THE ROLE OF MACROPHAGE POLARIZATION IN LIVER REPAIR FOLLOWING AN ACUTE INJURY

By

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ABSTRACT

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Acute liver failure is a condition in which the liver loses of function and the ability to repair. ALF is hallmarked by the inability of monocytes and macrophages to become classically activated. The mechanisms by which macrophages may contribute to the liver injury and their role in the subsequent repair are not well characterized.

The purpose of these studies was to determine the role of macrophages in acute liver injury and repair in response to carbon tetrachloride treatment. We found that compared to control mice, the ALT activity level was not significantly different in macrophage-depleted mice, while an increased area of necrosis was observed in macrophage-depleted mice 72hrs following injury. However, a decreased amount of Type I collagen staining was seen in macrophage-depleted mice while no change was seen in the mRNA levels of Type I collagen and factors related to collagen production. Collectively, these studies suggest that macrophages may play a role in the clearance of necrotic cells and collagen remodeling to aid in liver repair.

Macrophages are known to polarize into a pro-inflammatory phenotype termed M1 macrophages and an anti-inflammatory phenotype, M2 macrophages. Currently, the role of M1 and M2 macrophages in liver injury and repair is not clear. I have conducted preliminary studies that determined the validity and feasibility of an *in vitro* macrophage polarization and the localization of adoptively transferred macrophages to the liver into macrophage depleted mice following the induction of an acute liver injury.

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KEY TO ABBREVIATIONS

ALF	Acute Liver Failure
ALT	Alanine Aminotransferase
APAP	Acetaminophen
CCl_4	Carbon Tetrachloride
CCND1	Cyclin D1
ECM	Extracellular matrix
FGF2	Fibroblast growth factor 2
H&E	Hematoxylin and Eosin
HGF	Hepatocyte Growth Factor
HSCs	Hepatic Stellate Cells
IFNγ	Interferon gamma
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
I.P.	Intraperitoneal
LAP	Latency associated peptide
LPS	Lipopolysaccharide
LTBP	Latent TGF-beta binding protein
Ly6C	Lymphocyte antigen 6C
MMP	Matrix Metallopeptidase
MT-MMP	Membrane type -MMP
NF-κB	Nuclear Factor kappa-light-chain-enhancer of Activated B-cells
NKT	Natural Killer T-cells

NO	Nitric Oxide
PAI-1	Plasminogen Activator Inhibitor-1
PAMP	Pathogen Associated Molecular Pattern
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet derived growth factor
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
TGF-α	Transforming Growth Factor alpha
TGF-β	Transforming Growth Factor beta
T_H1	Type-1 T-Helper Cell
T_H2	Type-2 T-Helper Cell
TIMP	Tissue Inhibitor of Metalloproteinase
TNFα	Tumor Necrosis Factor alpha
TNFR-1	Tumor Necrosis Factor Receptor-1
TSP-1	Thrombospondin-1
uPA	Urokinase-type Plasminogen Activator

CHAPTER 1

INTRODUCTION

INTRODUCTION

Liver regeneration is an amazing process that has been known to occur since ancient times. Following partial hepatectomy, the liver will regenerate proportionally to the amount of liver removed (Kawasaki et al., 1992). The liver has also been shown to regenerate proportionally to the size of the animal following transplantation of a donor liver of a different sized animal (Vine & Kier, 1993). These observations have led to the desire to fully understand the cellular mechanisms behind liver regeneration in order to treat people suffering from various liver diseases that impair normal liver repair and regeneration. Most of the previous work done on liver repair has been focused on the process of hepatocyte proliferation and the molecular signals that mediate this process.

Hepatocytes are the main cell type that comprise the liver and are of critical importance for liver function and maintenance of homeostasis. Hepatocytes are responsible for glucose regulation, synthesis of a variety of proteins found in blood, synthesis and secretion of bile as well as drug and toxin metabolism. During liver repair, hepatocytes are the first cells in the liver to respond by synthesizing DNA and mitogenic stimuli for other cell types in the liver (Adachi et al., 1995). However, the trigger for hepatocyte synthesis itself remained elusive until 1984, when it was found in the serum of rats that had undergone partial hepatectomy. Hepatocyte Growth Fator (HGF) concentrations reach 20-fold above normal levels in the blood 1 hour following partial hepatectomy and reduce slowly until 72 hours (Naldini et al., 1991; Lindroos et al., 1991). HGF is known to play an essential role in liver development. Complete deletion of this gene in mice results in embryonic lethality (Schmidt et al., 1995). The source of HGF in the liver following partial hepatectomy is the result of the disruption in the biomatrix of the liver that contains a large amount of HGF (Matsumoto & Yamamoto,

1991). Once HGF is released into the serum, it is directly involved in the induction of hepatocyte mitosis (Kim et al., 1997).

Although much is known about hepatocyte proliferation during liver repair, much remains unclear about the roles that other non-parenchymal cells play in liver repair. In addition, much of what we know about liver repair has been determined from studies of partial hepatectomy. However, most instances of liver repair naturally occur as a result of mild and acute liver injury. Drug Induced Liver Injury (DILI) is a common cause of mild and acute liver injury but also accounts for half of the acute liver failure cases in the United States (Abboud & Kaplowitz, 2007). This suggests that there is a threshold for injury that can be repaired by normal liver regeneration and beyond this threshold, the liver fails to repair itself and results in failure (Chaudhuri et al., 2011). By illuminating the mechanisms normally involved in DILI repair, further research can then be conducted to identify deviations from this repair and potential therapeutic targets. My goal is to determine the role of macrophages in normal liver repair as a result of DILI.

Macrophages are innate immune cells of the myeloid lineage which play an important role in liver injury (Geissmann et al., 2010). They are phagocytic cells and are the first line of defense against bacteria and other infectious microbes (Liaskou et al., 2012). Macrophages are known to undergo classical and alternative activation (Laskin, 2009). During infection, macrophages and monocytes are induced by pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) to polarize and express pro-inflammatory cytokines during classical activation (Bieghs & Trautwein, 2013). Classically activated or M1 macrophages express pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6)

(Mantovani et al., 2004). TNFα and IL-6 have been shown to be important signals involved in early signaling pathways leading to liver regeneration (Fujiyoshi & Ozaki, 2011). The concentration of both cytokines increase in the liver following injury. And the ablation of both cytokines results in severe impairment of liver repair (Bourdi et al., 2007; Kovalovich et al., 2000). However, the mechanism for classical activation in DILI remains unclear.

Macrophages can also be alternatively activated by exposure to anti-inflammatory cytokines such as IL-4 and IL-13 (Loke et al., 2002). These alternatively activated macrophages or M2 macrophages express anti-inflammatory cytokines such as IL-10 and transforming growth factor- β (TGF-β) (Gong et al., 2012). Kupffer cells, resident liver macrophages, express more of an anti-inflammatory or M2-like phenotype (Bieghs & Trautwein, 2013). The liver serves as a filter for the products of the digestive system before nutrients and other macromolecules enter the systemic circulation (Vollmar & Menger, 2009). Kupffer cells phagocytose bacteria and other microbes that arrive from the gut and serve as the liver's first line of defense (Parker & Picut, 2005).

Following liver injury, monocytes infiltrate into the liver and differentiate into macrophages (Holt et al., 2008). The phenotype of these monocytes can be characterized by the level of Lymphocyte antigen 6C (Ly6C) expressed on the cell surface (Geissmann et al., 2003). $Ly6C^{hi}$ monocytes circulate in the blood, are recruited to sites of inflammation and express pro-inflammatory mediators (Gordon & Taylor, 2005). $Ly6C^{lo}$ monocytes also circulate throughout the body, but differentiate into resident tissue macrophages such as Kupffer cells. $Ly6C^{lo}$ monocytes are phenotypically anti-inflammatory and M2-like (Geissmann et al., 2003).

Ly6C^{hi} monocytes are recruited to the liver following toxicant exposure and contribute to the inflammatory response by expressing Thrombospondin-1 (TSP-1), TNFα and Nitric Oxide (NO) in the first 12 hours after toxicant exposure (Helk et al., 2013; Ramachandran et al., 2012). As necrotic hepatocyte debris accumulates in the liver, the M1-like cells phagocytose the debris which induces the macrophages to polarize into an M2-like phenotype (Ramachandran et al., 2012). The polarization of M1-like macrophages into M2-like macrophages could be due to the exposure of the M1-like macrophages to IL-4 that is expressed by Type-2 T-Helper cells (T_H2) and Natural Killer T-cells (NKT) and to TGF-β that is expressed by Kupffer cells (Gong et al., 2012; Pollard, 2009). By a combination of these two mechanisms, an increase in the population of Ly6C^{lo} or M2-like macrophages in the liver arises from the polarization of infiltrated Ly6C^{hi} monocytes (Ramachandran et al., 2012). In addition to anti-inflammatory factors, Ly6C^{lo} macrophages are known to express factors involved in scar resolution and tissue repair such as Matrix Metalloproteinase (MMP) 9 and 12 (Sobrevals et al., 2010).

The role of these different macrophage populations in liver repair is not well understood. Ly6C^{lo} macrophages may contribute to liver repair by their increased phagocytic activity and production of growth factor activators and ECM components. However factors produced by Ly6C^{hi} macrophages also induce hepatocyte proliferation although some cytokines may exacerbate the injury. In the following studies, I investigated the role of macrophages in liver injury, inflammation, hepatocyte regeneration and matrix deposition involved in liver repair. I also generated preliminary

data to support future studies to identify the role of M1 and M2 macrophages in liver repair.

CHAPTER 2

THE IMPACT OF MACROPHAGES ON MECHANISMS OF LIVER REPAIR FOLLOWING CARBON TETRACHLORIDE INDUCED LIVER INJURY

ABSTRACT

Acute Liver Failure is a condition in which the liver loses function as well as the ability to repair itself. There are a number of studies that implicate the dysfunction of macrophages in ALF. However, not much is known about the role of macrophages in normal liver repair. In order to determine if macrophages are involved in liver repair following an acute injury, macrophages were depleted from mice treated with carbon tetrachloride to induce an acute liver injury. The livers of the macrophage depleted mice had decreased mRNA levels of pro-inflammatory genes typically produced by macrophages. However, the ALT activity level of the control mice and macrophage-depleted mice were consistent between both groups while the areas of necrosis observed in the macrophage-depleted mice were increased at 72 hours following injury. This suggests that macrophages are involved in the clearance of dead hepatocytes following injury. We also found that macrophages may not be required for hepatocyte proliferation possibly due to compensatory redundant mechanisms.

This was determined by observing similar levels of PCNA staining of macrophage depleted and control mice. The mRNA levels of genes involved in hepatocyte proliferation were also unchanged in the macrophage depleted mice as compared to the control mice. A notable decrease in Type I collagen deposition was observed in the macrophage depleted mice. However, the mRNA levels of Type I collagen were consistent between groups. In addition, the mRNA levels of genes involved in ECM production were all unchanged by macrophage depletion. However, the decreased collagen deposition could be accounted for by a change in the activation states of TGF-β or other regulators of collagen deposition such as MMPs, MT-MMPs or TIMPS.

INTRODUCTION

Acute liver failure is a condition in which the liver undergoes a massive and rapid loss of function following injury. A variety of toxic agents can damage the liver sufficiently to cause ALF such as drugs, alcohol, viral hepatitis, autoimmune liver disease and septic shock. These agents induce hepatic damage that can lead to wide-spread hepatic necrosis. Normally, the liver has an incredible ability to repair itself. However, the livers of patients with ALF have lost the ability to carry out this essential function. Instead, patients have elevated levels of anti-inflammatory cytokines and decreased levels of pro-inflammatory cytokines in their serum which correlates with poor outcomes (Antoniades et al., 2006). In addition, ALF patients with adverse outcomes exhibit compensatory anti-inflammatory response syndrome in which their monocytes and macrophages lose the ability to become activated (Wasmuth et al., 2005). Together these data imply that the immune system and especially macrophages may play an important role in the pathogenesis of ALF (Antoniades et al., 2008). However, in order to fully understand the mechanisms that underlie ALF, it is important to also understand the role of macrophages in normal liver repair. The purpose of the following studies was to investigate the role of macrophages in liver repair.

Kupffer cells, the resident liver macrophages, serve as the first line of defense against pathogens arriving from the gut. In response to PAMPs and other molecular signals such as toxin-induced injury, macrophages can become classically activated to produce pro-inflammatory cytokines and anti-microbial superoxides. One mechanism by which macrophages may contribute to liver repair is through the production of the pro-inflammatory cytokines, TNFα and IL-6. Both cytokines are known to stimulate

hepatocyte proliferation and are necessary for liver repair (Cressman et al., 1996; Yamada et al., 1997). Studies have demonstrated that these cytokines prime hepatocytes to be more responsive to growth factors such as HGF and transforming growth factor-alpha (TGF-α) by inducing transcription factors involved in cell cycle progression (Columbia & Clinic, 1998; Libermann & Baltimore, 1990). For example, IL-6 activates the STAT3 pathway which increases Cyclin D1 (CCND1) expression, a key regulatory protein for cells to pass from G1 to S phase during mitosis (Li et al., 2002) .

Once primed, hepatocytes achieve a maximal level of mitotic activity in response to the mitogens HGF and TGF-α (Adachi et al., 1995; Tomiya et al., 2000). Macrophages can also regulate availability of these growth factors to hepatocytes by the production of MMPs and other factors that regulate extracellular matrix (ECM) remodeling (Valentin et al., 2009). The ECM is the structural scaffold of the liver and is mainly comprised of collagens Type I, III, IV and V as well as various glycoproteins and proteoglycans (Ashkenas et al., 1996). MMPs degrade collagens and thereby release HGF that was previously sequestered in the ECM (Skrtic et al., 1999).

Macrophages also regulate ECM synthesis by producing growth factors that stimulate collagen synthesis and stimulate proliferation of activated HSCs and fibroblasts. These growth factors include platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and TGF-β (Khalil et al., 1989). In addition to producing growth factors, macrophages can produce proteins that affect the activity of these growth factors, such as TSP-1 and MMPs which can activate latent-TGF-β.

Macrophages also aid in tissue recovery by clearing pathogens as well as cellular debris by phagocytosis. During this process, macrophages have the ability to

distinguish pathogens from necrotic and apoptotic cellular debris through binding of different cell surface receptors (Allen & Aderem, 1996). Following recognition, the target material is then engulfed by the cell and transported to the phagosome where it awaits fusion with a lysosome in order for the engulfed material to be fully degraded (Poon, Hulett, & Parish, 2010). However, it is not known if the phagocytic function of macrophages contributes to normal liver repair.

Although not much is known about the distinct functions of macrophages during ALF, there is evidence that the production of pro-inflammatory cytokines and macrophage activation may be essential to proper liver repair. In the following experiments, liver injury was produced by carbon tetrachloride in normal mice and macrophage depleted mice and mechanisms of liver repair were assessed. My hypothesis was that following liver injury caused by carbon tetrachloride treatment in mice, livers depleted of macrophages would not undergo proper liver repair.

MATERIALS AND METHOD

Animals

Mice used in these studies are all male C57BL/6 (Jackson Laboratories, Bar Harbor, ME) mice from 8 to 10 weeks of age. All mice were maintained on a 12-h light/dark cycle under controlled temperature (18-21ºC) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health.

Carbon Tetrachloride Treatment

Mice were treated by intraperitoneal injection with 1 ml/kg of carbon tetrachloride (CCl_4, Sigma Chemical Company). The carbon tetrachloride was diluted 1:10 in corn oil (Sigma Chemical Company) prior to injection.

RNA Isolation and qRT-PCR

Liver tissue was homogenized in 1mL of TRI reagent (Sigma Chemical Company, St. Louis, MO) and total RNA was isolated per manufacturer's instructions. Total RNA was quantified spectrophotometrically using the Infinite 200PRO (Tecan Group Ltd., Männedorf, Switzerland). 125ng of total RNA was reverse transcribed into cDNA in a volume of 12µL containing PCR buffer (166 mM $(NH_4)_2SO_4$, 50 mM β-mercaptoethanol, 67 µM EDTA, 0.67 M Tris buffer, pH 8.8, 0.8 mg/ml BSA, 5 mM $MgCl_2$), 1 mM each dNTP (Promega, Madison, WI), 10 units rRNasin (Promega), 125 ng oligo$(dT)_{15}$ (Promega) and 50 units of Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT; Promega). The cDNA reaction was heated to 37℃ for 55 min and then 70℃ for 5 min. Next, 10 µL of iTaq Universal SYBR Green Supermix (Bio-

Rad, Hercules, CA) and 5μM of each primer pair was added to each cDNA sample for a final volume of 20 μL. Real-time PCR was performed on an ABI 7900 real-time PCR instrument (Applied Biosystems, Foster City, CA). Primers used for PCR are shown in Table 1.

Immunohistochemistry and Immunofluorescence

Immunofluorescence was used to detect and quantify type I collagen and macrophages in 8μm frozen sections of liver. The sections were fixed in 4% formalin and then incubated with rat anti-CD68 antibody diluted 1:100 (Novus, Littleton, CO) or rat anti-F4/80 antibody diluted 1:25 (AbD Serotec, Raleigh, NC) and rabbit anti-type I collagen antibody diluted 1:400 (Millipore, Billerica, MA). The sections were then incubated with secondary antibodies conjugated to either Alexa Fluor 488 or Alexa Fluor 594 (Life Technologies, Grand Island, NY).

Proliferating cell nuclear antigen (PCNA) was detected in formalin-fixed, paraffin-embedded sections of liver using the Vectastain Elite ABC Kit and Vector DAB (Vector Laboratories, Burlingame, CA). Anti-PCNA antibody (1:8000, Abcam, Cambridge, MA) was added to the tissues and incubated overnight at 4 degrees C.

Macrophage Depletion

Mice were treated with 200 μl liposome encapsulated clodronate (ClodronateLiposomes.com, The Netherlands) or PBS-containing liposomes by i.p. injection. After 48 hours, the mice were treated with carbon tetrachloride.

Quantification of Necrosis

The area of necrosis was quantified in 15, 200X fields per hematoxylin and eosin-stained liver section using Image J Software (National Institutes of Health). The analysis was performed in a blinded fashion.

Alanine Aminotransferase Activity

Hepatocyte injury was evaluated by measuring the serum activity of alanine aminotransferase (ALT) (Pointe Scientific Inc., Canton, MI).

Statistics

Results are presented as the mean $\pm$ SEM. 5-7 mice were used for all *in vivo* studies. 3 mice were used for all *in vitro* studies. Data were analyzed by Analysis of Variance (ANOVA). Data expressed as a fraction were transformed by arc sine square root prior to analysis. Comparisons among group means were made using the Student-Newman-Keuls test. The criterion for significance was $p < 0.05$ for all studies.

Table 1: Summary of Primer Sequences used for qRT-PCR

Gene	Forward Primer	Reverse Primer
Rpl13a	5'-TCCTTGTTCCACTGTGCCTTG-3'	5'-TGCTTCCACATGTCCTCACAA-3'
Col1a1	5'-TTGACGGAAGGGCACCACCAG-3'	5'-GCACCACCACCCACGGAATCG-3'
MIP-2	5'-CTCAGACAGCGAGGCACATC-3'	5'-CCTCAACGGAAGAACCAAAGAG-3'
PDGF-B	5'-CCCACAGTGGCTTTTCATTT-3'	5'-GTGGAGGAGCAGACTGAAGG-3'
FGF2	5'-AGCGGCTCTACTGCAAGAAC-3'	5'-GCCGTCCATCTTCCTTCATA-3'
PAI-1	5'-AGTCTTTCCGACCAAGAGCA-3'	5'-ATCACTTGCCCCATGAAGAG-3'
α-SMA	5'-CCACCGCAAATGCTTCTAAGT-3'	5'-GGCAGGAATGATTTGGAAAGG-3'
iNOS	5'-TTCTGTGCTGTCCCAGTGAG-3'	5'-TGAAGAAAACCCCTTGTGCT-3'
IL-6	5'-ACCAGAGGAAATTTTCAATAGGC-3'	5'-TGATGCACTTGCAGAAAACA-3'
TNF-α	5'- TGGCTGTGACTCCCCTTCTTT-3'	5'-AGAGCTCAACACAAGCGTGGA-3'
TGF-β1	5'-CAACCCAGGTCCTTCCTAAA-3'	5'-GGAGAGCCCTGGATACCAAC-3'
MMP2	5'-GGGGTCCATTTTCTTCTTCA-3'	5'-CCAGCAAGTAGATGCTGCCT-3'
MMP3	5'CCCACCAAGTCTAACTCTCTGGAA-3'	5'-GGGTGCTGACTGCATCAAAGA-3'
MMP9	5'-CTGTCGGCTGTGGTTCAGT-3'	5'-AGACGACATAGACGGCATCC-3'
MMP13	5'-GGTCCTTGGAGTGATCCAGA-3'	5'-TGATGAAACCTGGACAAGCA-3'

RESULTS

The extent of macrophage accumulation in the liver after carbon tetrachloride was quantified by immunohistochemical staining of the macrophage cell surface markers, CD68 and F4/80. CD68 is a 110-kD transmembrane glycoprotein that is expressed in blood monocytes and resident tissue macrophages in mice and humans. (Holness & Simmons, 1993). F4/80 is a highly specific cell surface marker of resident tissue macrophages found in mice (Austyn & Gordon, 1981). Following carbon tetrachloride treatment, CD68 and F4/80 positive macrophages accumulated within centrilobular regions of liver (Figure 2-1).

In order to probe the role of macrophages in liver repair after injury, macrophages were depleted from the livers of mice prior to carbon tetrachloride treatment with liposomal clodronate. Treatment of mice with liposomal clodronate decreased hepatic macrophages 72 hours following carbon tetrachloride treatment (Figure 2-2).

ALT is a hepatocyte protein released into the blood after hepatocyte cell death. Its activity in serum is measured clinically to estimate the extent of liver injury (Karmen et al., 1954; Snell & Jenkins, 1959). ALT activity was not different between mice treated with liposomal clodronate and carbon tetrachloride and mice treated with liposomal PBS and carbon tetrachloride at 48 hours (Figure 2-3). At 72 hours after carbon tetrachloride, there was a modest decrease in ALT activity in mice treated with liposomal clodronate (Figure 2-3).

Next, the area of necrosis was quantified in sections of hematoxylin and eosin (H&E) stained liver. Liposomal clodronate treated mice had a significantly larger area of necrosis in the liver 72 hours after carbon tetrachloride when compared to mice treated

with liposomes containing PBS (Figure 2-4). This suggested that clearance of necrotic tissue may be defective in the mice depleted of macrophages.

Expression of pro-inflammatory genes was analyzed to determine if macrophage depletion affects their upregulation (Figure 2-5). TNF-α, inducible nitric oxide synthase (iNOS) and IL-6 were all ablated in liposomal clodronate treated mice as compared to mice treated with liposomal PBS.

Hepatocyte proliferation is another key process in liver repair. Genes involved in hepatocyte proliferation were analyzed to determine whether macrophages contribute to their upregulation. As shown in Figure 2-6 there was no difference in the levels of these genes between control mice and macrophage depleted mice. Proliferating cell nuclear antigen (PCNA) protein was quantified in liver sections following macrophage depletion and carbon tetrachloride treatment as a measure of hepatocyte proliferation. There was no significant difference in PCNA staining between the macrophage depleted and non-depleted mice (Figure 2-7).

A critical component of the tissue regeneration is synthesis of extracellular matrix, such as Type I collagen. Therefore, we stained livers of macrophage-depleted and control mice for Type I collagen using immunofluorescence, in order to determine if macrophages affect this critical process (Figure 2-8). Interestingly, hepatic Type I collagen was decreased in macrophage depleted mice as compared to control mice. We next verified this finding by analyzing the mRNA levels of Type I collagen (Figure 2-9A). Interestingly, the mRNA levels of this gene were not affected by macrophage depletion.

Since Type I collagen is mainly produced by hepatic stellate cells in the liver, the mRNA levels of other genes involved in hepatic stellate cell activation and collagen production were analyzed to identify any differences that may explain the difference seen in Type I collagen accumulation (Figure 2-9). All of the genes that were analyzed were unchanged in the macrophage depleted mice as compared to the control mice. Macrophages also produce members of the MMP family that are involved in the degradation of different components of the liver ECM. mRNA levels of MMP-13, a collagenase, and MMP-2, a gelatinase, were both significantly decreased as a result of macrophage depletion (Figure 2-10).

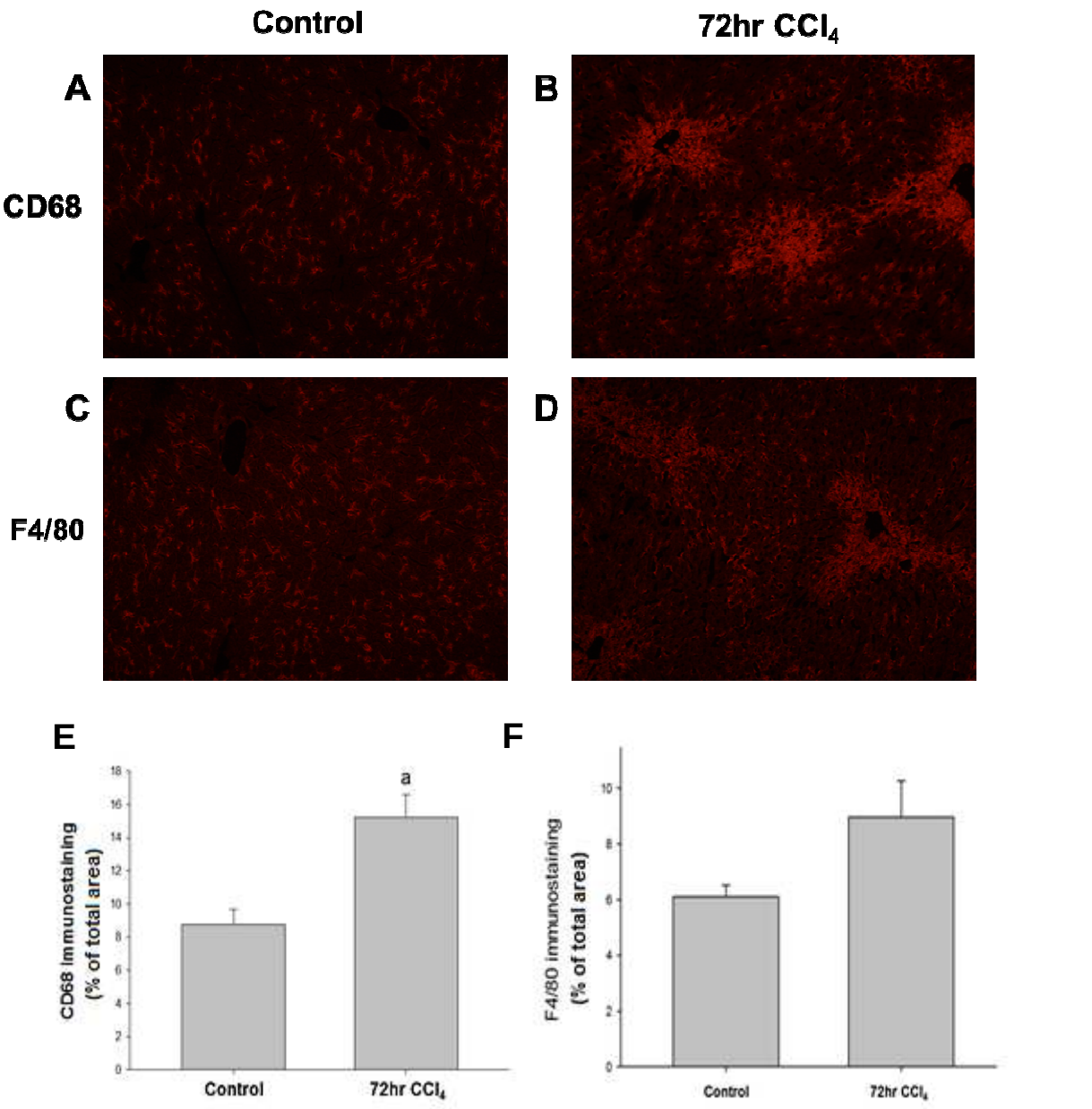

Figure 2-1: Macrophages accumulate in the liver following carbon tetrachloride treatment.

Male C57BL/6 mice were treated with either (A and C) vehicle or (B and D) 1 ml/kg carbon tetrachloride for 72 hours. Representative CD68 immunofluorescence in frozen liver sections from (A) vehicle or (B) carbon tetrachloride treated mice. Representative F4/80 immunofluorescence in livers sections from (C) vehicle or (D) carbon tetrachloride treated mice. Positive staining is shown in red for both CD68 and F4/80. The area of immunofluorescence of (E) CD68 and (F) F4/80 was quantified in liver sections by Image J analysis. [a]Significantly different from control mice.

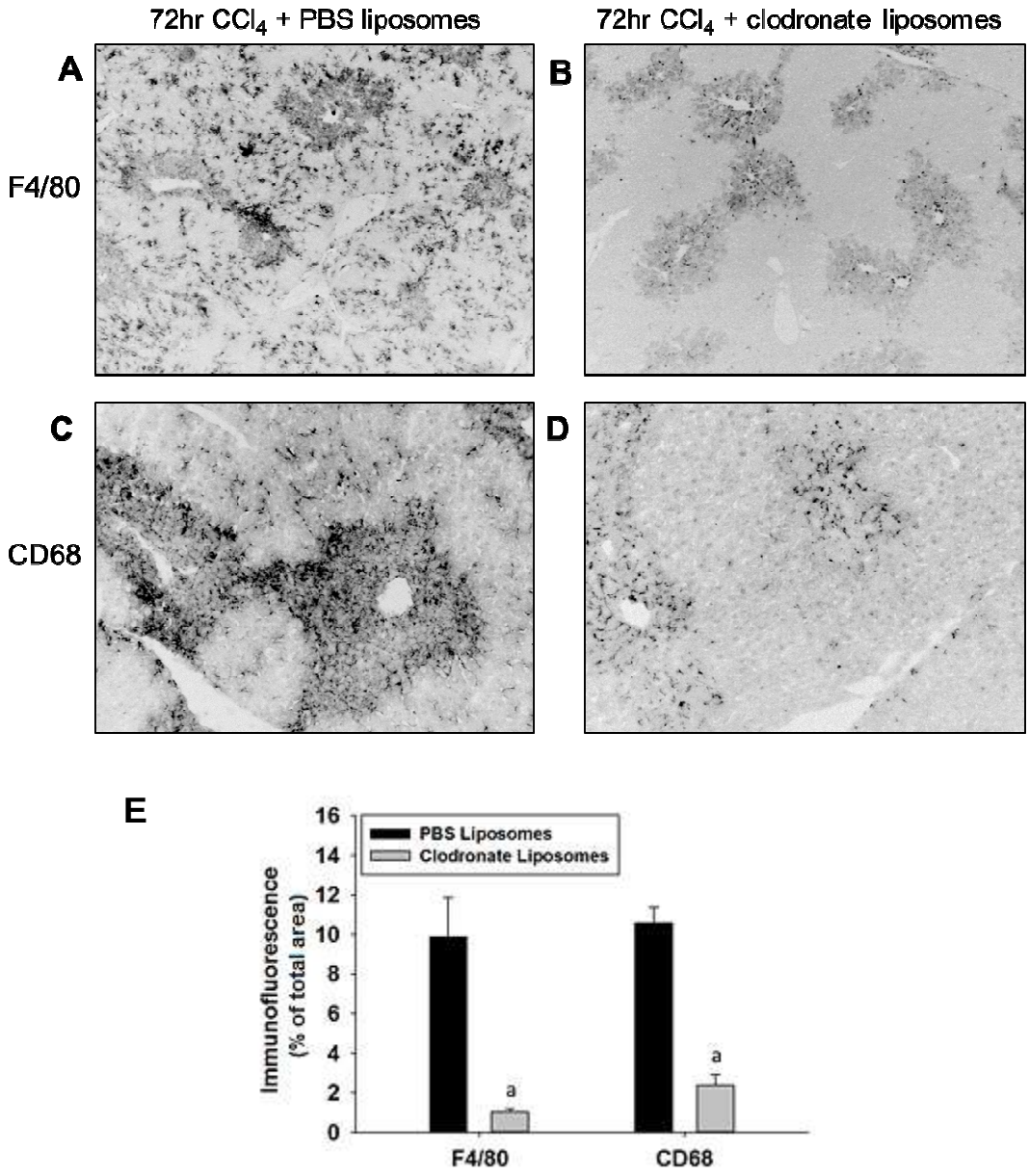

Figure 2-2: Macrophage depletion 72 hours after carbon tetrachloride treatment.

Male C57BL/6 mice were treated with either (A and C) PBS-containing liposomes or (B and D) clodronate-containing liposomes for 72 hours. Mice were then treated with carbon tetrachloride for 72 hours. Representative CD68 immunofluorescence in frozen liver sections, positive staining shown in black, from (A) PBS-containing liposome treated mice or (B) clodronate-containing liposome treated mice. Representative F4/80 immunofluorescence in livers sections, positive staining shown in black, from (C) PBS-containing liposome treated mice or (D) clodronate-containing liposome treated mice. Area of immunofluorescence of (E) CD68 and (F) F4/80 was quantified in liver sections. [a]Significantly different from PBS-containing liposome treated mice.

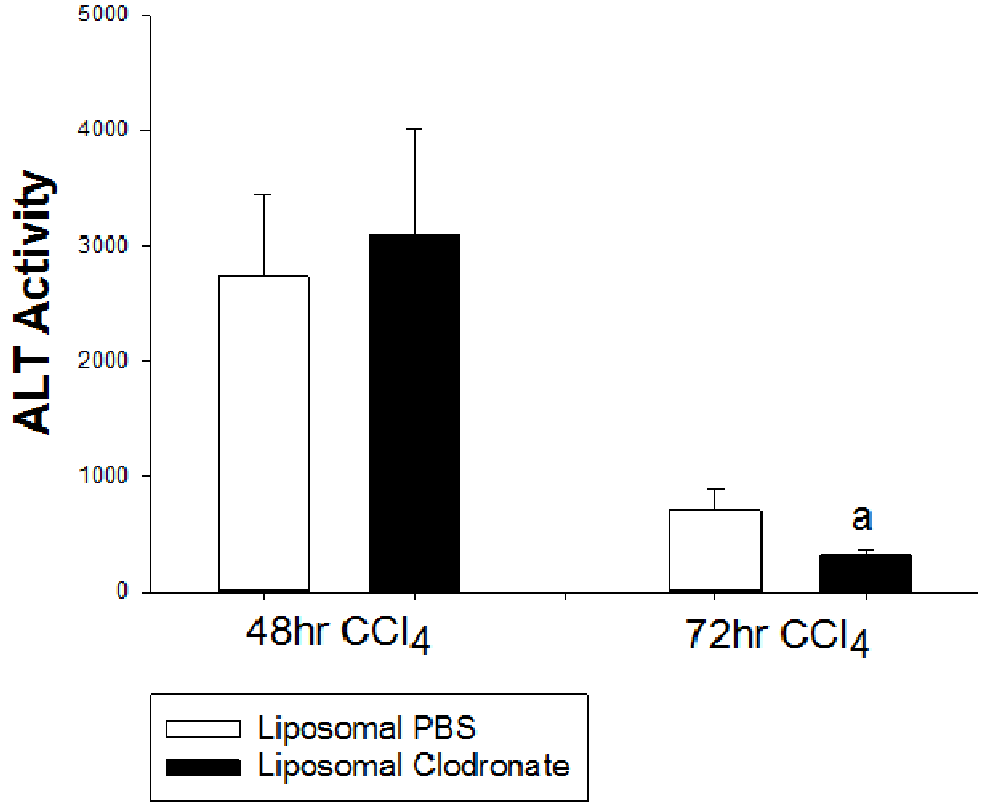

Figure 2-3: Serum ALT activity in mice following treatment with carbon tetrachloride.

Serum from mice treated with liposomal PBS or liposomal clodronate was collected and analyzed for ALT activity. [a]Significantly different from PBS-containing liposome treated mice.

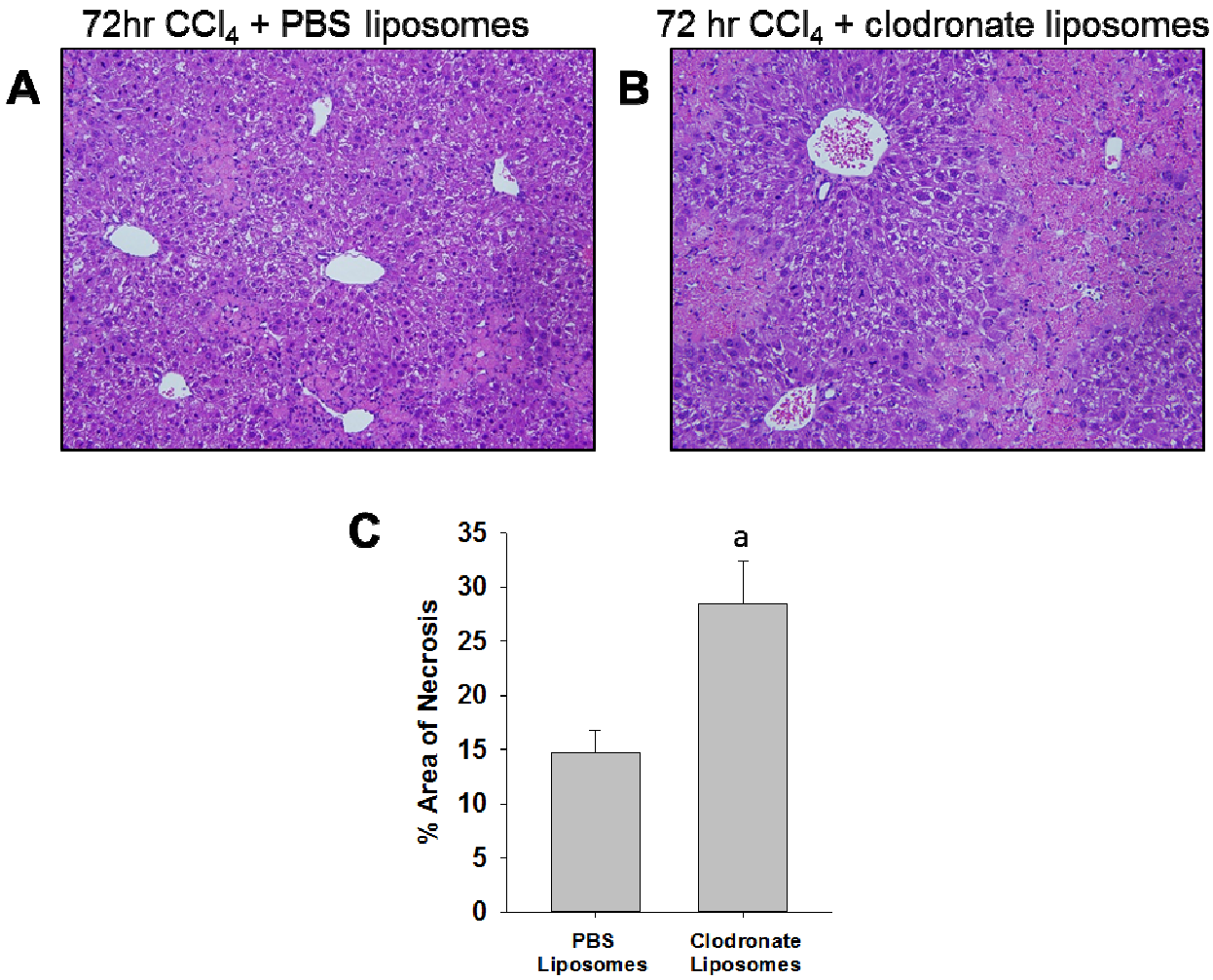

Figure 2-4: Quantification of necrotic areas following macrophage depletion and carbon tetrachloride treatment.

Male C57BL/6 mice were treated with either (A) PBS-containing liposomes or (B) clodronate-containing liposomes for 72 hours. Mice were then treated with carbon tetrachloride for 72 hours. Liver sections were stained with H&E and (C) area of necrosis was quantified using Image J analysis. [a]Significantly different from PBS-containing liposome treated mice.

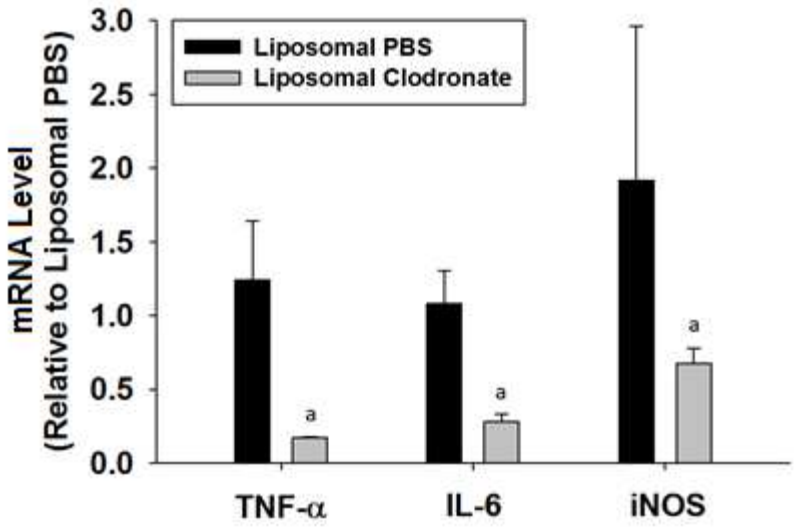

Figure 2-5: The effect of macrophage depletion on inflammatory mediators in the liver following carbon tetrachloride treatment.

mRNA levels of pro-inflammatory genes were measured in liver samples from mice following liposomal PBS or liposomal clodronate treatment prior to carbon tetrachloride treatment. mRNA expression was quantified 72 h after carbon tetrachloride treatment. [a]Significantly different from PBS-containing liposome treated mice.

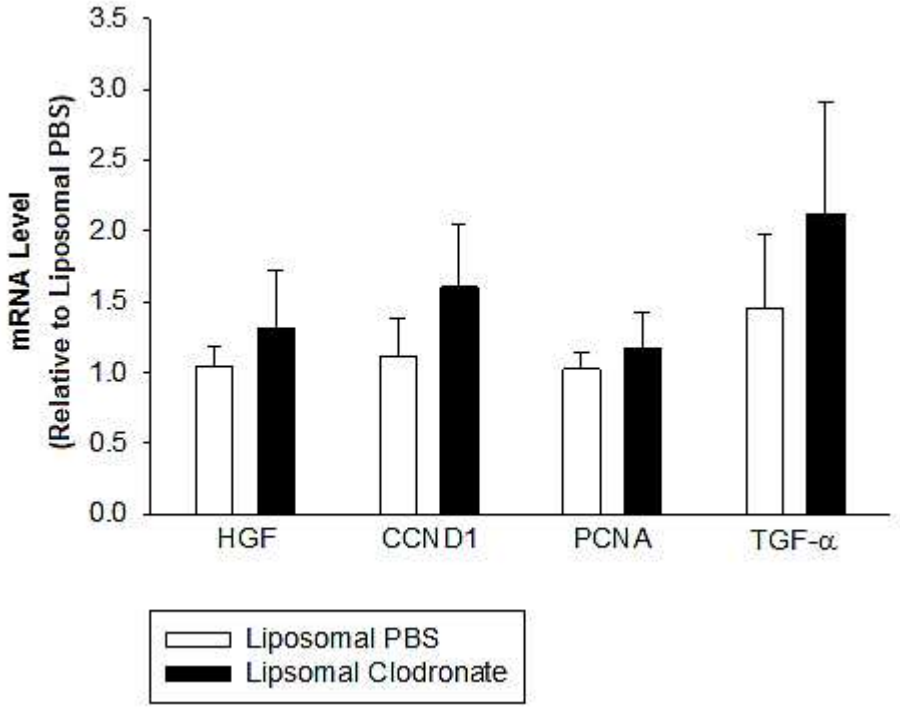

Figure 2-6: The effect of macrophage depletion on expression of genes associated with hepatocyte proliferation in the liver following carbon tetrachloride treatment.

mRNA levels of genes involved in hepatocyte proliferation from whole liver samples of mice following liposomal PBS or liposomal clodronate treatment prior carbon tetrachloride treatment. mRNA expression was quantified 72 h after carbon tetrachloride treatment.

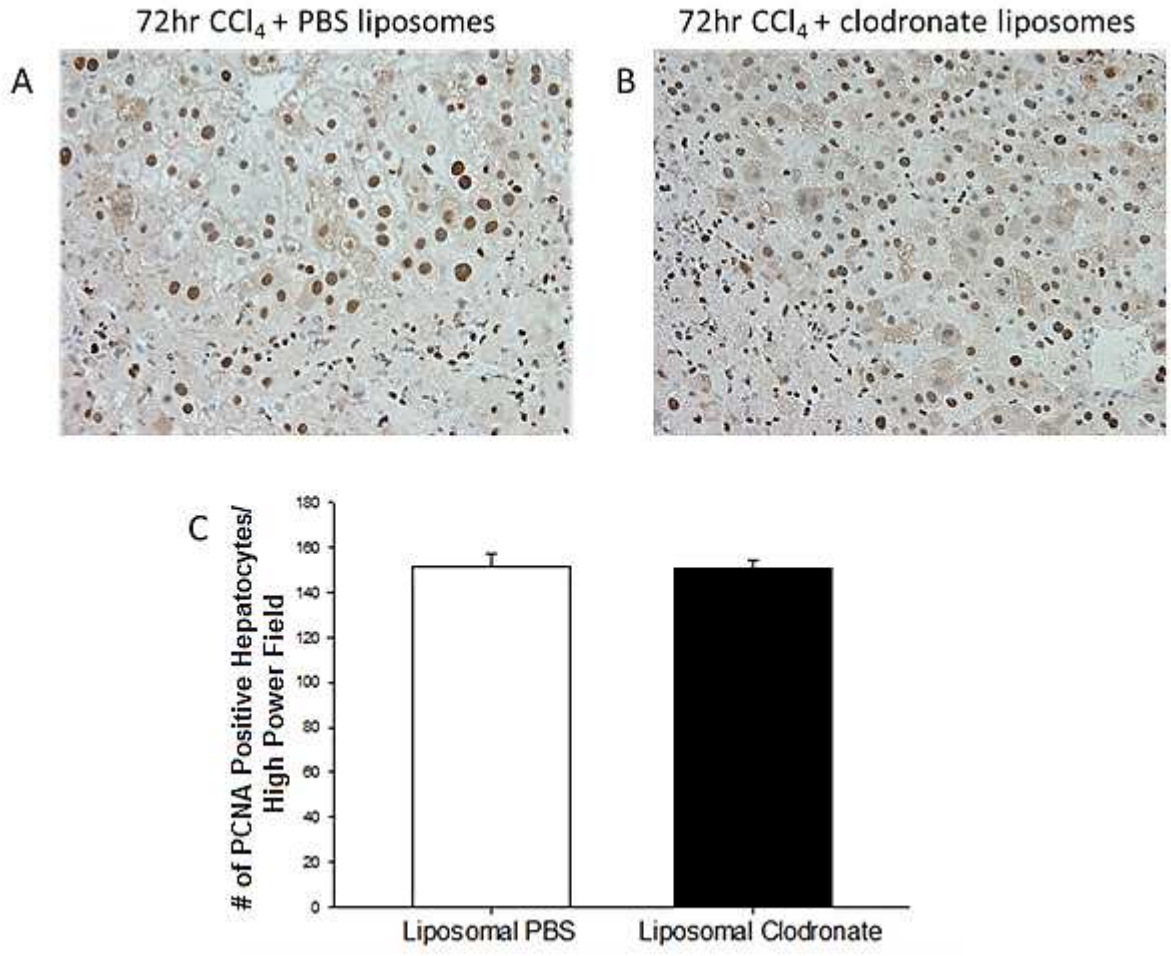

Figure 2-7: Quantification of PCNA staining of hepatocyte nuclei following macrophage depletion and carbon tetrachloride treatment.

Male C57BL/6 mice were treated with either (A) PBS-containing liposomes or (B) clodronate-containing liposomes for 72 hours. Mice were then treated with carbon tetrachloride for 72 hours. Paraffin embedded liver sections were stained for PCNA. (C) PCNA positive hepatocyte nuclei were quantified.

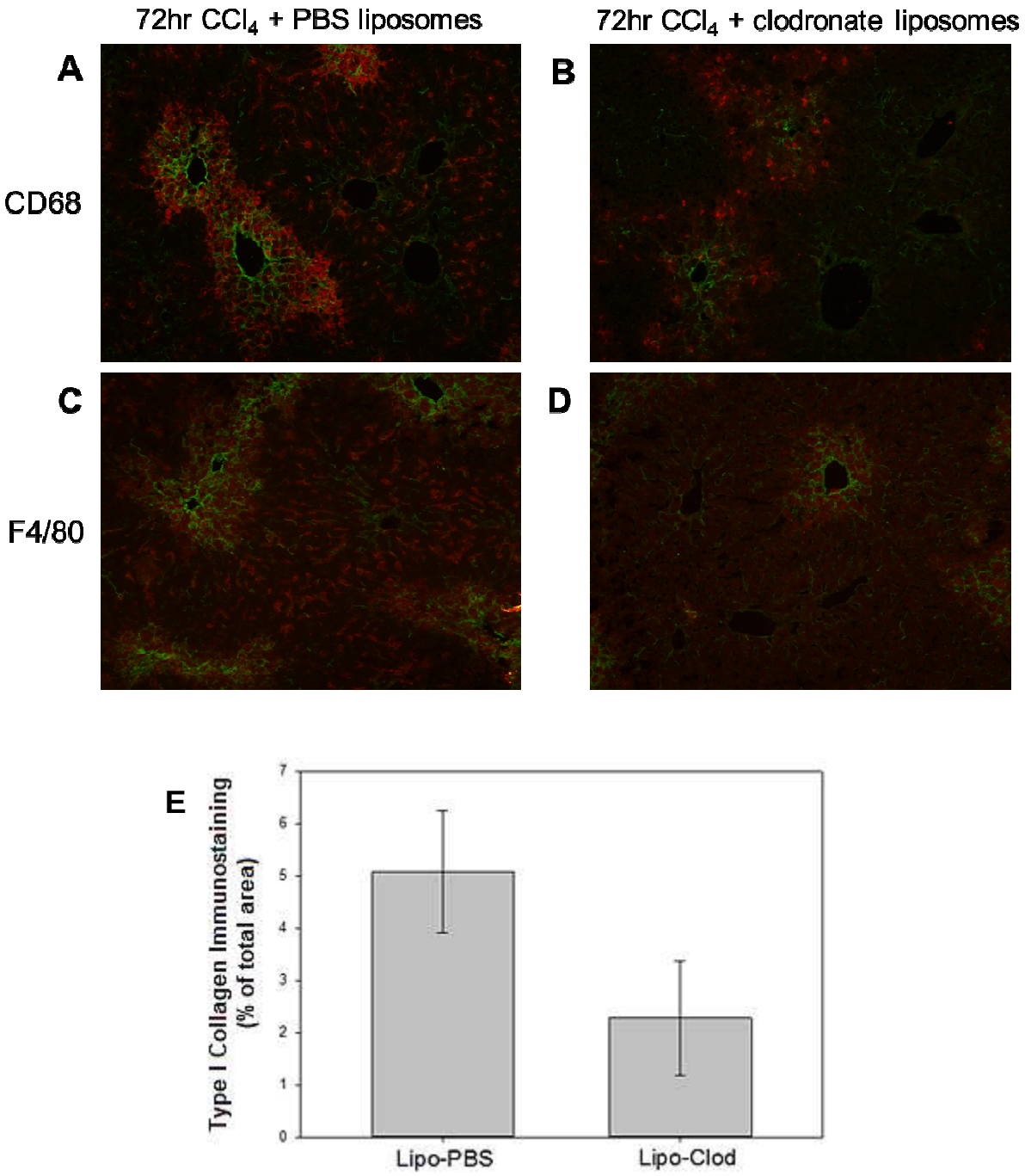

Figure 2-8: Quantification of Type I Collagen accumulation following macrophage depletion and carbon tetrachloride treatment.

Male C57BL/6 mice were treated with either (A) PBS-containing liposomes or (B) clodronate-containing liposomes for 72 hours. Mice were then treated with carbon tetrachloride for 72 hours. Representative CD68 immunofluorescence in frozen liver

Figure 2-8 (cont'd)

sections, positive staining shown in red, from (A) PBS-containing liposome treated mice or (B) clodronate-containing liposome treated mice. Representative F4/80 immunofluorescence in livers sections, positive staining shown in red, from (C) PBS-containing liposome treated mice or (D) clodronate-containing liposome treated mice. All frozen sections were also stained for Type I collagen with positive staining shown in green. (E) Area of Type I collagen positive staining was quantified using Image J software.

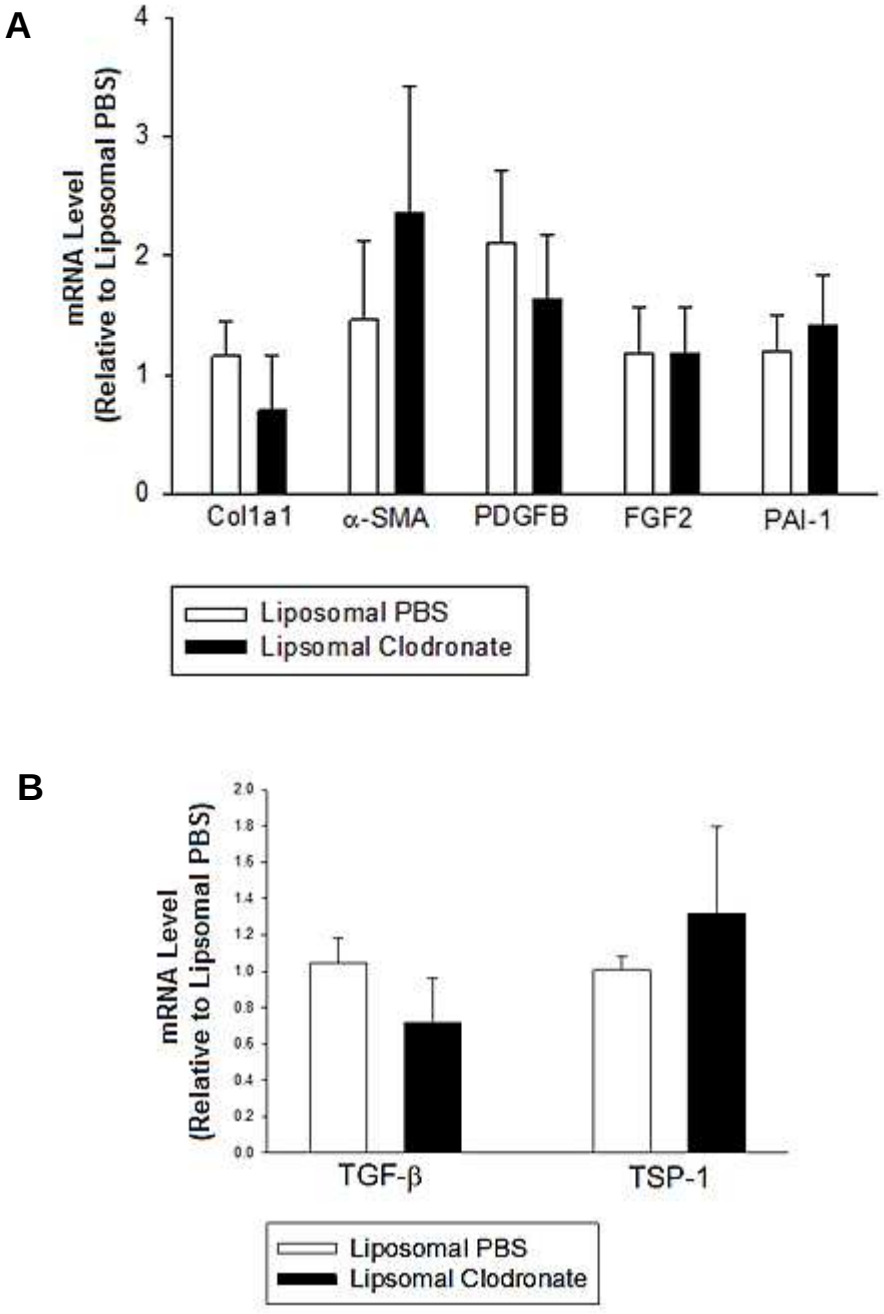

Figure 2-9: The effect of macrophage depletion on pro-fibrotic gene expression in the liver following carbon tetrachloride treatment.

mRNA levels of (A) genes involved in fibrosis and ECM repair and (B) TGF-β and TSP-1 in macrophage depleted livers as compared to control livers following 72 hours of carbon tetrachloride treatment.

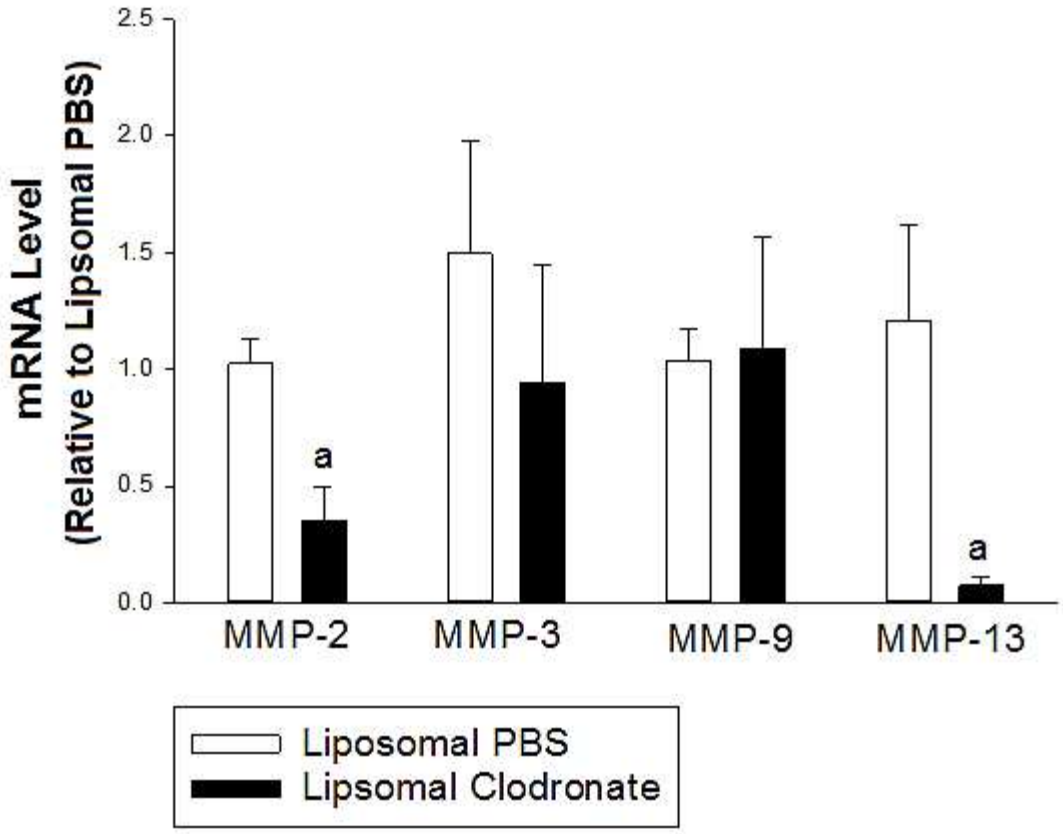

Figure 2-10: The effect of macrophage depletion on matrix metalloproteinase expression in the liver following carbon tetrachloride treatment.

mRNA levels matrix metalloproteinase proteins from whole liver samples of mice following liposomal PBS or liposomal clodronate treatment prior carbon tetrachloride treatment. mRNA expression was quantified 72 h after carbon tetrachloride treatment. [a]Significantly different from PBS-containing liposome treated samples.

DISCUSSION

M2 macrophages are known to produce anti-inflammatory cytokines that counteract the effects of the inflammatory response carried out by M1 macrophages (Duffield et al., 2005). Although the pro-inflammatory response is known to have a number of beneficial effects, it also has the potential to cause additional injury to the rest of the liver (Zimmermann et al., 2010). It was interesting to find that by depleting mice of macrophages, the ALT activity level and thus the level of hepatocyte injury was only modestly reduced when compared to non-depleted mice. Surprisingly, although the extent of liver injury as measured by ALT was modestly reduced, the area of necrosis was larger in the livers of mice depleted of macrophages when compared to non-depleted mice. This suggested that although the same level of hepatocyte death had occurred in the two groups, dead hepatocytes were not being cleared from the livers of macrophage depleted mice, and suggested that the phagocytic clearance of necrotic debris from the liver is primarily conducted by macrophages.

Another possibility is that the rate of hepatocyte proliferation in the livers of macrophage depleted mice is reduced as compared to the non-depleted mice. Macrophages produce the pro-inflammatory cytokines TNFα and IL-6 that have been shown to induce hepatocyte proliferation (Bradham et al., 1998; Zimmers, McKillop et al., 2003). Both cytokines were shown to be significantly decreased in the livers of macrophage depleted mice. However, the mRNA levels of genes involved in hepatocyte proliferation were unchanged in the macrophage depleted mice as compared to control mice. Also, the level of PCNA staining seen in the livers of macrophage depleted mice was not significantly different from control livers. Together, this showed that reducing the levels of TNFα and IL-6, by depleting macrophages, did not alter the rate of

hepatocyte proliferation. Since a number of redundant mechanisms control hepatocyte proliferation, it is possible that the decrease in TNFα and IL-6 were not significant enough to inhibit hepatocyte proliferation during normal liver repair.

Macrophages may also contribute to liver repair by the production of cytokines that induce production of various ECM components (Cordeiro-da-Silva et al., 2004). M2 macrophages are known to produce TGF-β, a potent inducer of collagen type I deposition, and other pro-fibrotic genes (Nunes et al., 1995). TGF-β is produced in a latent form, noncovalently associated with the latency associated peptide (LAP) (Koli et al., 2001). This complex is secreted from cells with LAP bound by disulfide linkages to latent TGF-β binding protein (LTBP) (Miyazono et al., 1991). LTBP localizes the complex to the ECM. In order to become biologically active, TGF-β is cleaved from LAP by proteins, such as plasmin, MMP-2, MMP-9 and TSP-1. Since macrophages are an important source of MMPs, these cells may affect matrix deposition by regulating activation of TGF-□. In addition to activating TGF-β, MMPs produced by macrophages can breakdown Type I collagen directly. It was interesting to find that in the livers of macrophage depleted mice, collagen type I was reduced at 72 hours following carbon tetrachloride treatment. This demonstrates that macrophages are involved in regulating the production of collagen Type I in the liver after an acute injury.

In order to determine the mechanism by which macrophages induce collagen production, the mRNA levels of genes that are classically known to be induced during fibrosis such as PDGF, FGF-2 and plasminogen activator inhibitor-1 (PAI-1) were investigated. However, all pro-fibrotic genes remained unchanged in macrophage depleted mice along with TGF-β. In contrast, MMP-2 and MMP-13 were significantly

decreased in the livers of macrophage depleted mice as compared to control mice. Since MMPs are known activators of TGF-β, it is possible that although the mRNA level of TGF-β was unchanged, the concentration of activated TGF-β in the liver following macrophage depletion was decreased as compared to control livers resulting in the decreased collagen production.

While a lack of TGF-β activation could be contributing to the decreased expression of Type I collagen in macrophage depleted livers, this effect should have been reflected by a decreased level of Col1a1 mRNA in the mice treated with clodronate liposomes. Instead, the mRNA levels of Col1a1 were unaffected by the depletion of macrophages. This suggests either a post-transcriptional inhibition of Col1a1 production or an increased degradation of Type I collagen protein. However, MMP-2 and MMP-13 mRNA expression were significantly decreased in macrophage depleted mice, while MMP-3 and MMP-9 mRNA levels were unchanged in the same mice. MMP-2 requires cleavage by membrane-type MMP-1 (MT-MMP-1) in order to gain activity. In addition, the family of tissue inhibitors of metalloproteinases (TIMPs) is known to inhibit MMP activity. Although the mRNA levels of MMP-2 and MMP-13 were lowered, the level of activity of MMPs could be increased by a decreased level of TIMPs or an increased level of MT-MMPS.

In summary, macrophages are involved in normal liver repair by phagocytic clearance of necrotic hepatocyte debris and regulation of ECM deposition. Although macrophage depletion did not exacerbate injury or decrease hepatocyte proliferation, the mechanisms by which macrophages regulate collagen deposition in normal liver repair will be an important topic for further investigation.

CHAPTER 3

MACROPHAGE POLARIZATION IN ACUTE LIVER INJURY: A PROSPECTIVE ADOPTIVE TRANSFER STUDY

ABSTRACT

The role of macrophage polarization in acute liver injury is not well understood. Macrophages have a high level of phenotypic plasticity and undergo polarization in order to carry out discrete functions as a part of the innate immune system. M1 macrophages are known to produce pro-inflammatory mediators such as IL-1, IL-6 and TNFα. On the other hand, M2 macrophages exhibit a high phagocytic activity and produce anti-inflammatory cytokines such as TGF-β and IL-10. In previous studies, M1 macrophages have been indirectly implicated in the exacerbation of liver injury following APAP treatment while cytokines produced by M2 macrophages have been shown to play a protective role against injury in this model. We are proposing to conduct a study that would directly demonstrate the involvement of M1 and M2 macrophages in liver injury and repair following acute exposure to a toxin. Mice will be depleted of macrophages and will receive either M1 or M2 macrophages by adoptive transfer following induction of acute liver injury. In order to determine the feasibility of this experiment, I have conducted the macrophage depletion, macrophage adoptive transfer, macrophage isolation and subsequent *in vitro* polarization. However, the ideal timeline in which the adoptive transfer should take place relative to the induction of acute injury has yet to be determined.

INTRODUCTION

Macrophage polarization is important for the inflammatory response and for the subsequent resolution of inflammation following hepatic injury (Edwards et al., 1992). M1 and M2 macrophages contribute to these processes by producing distinct classes of cytokines. Although there is indirect evidence that M1 macrophages exacerbate liver injury following exposure to a hepatotoxicant and that M2 macrophages protect the liver from injury, these distinct roles have not been shown directly (Bourdi et al., 2007; Ishida et al., 2002). The following studies are preliminary work in preparation for an experiment that would employ adoptive transfer to directly investigate the role of M1 and M2 macrophages on liver injury and repair following an acute exposure to a liver toxin.

Macrophages derive from the myeloid lineage and develop directly from the differentiation of monocytes. Monocytes are induced to polarize into an M1 phenotype by exposure to cytokines that typically activate T_H1 cells such as, interferon-gamma (IFNγ) and TNFα (Gordon & Taylor, 2005). M1 macrophages produce the pro-inflammatory factors IL-6, TNFα and nitric oxide (NO) (J. P. Edwards, Zhang, Frauwirth, & Mosser, 2006). Monocytes can also polarize into M2 macrophages after exposure to IL-4 and IL-13; cytokines that are involved in the initiation of differentiation and maintenance of T_H2 activation (Sica & Mantovani, 2012). M2 macrophages produce anti-inflammatory cytokines such as IL-10 and TGF-β (Sutterwala, Noel, Salgame, & Mosser, 1998). By the production of these cytokines, M1 and M2 macrophages can potentiate either a pro- or anti-inflammatory response by autocrine and paracrine signaling (Biswas & Mantovani, 2010). However, the distinct roles of M1 and M2 macrophage populations in the liver following acute injury remain unclear.

There has been some indirect evidence arguing that M1 macrophages contribute to liver injury while M2 macrophages aid in the resolution of liver inflammation and repair. In rodents depleted of TNF-α and IFNγ, pro-inflammatory cytokines that polarize monocytes into M1 macrophages, the level of injury in APAP treated mice was decreased as compared to control mice (Ishida et al., 2002; Morio et al., 2001). Also in IL-10 and IL-4 knockout mice as well as in wild type mice depleted of IL-13, the level of injury was increased as compared to controls following an acute liver injury (Bourdi et al., 2007; Yee, Bourdi, Masson, & Pohl, 2007). This suggests that cytokines produced by M1 macrophages exacerbate acute liver injury while cytokines produced by M2 macrophages protect the liver from injury.

In our studies, we will test this hypothesis by isolating macrophages from mice and polarizing them into either an M1 or M2 phenotype (Figure 3-0A). Next, separate mice will be depleted of macrophages and treated with a hepatotoxicant to induce acute liver injury (Figure 3-0B). These mice will then receive either M1 or M2 macrophages by adoptive transfer (Figure 3-0C). By polarizing M1 and M2 populations *in vitro* and adoptively transferring them into macrophage depleted mice that have been treated with a hepatotoxicant, this will directly demonstrate the effects of M1 and M2 macrophages in the liver following an acute injury.

The following preliminary work was necessary to ensure that each step of the experiment was yielding the expected results in order to maintain the integrity of the experiment as a whole. First, we determined that macrophages could be isolated and polarized *in vitro* properly and in adequate numbers (Figure 3-0A). Next, we determined that mice were in fact depleted of macrophages following an i.p. injection of liposomal

clodronate (Figure 3-0B). And finally we demonstrated the adoptive transfer of GFP-expressing macrophages into macrophage-depleted mice and their migration to the liver. The adoptive transfer of M1 and M2 polarized macrophages into the livers of mice depleted of macrophages following acute liver injury would provide a direct method to determine the effect of macrophage polarization following acute liver injury.

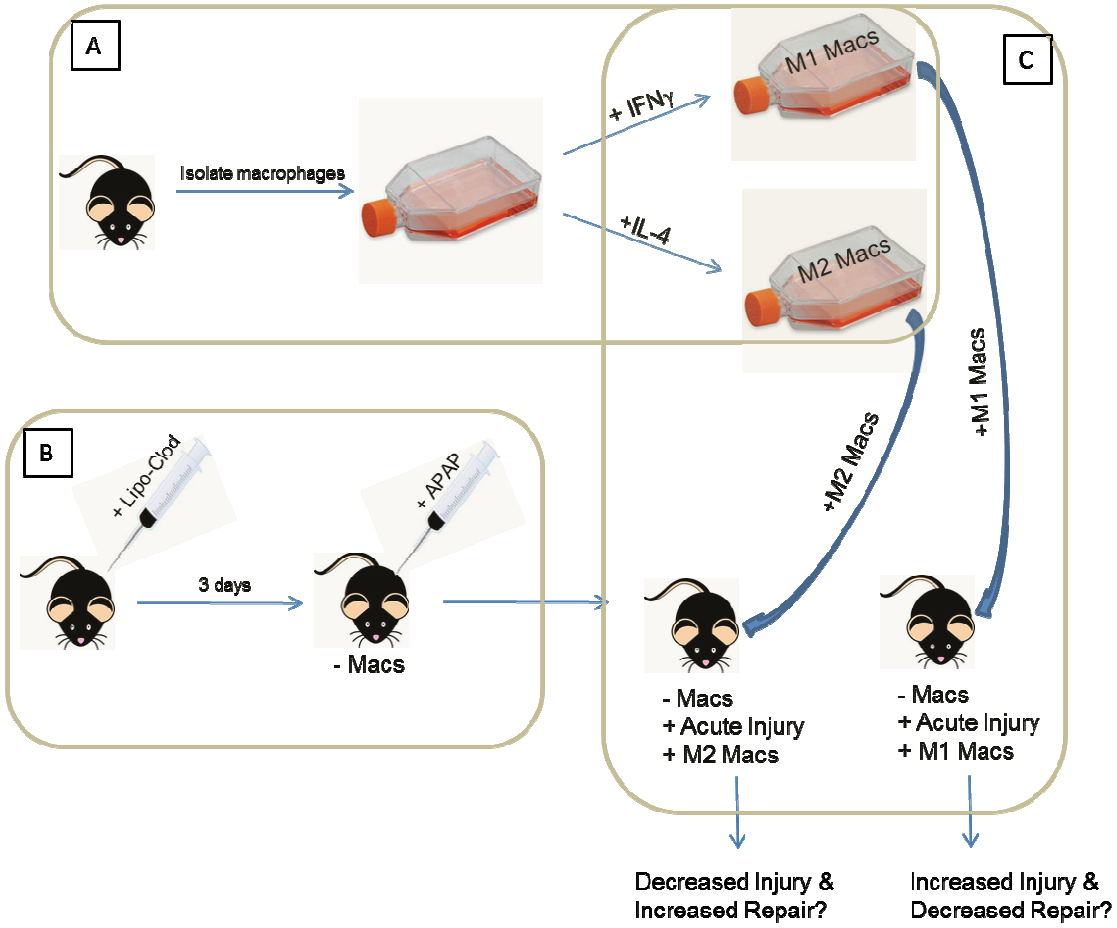

Figure 3-0: Adoptive transfer of M1 and M2 polarized macrophages into macrophage-depleted mice following acute liver injury. Bone marrow-derived macrophages will be isolated from male C57BL/6 mice and polarized into M1 and M2 macrophages *in vitro* (A). Mice will then be treated with liposomal clodronate to deplete macrophages or liposomal PBS as a control. The mice will also be treated with a hepatotoxic agent such as APAP or CCl_4 in order deplete macrophages (B). Mice previously depleted of macrophages will then have either M1 or M2 macrophages adoptively transferred by retro-orbital injection (C).

MATERIALS AND METHODS

Animals

Mice used in these studies were male C57BL/6 (Jackson Laboratories, Bar Harbor, ME) and GFP-expressing mice (C57BL/6-Tg(ACTB-EGFP)131Osb/LeySopJ, Jackson Laboratories) from 8 to 10 weeks of age were used in this study. All mice were maintained on a 12-h light/dark cycle under controlled temperature (18-21ºC) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health.

Macrophage Depletion

Mice were treated with 200µl of liposome encapsulated clodronate (ClodronateLiposomes.com, The Netherlands) or PBS-containing liposomes by i.p. injection.

Macrophage Adoptive Transfer

Peritoneal macrophages were isolated from GFP mice 72 hours after i.p. injection with 2 mL of 3% Brewer's thioglycollate. Macrophages were isolated as previously described (Zhang, Gonclaves, & Mosser, 2008). $5x10^6$ macrophages per mouse were injected retro-orbitally into 5 mice.

In vitro Bone Marrow-derived Macrophage Polarization

Bone marrow-derived macrophages were cultured as previously described (Zhang et al., 2008). Briefly, bone marrow cells were isolated from the femurs of C57BL/6 mice by flushing with 5mL of sterile PBS with 2% FBS. Following centrifugation at 50g for 10 minutes at RT, bone marrow cells were resuspended and plated in complete growth media (DMEM +10% FBS +10 ng/ml M-CSF) at a concentration of $4x10^4$ cells/ mL. Cells were incubated in 37℃ and 5% CO2 . On day 3, the old complete media was aspirated and new complete growth media was added to each plate. On day 7, media was changed to either M1 differentiation media (DMEM +10% FBS +50ng/mL IFNγ) or M2 differentiation media (DMEM +10%FBS +15ng/mL IL-4). M1 macrophages were incubated for 24 hours and M2 macrophages were 48 hours prior to characterization.

RNA Isolation and qRT-PCR

Macrophages were lysed in 500μL of TRI reagent (Sigma Chemical Company, St. Louis, MO) and total RNA was isolated per manufacturer's instructions. Methods for qRT-PCR were conducted as previously described in Chapter 2, Materials and Methods Section, pages 9-10. PCR primers were used as follows: mouse iNOS forward: 5'-TTCTGTGCTGTCCCAGTGAG-3' and reverse: 5'-TGAAGAAAACCCCTTGTGCT-3'; mouse Arg1 forward: 5'-TTTTTCCAGCAGACCAGCTT-3' and reverse: 5'-AGAGATTATCGGAGCGCCTT-3'; mouse TNFα forward: 5'-AGGGTCTGGGCCATAGAACT-3' and reverse: 5'-CCACCACGCTCTTCTGTCTAC-3'; mouse TGF-β forward: 5'-CAACCCAGGTCCTTCCTAAA-3' and reverse: 5'-GGAGAGCCCTGGATACCAAC-3'.

Immunohistochemistry and Immunofluorescence

Immunofluorescence was used to detect and quantify macrophages in 8μm frozen sections of liver. The sections were fixed in 4% formalin and then incubated with rat anti-CD68 antibody diluted 1:100 (Novus, Littleton, CO) or rat anti-F4/80 antibody diluted 1:25 (AbD Serotec, Raleigh, NC) and chicken anti-GFP antibody diluted 1:500 (Aves Labs, Tigard, OR). The sections were then incubated with secondary antibodies conjugated to either Alexa Fluor 488 or Alexa Fluor 594 (Life Technologies, Grand Island, NY).

Statistics

Results are presented as the mean $\pm$ SEM. 5-7 mice were used for all *in vivo* studies. 3 mice were used for all *in vitro* studies. Data were analyzed by unpaired Student's t-test. The criterion for significance was $p < 0.05$ for all studies.

RESULTS

In order to carry out the adoptive transfer experiment, macrophages were first depleted from mice by i.p. injection of liposomal clodronate (Figure 3-1). To confirm depletion of macrophages in the liver, macrophages were detected by immunofluorescent staining of the macrophage markers CD68 and F4/80 as described in Chapter 2. CD68 and F4/80-positive macrophages were detected in liver sections from mice treated with PBS-containing liposomes (Figure 3-1A and 3-1C). In contrast, staining of both markers was decreased in livers following liposomal clodronate treatment (Figure 3-1B and 3-1D).

Next, we determined whether macrophages injected retro-orbitally migrate to the liver of macrophage-depleted mice. For this study, macrophages were isolated from mice that ubiquitously express green fluorescent protein (GFP) in all cell types, and injected retro-orbitally into macrophage depleted mice. Adoptively transferred macrophages were then detected in the liver by co-immunofluorescence staining for GFP and the macrophage markers CD68 and F4/80. As shown in Figure 3-2, GFP expressing cells in the liver also expressed F4/80 (Figure 3-2A) or CD68 (Figure3-2B) indicating that the macrophages administered to mice by retro-orbital injection repopulated the livers of macrophage-depleted mice.

Finally, M1 and M2 macrophages were polarized *in vitro* from bone-marrow derived macrophages. Bone marrow cells were isolated from the femur of C57BL/6 mice. Hematopoietic stem cells were then differentiated into macrophages by incubation with monocyte colony stimulating factor (M-CSF). And the mature macrophages were then polarized into either M1 or M2 cells by IFNγ and IL-4 treatment, respectively. Both the M1 and M2 cultures were analyzed for specific markers indicating both classical and

alternative activation. As expected, mRNA levels of iNOS, TNFα and HIF-1α were highest in M1 macrophages (Figures 3-3A, B & C), whereas mRNA levels of HIF-2α and TGF-β were highest in M2 macrophages (Figures 3-3D & E).

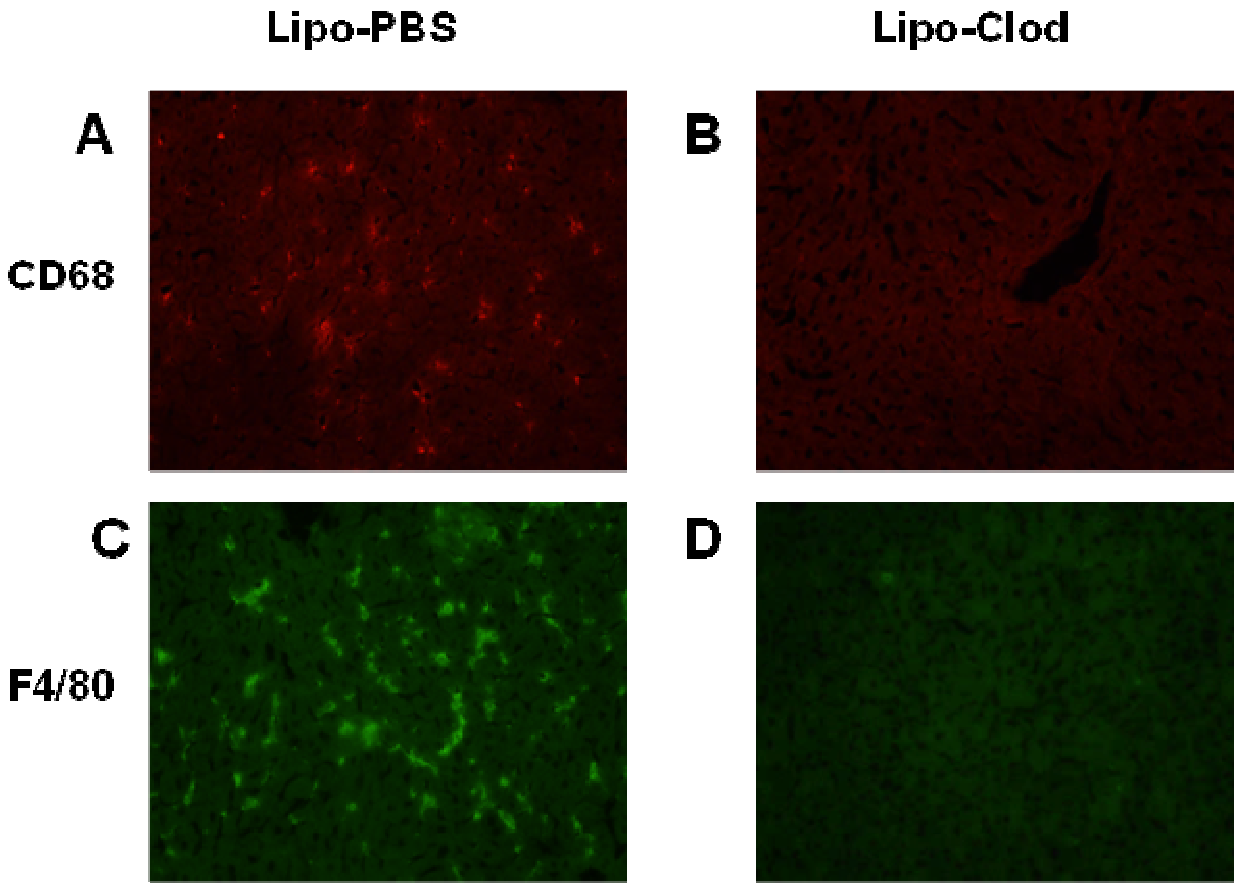

Figure 3-1: Hepatic macrophage depletion in the liver following liposomal clodronate treatment. Male C57BL/6 mice were treated with either (A and C) PBS-containing liposomes or (B and D) clodronate-containing liposomes for 72 hours. Representative CD68 immunofluorescence (green staining) in frozen liver sections from (A) PBS-containing liposomes or (B) clodronate-containing liposomes. Representative F4/80 immunofluorescence (red staining) in livers sections from (C) PBS-containing liposomes or (D) clodronate-containing liposomes.

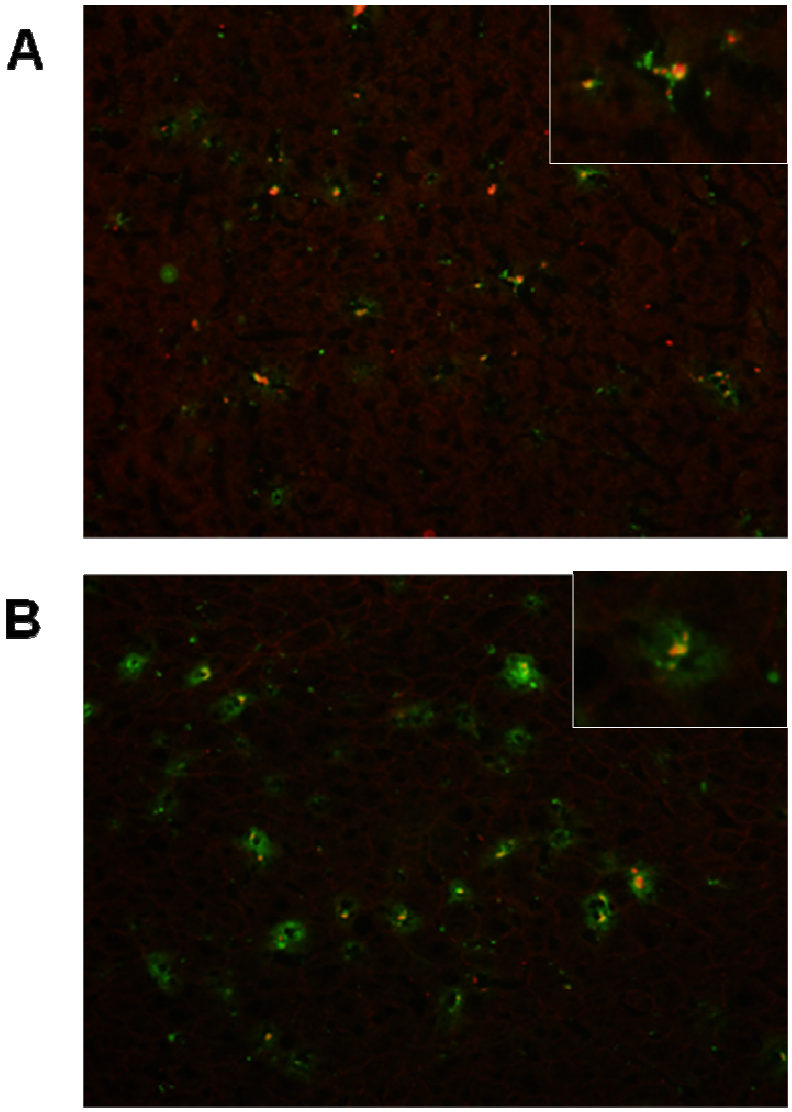

Figure 3-2: Adoptive transfer of GFP-expressing macrophages into macrophage depleted mice. Male C57BL/6 mice were treated with 200µL of liposomal clodronate for 72 hours to deplete macrophages. Thioglycollate-elicited peritoneal macrophages were isolated from GFP-expressing mice and adoptively transferred into macrophage-depleted mice by retro-orbital injection. Macrophages and GFP were then detected by immunofluorescence. (A) Representative F4/80 (red staining) and GFP (green staining) immunofluorescence in liver sections. (B) Representative CD68 (red staining) and GFP (green staining) immunofluorescence in liver sections. Yellow indicates co-localization of GFP with macrophage markers.

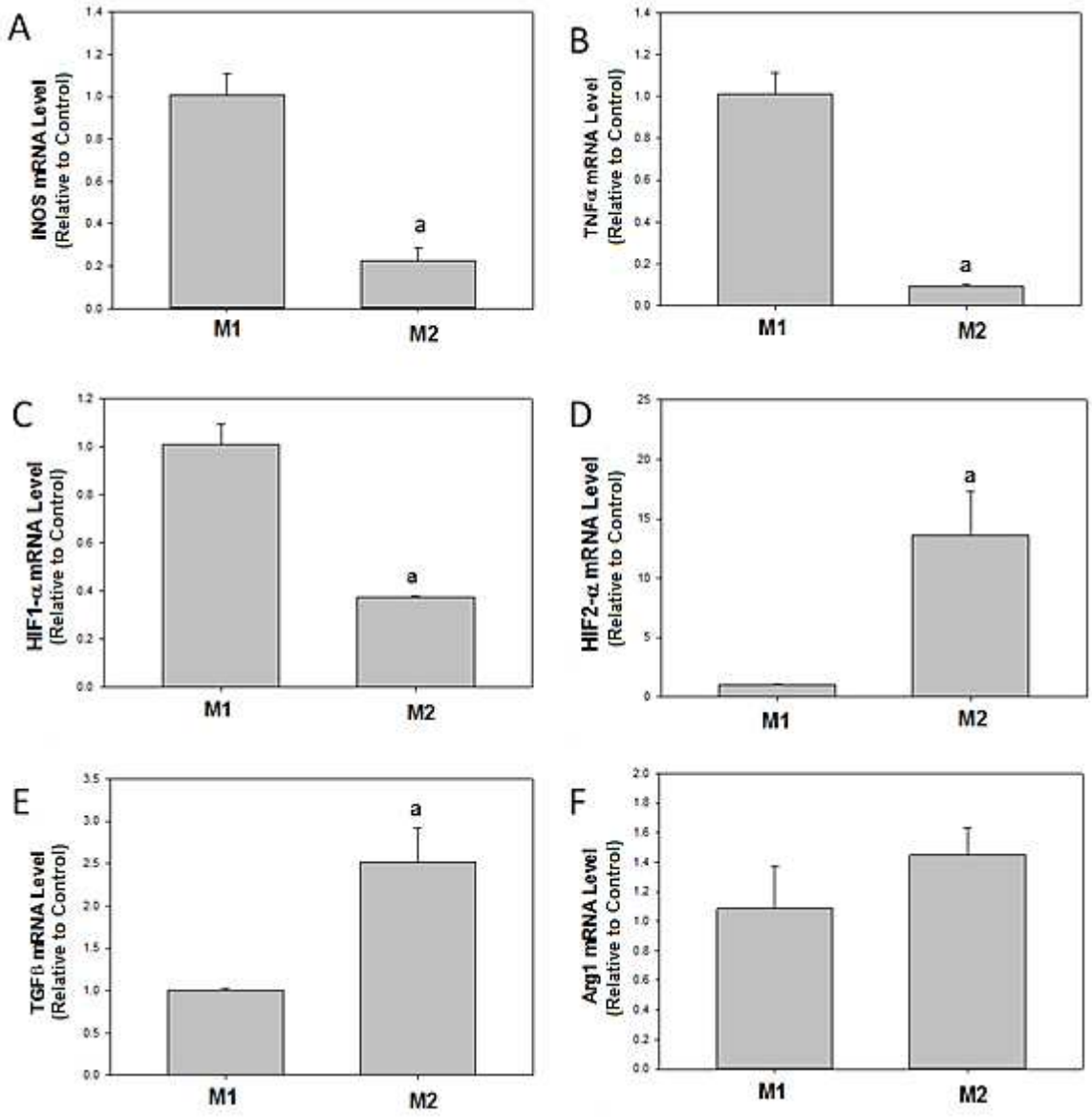

Figure 3-3: Polarization of bone marrow derived macrophages *in vitro*.

Macrophages were isolated, differentiated and polarized, as previously described. Quantitative real-time PCR was used to measure mRNA levels of the indicated genes. Data represent means ± SEM, n=3. [a]Significantly different from M1 macrophages.

DISCUSSION

In the future, the adoptive transfer of M1 and M2 macrophages is highly feasible. I have demonstrated that macrophages can be successfully depleted from the liver and that macrophages injected retro-orbitally into a host mouse, will migrate to the liver. Also M1 and M2 macrophages can be successfully differentiated and polarized *in vitro.*

However, the ideal time for the adoptive transfer to take place relative to the induction of acute liver injury is another factor that will require optimization prior to conducting the study. Other cell types in the liver produce chemotactic factors in order to induce monocyte migration into the liver following injury. Circulating macrophages infiltrate into the liver by the binding of chemokine (C-C motif) ligand 2 (CCL2) also called, monocyte chemoattractant protein-1 (MCP-1) to the C-C chemokine receptor type 2 (CCR2) (Karlmark, Wasmuth, & Trautwein, 2008). Activated stellate cells, hepatocytes, macrophages and endothelial cells produce CCL2 following acute liver injury (Baeck et al., 2012). For this reason, macrophages may migrate to the liver in larger numbers if the adoptive transfer follows the induction of the liver injury. However, depleting the liver of macrophages prior to causing acute injury may cause a higher degree of liver injury than would be present by having the adoptive transfer precede the induction of acute liver injury (Ju et al., 2002). More studies will need to be conducted in order to determine the ideal time course of this experiment.

Another confounding factor could include the high degree of phenotypic plasticity of macrophages. Macrophage phenotype can be altered by a change in the cytokine milieu in the extracellular environment (Davis, Tsang, & Qiu, 2013). For example, M1 macrophages can switch to an M2 phenotype following exposure to IL-4, IL-13 or after phagocytosis of necrotic tissue (Ramachandran et al., 2012). While this property of

macrophages aids in the *in vitro* polarization, this property may be a problem once *in vivo.* Since the cytokine milieu of the liver and circulatory system changes throughout the progression of acute liver injury and subsequent repair, it is possible that macrophages may undergo another phenotypic switch at different stages of the acute liver injury and repair (Porcheray et al., 2005). It will be important to determine if this occurs. This in itself may provide insight into the mechanisms that drive macrophage polarization during liver injury and repair.

In summary, this experiment provides a method to test the effects of macrophage polarization in the liver following acute injury. This experiment also provides a method to test the impact of macrophage polarization on liver repair.

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