflammation be balanced. The endogenous regulation of leukocyte MMP expression and activity occurs at various levels such as the transcriptional, post-transcriptional, protein synthesis, zymogen secretion and proMMP activation[44][63]. Activation of the proMMP requires the removal of the propeptide by dissociation of the cysteine from the Zn^{2+} ion [64]. This mechanism of freeing the active site is designated the “cysteine-switch” and it can be achieved either by proteolytic cleavage of the propeptide or by a redox reaction between the thiol cysteine group of the propetide and ROS or RNS[65-68]. Another extremely important level of MMP regulation is the inhibition of its activity by endogenously expressed proteins that form specific 1:1 stoichiometric complexes with MMPs and interfere with their access to the substrate [69]. This family of endogenous MMP inhibitors is known as the tissue

inhibitors of metalloproteinases (TIMP) and they to play an essential role the outcome of inflammation [69].

TIMP-1 is an inhibitor of several MMPs and is a particularly potent inhibitor of MMP-9 [70]. Due to the MMP-9's critical role in hepatic I/R injury, TIMP-1 is of special interest to us. TIMP-1 has been implicated as a key regulator of inflammation in several pathological settings like bleomycin induced acute lung injury, experimental autoimmune encephalomyelitis, and focal cerebral ischemia [70][71]. While TIMP-1 has been detected in liver transplant recipients and in steatotic rat liver I/R injury, very little is known about the role of TIMP-1 in hepatic I/R injury [21, 72, 73]. Therefore, in chapter IV we studied the functional significance of TIMP-1 in a well-established mouse model of 90 min partial liver warm IRI. The absence of TIMP-1 led to an amplification of MMP-9 expression and activity at 6 hours and especially 48 hours after I/R injury. Associated to the increased MMP-9 activity, the absence of TIMP-1 led to massive neutrophil and macrophage infiltration in periportal areas, which, as expected, correlated with severe liver damage after I/R injury. Consequently, TIMP-1 deficient mice were unable to recover from the severe injury and evidenced a considerably high 7-day mortality rate. While all wild-type mice survived up to 7 days after I/R injury, only 3 out of 8 TIMP-1^{-/-} mice survived during this period. Interestingly, after 7 days of I/R injury, MMP-9 activity leukocyte infiltration and tissue injury remained elevated in the absence of TIMP-1, while in WT mice, liver injury, leukocyte infiltration and MMP-9 activity were minimal. These findings provide strong support to the view that TIMP-1 has an important hepatoprotective role in hepatic I/R injury and demonstrate the significance of this level of MMP regulation in the overall dynamics of MMPs in the inflammatory process. Moreover, they evidence a critical role for TIMP-1 in the recruitment of leukocytes to damaged livers, which is likely mediated by regulation of MMP-9 expression/activation. As a response to the increased presence of MMP-9, the liver intensifies the production of TIMP-1 in an attempt to control leukocyte infiltration and the consequent damage to the organ.

Interestingly, despite the significant differences observed at all other time-points in the study, TIMP-1 deficient animals and wild-type controls evidenced similar levels of injury, leukocyte infiltration, and MMP-9 activity after 24 hours of I/R injury. Though puzzling, it is possible that these results may be explained by an inactivation and degradation of TIMP-1 protein that occurs in wild-type animals, abrogating the hepatoprotective effect of TIMP-1 and rendering them similar to animals deficient in TIMP-1 at 24 hours after I/R injury. In this regard, it has been suggested that one of the many targets of the ROS produced by infiltrating leukocytes are the endogenously expressed anti-proteases [74]. Since hepatic

I/R injury is characterized by massive leukocyte infiltration, it is possible that the ROS mediated oxidation and deactivation of TIMP-1 may protect MMP-9 from inhibition in the leukocytes immediate microenvironment and enhance leukocyte migration. In fact, a study by Brown and colleagues has shown that peroxynitrate and nitric oxide, both abundantly present in liver I/R injury, can oxidize TIMP-1 and consequently increase MMP activity [75]. The same effect on TIMP-1 has been observed for HOCl derived from neutrophil myeloperoxidase (MPO) activity [76]. Therefore, in future studies it would be interesting to verify, whether TIMP-1 protein is in fact oxidized and its integrity compromised throughout the development of hepatic I/R injury. If confirmed, this would provide yet another level of intricate regulation influencing the delicate balance between MMPs and TIMPs during hepatic I/R injury.

Associated to their high mortality rate, TIMP-1 deficient mice exhibited a high degree of both necrotic and apoptotic cell death. TIMP-1 deficiency led to elevated hepatocyte levels of cleaved caspase-3 and a virtual absence of anti-apoptotic Bcl-2 and pro-survival phosphorylated AKT when compared to liver of wild-type mice in hepatic I/R injury. These results together with the expression of TIMP-1 in the surviving hepatic parenchyma of wild-type mice, suggested a potential role for TIMP-1 in conferring resistance to cell death during liver I/R injury. In support of this notion, TIMP-1 has been identified as capable of promoting the survival of a wide variety of cells including, lymphoma cells, b-cells and breast epithelial cells independently from its role inhibiting MMPs [77]. However, it may also be possible that a more intense MMP mediated ECM breakdown and loss of cellular anchorage is affecting cell survival signaling and leading to "Anoikis". However, our data is far from conclusive on this subject and in the near future we intend to extend our research to an in vitro analysis of the role of TIMP-1 in hepatocyte survival.

The liver has a remarkable ability to regenerate after injury or loss of cellular mass [78]. Upon the onset of certain signals, hepatocytes, which are normally quiescent in the adult liver, undergo a process of cell division and proliferation that substitutes the injured and dead hepatocytes and repopulates the liver [79, 80]. Liver regeneration is an extremely intricate and tightly regulated process that is dependent on the outcome of multiple different cellular processes such as angiogenesis, inflammation, and metabolism [81]. After hepatic injury resulting from the 90 min warm hepatic I/R injury model, wild-type mice undergo a process of recovery and regeneration until complete recovery. Therefore, in addition to the overwhelming hepatocyte cell death observed in TIMP-1 deficient animals, the inability of hepatocytes to undergo cell division and proliferate could explain the acute liver failure and consequent animal death observed in TIMP-1 deficient mice. Additionally,

there is a rapidly growing body of evidence that TIMP-1 has the ability to promote or inhibit cell proliferation. Thus we decided to evaluate the regenerative abilities of TIMP-1^{-/-} livers after hepatic I/R injury. In chapter IV we show that all hepatocyte cell cycle and regenerative markers evaluated (Cyclin D and E, BrdU incorporation and PCNA) were significantly impaired in the livers of TIMP-1 deficient mice, suggesting that the death of these animals is the result of their inability to regenerate after hepatic I/R injury. It is well established that the hepatocyte growth factor (HGF)/c-met signaling pathway is required for liver to efficiently regenerate and repair [82, 83]. In this regard, some studies have shown that TIMP-1 can have a pro-proliferative effect on hepatocytes by inhibiting the proteolytic shedding of c-met from the cell membrane and maintaining its signaling cascade active [84]. On the other hand, one study has reported that TIMP-1 deficiency facilitates an increased ADAM-dependent activation of HGF and consequently an accelerated regeneration after 70% partial hepatectomy [85]. Thus, we evaluated HGF expression and the integrity of c-met in livers of TIMP-1 deficient mice. Indeed, while HGF expression was unaltered in both the presence and absence of TIMP-1, c-met proteolytic shedding was markedly enhanced in TIMP-1 deficient livers, suggesting that in the absence of TIMP-1 there may be an MMP-dependent degradation of c-met that renders livers incapable of adequately regenerating and more susceptible to acute liver injury. Hence, this provides us with potential novel role for TIMP-1 in hepatic I/R injury in addition to its regulation of MMP dependent leukocyte recruitment and infiltration. Moreover, these results were obtained in a distinctive model to those normally used in the study of hepatic regeneration and are therefore, a valuable contribution to a better understanding on the role of proteases and their endogenous inhibitors in the hepatic regenerative process. Nevertheless, further research efforts are necessary to confirm this mechanism and identify if this is an MMP-9 dependent effect, if there are other molecules involved in the process, or if TIMP-1 has the ability to stimulate hepatocyte proliferation by binding to a cell receptor and initiating a signaling cascade.

Altogether the results in Chapter IV support the notion that TIMP-1's ability to tightly regulate MMP provides it with a critical hepatoprotective role in hepatic I/R injury. They confirm that in the absence of regulation, the uncontrolled activity of MMPs, specifically MMP-9, can lead to massive leukocyte infiltration, exacerbated liver injury, impairment of hepatic recovery and regeneration and an increased susceptibility to acute liver I/R injury that ultimately leads to organ failure and death.

To further understand the multiple levels of MMP regulation during hepatic I/R injury, in chapter V, we evaluated whether iNOS expression can regulate the activation of MMP-9 in liver I/R injury. Therefore iNOS deficient mice and mice treated with specific iNOS inhibitors were used in the well-established model of 70% partial warm liver I/R injury. iNOS has been implicated in multiple inflammatory settings where it is known as a mediator of cellular injury. We and others have shown that iNOS expression correlates with the degree of hepatic I/R injury. The up-regulation of iNOS expression in hepatic I/R injury can lead to an excessive production of NO, which can react with several ROS and generate RNS that further contribute to cellular injury [86]. Moreover, several recent studies have suggested that NO can contribute to MMP activation by peptide nitrosilation and oxidation [68, 87]. In our study we observed that both iNOS gene knock down and iNOS chemical inhibition were protective to livers subject to hepatic I/R injury. Associated to liver protection, iNOS deficiency profoundly disrupted leukocyte recruitment in hepatic I/R injury. In WT mice with elevated levels of leukocyte infiltration, macrophages expressed both MMP-9 and iNOS but neutrophils expressed solely MMP-9. However, MMP-9⁺ neutrophils located to areas with elevated iNOS expression. In addition, iNOS deficiency and iNOS inhibition down-regulated MMP-9 activity in hepatic I/R injury. Together these results suggest that iNOS may indeed influence MMP-9 activity. Hence, we dissected the functional significance of this mechanism in a series of *in vitro* studies. These studies showed that neutrophil and macrophage derived MMP-9 activity, as detected by zymography, was significantly up-regulated when cells were treated with various concentrations of NO donors. Stimulated macrophages expressing iNOS were able to induce MMP-9 mediated neutrophil transmigration across a FN coated membrane. This migration was abrogated by pharmacological MMP-9 activity inhibition, providing evidence of the specificity of the iNOS mediated promotion of cell migration via the NO, or derived RNS, activation of MMP-9.

An important aspect that will be interesting to research further in the near future is the effect of iNOS expression, and production of NO, on the integrity of TIMP-1, MMP-9's major endogenous inhibitor. Interestingly, in chapter IV we observed that, in contrast to all other evaluated time-points, at 24 hours post-reperfusion, wild-type mice presented the same levels of MMP-9 activity, leukocyte infiltration and liver injury as animals deficient in TIMP-1. In the discussion above we hypothesized that this observation might be the result of an oxidative degradation of TIMP-1 by ROS and RNS produced by the infiltrating leukocytes. Indeed in figure 1 of chapter V we confirm that iNOS expression is at its highest point in the period from 6 to 24 hours after reperfusion. Multiple studies in recent years have suggested that leukocyte produced ROS aid leukocyte migration via the

oxidative degradation of the endogenously expressed anti-proteases [74]. Moreover, studies have pointed to the specific inactivation of TIMP-1 by peroxynitrate and other elements of the nitric oxide pathway [75, 88]. Therefore, it is possible that an elevated iNOS expression may promote MMP-9 activity by mediating both the removal of the pro-peptide, as shown here, and the degradation of its inhibitor TIMP-1.

The study in chapter V provides us with adequate evidence for iNOS mediated MMP-9 activity in inflammation, via the production of nitric oxide. It provides us with further evidence on the complexity of MMP regulation during the inflammatory response in hepatic I/R injury. Finally, it also provides strong evidence for the deleterious role that excessive iNOS expression and NO production has in pathogenesis of hepatic I/R injury.

In conclusion, the studies performed in this thesis focus on the mechanisms of leukocyte transmigration through the vasculature and ECM barriers during hepatic I/R injury. Inflammatory leukocyte infiltration and leukocyte-mediated hepatocellular injury are major features of hepatic I/R injury [29]. However, our current understanding of the mechanisms that guide leukocyte migration in hepatic I/R injury is insufficient and remains a major challenge for the development of novel and targeted therapeutic strategies. Our studies support an important role for FN expression on the hepatic vasculature in the early moments after I/R injury. We demonstrate that FN- α 4 β 1 interactions promote leukocyte infiltration by up-regulating the expression of MMP-9 and MMP14, via the p38 MAPK signaling pathway. Moreover, the increased expression of these two key MMPs is associated to an increase of liver injury and a decline in liver function and graft recipient survival. We also show that FN- α 5 β 1 interactions up-regulate MMP-9 expression and subsequently promote leukocyte infiltration and tissue injury. A tight regulation of MMP activity is essential to avoid excessive injurious effects on the tissue during an inflammatory response. In this thesis we establish that MMP-9's major endogenous inhibitor, TIMP-1, plays a critical role in regulating MMP-9 activity, leukocyte infiltration and liver injury during hepatic I/R injury. Moreover, we demonstrate for the first time that TIMP-1 deficiency leads to lethal hepatic I/R injury by rendering livers unable to regenerate and recover from hepatic I/R injury, suggesting that TIMP-1 is vital for hepatocyte survival and proliferation. Finally, our studies verify that the up-regulation of pro-inflammatory mediator iNOS in liver I/R injury can promote the NO mediated activation of leukocyte expressed MMP-9, providing an additional regulation mechanism for MMP-9 mediated leukocyte infiltration. Altogether these results provide us with novel mechanistic insights on leukocyte transmigration in hepatic I/R injury.

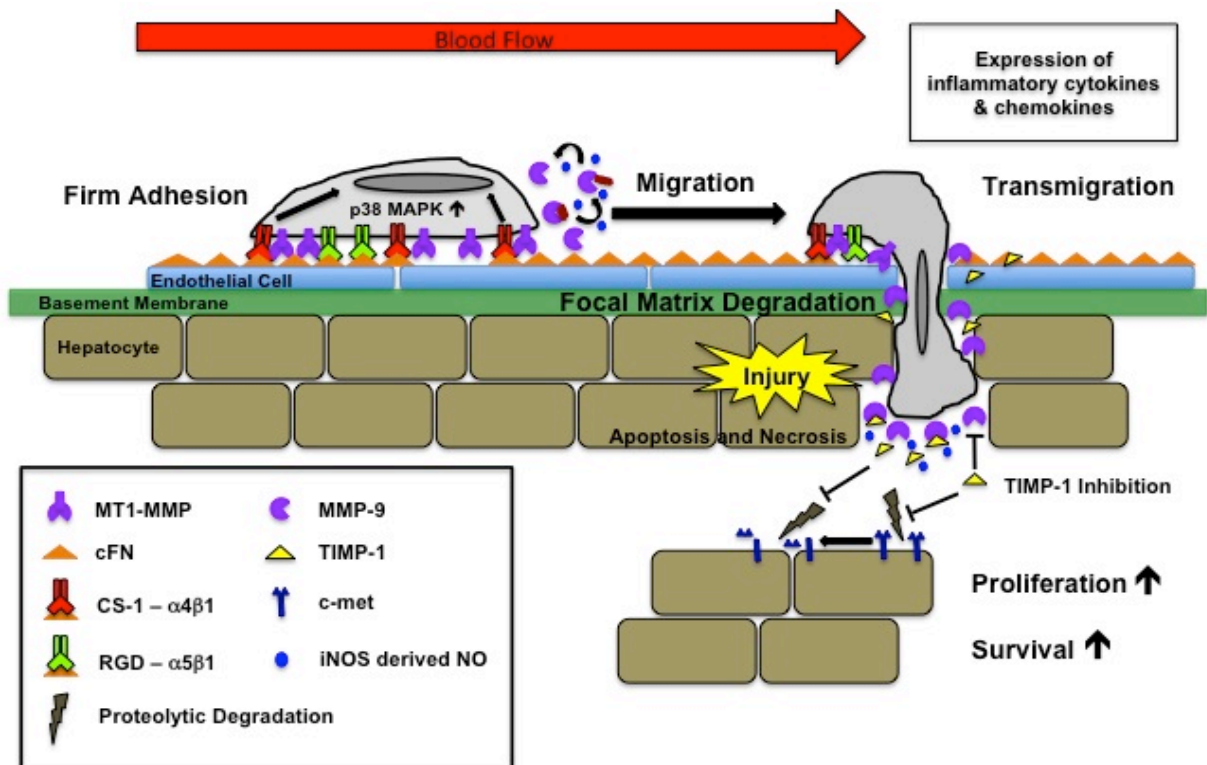


Figure 1 - Upon the onset of I/R injury, endothelial cells begin to express cellular fibronectin, which promotes leukocyte firm adhesion via the interactions between leukocyte activated $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins and the CS-1 and RGD sequences of cellular fibronectin, respectively. FN-leukocyte interactions, via its integrin receptors $\alpha 4\beta 1$ and $\alpha 5\beta 1$, up-regulate MMP-9 and MT1-MMP expression, which have the ability to promote focal matrix degradation events. Activated MMP-9 facilitates leukocyte migration and transmigration through the endothelium and matrix barriers to sites of inflammatory injury. In the process, infiltrating leukocytes mediate further injury to the hepatic parenchyma, promoting hepatocyte apoptosis and necrosis. MMP-9 activity can be upregulated by the increased presence of iNOS-derived nitric oxide (NO), produced during the acute phase of I/R injury. In response to the increased leukocyte expression of MMP-9, the endogenous expression of TIMP-1 is increased. TIMP-1 is a highly specific inhibitor of MMP-9 activity and thus dampens MMP-9 promoted leukocyte infiltration to the liver and stimulates hepatocyte survival. Moreover, TIMP-1 inhibits the proteolytic degradation of c-met (hepatocyte growth factor receptor) from the cell membrane, which may contribute to hepatocyte proliferation and facilitate liver regeneration and recovery after hepatic I/R injury.

REFERENCES

1. Morwood, S.R. and L.B. Nicholson, 2006. *Modulation of the immune response by extracellular matrix proteins*. Arch Immunol Ther Exp (Warsz). **54**(6): p. 367-74.
2. Coito, A.J. and J.W. Kupiec-Weglinski, 2000. *Extracellular matrix proteins in organ transplantation*. Transplantation. **69**(12): p. 2465-73.
3. Coito, A.J., M. de Sousa, and J.W. Kupiec-Weglinski, 2000. *Fibronectin in immune responses in organ transplant recipients*. Dev Immunol. **7**(2-4): p. 239-48.
4. Elices, M.J., et al., 1994. *Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature*. J Clin Invest. **93**(1): p. 405-16.
5. McFadden, J.P., et al. *Psoriasis and extra domain A fibronectin loops*. Br J Dermatol. **163**(1): p. 5-11.
6. Coito, A.J., et al., 1997. *Expression of fibronectin splicing variants in organ transplantation: a differential pattern between rat cardiac allografts and isografts*. Am J Pathol. **150**(5): p. 1757-72.
7. Coito, A.J., et al., 1994. *The expression of extracellular matrix proteins during accelerated rejection of cardiac allografts in sensitized rats*. Transplantation. **57**(4): p. 599-605.
8. Jarnagin, W.R., et al., 1994. *Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis*. J Cell Biol. **127**(6 Pt 2): p. 2037-48.
9. Amersi, F., et al., 2003. *Fibronectin-alpha 4 beta 1 integrin-mediated blockade protects genetically fat Zucker rat livers from ischemia/reperfusion injury*. Am J Pathol. **162**(4): p. 1229-39.
10. Kolachala, V.L., et al., 2007. *Epithelial-derived fibronectin expression, signaling, and function in intestinal inflammation*. J Biol Chem. **282**(45): p. 32965-73.
11. Barczyk, M., S. Carracedo, and D. Gullberg. *Integrins*. Cell Tissue Res. **339**(1): p. 269-80.
12. Liao, Y.F., et al., 2002. *The EIIIA segment of fibronectin is a ligand for integrins alpha 9beta 1 and alpha 4beta 1 providing a novel mechanism for regulating cell adhesion by alternative splicing*. J Biol Chem. **277**(17): p. 14467-74.
13. Guan, J.L. and R.O. Hynes, 1990. *Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1*. Cell. **60**(1): p. 53-61.
14. Huo, Y., A. Hafezi-Moghadam, and K. Ley, 2000. *Role of vascular cell adhesion molecule-1 and fibronectin connecting segment-1 in monocyte rolling and adhesion on early atherosclerotic lesions*. Circ Res. **87**(2): p. 153-9.
15. Matias-Roman, S., et al., 2005. *Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium*. Blood. **105**(10): p. 3956-64.
16. Yakubenko, V.P., et al., 2000. *Differential induction of gelatinase B (MMP-9) and gelatinase A (MMP-2) in T lymphocytes upon alpha(4)beta(1)-mediated adhesion to VCAM-1 and the CS-1 peptide of fibronectin*. Exp Cell Res. **260**(1): p. 73-84.
17. Lehnert, K., et al., 1998. *MAdCAM-1 costimulates T cell proliferation exclusively through integrin alpha4beta7, whereas VCAM-1 and CS-1 peptide use alpha4beta1: evidence for "remote" costimulation and induction of hyperresponsiveness to B7 molecules*. Eur J Immunol. **28**(11): p. 3605-15.
18. Yanaka, K., et al., 1996. *Neuronal protection from cerebral ischemia by synthetic fibronectin peptides to leukocyte adhesion molecules*. J Cereb Blood Flow Metab. **16**(6): p. 1120-5.
19. Jackson, D.Y., et al., 1997. *Potent alpha 4 beta 1 peptide antagonists as potential anti-inflammatory agents*. J Med Chem. **40**(21): p. 3359-68.

20. Bearz, A., et al., 1998.*Fibronectin binding promotes a PKC-dependent modulation of NF-kappa B in human T cells.* Biochem Biophys Res Commun. **243**(3): p. 732-7.
21. Moore, C., et al., 2007.*Fibronectin-alpha4beta1 integrin interactions regulate metalloproteinase-9 expression in steatotic liver ischemia and reperfusion injury.* Am J Pathol. **170**(2): p. 567-77.
22. Elices, M.J., et al., 1990.*VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site.* Cell. **60**(4): p. 577-84.
23. Barton, G.M., 2008.*A calculated response: control of inflammation by the innate immune system.* J Clin Invest. **118**(2): p. 413-20.
24. Matsuyama, T., et al., 1989.*Activation of CD4 cells by fibronectin and anti-CD3 antibody. A synergistic effect mediated by the VLA-5 fibronectin receptor complex.* J Exp Med. **170**(4): p. 1133-48.
25. Shang, X.Z., B.J. Lang, and A.C. Issekutz, 1998.*Adhesion molecule mechanisms mediating monocyte migration through synovial fibroblast and endothelium barriers: role for CD11/CD18, very late antigen-4 (CD49d/CD29), very late antigen-5 (CD49e/CD29), and vascular cell adhesion molecule-1 (CD106).* J Immunol. **160**(1): p. 467-74.
26. Lishko, V.K., V.P. Yakubenko, and T.P. Ugarova, 2003.*The interplay between integrins alphaMbeta2 and alpha5beta1 during cell migration to fibronectin.* Exp Cell Res. **283**(1): p. 116-26.
27. Hynes, R.O., 1992.*Integrins: versatility, modulation, and signaling in cell adhesion.* Cell. **69**(1): p. 11-25.
28. Koivunen, E., B. Wang, and E. Ruoslahti, 1995.*Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins.* Biotechnology (N Y). **13**(3): p. 265-70.
29. Coito, A.J.*Leukocyte transmigration across endothelial and extracellular matrix protein barriers in liver ischemia/reperfusion injury.* Curr Opin Organ Transplant.
30. Lee, W.Y. and P. Kubes, 2008.*Leukocyte adhesion in the liver: distinct adhesion paradigm from other organs.* J Hepatol. **48**(3): p. 504-12.
31. Sorokin, L.*The impact of the extracellular matrix on inflammation.* Nat Rev Immunol. **10**(10): p. 712-23.
32. Lock, J.G., B. Wehrle-Haller, and S. Stromblad, 2008.*Cell-matrix adhesion complexes: master control machinery of cell migration.* Semin Cancer Biol. **18**(1): p. 65-76.
33. Makarem, R., et al., 1994.*Competitive binding of vascular cell adhesion molecule-1 and the HepII/IIICS domain of fibronectin to the integrin alpha 4 beta 1.* J Biol Chem. **269**(6): p. 4005-11.
34. Manicone, A.M. and J.K. McGuire, 2008.*Matrix metalloproteinases as modulators of inflammation.* Semin Cell Dev Biol. **19**(1): p. 34-41.
35. Segarra, M., et al., 2005.*Dual function of focal adhesion kinase in regulating integrin-induced MMP-2 and MMP-9 release by human T lymphoid cells.* FASEB J. **19**(13): p. 1875-7.
36. Esparza, J., et al., 1999.*Fibronectin upregulates gelatinase B (MMP-9) and induces coordinated expression of gelatinase A (MMP-2) and its activator MT1-MMP (MMP-14) by human T lymphocyte cell lines. A process repressed through RAS/MAP kinase signaling pathways.* Blood. **94**(8): p. 2754-66.
37. Hamada, T., et al., 2008.*Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury.* Hepatology. **47**(1): p. 186-98.
38. Opdenakker, G., P.E. Van den Steen, and J. Van Damme, 2001.*Gelatinase B: a tuner and amplifier of immune functions.* Trends Immunol. **22**(10): p. 571-9.
39. Ram, M., Y. Sherer, and Y. Shoenfeld, 2006.*Matrix metalloproteinase-9 and autoimmune diseases.* J Clin Immunol. **26**(4): p. 299-307.
40. Marchant, D. and B.M. McManus, 2009.*Matrix metalloproteinases in the pathogenesis of viral heart disease.* Trends Cardiovasc Med. **19**(1): p. 21-6.

41. Viappiani, S., M. Sariahmetoglu, and R. Schulz, 2006. *The role of matrix metalloproteinase inhibitors in ischemia-reperfusion injury in the liver*. *Curr Pharm Des*. **12**(23): p. 2923-34.
42. Hamada, T., et al., 2008. *Cyclooxygenase-2 deficiency enhances Th2 immune responses and impairs neutrophil recruitment in hepatic ischemia/reperfusion injury*. *J Immunol*. **180**(3): p. 1843-53.
43. Kuriyama, N., et al. *Tenascin-C: A novel mediator of hepatic ischemia and reperfusion injury*. *Hepatology*. **54**(6): p. 2125-36.
44. Sternlicht, M.D. and Z. Werb, 2001. *How matrix metalloproteinases regulate cell behavior*. *Annu Rev Cell Dev Biol*. **17**: p. 463-516.
45. Barbolina, M.V. and M.S. Stack, 2008. *Membrane type 1-matrix metalloproteinase: substrate diversity in pericellular proteolysis*. *Semin Cell Dev Biol*. **19**(1): p. 24-33.
46. Itoh, Y., 2006. *MT1-MMP: a key regulator of cell migration in tissue*. *IUBMB Life*. **58**(10): p. 589-96.
47. Rajavashisth, T.B., et al., 1999. *Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques: evidence for activation by proinflammatory mediators*. *Circulation*. **99**(24): p. 3103-9.
48. Kontinen, Y.T., et al., 1998. *New collagenolytic enzymes/cascade identified at the pannus-hard tissue junction in rheumatoid arthritis: destruction from above*. *Matrix Biol*. **17**(8-9): p. 585-601.
49. Graesser, D., et al., 1998. *The interrelationship of alpha4 integrin and matrix metalloproteinase-2 in the pathogenesis of experimental autoimmune encephalomyelitis*. *Lab Invest*. **78**(11): p. 1445-58.
50. Teesalu, T., A.E. Hinkkanen, and A. Vaheri, 2001. *Coordinated induction of extracellular proteolysis systems during experimental autoimmune encephalomyelitis in mice*. *Am J Pathol*. **159**(6): p. 2227-37.
51. Deschamps, A.M., et al., 2005. *Trafficking of the membrane type-1 matrix metalloproteinase in ischemia and reperfusion: relation to interstitial membrane type-1 matrix metalloproteinase activity*. *Circulation*. **111**(9): p. 1166-74.
52. Deschamps, A.M., et al., 2008. *Interruption of endothelin signaling modifies membrane type 1 matrix metalloproteinase activity during ischemia and reperfusion*. *Am J Physiol Heart Circ Physiol*. **294**(2): p. H875-83.
53. Chang, D.I., et al., 2003. *Activation systems for latent matrix metalloproteinase-2 are upregulated immediately after focal cerebral ischemia*. *J Cereb Blood Flow Metab*. **23**(12): p. 1408-19.
54. Yang, Y., et al., 2007. *Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat*. *J Cereb Blood Flow Metab*. **27**(4): p. 697-709.
55. Holmbeck, K., et al., 1999. *MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover*. *Cell*. **99**(1): p. 81-92.
56. Hynes, R.O., 2002. *Integrins: bidirectional, allosteric signaling machines*. *Cell*. **110**(6): p. 673-87.
57. Ono, K. and J. Han, 2000. *The p38 signal transduction pathway: activation and function*. *Cell Signal*. **12**(1): p. 1-13.
58. King, L.A., et al., 2009. *Role of p38 and JNK in liver ischemia and reperfusion*. *J Hepatobiliary Pancreat Surg*. **16**(6): p. 763-70.
59. Kobayashi, M., et al., 2002. *P38 mitogen-activated protein kinase inhibition attenuates ischemia-reperfusion injury of the rat liver*. *Surgery*. **131**(3): p. 344-9.
60. Mainiero, F., et al., 2000. *RAC1/P38 MAPK signaling pathway controls beta1 integrin-induced interleukin-8 production in human natural killer cells*. *Immunity*. **12**(1): p. 7-16.
61. Frisch, S.M. and R.A. Screaton, 2001. *Anoikis mechanisms*. *Curr Opin Cell Biol*. **13**(5): p. 555-62.

62. Taddei, M.L., et al. *Anoikis: an emerging hallmark in health and diseases*. J Pathol. **226**(2): p. 380-93.
63. Clutterbuck, A.L., et al., 2009. *Targeting matrix metalloproteinases in inflammatory conditions*. Curr Drug Targets. **10**(12): p. 1245-54.
64. Van Wart, H.E. and H. Birkedal-Hansen, 1990. *The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family*. Proc Natl Acad Sci U S A. **87**(14): p. 5578-82.
65. Murphy, G., et al., 1994. *Regulation of matrix metalloproteinase activity*. Ann N Y Acad Sci. **732**: p. 31-41.
66. Tallant, C., A. Marrero, and F.X. Gomis-Ruth. *Matrix metalloproteinases: fold and function of their catalytic domains*. Biochim Biophys Acta. **1803**(1): p. 20-8.
67. Okamoto, T., et al., 1997. *Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide*. Arch Biochem Biophys. **342**(2): p. 261-74.
68. McCarthy, S.M., et al., 2008. *Nitric oxide regulation of MMP-9 activation and its relationship to modifications of the cysteine switch*. Biochemistry. **47**(21): p. 5832-40.
69. Brew, K., D. Dinakarpandian, and H. Nagase, 2000. *Tissue inhibitors of metalloproteinases: evolution, structure and function*. Biochim Biophys Acta. **1477**(1-2): p. 267-83.
70. Kim, K.H., et al., 2005. *Tissue inhibitor of metalloproteinase-1 deficiency amplifies acute lung injury in bleomycin-exposed mice*. Am J Respir Cell Mol Biol. **33**(3): p. 271-9.
71. Fujimoto, M., et al., 2008. *Tissue inhibitor of metalloproteinases protect blood-brain barrier disruption in focal cerebral ischemia*. J Cereb Blood Flow Metab. **28**(10): p. 1674-85.
72. Kuyvenhoven, J.P., et al., 2004. *Plasma MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 during human orthotopic liver transplantation. The effect of aprotinin and the relation to ischemia/reperfusion injury*. Thromb Haemost. **91**(3): p. 506-13.
73. Kuyvenhoven, J.P., et al., 2004. *Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection*. Transplantation. **77**(11): p. 1646-52.
74. Jaeschke, H. *Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts*. J Gastroenterol Hepatol. **26 Suppl 1**: p. 173-9.
75. Brown, D.J., et al., 2004. *Elements of the nitric oxide pathway can degrade TIMP-1 and increase gelatinase activity*. Mol Vis. **10**: p. 281-8.
76. Wang, Y., et al., 2007. *Myeloperoxidase inactivates TIMP-1 by oxidizing its N-terminal cysteine residue: an oxidative mechanism for regulating proteolysis during inflammation*. J Biol Chem. **282**(44): p. 31826-34.
77. Guedez, L., et al., 2001. *Tissue inhibitor of metalloproteinase-1 alters the tumorigenicity of Burkitt's lymphoma via divergent effects on tumor growth and angiogenesis*. Am J Pathol. **158**(4): p. 1207-15.
78. Michalopoulos, G.K. and M.C. DeFrances, 1997. *Liver regeneration*. Science. **276**(5309): p. 60-6.
79. Fausto, N., J.S. Campbell, and K.J. Riehle, 2006. *Liver regeneration*. Hepatology. **43**(2 Suppl 1): p. S45-53.
80. Riehle, K.J., et al. *New concepts in liver regeneration*. J Gastroenterol Hepatol. **26 Suppl 1**: p. 203-12.
81. Mohammed, F.F. and R. Khokha, 2005. *Thinking outside the cell: proteases regulate hepatocyte division*. Trends Cell Biol. **15**(10): p. 555-63.
82. Nakamura, T., K. Sakai, and K. Matsumoto. *Hepatocyte growth factor twenty years on: Much more than a growth factor*. J Gastroenterol Hepatol. **26 Suppl 1**: p. 188-202.

83. Huh, C.G., et al., 2004. *Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair*. Proc Natl Acad Sci U S A. **101**(13): p. 4477-82.
84. Schelter, F., et al. *Tumor cell-derived Timp-1 is necessary for maintaining metastasis-promoting Met-signaling via inhibition of Adam-10*. Clin Exp Metastasis. **28**(8): p. 793-802.
85. Mohammed, F.F., et al., 2005. *Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell cycle via HGF activation in murine liver regeneration*. Hepatology. **41**(4): p. 857-67.
86. Abu-Amara, M., et al. *Liver ischemia/reperfusion injury: processes in inflammatory networks--a review*. Liver Transpl. **16**(9): p. 1016-32.
87. Illing, N. and J. Errington, 1991. *The spoIIA operon of Bacillus subtilis defines a new temporal class of mother-cell-specific sporulation genes under the control of the sigma E form of RNA polymerase*. Mol Microbiol. **5**(8): p. 1927-40.
88. Frears, E.R., et al., 1996. *Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite*. FEBS Lett. **381**(1-2): p. 21-4.