# ROLE OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 DURING LIVER REGENERATION

by

Stephanie Wyler

A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in Biology

Boise State University

May 2015

BOISE STATE UNIVERSITY GRADUATE COLLEGE

# DEFENSE COMMITTEE AND FINAL READING APPROVALS

of the thesis submitted by

Stephanie Wyler

Thesis Title: Role of Monocyte Chemoattractant Protein-1 During Liver Regeneration

Date of Final Oral Examination: 16 March 2015

The following individuals read and discussed the thesis submitted by student Stephanie Wyler, and they evaluated her presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

| | |
|---|---|
| Kristen A. Mitchell, Ph.D. | Chair, Supervisory Committee |
| Kenneth A. Cornell, Ph.D. | Member, Supervisory Committee |
| Julia Thom Oxford, Ph.D. | Member, Supervisory Committee |

The final reading approval of the thesis was granted by Kristen A. Mitchell, Ph.D., Chair of the Supervisory Committee. The thesis was approved for the Graduate College by John R. Pelton, Ph.D., Dean of the Graduate College.

# DEDICATION

This thesis is dedicated my family and colleagues. First and foremost to my brother, James Wyler. Jimmy, your support has been instrumental in all of my achievements and your quest for knowledge is truly an inspiration. Also to my mother. Your memory lives on with me in my sense of curiosity and wonder. I would like to recognize my father, Gary Wyler, and brother, Andrew Wyler, for their support and encouragement as well.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Kristen Mitchell, for her guidance in my scientific inquiry. Her passion for scientific excellence has made this project an amazing experience. I would like to thank Dr. Julia Oxford and Dr. Cheryl Jorcyk for their support as members of my thesis committee.

No scientific knowledge can be advanced without the support of a scientific community, and for that I would like to acknowledge the wonderful experience of inquiry at Boise State University in the Mitchell Lab. Thank you to all my colleagues including Chris Horras, Ashley McCartney, Wendy Harvey, Shawna D'Ingillo, Cheri Lamb, and Eva Amouzougan. I would also like to thank Eric Tretter for his assistance with editing and reviewing this thesis.

This project was supported by the following grants from the National Institutes of Health: R15DK088749, P20GM103408, 1UL1RR025014, and P20GM109095 (COBRE in Matrix Biology at Boise State), and by start-up funds from the State of Idaho and Boise State University.

## ABSTRACT

Liver regeneration is a complex process that requires the coordinated expression of cytokines and growth factors. One well-studied model of liver regeneration is partial hepatectomy (PH), in which removal of 70% of the liver initiates compensatory hepatocyte proliferation. PH-induced liver regeneration requires the activation of resident macrophages (Kupffer cells), which produce cytokines that drive hepatocyte proliferation. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that is known to activate macrophages and recruit monocytes during tissue injury. The goal of this study was to determine how MCP-1 contributes to macrophage activation during liver regeneration. Results indicate that hepatic and plasma MCP-1 levels increased within 12 hr after PH and correlated with hepatic recruitment of cells expressing the MCP-1 receptor, CCR2. Nevertheless, hepatocyte proliferation was comparable in MCP-1 knockout and wild-type mice, as was the expression of Kupffer cell-derived cytokines. Furthermore, hepatic recruitment of $CCR2^+$ cells was similar in MCP-1 knockout and wild-type mice, which suggests that other chemokines may efficiently recruit $CCR2^+$ cells in the absence of MCP-1. CCR2 appears to be required for optimal regeneration, as CCR2 knockout mice had levels of hepatocyte proliferation that were 50% lower than wild-type mice 36 hr after PH. We conclude that MCP-1 is not required for macrophage activation during PH-induced liver regeneration. Future studies should instead focus on mechanisms by which CCR2 signaling events and the hepatic recruitment of CCR2-expressing cells facilitates hepatocyte proliferation during liver regeneration.

TABLE OF CONTENTS

LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| BEC | Biliary Epithelial Cells |
| BM | Bone Marrow |
| BRDU | Bromodeoxyuridine |
| CCR2 | CC Chemokine Receptor-2 |
| HSC | Hepatic Stellate Cells |
| IL-1 | Interleukin- 1 |
| IL-6 | Interleukin- 6 |
| KC | Kupffer Cells |
| LPS | Lipopolysaccharide |
| LSEC | Liver Sinusoid Endothelial Cells |
| MCP-1 | Monocyte Chemoattractant Protein-1 |
| PAB | Phosphate Buffered Saline with Sodium Azide and Bovine Serum Albumin |
| PH | Partial Hepatectomy |
| RBC | Red Blood Cell |
| TNFα | Tumor Necrosis Factor- Alpha |

# CHAPTER 1: INTRODUCTION

## Overview

Liver regeneration is an amazing phenomenon, in which removal of a portion of the liver induces remaining cells to proliferate to restore organ mass. The capacity of the liver to regenerate has been recognized for thousands of years. In fact, it is referenced in the Greek myth of Prometheus, who was a titan accused of stealing fire from the gods of Mount Olympus. As punishment for his treachery, Prometheus was tied to a rock, and his liver was eaten by an eagle each day only to grow back during the night, thus causing perpetual torment (Power & Rasko, 2008).

While the human liver does not actually regenerate overnight, it does have the capacity to restore its mass and function within just a few months after widespread damage or tissue loss (Aoki et al., 2011). The physiology of liver regeneration has been extensively investigated, but it is still not completely understood. For example, the precise mechanisms that initiate and terminate regeneration have yet to be identified. Furthermore, the identification of these mechanisms is confounded by pathological factors, such as inflammation or viral disease, which often accompany human liver disease.

## Liver Anatomy and Physiology

The liver performs several essential physiological functions. One major function of the liver is to synthesize proteins. In fact, almost all of the major plasma proteins are

produced in the liver. These proteins include albumin, which is essential for maintaining osmotic pressure in the circulatory system, as well as transferrin, prothrombin, fibrinogen, lipoproteins and complement proteins. The liver also functions in regulation of lipid, protein and glucose metabolism (Tacke, Luedde, & Trautwein, 2009), and it is richly populated with immune cells, allowing it to participate in both innate and adaptive immune responses (Tacke et al., 2009). Finally, perhaps the most recognized function of the liver is detoxification of exogenous and endogenous compounds via the induction of a wide array of xenobiotic metabolizing enzymes, such as the cytochrome P450 enzyme family (Hakkola, Tanaka, & Pelkonen, 1998).

## Blood Flow Through the Liver

The liver is a highly perfused organ. Nutrient-rich blood from the gastrointestinal tract is routed to the liver through the hepatic portal vein, whereas oxygen-rich blood is delivered to the liver through the hepatic artery (Fig. 1.1). Portal blood mixes with arterial blood in small, permeable capillaries, known as sinusoids, which permeate the liver. Sinusoids deliver blood to the hepatocytes, enabling them to carry out metabolic processes. Blood then empties into central veins that coalesce into the hepatic vein, which returns blood to the systemic circulation (Wheatley et al., 1997).

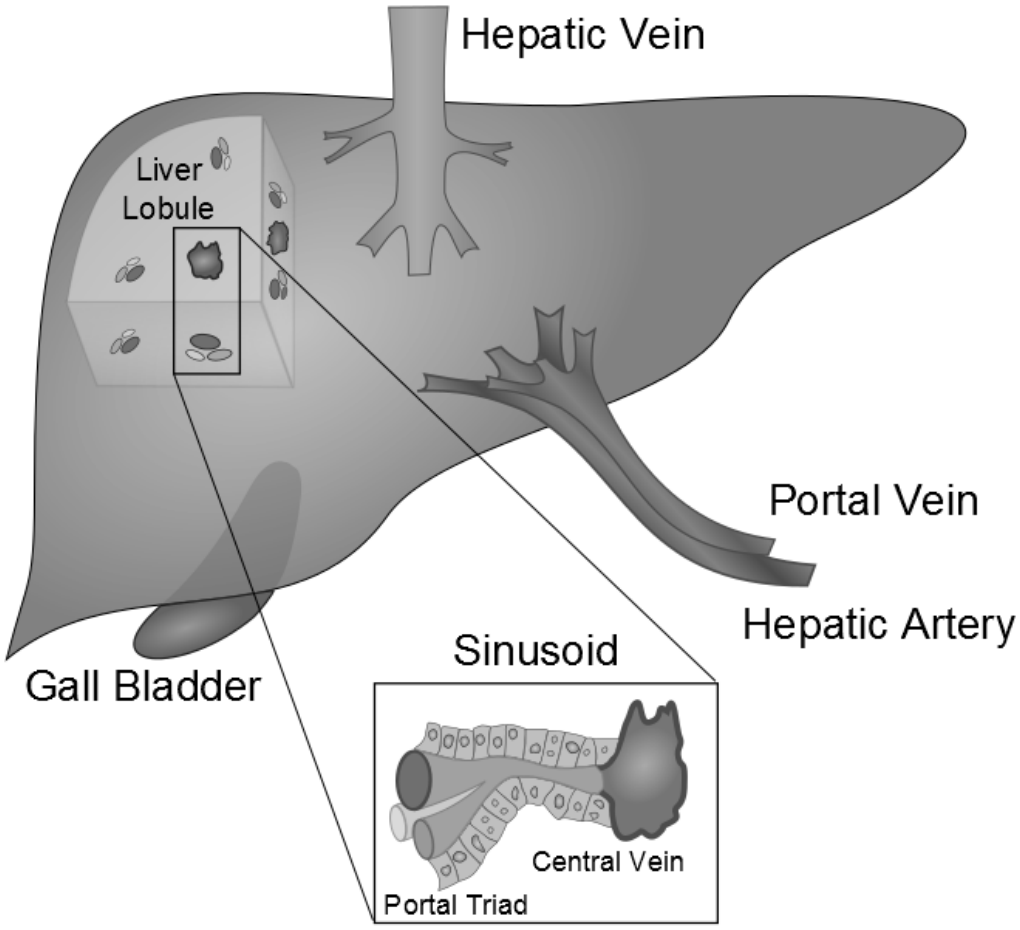


**Figure 1.1 Blood Flow Through the Liver.** Oxygenated blood is carried to the liver through the hepatic artery. Nutrient-rich, oxygen-poor blood is delivered from the intestines to the liver though the portal vein. Blood mixes in small, permeable capillaries known as sinusoids. The sinusoids allow blood to flow from the portal triads to the central veins, which coalesce and drain into the hepatic vein, which returns blood to the systemic circulation.

Bile Production

In addition to receiving a dual blood supply, another interesting feature of the liver is its role in producing bile. Hepatocytes use cholesterol to synthesize bile, which helps emulsify fats in the small intestine (Chiang, 2009). Bile is collected from all areas of the liver through bile ductules, which coalesce and exit the liver through the common bile duct. The liver architecture is divided into lobules, with each lobule receiving blood

and producing bile. The lobules intersect each other at structures called portal triads, which consist of branches of the hepatic portal vein and hepatic artery, as well as a bile ductule (Fig. 1.1). The abundance of portal triads throughout the liver ensures adequate perfusion of blood and efficient collection of bile.

## Hepatocytes

The most common cell in the liver is the parenchymal hepatocyte, which comprises 60% of all liver cells and 80% of the liver volume (Ramadori & Saile, 2002). Hepatocytes are a specialized type of epithelial cell that stack tightly together to form plates that are separated by sinusoids. Hepatocytes are polarized and have distinct apical, lateral, and basal surfaces that aid in uptake and secretion of substances. The basal surface of the hepatocyte faces the sinusoid and contains microvilli that increase surface area to facilitate exchange of materials. The apical surface also has many microvilli and faces the bile canaliculi (Ramadori & Saile, 2002). The lateral surfaces of hepatocytes are connected by gap junctions to allow the flow of molecules between cells. Hepatocytes perform the bulk of liver functions including detoxification and plasma protein production.

## Nonparenchymal Cells

While the most abundant type of cell in the liver is the parenchymal hepatocyte, other non-parenchymal cells exist and are important for liver homeostasis. Non-parenchymal cells comprise 35% of all liver cells and include sinusoidal endothelial cells, resident macrophages, hepatic stellate cells, and biliary epithelial cells, which are also called cholangiocytes (Fig. 1.2). Other less abundant non-parenchymal cells include

immune cells, such as resident T cells and natural killer cells. Non-parenchymal cells contribute to liver homeostasis through diverse mechanisms, such as regulation of blood flow, bile transport, vitamin A storage, and immune surveillance.

### Liver Sinusoid Endothelial Cells

Liver sinusoid endothelial cells line the capillary sinusoids. These cells are highly fenestrated, which allows blood to easily exit the capillary lumen and bathe the surrounding sheets of hepatocytes, where nutrients and soluble factors are exchanged. Following liver injury, these cells are capable of proliferating, and additional progenitors are recruited from the bone marrow (DeLeve, 2013). During liver regeneration, sinusoid endothelial cells not only proliferate, but also secrete soluble mediators, such as hepatocyte growth factor, which is a mitogen for hepatocyte proliferation.

### Hepatic Stellate Cells

Hepatic stellate cells (HSC) reside between the LSEC and hepatocytes in an area referred to as the space of Disse. In the healthy liver, HSCs store 80% of the body's vitamin A as retinol esters (Friedman, 2008). However, liver injury may induce these cells to assume a myofibroblast-like phenotype, characterized by the production of extracellular matrix proteins, such as collagen type I. Indeed, HSCs are central to the wound healing response, and inappropriate activation of these cells plays a central role in the development of liver fibrosis (Kawser, Iredale, Winwood, & Arthur, 1998). During liver regeneration, HSCs are thought to be an abundant producer of hepatocyte growth factor (Skrtic et al., 1997).

Biliary Epithelial Cells

Biliary epithelial cells are non-parenchymal cells that line the bile ducts that transport bile to the small intestine (Katayanagi, Kono, & Nakanuma, 1998). These cells are cuboidal epithelial cells that have been shown to secrete cytokines (Isse, Harada, & Nakanuma, 2007; Katayanagi et al., 1998). These cells have been shown to proliferate and recruit leukocytes during several pathological conditions of the liver (Isse et al., 2007).

Immune Cells of the Liver

Immune cells are an important component of the liver. Because blood is delivered directly from the gastrointestinal tract to the liver, the liver is tasked with removing foreign substances and potential pathogens from the blood before it reaches the systemic circulation. Resident immune cells, such as T and B lymphocytes, natural killer cells, and macrophages, are crucial for removing pathogens and initiating an adaptive immune response, if necessary.

Resident macrophages in the liver, also known as Kupffer cells (KC), line the sinusoids and scavenge particulate debris in blood that enters the liver. They are derived from circulating monocytes. These cells play a critical role in detoxifying blood from the portal circulation. In fact, large periportal KC are the first cells to encounter pathogens that reach the liver through the portal vein. Upon contact with pathogens, KC become activated and secrete soluble mediators, such as tumor necrosis factor-α (TNFα), prostaglandin-$E_2$, and interleukin (IL)-1. Smaller KC are located near the central vein of liver lobules (Bilzer, Roggel, & Gerbes, 2006). Although KC typically reside in the liver for up to 14 months, they are capable of replication (Dory et al., 2003). Also, KC

numbers can be replenished when circulating monocytes enter the liver and differentiate into macrophages. During liver regeneration, macrophages are critical sources of IL-6 and TNFα, which are necessary for hepatocyte proliferation (Selzner et al., 2003).

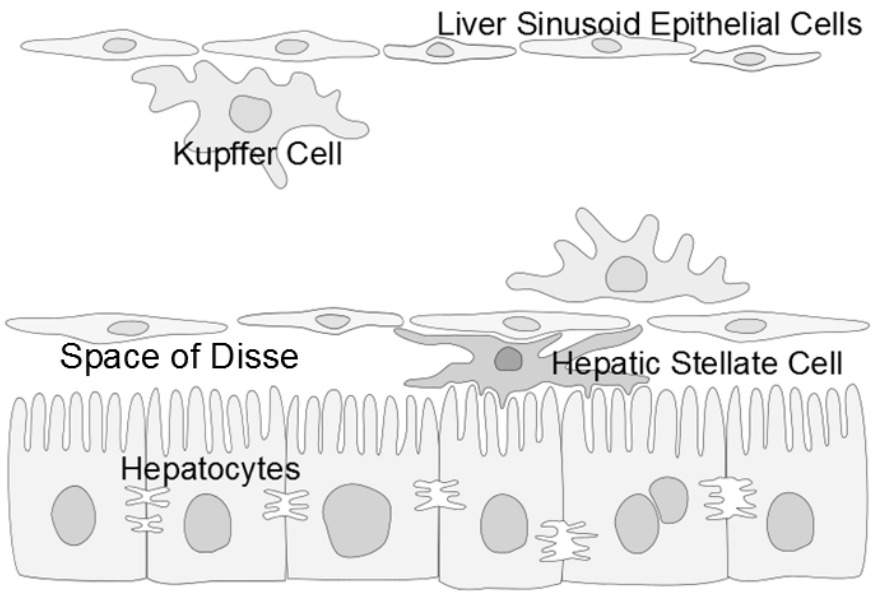


**Figure 1.2 Cells of the Liver.** The liver is comprised of several cell types. Parenchymal hepatocytes are arranged in sheets that are lined by sinusoidal capillaries. The sinusoidal epithelium is highly fenestrated and is lined with endothelial cells. Hepatic stellate cells are non-parenchymal cells found in the space of Disse between the hepatocytes and sinusoids. Macrophages known as Kupffer cells line the sinusoidal endothelium.

## Regenerative Ability of the Liver

The liver is constantly exposed to foreign material and potentially toxic chemicals, as it receives blood from the stomach and intestines. Nevertheless, mammalian hepatocytes typically have low turnover (Malato et al., 2011). However, hepatocyte proliferation may be induced depending on the severity of the insult. The regenerative capacity of the liver may have evolved as protection against ingested

toxicants (Taub, 2004). This regenerative process appears to depend on a complex symphony of soluble mediators and cellular events and has been well studied in animal models of partial hepatectomy (PH), in which a substantial portion (usually 70-80%) of the liver is resected. After PH, resident nonparenchymal cells produce soluble mediators that signal the hepatocytes, as well as other cells in the liver, to proliferate in a fairly synchronized manner and restore liver mass.

It is a hotly debated issue whether stem cells exist in the liver, and if so, how extensively they contribute to regeneration. Observation of hepatocytes moving from periportal to centrilobular locations during maturation has been interpreted by some to be evidence of a stem cell population in the periportal region of the lobule (Sigal et al., 1995). Despite the continual research to discover a population of stem cells in the liver, it has been shown that existing, mature hepatocytes can replicate and restore liver mass without the involvement of stem cells (Sharma, Cantz, Manns, & Ott, 2006).

One model system that demonstrates the proliferative capacity of mature hepatocytes is fumarylacetoacetate hydrolase-deficient mice (Overturf, AlDhalimy, Ou, Finegold, & Grompe, 1997). Fumarylacetoacetate hydrolase is an enzyme that catalyzes the hydrolysis of 4-fumarylacetoacetate into fumarate and acetoacetate. Without this enzyme, buildup of toxic metabolites leads to progressive liver damage and cell loss. When a small number of healthy adult hepatocytes were transplanted into the liver of fumarylacetoacetate hydrolase-deficient mice, the cells completely repopulated the liver and restored liver function (Overturf et al., 1997). In fact, it was found that the cells could be serially transplanted from one enzyme-deficient mouse to another, which demonstrates the nearly limitless proliferative capacity of adult hepatocytes.

Liver regeneration following PH is referred to as compensatory regeneration, in which cells proliferate to restore lost organ mass. Compensatory hyperplasia occurs when large areas of the liver are damaged or physically removed. A physiologically relevant event that could lead to compensatory hyperplasia is widespread liver necrosis due to the effects of a toxicant. However, in the laboratory, surgical resection of the liver is typically preferred to chemical-induced necrosis for inducing regeneration because the excessive inflammatory response associated with necrosis can be a confounding factor in understanding normal regenerative processes.

Compensatory regeneration should not be confused with two other types of regeneration: mitogen-induced hyperplasia and leading edge regeneration. Mitogen-induced hyperplasia refers to hepatocyte proliferation that occurs in response to mitogens in the absence of tissue injury or loss (Columbano et al., 1990). In this type of hyperplasia, hepatocytes must overcome normal growth control mechanisms to proliferate, and under such conditions, the increased liver mass is called “augmentative hepatomegaly” (Michalopoulos, 2013). When the mitogen is removed, the original organ mass is restored as excess cells undergo apoptosis (Columbano, Leddacolumbano, Lee, Rajalakshmi, & Sarma, 1987). Whereas the liver can undergo compensatory regeneration and mitogen-induced hyperplasia, it does not participate in leading-edge regeneration, in which only those cells that are positioned along a damaged edge of a tissue are capable of dividing. A classic example of leading edge regeneration is limb regeneration in salamanders, in which amputation of the limb results in regrowth of the entire limb. This process is also analogous to limb development during embryogenesis (Nacu & Tanaka, 2011).

## PH Model of Liver Regeneration

The 70% PH model has been extensively used to investigate mechanisms of liver regeneration (Michalopoulos, 2010). In mice, 70% PH consists of surgical resecting three of the five main liver lobes (Greene & Puder, 2003). Following resection, parenchymal and non-parenchymal cells in the remnant liver proliferate in a fairly synchronized manner. Peak DNA synthesis in hepatocytes occurs within 24 hours in rats and 36 hours in mice (Mao, Glorioso, & Nyberg, 2014; Michalopoulos & DeFrances, 1997). The precise mechanisms by which liver regeneration is regulated, including the onset and termination of regeneration, are poorly understood. Once the mass of the liver is restored, cells presumably receive signals that terminate their proliferation. The proliferative process typically results in a slight overproduction of liver cells but is followed by a wave of apoptosis, which restores the liver to within 5% of its previous mass (Michalopoulos, 2013).

## Phases of Liver Regeneration

Liver regeneration can be divided into two phases, referred to as priming and progression (Fig. 1.3), both of which are required for complete regeneration (Fausto, 2000). Priming refers to a collection of events that culminate in the movement of quiescent liver cells into the cell cycle, whereas progression refers to those events that facilitate continued progression through the cell cycle, resulting in actual cell division.

### Priming and Progression of Liver Regeneration

Hepatocyte priming is mediated by the production of TNFα and IL-6 by Kupffer cells (Baier et al., 2005). These soluble mediators bind to their cognate receptors on

hepatocytes and induce the activity of transcription factors, such as NF-kB, STAT3, AP1, and CF/EBP-beta (Kountouras, Boura, & Lygidakis, 2001). Changes in hepatocyte gene expression prepare these cells to respond to growth factors produced during the progression phase of regeneration. For example, hepatocytes upregulate c-Met, which is the receptor for hepatocyte growth factor, and EGFR, which is the receptor for epidermal growth factor (Su, Guidotti, Pezacki, Chisari, & Schultz, 2002). During the progression phase of regeneration, hepatocyte replication is dependent on the availability of these growth factors, as well as other soluble mediators (Fausto, Campbell, & Riehle, 2006).

### Termination of Liver Regeneration

Termination of liver regeneration is precisely regulated, yet the mechanisms that halt hepatocyte proliferation are poorly understood. Previous research implicated TGF-β due to its well known antiproliferative activity, but this has not been definitely proven (Macias-Silva, Li, Leu, Crissey, & Taub, 2002). Recent evidence suggests a possible role for caspases, which are cysteine-aspartic proteases important for apoptosis, necrosis, and inflammation (Alnemri et al., 1996). For example, caspase recruitment domain-containing protein-11 was recently found to be upregulated during termination of liver regeneration (Nygard et al., 2012). Nevertheless, the exact molecular mechanisms of this termination phase remain unclear.

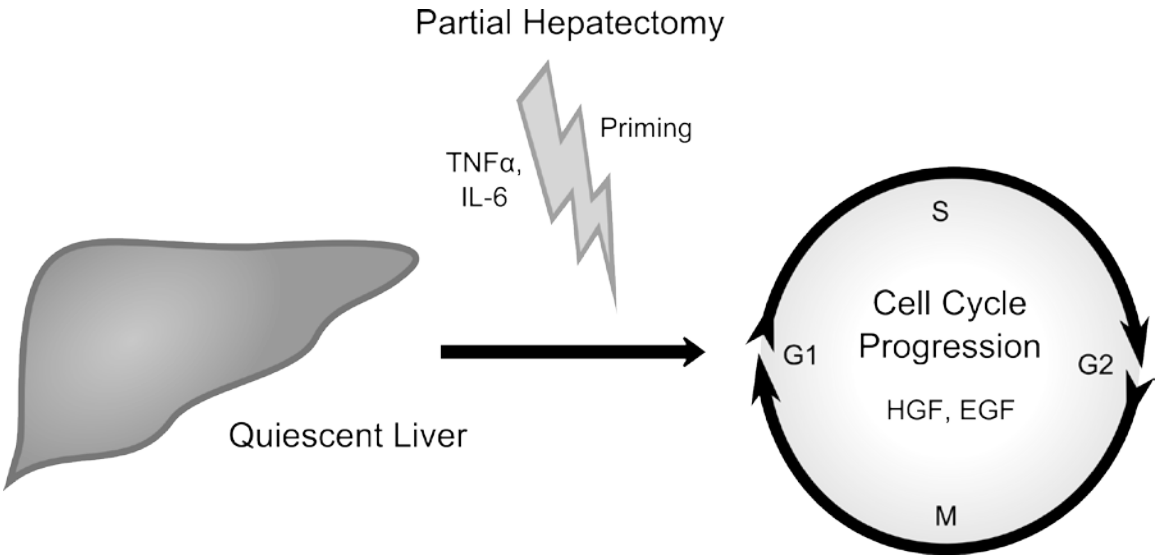


**Figure 1.3 Phases of Liver Regeneration.** In the healthy liver, hepatocytes are quiescent. Removal of a substantial portion of the liver through PH induces macrophages to produce TNFα and IL-6, which primes the hepatocytes for subsequent progression through the cell cycle, which is dependent on growth factors, such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF).

Monitoring Liver Regeneration

Several methods can be used to monitor progression of liver regeneration. A common measure is liver weight and the liver-body weight ratio. While this method is straightforward, it can be influenced by factors not directly related to regeneration, such as glycogen and lipid content in the liver, as well as blood volume. A commonly used approach for measuring liver regeneration is quantifying the number of proliferating hepatocytes. This is often accomplished through the administration of the nucleotide analog bromodeoxyuridine (BrdU), which is incorporated into the newly synthesized DNA strand in place of thymidine (Assy & Minuk, 1997). BrdU incorporation indicates that a hepatocyte has progressed to S-phase of the cell cycle and has successfully duplicated its DNA. BrdU incorporation is typically quantified in hepatocytes using flow cytometry or immunohistochemistry.

## Role of Macrophages in Liver Regeneration

### Macrophage Numbers Increase Following PH

In healthy liver tissue, KC make up the largest population of nonparenchymal cells. These liver cells maintain their population by self-renewal and also via recruitment of monocytes from the bone marrow or spleen (Naito, Hasegawa, & Takahashi, 1997). Following PH, the number of KC in the liver increases within 1 hour (Baier et al., 2005). There is another increase in the number of KC 6-12 hr following PH, and this increase is comprised mainly of activated cells (Baier et al., 2005). It has been suggested that the early increase in macrophage number is due to the recruitment of monocytes, which are macrophage precursors, from the bone marrow (Minamino et al., 2012). The second increase is believed to result from proliferation of activated resident macrophages (Ukai et al., 1990).

### Macrophages Become Activated After PH

It is hypothesized that changes in blood flow through the liver may be involved in priming hepatocytes after PH (Michalopoulos, 2010; Mochida, Ohta, Ogata, & Fujiwara, 1992). After removal of 70% of the liver, blood from the portal vein and hepatic artery is still being delivered to the liver at normal flow rates, despite the fact that the organ volume is markedly decreased, which results in increased intrahepatic pressure. Increased blood flow to the liver enhances the delivery of molecules that can potentially activate resident KC, including bacterial lipopolysaccharide (LPS), which is known to activate macrophages (Shiratori et al., 1996). In fact, LPS is a well-known inducer of macrophage-derived IL-6 and TNFα, both of which are necessary for priming of

hepatocytes immediately following liver regeneration (Fujihara et al., 2003). Increased intrahepatic pressure following PH can also stimulate KC to secrete vasoactive factors, such as heme oxygenase (HO)-1 and endothelial nitric oxide synthase (eNOS) (Abshagen, Eipel, Kalff, Menger, & Vollmar, 2008). Kupffer cells are critical for regulating vascular permeability in the liver (Abshagen et al., 2008). When KC are depleted, blood flow in liver sinusoids during regeneration is decreased (Abshagen et al., 2008). Hence, KC-mediated changes in microcirculation may be critical to initiation of liver regeneration via increased intrahepatic pressure or enhanced delivery of LPS.

Macrophages Mediate the Priming Phase of Liver Regeneration

Cells of the monocyte-macrophage lineage are necessary for priming hepatocytes during liver regeneration through the production of IL-6 and TNFα (Taub, 2004). First, TNFα is secreted by KC in response to activation by the bacterial antigen, LPS. TNFα functions in an autocrine fashion to stimulate IL-6 secretion. IL-6 binds to receptors on the cell surface of hepatocytes. This leads to activation of STAT3, which is a transcription factor that drives the expression of immediate early genes that drive hepatocyte cell cycle progression. These events are summarized in Figure 1.4.

Numerous studies demonstrate that monocyte/macrophage-derived TNFα and IL-6 are necessary for normal liver regeneration to proceed. For example, in one study, mice were irradiated to destroy their immune cell populations, including monocytes and macrophages (Sudo et al., 2008). Immune cells were repopulated through the transfer of bone marrow from wild-type, TNFα knockout, or IL-6 knockout mice, and the mice were subjected to PH. Mice that received bone marrow from TNFα or IL-6 knockout mice had

decreased rates of hepatocyte DNA synthesis compared to those that received transplants from wild-type mice.

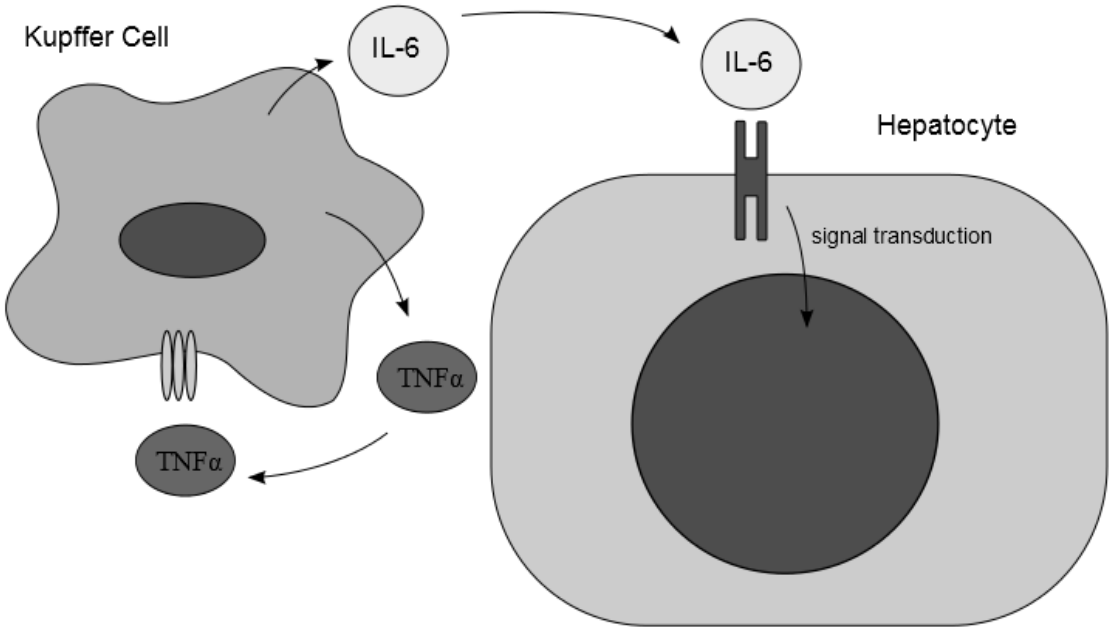


**Figure 1.4 KC Prime Hepatocytes to Enter the Cell Cycle.** Upon activation, KC secrete TNFα, which stimulates the secretion of IL-6. IL-6 binds to receptors on hepatocytes and stimulates expression of genes necessary for hepatocyte cell cycle progression.

### Role of MCP-1 in Monocyte Recruitment and Macrophage Activation

During inflammatory responses, resident macrophages in injured tissue become activated, and additional monocytes are recruited from the bone marrow and circulation to the injured tissue, where they differentiate into activated macrophages. Monocyte recruitment occurs in response to chemokines, which are proteins that regulate white blood cell trafficking as well as inflammation (Mantovani et al., 2004; Rossi & Zlotnik, 2000). Chemokines are categorized based on the position of cysteine (C) residues at the

N-terminus: CC, CXC, C, and CX3C (Deshmane, Kremlev, Amini, & Sawaya, 2009; Mantovani et al., 2004).

The first human CC chemokine discovered was monocyte chemoattractant protein-1 (MCP-1), also known as CC-chemokine ligand-2 (CCL2) (Yadav, Saini, & Arora, 2010). Regulated at the transcriptional level, MCP-1 expression is induced by a variety of stimuli, including LPS and changes in blood flow (Taub, 2004; Uguccioni, Dapuzzo, Loetscher, Dewald, & Baggiolini, 1995). During liver regeneration, MCP-1 appears to be primarily produced by Kupffer cells, but it can also be secreted by hepatic stellate cells and endothelial cells (Marra, 2002).

MCP-1 works through a G-protein coupled receptor, CCR2, which is expressed on the surface of many leukocytes, including monocytes, T-cells, and NK cells (Deshmane et al., 2009). Although at least three MCP-1 receptors exist, CCR2 is the primary receptor for MCP-1 in mice (Charo et al., 1994). CCR2 is expressed on Kupffer cells (Deshmane et al., 2009), which can be activated and recruited to sites of inflammation by MCP-1 (Boring et al., 1997; Kurihara, Warr, Loy, & Bravo, 1997).

In the liver, MCP-1 expression is increased during chronic hepatitis, inflammation and fibrosis (Czaja, Geerts, Xu, Schmiedeberg, & Ju, 1994; Marra, 2002; Marra et al., 1998), and inactivation of MCP-1 has been shown to attenuate liver injury by inhibiting macrophage recruitment (Imamura, Ogawa, Sasaguri, Chayama, & Ueno, 2005; Zamara et al., 2007). Impaired monocyte migration has also been reported for mice that lack CCR2 (Boring et al., 1997). However, little is known about the consequences of MCP-1/CCR2 signaling during PH-induced liver regeneration.

## Hypothesis

Given the significance of MCP-1 to monocyte recruitment and macrophage activation, we hypothesized that this chemokine is important for activating monocytes and resident KC during liver regeneration. We took advantage of the availability of transgenic mice to determine how MCP-1 expression and CCR2 expression contribute to hepatocyte proliferation during PH-induced liver regeneration. The specific aims of the study were as follows:

1) To characterize MCP-1 expression during liver regeneration in wild-type mice.
2) To determine if MCP-1/CCR2 is required for monocyte recruitment, macrophage activation, and hepatocyte proliferation during liver regeneration.

# CHAPTER 2: MATERIALS AND METHODS

## Mice

Female C57Bl/6 (wild-type), MCP-1 knockout (KO) (strain B6.129S4-Ccl2$^{tm1Rol}$/J), and CCR2 KO (strain B6.129S4-Ccr2$^{tm1Ifc}$/J) mice were purchased from the Jackson Lab (Bar Harbor, ME) and used at 8-9 weeks of age. MCP-1 and CCR2 KO mice are fertile and display no gross physical or behavioral abnormalities. Furthermore, numbers of macrophages (peritoneal, alveolar, and Kupffer cells) in these KO mice are similar to levels found in wild type mice (Boring et al., 1997; Lu et al., 1998). Mice were housed in a temperature-controlled facility on a 12-hr light/dark cycle with unlimited access to food and water. Mice were anesthetized with inhaled isoflurane, and 70% PH was performed as previously described (Mitchell, Lockhart, Huang, & Elferink, 2006). After euthanasia, blood was collected at the axillary plexus, plasma was recovered by centrifugation, and remnant liver tissue was collected. The experimental protocol was approved by the Animal Studies Subcommittee and the Research and Development Committee of the Boise VA Medical Center (Boise, ID), where the animals were housed. 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.

## Measurement of MCP-1 Levels

At each time point, tissues from 3–5 mice were used to measure MCP-1 mRNA levels in the liver and MCP-1 protein levels in liver homogenates and plasma. To measure hepatic MCP-1 mRNA levels, total RNA was isolated from frozen liver tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Transcript levels were measured by quantitative real-time PCR (qRT-PCR) at the Molecular Genomics Core at the University of Texas Medical Branch (Galveston, TX). To measure MCP-1 protein levels in the liver, frozen liver tissue was homogenized in PBS containing 0.5% Triton X-100 0.05% sodium azide, and protease inhibitors. MCP-1 levels were measured in liver homogenates by ELISA (Thermo Scientific Pierce, Rockford, IL) and expressed as pg per mg liver protein, based on a Lowry protein assay (Bio-Rad, Hercules, CA). Plasma levels of MCP-1 were measured using a Cytometric Bead Array (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Unpooled plasma samples were run in duplicate.

## Quantification of F4/80$^+$ Cells by Immunohistochemistry

Paraffin-embedded liver tissue was incubated with proteinase K for 3 minutes to retrieve antigen. Tissue was then incubated overnight with a biotinylated rat-anti-mouse F4/80 antibody (Invitrogen, Carlsbad, CA) and visualized using an anti-rat HRP-DAB Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN). Eight fields of 400X magnification were counted from each mouse.

### Measurement of TNFα and IL-6 Production by ELISA

Levels of TNFα and IL-6 were measured in unpooled plasma samples using sandwich enzyme-linked immunosorbent assay kits (Pierce Biotech, Rockford, IL). The assays were performed according to the manufacturer's instructions, and all samples were run in duplicate. The lower limit of detection for the assays was 50 pg/ml.

### Measurement of Stat3 Activation by Western Blot

Frozen liver tissue was mechanically homogenized as described elsewhere (Mitchell et al., 2006). Cellular debris was pelleted at 10,000 X *g*, and protein concentration was determined by using a Lowry protein assay (Bio-Rad, Hercules, CA). Homogenates (25-50 μg protein/lane) were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes for western blot analysis with anti-STAT3 and anti-phosphorylated STAT3 antibodies (Cell Signaling Technology, Danvers, MA).

### BrdU Labeling and Detection

To measure hepatocyte proliferation, mice were injected intraperitoneally with 50 mg/kg bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) two hours prior to euthanasia. In a separate experiment, continuous BrdU labeling was achieved by administering BrdU in the drinking water (0.8 mg/ml). Water bottles containing BrdU were protected from light and replenished daily until mice were killed 6 days postoperatively. After euthanasia, fresh liver tissue was fixed in UltraLight™ fixative (Bi-Biomics, Nampa, ID), paraffin-embedded, and processed for immunohistochemical staining. Tissue sections (5-um) were incubated with a biotinylated anti-BrdU antibody (Invitrogen, Carlsbad, CA) followed by avidin-conjugated horseradish peroxidase and the

substrate 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin. For each animal, at least five random 400X fields were examined, and a total of 800-1000 nuclei were counted. The number of brown (DAB)-stained nuclei ($BrdU^+$) was expressed as a percentage of total number of nuclei.

### Detection of $CCR2^+$ Cells by Immunofluorescence Staining

Paraffin-embedded tissue sections (5-μm) were processed and cut for immunofluorescence staining. Antigen retrieval was carried out in sodium citrate buffer at 95°C for 15 minutes. Sections were incubated with a rabbit monoclonal anti-CCR2 antibody (Novus, Littleton, CO) followed by a FITC-conjugated goat anti-rabbit antibody (BD Biosciences, San Diego, CA). $CCR2^+$ cells were visualized using a fluorescent microscope.

### Detection of $CCR2^+$ Cells by Flow Cytometry

Nonparenchymal cells were isolated from the liver and analyzed by flow cytometry. Briefly, livers were perfused with Hank's Buffered Salt Solution (HBSS) containing 50 mM EDTA, transferred to RPMI 1640 with 2.5% FBS, minced into a slurry, and poured through a nylon cell strainer. Samples were centrifuged at 60 x *g* for 1 min at room temperature (RT), and pellets containing hepatocytes were discarded. Samples were then centrifuged at 500 x *g* for 10 min at room temperature. Resulting pellets were re-suspended in Percoll in RPMI 1640 without FBS and centrifuged at 850 x *g*, 30 min at room temperature. Pellets were depleted of red blood cells by hypotonic lysis, and remaining cells were resuspended in PBS containing 1% fetal bovine serum. Cells were then incubated with $F_c$-Receptor Block (BD Biosciences, San Jose, CA) for 10

min before staining with a rabbit monoclonal anti-CCR2 antibody (Novus, Littleton, CO) followed by a FITC-conjugated goat anti-rabbit antibody (BD Biosciences, San Diego, CA). Stained cells were analyzed on an Accuri C6 flow cytometer (Ann Arbor, MI). At least 50,000 events (viable cells) were collected from unpooled samples and analyzed using Accuri CFlow Plus software.

## Statistical Analysis

Data were analyzed using Prism (version 4.0, GraphPad Software, San Diego, CA). Data were evaluated by one-way ANOVA followed by a Dunnett's post-hoc test, or by two-way ANOVA and Bonferroni post-hoc test. Data were considered significantly different at $p \leq 0.05$.

# CHAPTER 3: RESULTS

## MCP-1 Levels Increase After PH

To examine MCP-1 production after PH, MCP-1 mRNA levels were quantified in the remnant liver, and protein levels were measured in liver homogenates and plasma. Hepatic MCP-1 mRNA levels peaked 90 minutes after PH (Fig. 3.1A), followed by increased MCP-1 protein expression in the regenerating liver 4 hr and 6 hr after PH (Fig. 3.1B). A four-fold increase in circulating MCP-1 was detected in the plasma 12 hr after PH (Fig. 3.1C).

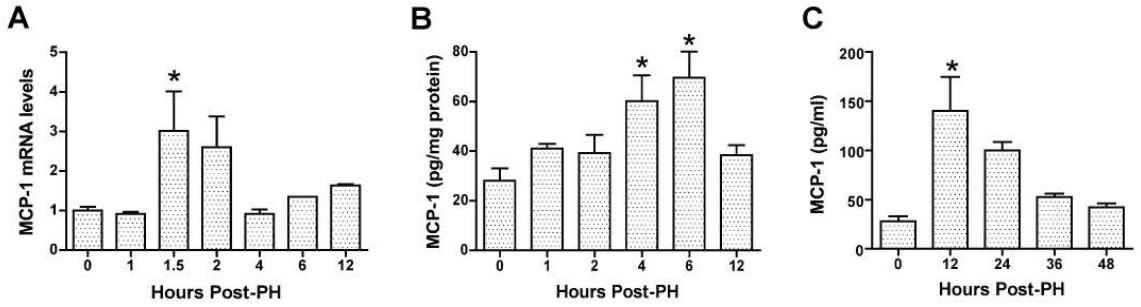


**Figure 3.1 MCP-1 Levels Increase After PH.** *A:* MCP-1 mRNA levels in the regenerating liver at the indicated times after PH. MCP-1 mRNA levels (mean +/- SEM) are expressed as fold-induction relative to expression of 18S rRNA in the same samples (n=3). *B:* MCP-1 protein levels in the liver after PH. MCP-1 levels (mean +/- SEM) in liver homogenates were normalized to the total amount of protein in each sample (n=3-5). *C:* MCP-1 protein levels (mean +/- SEM) in the plasma after PH (n=3-6). * $P < 0.05$ when compared to 0-hr group based on one-way ANOVA followed by Dunnett's test.

## MCP-1 is Not Required for Recruitment of Hepatic F4/80$^+$ Macrophages

It has been demonstrated that hepatic-derived MCP-1 stimulates the expansion and egress of a population of F4/80$^+$ monocytes in the bone marrow (Crane, Hokeness-Antonelli, & Salazar-Mather, 2009). Hence, we hypothesized that MCP-1 is important for

recruiting bone marrow-derived monocytes to the regenerating liver and that numbers of hepatic macrophages would be decreased in the absence of MCP-1. Using wild type and MCP-1 knockout (KO) mice, we performed a cursory examination of liver macrophages based on expression of F4/80 antigen. Contrary to our hypothesis, no changes in F4/80 expression were detected in the regenerating liver of wild type and MCP-1 KO mice (Fig. 3.2).

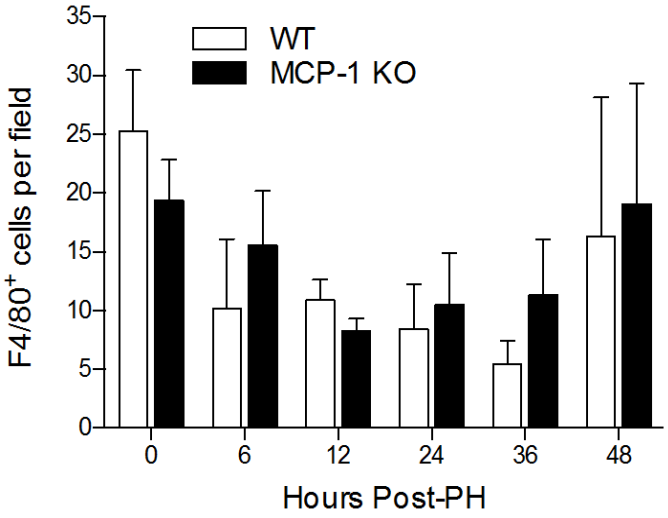


**Figure 3.2 Number of Macrophages is Similar in the Liver of Wild Type and MCP-1 KO Mice.** Data represent average number of F4/80$^+$ cells (+/- SEM) in the regenerating liver of wild type and MCP-1 knockout mice at the indicated times after PH. The number of F4/80$^+$ cells was counted in 8 separate 40x fields per mouse from 3-5 mice per treatment group.

**MCP-1 is Not Required for TNFα or IL-6 Production During Liver Regeneration**

During the priming phase of liver regeneration, the production of TNFα and IL-6 is attributed to activated Kupffer cells (Taub, 2004). Kupffer cells express the MCP-1 receptor, CCR2, and have been shown to become activated in response to MCP-1 in other model systems (Marra, 2002). Hence, we hypothesized that MCP-1 may influence the production of Kupffer cell-derived cytokines during liver regeneration. However,

measurement of plasma cytokine levels revealed no difference in TNFα or IL-6 production between wild type and MCP-1 KO mice (Fig. 3.3). Hepatic mRNA levels of these cytokines were below the limit of detection (data not shown).

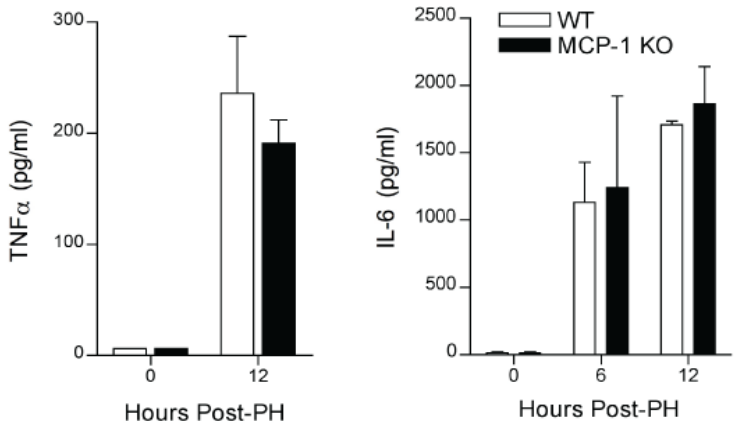


**Figure 3.3 Levels of Kupffer Cell-Derived Cytokines are Similar in Wild Type and MCP-1 KO Mice.** Data represent plasma levels (mean +/- SEM) of TNFα and IL-6 in wild type and MCP-1 KO mice at the indicated times after PH. Cytokines were measured in unpooled plasma samples by ELISA (n=3-5) and run in duplicate.

## MCP-1 is Not Required for Priming of Hepatocytes During Liver Regeneration

The production of TNFα and IL-6 by Kupffer cells is implicated in priming hepatocytes for cell cycle progression (Aldeguer et al., 2002). Upon binding to its cognate receptor on hepatocytes, IL-6 activates Stat3 signaling pathways, leading to gene expression that facilitates hepatocyte proliferation. Hence, Stat3 activation is a pivotal step in regeneration that is likely to be dependent on macrophage activation. Our results indicate that hepatic levels of phosphorylated Stat3 increased 6 and 12 hr after PH in both wild type and MCP-1 KO mice, suggesting that MCP-1 is not necessary for hepatocyte priming during liver regeneration (Fig. 3.4).

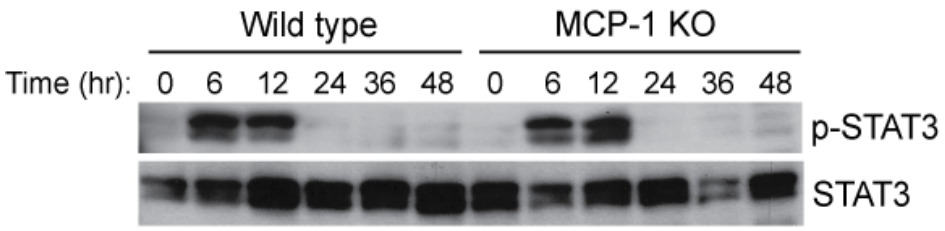


**Figure 3.4 MCP-1 is Not Required for Stat3 Activation During Liver Regeneration.** Representative western blot analysis of Stat3 and phosphorylated-Stat3 proteins in the regenerating liver of wild type and MCP-1 KO mice at the indicated times after PH (20 μg protein/lane).

### MCP-1 is Not Required for Hepatocyte Proliferation After PH

Finally, we investigated the ramifications of MCP-1 production on the regenerative capacity of the liver. No differences in liver-body weight ratios were detected between wild type and MCP-1 KO mice (Fig. 3.5A). Pulse labeling with BrdU revealed that robust hepatocyte proliferation occurred 36 and 48 hr after PH in wild-type mice and was not suppressed in MCP-1 KO mice (Fig. 3.5B). Likewise, continuous BrdU administration revealed no overt differences in BrdU incorporation in the regenerating liver of wild type and MCP-1 KO mice 6 days post-PH (Fig. 3.5C and 3.5D).

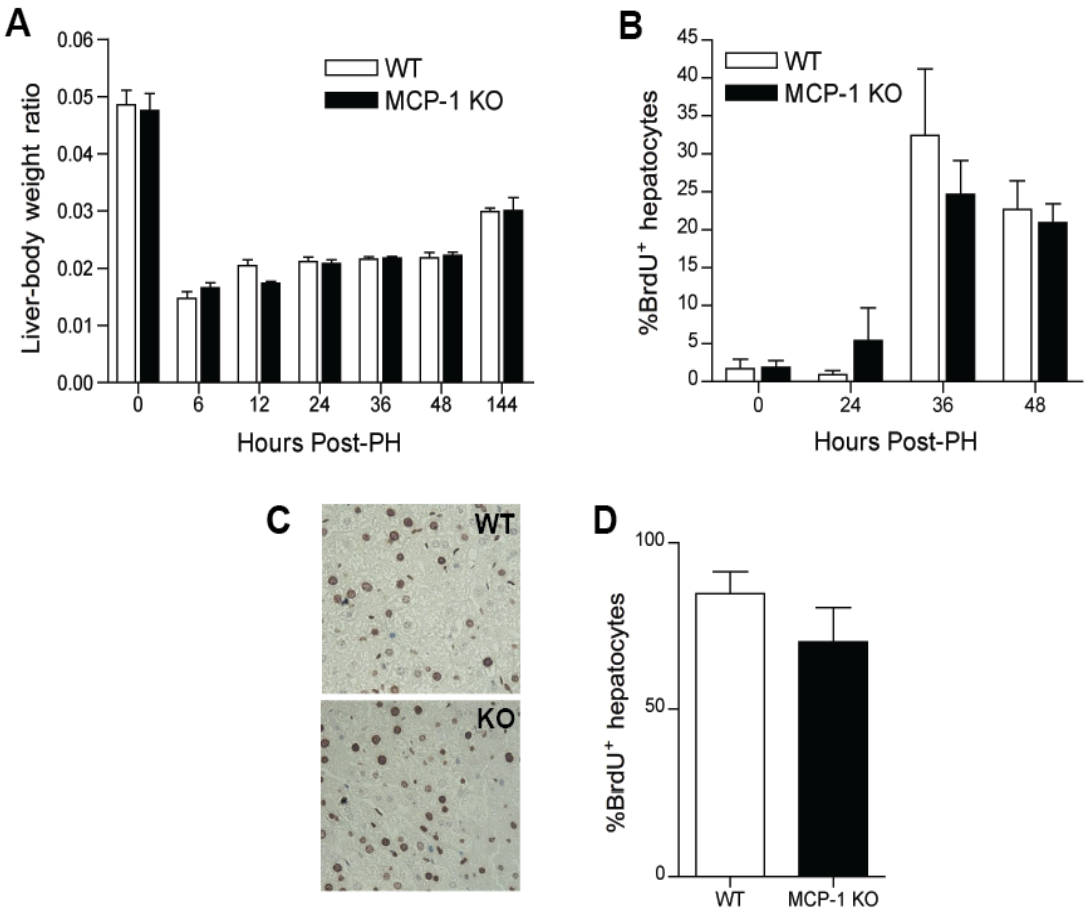


**Figure 3.5 MCP-1 is Not Required for Hepatocyte Proliferation After PH.** *A:* Liver-body weight ratios (mean +/- SEM) of wild type and MCP-1 KO mice at the indicated times after PH (n=4-10). *B:* Percentage (mean +/- SEM) of $BrdU^+$ hepatocyte nuclei from wild type or MCP-1 KO mice at the indicated times after PH. Mice were pulsed with BrdU 2 hr prior to euthanasia at the indicated time points (n=6-10). *C:* Representative BrdU incorporation in regenerating liver tissue from wild type and MCP-1 KO mice 6 days after PH; BrdU was added to the drinking water continuously for 6 days. *D:* Cumulative BrdU incorporation in the regenerating liver of wild type and MCP-1 KO mice administered BrdU in their drinking water for 6 days post-PH. Data represent the percentage (mean +/- SEM) of $BrdU^+$ hepatocyte nuclei from 4 mice per treatment group.

### MCP-1 is Not Required for Recruitment of $CCR2^+$ Cells to the Regenerating Liver

In addition to activating resident macrophages, MCP-1 may also recruit cells, such as bone-marrow-derived monocytes, to the damaged liver (Liaskou et al., 2012). To investigate the possibility that cell recruitment was diminished in the absence of MCP-1, the prevalence of $CCR2^+$ cells in the regenerating liver was measured.

Immunofluorescence staining revealed equivalent numbers of $CCR2^+$ cells in the liver of wild-type and MCP-1 KO mice 12 hr after PH (Fig. 3.6A). This finding was confirmed using flow cytometry, which revealed no changes in either the percent or total number of $CCR2^+$ non-parenchymal cells in wild-type and MCP-1 KO mice (Fig. 3.6B).

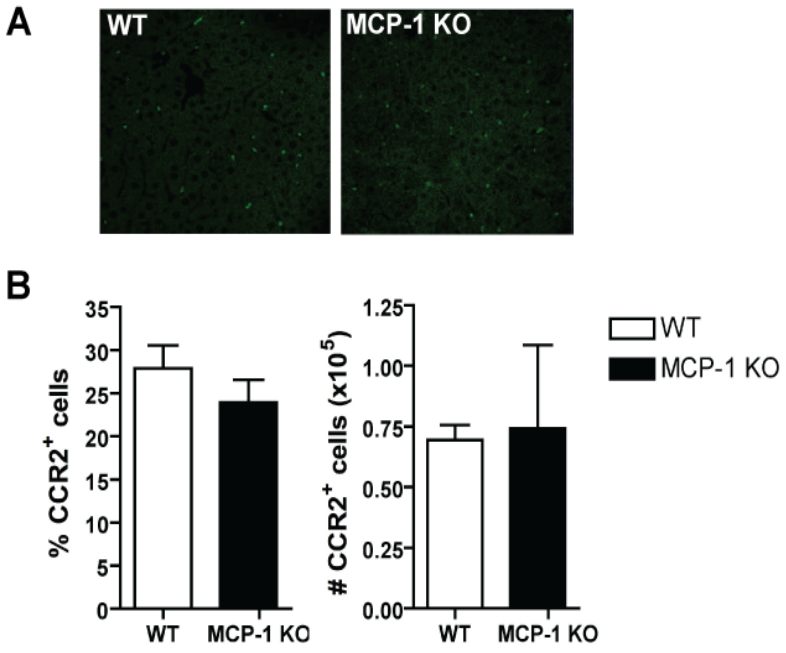


**Figure 3.6 MCP-1 is Not Required for Recruitment of $CCR2^+$ Cells to the Regenerating Liver.** *A:* Fluorescence microscopy was used to identify CCR2 expression in the liver of wild type and MCP-1 KO mice 12 hr after PH. *B:* Flow cytometry was used to measure CCR2 expression in non-parenchymal cells isolated from the regenerating liver of wild type and MCP-1 KO mice 12 hour after PH. Data represent the mean percentage and number (±SEM) of $CCR2^+$ cells (n=4).

## Hepatocyte Proliferation is Suppressed in CCR2-Deficient Mice

While MCP-1 is the most potent ligand of CCR2, there is redundancy in chemokine signaling such that MCP-3 and MCP-5 also bind this receptor, which could explain why liver regeneration was not impaired in the absence of MCP-1. To investigate the collective contribution of all CCR2 chemokines to regeneration, we compared PH-induced hepatocyte proliferation between CCR2 KO and wild-type mice. As shown in

Figure 3.7, levels of BrdU incorporation were reduced by about 40% in CCR2 KO mice 36 hr after PH, although no statistically significant suppression was observed at 48 hr Reduced hepatocyte proliferation at 36 hr did not coincide with diminished liver-body weight ratios, which remained similar in CCR2 KO and wild-type mice at every time point tested.

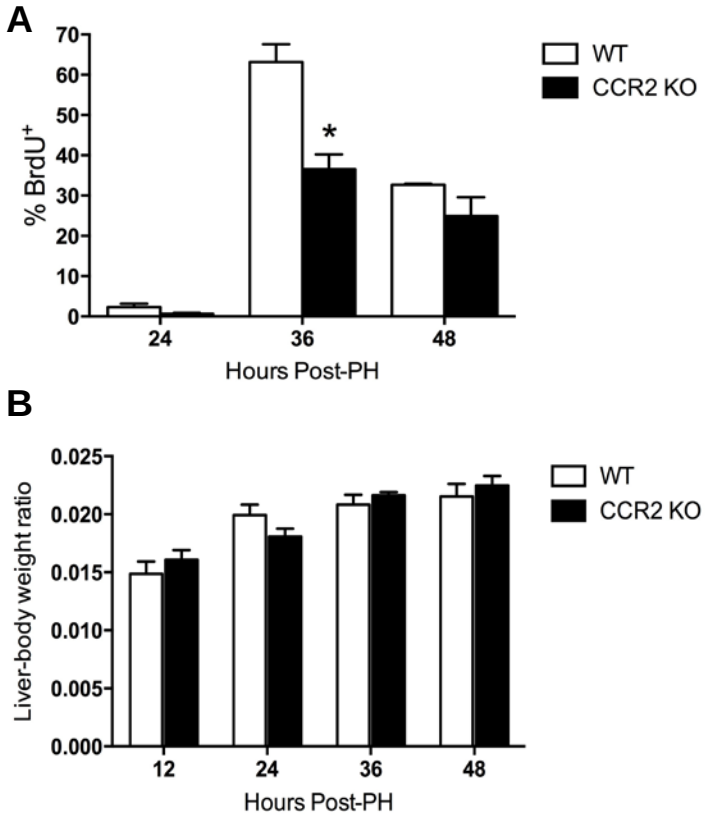


**Figure 3.7 Hepatocyte Proliferation is Suppressed in CCR2-Deficient Mice.** *A:* Percentage (mean +/- SEM) of $BrdU^+$ hepatocyte nuclei from wild type or CCR2 KO mice at the indicated times after PH. Mice were pulsed with BrdU 2 hr prior to euthanasia at the indicated time points (n=3-4). *B:* Liver-body weight ratios (mean +/- SEM) of wild type and CCR2 KO mice at the indicated times after PH. * $p<0.05$ when compared to wild-type mice at same time point.

# CHAPTER 4: DISCUSSION

Macrophage activation is a crucial component of the early phase of liver regeneration following PH (Taub, 2004). Given the well-characterized role of MCP-1 in activating macrophages and promoting macrophage infiltration, we hypothesized that MCP-1 would be important for macrophage activation during PH-induced liver regeneration. However, data presented herein refute this hypothesis and demonstrate that, despite increased hepatic levels of MCP-1 after PH, this chemokine is not required for the production of macrophage-derived inflammatory cytokines or for hepatocyte proliferation.

Increased MCP-1 expression has been observed in numerous models of liver injury and disease, such as administration of concanavalin A, carbon tetrachloride, and acetaminophen (Ajuebor, Hogaboam, Le, & Swain, 2003; James et al., 2005; Zamara et al., 2007). Our results indicate that MCP-1 transcript levels increased just 90 min after PH, which confirms another report (Su et al., 2002). Peak plasma levels of MCP-1 were detected at 12 hr in our study and were only slightly above basal levels. It is unlikely that robust expression of MCP-1 occurs in the plasma before this time, as a recent report by Sgroi et al. demonstrates only scant levels of MCP-1 4 hr post-PH (Sgroi et al., 2011). Although Kupffer cells are a known source of MCP-1, it is likely that multiple cellular sources of MCP-1 exist in the regenerating liver (Hildebrand et al., 2006). For instance, in a rat model of PH-induced liver regeneration, elevated MCP-1 mRNA levels were detected in hepatocytes, biliary epithelial cells, sinusoidal endothelial cells, Kupffer cells,

pit cells, and hepatic oval cells (Chen, Xu, Zhang, & Ma, 2010). It is also possible that MCP-1 production could be attributed to inflammatory cells that infiltrate the liver after PH, such as bone marrow-derived monocytes/macrophages. In fact, an intriguing report by Crane et al. (2009) indicates that $F4/80^{+}$ bone marrow cells are an abundant source of MCP-1, as well as MCP-3 and MCP-5, during infection with murine cytomegalovirus (Crane et al., 2009). Hence, during PH-induced liver regeneration, bone-marrow-derived monocytes could be an important source of MCP-1 in the bone marrow, during transit to the liver, or upon infiltration into hepatic tissue.

MCP-1 expression has been shown to directly correlate with the number of infiltrating monocytes and macrophages in the liver (Marra et al., 1998), and inactivation of MCP-1 reportedly attenuates liver injury by inhibiting macrophage recruitment (Imamura et al., 2005; Marra et al. 2008, Zamara et al., 2007). However, we found no difference in expression of the macrophage marker F4/80 in wild-type and MCP-1 knockout mice. Furthermore, plasma levels of TNFα and IL-6 were also similar, which indicates that macrophage activation was likely unaffected by the absence of MCP-1. IL-6 has been studied extensively as an important initiator of hepatocyte proliferation after PH, as regeneration is impaired and survival is reduced in IL-6-deficient mice subjected to PH (Blindenbacher et al., 2003; Kovalovich et al., 2000). Studies with IL-6 bone marrow chimeric mice demonstrate that, during liver regeneration, IL-6 is produced by bone marrow-derived, resident macrophages, rather than bone-marrow-derived stem cells that enter the liver and differentiate into parenchymal cells (Aldeguer et al., 2002). This leads us to conclude that MCP-1 is probably not required for Kupffer cell activation after PH.

During the priming phase of regeneration, IL-6 activates the signal transducer and activator of transcription-3 (STAT3) pathway, which upregulates immediate-early gene expression in hepatocytes and promotes responsiveness to growth factors (Li, Liang, Kellendonk, Poli, & Taub, 2002; Terui & Ozaki, 2005). Given that levels of STAT3 phosphorylation were similar in wild-type and MCP-1 knockout mice, it stands to reason that hepatocyte priming proceeds sufficiently despite the absence of MCP-1. Furthermore, continuous and pulse BrdU labeling revealed similar levels of hepatocyte proliferation in wild-type and MCP-1 knockout mice, which confirms that MCP-1 is dispensable for liver regeneration.

Results from this study demonstrate comparable numbers of $CCR2^{+}$ cells in the liver of wild-type and MCP-1 knockout mice. We speculate that this was due to the activity of other CCR2 ligands, such as MCP-3 or MCP-5, which could essentially compensate for the absence of MCP-1. However, the observation that liver regeneration was not overtly suppressed in CCR2 knockout mice renders this notion somewhat irrelevant. In CCR2 KO mice, hepatocyte proliferation was suppressed 36 hr post-PH. However, this did not coincide with decreased liver-body weight ratios, and BrdU incorporation was comparable between CCR2 KO and wild-type mice at 48 hr post-PH. The suppression at 36 hr may result from diminished macrophage activity in the absence of CCR2, either through decreased monocyte recruitment to the liver or reduced activation of resident Kupffer cells. Crane et al. (2009) demonstrated that CCR2 activation in the bone marrow initiates the egress of CCR2+ monocytes into the circulation and to the liver. Furthermore, they demonstrated that the $CCR2^{+}$ $Ly6C^{high}$ inflammatory subpopulation of monocytes/macrophages accumulated in the bone marrow

during MCMV infection, and that the number of inflammatory monocytes/macrophages was diminished in CCR2-deficient mice. Hence, it is possible that monocyte recruitment and/or macrophage activation is reduced in CCR2 knockout mice, leading to a transient suppression of hepatocyte proliferation. Results from this study highlight the need to further investigate CCR2 signaling, as well as other chemokines receptors expressed in the bone marrow, as this could be a driving force that initiates the release of inflammatory monocytes/macrophages that are destined for the regenerating liver.

Future studies could include elucidating the role of $CCR2^{+}$ cells in the regenerative process by isolating them and studying gene expression at various times following PH. Furthermore, the heterogeneity among this $CCR2^{+}$ population can be further characterized using flow cytometry to determine if a specific subpopulation is critical to the regenerative process. Results from this project further our understanding of MCP-1/CCR2 signaling as it relates to inflammation and injury in the liver. This is an important endeavor for advancing our knowledge of liver disease and developing new therapeutic strategies to enhance recovery from liver disease.

# REFERENCES

Abshagen, K., Eipel, C., Kalff, J. C., Menger, M. D., & Vollmar, B. (2008). Kupffer Cells are Mandatory for Adequate Liver Regeneration by Mediating Hyperperfusion via Modulation of Vasoactive Proteins. *Microcirculation, 15*(1), 37-47.

Ajuebor, M. N., Hogaboam, C. M., Le, T., & Swain, M. G. (2003). C-C Chemokine Ligand 2/Monocyte Chemoattractant Protein-1 Directly Inhibits NKT Cell IL-4 Production and is Hepatoprotective in T Cell-Mediated Hepatitis in the Mouse. *Journal of Immunology, 170*(10), 5252-5259.

Aldeguer, X., Debonera, F., Shaked, A., Krasinkas, A. M., Gelman, A. E., Que, X. G., Olthoff, K. M. (2002). Interleukin-6 from Intrahepatic Cells of Bone Marrow Origin is Required for Normal Murine Liver Regeneration. *Hepatology, 35*(1), 40-48.

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., & Yuan, J. Y. (1996). Human ICE/CED-3 Protease Nomenclature. *Cell, 87*(2), 171-171.

Aoki, T., Imamura, H., Matsuyama, Y., Kishi, Y., Kobayashi, T., Sugawara, Y., Kokudo, N. (2011). Convergence Process of Volumetric Liver Regeneration After Living-Donor Hepatectomy. *Journal of Gastrointestinal Surgery, 15*(9), 1594-1601.

Assy, N., & Minuk, G. Y. (1997). Liver Regeneration: Methods for Monitoring and Their Applications. *Journal of Hepatology, 26*(4), 945-952.

Baier, P. K., Baumgartner, U., Hempel, S., Wolff-Vorbeck, G., von Dobschuetz, E., & Hopt, U. T. (2005). Kupffer Cells Infiltrate Liver Tissue Early After Ischemia-Reperfusion and Partial Hepatectomy. *European Surgical Research, 37*(5), 290-297.

Bilzer, M., Roggel, F., & Gerbes, A. L. (2006). Role of Kupffer Cells in Host Defense and Liver Disease. *Liver International, 26*(10), 1175-1186.

Blindenbacher, A., Wang, X. Y., Langer, I., Savino, R., Terracciano, L., & Heim, M. H. (2003). Interleukin 6 is Important for Survival After Partial Hepatectomy in Mice. *Hepatology, 38*(3), 674-682.

Boring, L., Gosling, J., Chensue, S. W., Kunkel, S. L., Farese, R. V., Broxmeyer, H. E., & Charo, I. F. (1997). Impaired Monocyte Migration and Reduced Type 1 (Th1) Cytokine Responses in C-C Chemokine Receptor 2 Knockout Mice. *Journal of Clinical Investigation, 100*(10), 2552-2561.

Charo, I., Myers, S., Herman, A., Franci, C., Connolly, A., & Coughlin, S. (1994). Molecular Cloning and Functional Expression of Two Monocyte Chemoattractant Protein 1 Receptors Reveals Alternative Splicing of the Carboxyl-Terminal Tails. *Proc Natl Acad Sci U S A, 91*(7), 2752-2756.

Chen, X. G., Xu, C. S., Zhang, F. C., & Ma, J. (2010). Comparative Analysis of Expression Profiles of Chemokines, Chemokine Receptors, and Components of Signaling Pathways Mediated by Chemokines in Eight Cell Types During Rat Liver Regeneration. *Genome, 53*(8), 608-618.

Chiang, J. Y. L. (2009). Bile Acids: Regulation of Synthesis. *Journal of Lipid Research, 50*(10), 1955-1966.

Columbano, A., Leddacolumbano, G. M., Ennas, M. G., Curto, M., Chelo, A., & Pani, P. (1990). Cell Proliferation and Promotion of Rat-Liver Carcinogenesis - Different Effect of Hepatic Regeneration and Mitogen Induce Hyperplasia on the Development of Enzyme-Altered Foci. *Carcinogenesis, 11*(5), 771-776. doi: 10.1093/carcin/11.5.771

Columbano, A., Leddacolumbano, G. M., Lee, G., Rajalakshmi, S., & Sarma, D. S. R. (1987). Inability of Mitogen-Induced Liver Hyperplasia to Support the Induction of Enzyme-Altered Islands Induce by Liver Carcinogens. *Cancer Research, 47*(21), 5557-5559.

Crane, M. J., Hokeness-Antonelli, K. L., & Salazar-Mather, T. P. (2009). Regulation of Inflammatory Monocyte/Macrophage Recruitment from the Bone Marrow during Murine Cytomegalovirus Infection: Role for Type I Interferons in Localized Induction of CCR2 Ligands. *Journal of Immunology, 183*(4), 2810-2817.

Czaja, M. J., Geerts, A., Xu, J., Schmiedeberg, P., & Ju, Y. (1994). Monocyte Chemoattractant Protein-1 (MCP-1) Expression Occurs in Toxic Rat Liver Injury and Human Liver Disease. *Journal of Leukocyte Biology, 55*(1), 120-126.

DeLeve, L. D. (2013). Liver Sinusoidal Endothelial Cells and Liver Regeneration. *Journal of Clinical Investigation, 123*(5), 1861-1866.

Deshmane, S. L., Kremlev, S., Amini, S., & Sawaya, B. E. (2009). Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *Journal of Interferon and Cytokine Research, 29*(6), 313-326.

Dory, D., Echchannaoui, H., Letiembre, M., Ferracin, F., Pieters, J., Adachi, Y., Akashi, S., Zimmerli, W., Landmann, R. (2003) Generation and Functional Characterization of a Clonal Murine Periportal Kupffer Cell Line from *H-2Kb*–tsA58 Mice. *J Leukoc Biol. 4(1):49-59.*

Fausto, N. (2000). Liver Regeneration. *Journal of Hepatology, 32*, 19-31.

Fausto, N., Campbell, J. S., & Riehle, K. J. (2006). Liver Regeneration. *Hepatology, 43*(2), S45-S53.

Friedman, S. L. (2008). Hepatic Stellate Cells: Protean, Multifunctional, and Enigmatic Cells of the Liver. *Physiological Reviews*, *88*(1), 125–172.

Fujihara, M., Muroi, M., Tanamoto, K., Suzuki, T., Azuma, H., & Ikeda, H. (2003). Molecular Mechanisms of Macrophage Activation and Deactivation by Lipopolysaccharide: Roles of the Receptor Complex. *Pharmacology & Therapeutics, 100*(2), 171-194.

Greene, A. K., & Puder, M. (2003). Partial Hepatectomy in the Mouse: Technique and Perioperative Management. *Journal of Investigative Surgery, 16*(2), 99-102.

Hakkola, J., Tanaka, E., & Pelkonen, O. (1998). Developmental Expression of Cytochrome P450 Enzymes in Human Liver. *Pharmacology & Toxicology, 82*(5), 209-217.

Hildebrand**,** F., Hubbard, W.J., Choudhry, M.A., Frink, M., Pape, H.C., Kunkel, S.L., Chaudry I.H. (2006) Kupffer Cells and their Mediators: the Culprits in Producing Distant Organ Damage After Trauma-Hemorrhage. *Am J Pathol*; 169(3):784-94.

Imamura, M., Ogawa, T., Sasaguri, Y., Chayama, K., & Ueno, H. (2005). Suppression of macrophage infiltration inhibits activation of hepatic stellate cells and liver fibrogenesis in rats. *Gastroenterology, 128*(1), 138-146.

Isse, K., Harada, K., & Nakanuma, Y. (2007). IL-8 Expression by Biliary Epithelial Cells is Associated with Neutrophilic Infiltration and Reactive Bile Ductules. *Liver International, 27*(5), 672-680.

James, L. P., Simpson, P. M., Farrar, H. C., Kearns, G. L., Wasserman, G. S., Blumer, J. L., Pediat Pharmacology Res Unit, N. (2005). Cytokines and Toxicity in Acetaminophen Overdose. *Journal of Clinical Pharmacology, 45*(10), 1165-1171.

Katayanagi, K., Kono, N., & Nakanuma, Y. (1998). Isolation, Culture and Characterization of Biliary Epithelial Cells from Different Anatomical Levels of the Intrahepatic and Extrahepatic Biliary Tree From a Mouse. *Liver, 18*(2), 90-98.

Kawser, C. A., Iredale, J. P., Winwood, P. J., & Arthur, M. J. P. (1998). Rat Hepatic Stellate Cell Expression of Alpha 2-Macroglobulin is a Feature of Cellular Activation: Implications for Matrix Remodelling in Hepatic Fibrosis. *Clinical Science, 95*(2), 179-186.

Kountouras, J., Boura, P., & Lygidakis, N. J. (2001). Liver Regeneration After Hepatectomy. *Hepato-Gastroenterology, 48*(38), 556-562.

Kovalovich, K., DeAngelis, R. A., Li, W., Furth, E. E., Ciliberto, G., & Taub, R. (2000). Increased Toxin-Induced Liver Injury and Fibrosis in Interleukin-6-Deficient Mice. *Hepatology, 31*(1), 149-159.

Kurihara, T., Warr, G., Loy, J., & Bravo, R. (1997). Defects in Macrophage Recruitment and Host Defense in Mice Lacking the CCR2 Chemokine Receptor. *Journal of Experimental Medicine, 186*(10), 1757-1762.

Li, W., Liang, X. P., Kellendonk, C., Poli, V., & Taub, R. (2002). STAT3 Contributes to the Mitogenic Response of Hepatocytes During Liver Regeneration. *Journal of Biological Chemistry, 277*(32), 28411-28417.

Liaskou, E., Wilson, D., & Oo, Y. (2012) Innate Immune Cells in Liver Inflammation. *Mediators of Inflammation*, vol. 2012

Lu, B., Rutledge, B. J., Gu, L., Fiorillo, J., Lukacs, N. W., Kunkel, S. L., Rollins, B. J. (1998). Abnormalities in Monocyte Recruitment and Cytokine Expression in Monocyte Chemoattractant Protein 1-Deficient Mice. *Journal of Experimental Medicine, 187*(4), 601-608.

Macias-Silva, M., Li, W., Leu, J. I., Crissey, M. A. S., & Taub, R. (2002). Up-Regulated Transcriptional Repressors SnoN and Ski Bind Smad Proteins to Antagonize Transforming Growth Factor-Beta Signals During Liver Regeneration. *Journal of Biological Chemistry, 277*(32), 28483-28490.

Malato, Y., Naqvi, S., Schurmann, N., Ng, R., Wang, B., Zape, J., Willenbring, H. (2011). Fate Tracing of Mature Hepatocytes in Mouse Liver Homeostasis and Regeneration. *Journal of Clinical Investigation, 121*(12), 4850-4860.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The Chemokine System in Diverse Forms of Macrophage Activation and Polarization. *Trends in Immunology, 25*(12), 677-686.

Mao, S. A., Glorioso, J. M., & Nyberg, S. L. (2014). Liver Regeneration. *Translational Research, 163*(4), 352-362.

Marra, F. (2002). Chemokines in Liver Inflammation and Fibrosis. *Frontiers in Bioscience, 7*, D1899-D1914.

Marra, F., DeFranco, R., Grappone, C., Milani, S., Pastacaldi, S., Pinzani, M., Gentilini, P. (1998). Increased Expression of Monocyte Chemotactic Protein-1 During

Active Hepatic Fibrogenesis - Correlation with Monocyte Infiltration. *American Journal of Pathology*, *152*(2), 423-430.

Michalopoulos, G. K. (2010). Liver Regeneration After Partial Hepatectomy Critical Analysis of Mechanistic Dilemmas. *American Journal of Pathology*, *176*(1), 2-13.

Michalopoulos, G. K. (2013). Principles of Liver Regeneration and Growth Homeostasis. *Comprehensive Physiology*, *3*(1), 485-513. doi: 10.1002/cphy.c120014

Michalopoulos, G. K., & DeFrances, M. C. (1997). Liver Regeneration. *Science*, *276*(5309), 60-66.

Minamino, T., Ito, Y., Ohkubo, H., Hosono, K., Suzuki, T., Sato, T., Majima, M. (2012). Thromboxane A(2) Receptor Signaling Promotes Liver Tissue Repair After Toxic Injury Through the Enhancement of Macrophage Recruitment. *Toxicology and Applied Pharmacology*, *259*(1), 104-114.

Mitchell, K. A., Lockhart, C. A., Huang, G. M., & Elferink, C. J. (2006). Sustained Aryl Hydrocarbon Receptor Activity Attenuates Liver Regeneration. *Molecular Pharmacology*, *70*(1), 163-170.

Mochida, S., Ohta, Y., Ogata, I., & Fujiwara, K. (1992). Gut-Derived Substances in Activation of Hepatic Macrophages After Partial-Hepatectomy in Rats. *Journal of Hepatology*, *16*(3), 266-272.

Nacu, E., & Tanaka, E. M. (2011). Limb Regeneration: A New Development? *Annual Review of Cell and Developmental Biology*, *Vol 27*, *27*, 409-440.

Naito, M., Hasegawa, G., & Takahashi, K. (1997). Development, Differentiation, and Maturation of Kupffer cells. *Microscopy Research and Technique*, *39*(4), 350-364.

Nygard, I., Mortensen, K., Hedegaard, J., Conley, L., Kalstad, T., Bendixin, C., & Revhaug, A. (2012). The Genetic Regulation of the Terminating Phase of Liver Regeneration. *Comparative Hepatology*, *11*(3).

Overturf, K., AlDhalimy, M., Ou, C. N., Finegold, M., & Grompe, M. (1997). Serial Transplantation Reveals the Stem-Cell-Like Regenerative Potential of Adult Mouse Hepatocytes. *American Journal of Pathology, 151*(5), 1273-1280.

Power, C., & Rasko, J. E. J. (2008). Whither Prometheus' Liver? Greek Myth and the Science of Regeneration. *Annals of Internal Medicine, 149*(6), 421-W479.

Ramadori, G., & Saile, B. (2002). Mesenchymal Cells in the Liver - One Cell Type or Two? *Liver*, *22*(4), 283-294.

Rossi, D., & Zlotnik, A. (2000). The Biology of Chemokines and their Receptors. *Annual Review of Immunology*, *18*, 217-243.

Selzner, N., Selzner, M., Odermatt, B., Tian, Y. H., Van Rooijen, N., & Clavien, P. A. (2003). ICAM-1 Triggers Liver Regeneration Through Leukocyte Recruitment and Kupffer Cell-Dependent Release of TNF-Alpha/IL-6 in Mice. *Gastroenterology, 124*(3), 692-700.

Sgroi, A., Gonelle-Gispert, C., Morel, P., Baertschiger, R. M., Niclauss, N., Mentha, G., Buhler, L. (2011). Interleukin-1 Receptor Antagonist Modulates the Early Phase of Liver Regeneration after Partial Hepatectomy in Mice. *Plos One*, *6*(9).

Sharma, A. D., Cantz, T., Manns, M. P., & Ott, M. (2006). The Role of Stem Cells in Physiology, Pathophysiology, and Therapy of the Liver. *Stem Cell Reviews, 2*(1), 51-58.

Shiratori, Y., Hongo, S., Hikiba, Y., Ohmura, K., Nagura, T., Okano, K., Omata, M. (1996). Role of Macrophages in Regeneration of Liver. *Digestive Diseases and Sciences*, *41*(10), 1939-1946.

Sigal, S. H., Gupta, S., Gebhard, D. F., Holst, P., Neufeld, D., & Reid, L. M. (1995). Evidence for a Terminal Differentiatin Process in the Rat Liver. *Differentiation, 59*(1), 35-42.

Skrtic, S., Wallenius, V., Ekberg, S., Brenzel, A., Gressner, A. M., & Jansson, J. O. (1997). Insulin-Like Growth Factors Stimulate Expression of Hepatocyte Growth Factor but not Transforming Growth Factor Beta(1) in Cultured Hepatic Stellate Cells. *Endocrinology*, *138*(11), 4683-4689.

Su, A., Guidotti, L., Pezacki, J., Chisari, F., Schultz, P. (2002). Gene Expression During the Priming Phase of Liver Regeneration After Partial Hepatectomy in Mice. *Proceedings of the National Academy of Sciences, 99*(17), 11181-11186.

Sudo, K., Yamada, Y., Saito, K., Shimizu, S., Ohashi, H., Kato, T., Seishima, M. (2008). TNF-Alpha and IL-6 Signals From the Bone Marrow Derived Cells are Necessary for Normal Murine Liver Regeneration. *Molecular Basis of Disease, 1782*(11), 671-679.

Tacke, F., Luedde, T., & Trautwein, C. (2009). Inflammatory Pathways in Liver Homeostasis and Liver Injury. *Clinical Reviews in Allergy & Immunology, 36*(1), 4-12.

Taub, R. (2004). Liver Regeneration: From Myth to Mechanism. *Nature Reviews Molecular Cell Biology, 5*(10), 836-847.

Terui, K., & Ozaki, M. (2005). The Role of STAT3 in Liver Regeneration. *Drugs of Today, 41*(7), 461-469.

Uguccioni, M., Dapuzzo, M., Loetscher, M., Dewald, B., & Baggiolini, M. (1995). Actions of the Chemotactic Cytokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1-Alpha and MIP-1-Beta on Human Monocytes. *European Journal of Immunology, 25*(1), 64-68.

Ukai, K., Terashima, K., Imai, Y., Shinzawa, H., Okuyama, Y., Takahashi, T., & Ishikawa, M. (1990). Proliferation Kinetics of Rat Kupffer Cells After Partial-Hepatectomy - Immunohistochemical and Ultrastructural Analysis. *Acta Pathologica Japonica, 40*(9), 623-634.

Wheatley, A. M., Rieder, H. U., Kuznetsova, L. V., Zhang, X. Y., Seiler, C., & Mathie, R. T. (1997). Effect of Liver Transplantation on Hepatic Hemodynamics: Methodology and Mechanisms. *Digestive Surgery, 14*(5), 344-354.

Yadav, A., Saini, V., & Arora, S. (2010). MCP-1: Chemoattractant with a Role Beyond Immunity: A Review. *Clinica Chimica Acta, 411*(21-22), 1570-1579.

Zamara, E., Galastri, S., Aleffi, S., Petrai, I., Aragno, M., Mastrocola, R., Marra, F. (2007). Prevention of Severe Toxic Liver Injury and Oxidative Stress in MCP-1-Deficient Mice. *Journal of Hepatology*, *46*(2), 230-238.