Acetaminophen-induced Liver Injury:

Characterization of Infiltrating Macrophages and CX3CR1 Associated Recruitment

By

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A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

In partial fulfillment of the requirements

For the degree of

Master of Science

Joint Graduate Program in Toxicology

Written under the direction of

Professor Debra L Laskin

And approved by

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New Brunswick, New Jersey

JANUARY, 2016

Abstract of the Thesis

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Toxic doses of the analgesic acetaminophen (APAP) cause hepatic necrosis, a response mediated, in part, by inflammatory macrophages. Herein we characterized macrophages responding to APAP. Infiltrating myeloid cells were identified as $CD11b^+$, $Ly6G^+$ (granulocytic) or $Ly6C^+$ (monocytic) subsets. $Ly6C^{hi}$ macrophages are pro-inflammatory, while $Ly6C^{lo}$ are anti-inflammatory. The origin of liver macrophages and mechanisms mediating their recruitment were analyzed utilizing GFP^+ bone marrow chimeric mice and $CX3CR1^{+/GFP}$ reporter mice. Following APAP administration, we observed increased numbers of GFP^+CD11b^+ cells in livers of chimeric mice at all post treatment times. Conversely, $CX3CR1^+CD11b^+$ cells were observed mainly at later times. GFP^+ bone marrow-derived $Ly6C^{hi}$ cells consisted of mature and immature cells as measured by F4/80 expression. Increased numbers of mature anti-inflammatory $Ly6C^{lo}$ bone marrow-derived macrophages were observed after APAP, which was correlated with a decrease in immature anti-inflammatory $Ly6C^{lo}$ macrophages at the later times. Analysis of coexpression of F4/80 and $CX3CR1^+$ showed that mature macrophages were selectively recruited at later times after APAP. This suggests that $CX3CR1^+$ recruitment involves anti-inflammatory cell recruitment, as well as multiple M2--like macrophage subpopulations. To further assess the phenotypes of GFP^+ bone marrow-

derived and CX3CR1^{+} macrophages, cells were examined for expression of pro- and anti-inflammatory markers. Confocal microscopy confirmed the accumulation of bone marrow-derived pro-inflammatory GFP^{+} macrophages in the liver which expressed the inflammatory enzyme, COX-2. This correlated with Ly6C^{hi} macrophage subpopulations in GFP^{+} bone marrow chimeric mice. Reduced COX-2 expression was observed on CX3CR1^{+} macrophages, indicating that COX-2^{+} cell accumulation is independent of CX3CR1. Bone marrow-derived anti-inflammatory macrophages expressing the anti-oxidative enzyme HO-1 and mannose receptor were present in the liver at similar times as Ly6C^{lo} macrophages. Interestingly, CX3CR1^{+} macrophages were found to only express HO-1, but not MR suggesting that the Ly6C^{lo} subpopulation recruited to the liver in CX3CR1$^{+/GFP}$ reporter mice after APAP represents a unique subpopulation of anti-inflammatory macrophages. Overall, these studies demonstrate that bone marrow provides distinct pro- and anti-inflammatory macrophage subpopulations that employ chemokine receptors, such as CX3CR1 to aid in their trafficking to the liver. Furthermore, CX3CR1 associated recruitment includes both pro- and anti-inflammatory macrophages.

DEDICATION

I dedicate this work to my family and friends.

I would never able to achieve anything without your support.

Even in the most difficult hours, you are always by my side.

Thank you all for being there for me,

I can only hope to return the kindness someday.

ACKNOWLEDGEMENTS

The quantity of people whom deserve my acknowledgment is too numerous to list here. The appreciation and gratitude I am able to express here is also criminally minuscule compared to the helps that I received. First and above all, the sacrifice of my family made during this time is beyond any description. I can never be where I am without them. They went above and beyond their duties as families and I will be forever grateful.

Of course, there are many impressive colleagues whom I am fortunate enough to work with and desire nothing but my most sincere gratitude. This includes every components of my lab. Their willingness to share their skills and expertise are critical to my progress. Special recognition goes to my fellow graduate students, Mary Francis and Alessandro Venosa. They were always there whenever I needed them the most. They are always able to provide answers, advice, or support. It is hard to regard them without my upmost gratitude after the time we spent together, working as fellow researchers, as colleagues, and as dear friend.

The path to a Master is impossible without many great people generously providing supports. There are many difficulties I face along the way. Thankfully, there are several great figures that I have the honor to learn from. A community of exceptional faculties and stuffs offer by the program provided the nothing but most excellent education. In addition, I need to thanks Cristina Dragomir for introduce me into the world of research. She teaches me some of the most valuable knowledges and skills that I hold dear and guide me even today. It is not an exaggeration to say there is not a single progress I made without Cristina's fingerprints. In addition, Miil Mandal also generously shared her knowledge and experience whenever I am in need of assistance. Mili worked with upmost efficiency and yet somehow abled to maintain positive attitude even in the face of tremendous stress. It is nothing less than a privilege to learn from these great scientists and I am honored to work alone side both of you. Special recognition goes to

my committee members, Dr. Lauren Aleksunes who have assisted me every steps of the way, as well as guided me growing as a scientist. Thanks to Dr. Carol Gardner for acting as the lab guardian throughout my time in the lab. She is always there to help, offering advice or share interesting tales from her experience. Lastly, I would like to thank my advisor, Dr. Debra Laskin. You offer nothing but your best assistance and support in every aspect of my graduate school path. I understand I am not an easy student to have. The amount of mistake I make is nothing but staggering. Thank you for not giving up on me where the others would have. Thank you for an unforgettable experience learning to be a researcher.

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ABBREVIATIONS

APAP	Acetaminophen
ApoE	Apolipoprotein E
Arg-1	Arginase-1
BPI	Bactericidal/Permeability-Increasing Protein
CCL13	Chemokine (C-C motif) Ligand 13
CCL2	Chemokine (C-C motif) Ligand 2
CCL5	Chemokine (C-C motif) Receptor 5
CCL7	Chemokine (C-C motif) Ligand 7
CCL8	Chemokine (C-C motif) Ligand 8
CCR2	Chemokine (C-C motif) Receptor 2
CD11b	Cluster of Differentiation Molecule 11B
COX-2	Cyclooxygenase-2
CX3CL1/ Fractalkine	Chemokine (C-X3-C motif) Ligand 1
CX3CR1	Chemokine (C-X3-C Motif) Receptor 1
CXCL1	Chemokine (C- X3-C motif) Ligand 1
CYP2E1	Cytochrome P450 Isoform 2E1
DAMPS	Damage-Associated Molecular Patterns
GFP	Green Flourescent Protein
F4/80	Epidermal Growth Factor (EGF)-like Module-Containing Mucin-like Hormone Receptor-like 1
Fizz-1	Resistin-like Molecule Alpha
GM-CSF	Granulocyte macrophage colony-stimulating factor
HGF	Hepatocyte Growth Factor
HMGB1	High Mobility Group Box 1

HO-1	Heme Oxygenase-1
ICAM	Intercellular Adhesion Molecule 1
IFN-γ	Interferon Gamma
IGFBP1	Insulin-Like Growth Factor Binding Protein
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-4/IL13	Interleukin-4/13
IL-6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
IRF	Interferon Regulatory Factors
IRF5	Interferon Regulatory Factor 5
IκBα	NF-κB Inhibitor Alpha
LPS	Lipopolysaccharide
LXA_4	Lipoxin A_4
Ly6C	Lymphocyte Antigen 6C
Ly6G	Lymphocyte Antigen 6G
MCP-1	Monocyte Chemoattractant Protein-1
MDSC	Myeloid-Derived Suppressor Cells
MMP9	Metalloproteinase 9
MR	Mannose Receptor
NAC	N-acetylcysteine
NAPQI	N-Acetyl-p-Benzoquinone Imine
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
PAMPS	Pathogen-Associated Molecular Patterns
PDGF	Platelet-Derived Growth Factor

PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGH^2	Prostaglandin H2
PRR	Pattern Recognition Receptor
PSGL1	P-Selectin Glycoprotein Ligand 1
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
STAT1	Signal Transducers and Activators of Transcription 1
STAT3	Signal Transducers and Activators of Transcription 3
STAT6	Signal Transducers and Activators of Transcription 6
SULT	Sulfotransferases
TGFβ	Transforming Growth Factor Beta
Th1	T-Helper Cell 1
TIR	Intracellular Toll–IL-1 Receptor
TLRs	Toll Like Receptors
TNF-α	Tumor Necrosis Factor Alpha
TTP	Tristetraprolin
UGT	UDP-Glucuronosyltransferases
VEGF	Vascular Endothelial Growth-Factor
Ym-1	Chitinase-3-like 3

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INTRODUCTION

Macrophages

Macrophages are mononuclear phagocytes that are critical in innate immune responses. They are found throughout the body and play important roles in tissue defense and maintenance of homeostasis. Resident tissue macrophages function as sentinels of the tissue, responding rapidly to pathogens and debris. Evidence suggests that resident tissue macrophages originate mainly from the fetal liver and embryonic yolk sac (Takahashi, 1989, Naito, 1997, Ginhoux, 2010, Hoeffel, 2012). Based on their anatomical location, resident macrophages specialize, exhibiting distinct functions depending on the needs of the tissue. Examples of this heterogeneity include: bone macrophages or osteoclasts, which are instrumental in bone resorption; alveolar macrophages in the lung, responsible for removing inhaled particles and pathogens; liver Kupffer cells, which are specialized for destroying gut derived pathogens, detoxification of harmful molecules and the removal of debris from the circulation; intestinal macrophages, which are important in combating pathogens and establishing tolerance to gut flora and food antigens; spleen macrophages in red pulp, which recycle aged erythrocytes and marginal zone macrophages which remove particulates from the circulation; Langerhans cells in the skin, which are highly proficient in the recognition and clearance of pathogens; and microglia, resident macrophages of the brain, which constantly survey and maintain the central nervous system (CNS) microenvironment. (Väänänen, 2010, Laskin, 2011, Gordon, 2005, Hanisch, 2007)

The bone marrow is a major source of inflammatory macrophages. Under homeostatic conditions, monocytes released from the bone marrow remain inactive and patrol the circulation. However, during a pathological event, pathogens and injured tissue release damage-associated molecular patterns (DAMPs) and/or pathogen-associated molecular patterns (PAMPs) to initiate inflammation. Once inflammation is

started, chemokines and activation signals are released from the inflamed site. Circulating monocytes identify the signal and migrate toward the inflamed tissue. Once within tissue, monocytes infiltrate into the tissue and mature into macrophages, which contribute first to promoting inflammation and later to suppressing inflammation and initiation of wound repair. [Strauss-Ayali, 2007]

Due to the remarkable plasticity of macrophages, several populations have been identified based on their expression of proteins, such as lymphocyte antigen 6C (Ly6C), a cell surface glycoprotein, as well as functional profiles [Ingersoll, 2010]. One population has been identified as classically activated, pro-inflammatory/cytotoxic $Ly6C^{hi}$ M1 macrophage and a second as alternatively activated, anti-inflammatory, wound repair $Ly6C^{lo}$ M2 macrophages. The balance between the M1 and M2 macrophage populations dictates the outcome of inflammation.

Classical Activation of Macrophages

Classically activated M1 macrophages are essential in combating pathogens and are critical in host defense. Mice deficient in the critical M1 activation cytokine, interferon gamma (IFN-γ) or its receptor, as well as mice treated with anti-IFN-γ antibody exhibit more severe bacterial infections [Benoit, 2008]. M1 macrophages are also important in tumor cell surveillance and removal. Mice with suppressed nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a nuclear transcription factor regulating expression of many M1 functional genes, are unable to inhibit tumor growth [Saccani, 2006]. There are three major M1 macrophage activators: IFN-γ, pattern recognition receptor (PRR) ligands, and granulocyte macrophage colony-stimulating factor (GM-CSF) [Martinez, 2014]. IFN-γ is mainly produced by T helper 1 (Th1) lymphocytes. Upon binding to macrophages, IFN-γ activates signal transducers and activators of transcription1 (STAT1) and interferon regulatory factors (IRF) pathways leading to the production of pro-inflammatory proteins including interleukin-6 (IL-6), inducible nitric

oxide synthase (iNOS), chemokine (C-C motif) ligand 5 (CCL5) and chemokine (C-X-C motif) ligand 1 (CXCL1) (Hu, 2009, Xu, 2009). PRR ligands, such as lipopolysaccharide (LPS), bind to toll like receptors (TLRs) and activate NF-κB resulting in upregulation of expression of tumor necrosis factor alpha (TNF-α), IL-1, IL-12, and pro-inflammatory chemokines [Kawai, 2011]. GM-CSF binding to macrophages also activates NF-κB, as well as interferon regulatory factor 5 (IRF5) [Martinez, 2014]. Once activated, M1 macrophages release cytotoxic mediators such as proteases, reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as pro-inflammatory cytokines. In addition, M1 macrophages produce inflammatory lipid mediators, which are instrumental to the inflammatory process; these are generated in large part by the enzyme, cyclooxygenase-2 (COX-2) [Shimizu, 2009]. COX-2 converts arachidonic acid into prostaglandin H_2 (PGH_2). PGH_2 is then metabolized into various pro-inflammatory eicosanoids that contribute to the symptoms of inflammation such as fever and vessel dilation. Up-regulation of COX-2 corresponds to the onset of inflammatory response; however, continued expression of COX-2 expression coinciding with the resolution of inflammation suggests a possible anti-inflammatory role of COX-2 [Rajakariar, 2006]. Studies also suggest that one of the PGH_2 derivatives, prostaglandin E_2 can deactivate NF-κB and cause negative feedback to resolve inflammation [Gomez, 2005]. While M1 macrophages are critical in host defense, over activation of these cells have been associated with inflammatory diseases such as rheumatoid arthritis, type 2 diabetes and atherosclerosis [Baker, 2011].

Alternative Activation of Macrophages

Many signaling pathways activated during inflammation are controlled by negative feedback. NF-κB, for example, also produces negative regulators NF-κB inhibitor alpha (IκBα) and A20 that disable NF-κB DNA binding [Ruland, 2011]. Another example, tristetraprolin (TTP), a protein that is produced along with TNF-α, destabilizes

TNF-α mRNA and limits TNF-α protein production [Sanduja, 2011]. As the production of M1 cytokines is down regulated, M2 macrophages become dominant during the resolution phase of inflammation. M2 macrophages are activated by a combination of cytokines IL-4/IL13, or IL-10 through STAT3 and STAT6 pathways [Sica, 2007]. Activation of STAT3 and STAT6 pathways leads to the expression of anti-inflammatory proteins such as arginase-1 (Arg-1), mannose receptor (MR), resistin-like molecule alpha (Fizz-1) and chitinase-like 3 (Ym-1) (Junttila, 2008, Nair, 2005, Taylor, 2005). In mice infected with *Toxoplasma gondii*, both STAT3 and STAT6 are activated resulting in up regulation of Arg-1. These animals are unable to initiate inflammatory reactions and clear infectious agents [Butcher, 2011]. Another important role of M2 macrophage is to remove cellular debris. M2 macrophages express high levels of receptors such as MR to promote the clearance of necrotic cell fragments [Martinez, 2006]. Mice lacking MR exhibit difficulties in clearing cellular debris and proteins after inflammation [Taylor, 2005]. M2 macrophages also produce growth factors promoting angiogenesis and tissue repair, such as vascular endothelial growth factor (VEGF) and transforming growth factor (TGF) (Luo, 2006, Ogata, 2006). The accumulation of alternatively activated macrophages shifts the balance toward down regulating inflammation and resolving tissue injury. However, in chronic inflammation, M2 macrophage over activation has been associated with fibrosis and tumor progression (Sica, 2006, Murray, 2011).

Based on the activities and method of activations, M2 macrophages were subdivided into M2a, M2b, and M2c subpopulations (Orme, 2012, Mosser, 2008, Mantovani, 2004). M2a are specialized in profibrotic function during chronic inflammation (Anders, 2011, Mann, 2011). They are induced by anti-inflammatory cytokines such as IL-33, IL-4 and IL-13 (Nelson, 2011, Mantovani, 2004). M2b are immunoregulatory M2 macrophages that are activated through the binding of immune complex, LPS, or IL-1 (Orme, 2012, Benoit, 2008, Mantovani, 2004). Finally, M2c macrophages are important

for tissue repair and are active by IL-10 and glucocorticoid Mantovani, 2004]. M2c secretes anti-inflammatory cytokine and growth factors such as IL-10 and TGF-β that are critical in matrix deposition and tissue remodeling. (Lu, 2013, Zizzo, 2012, Benoit, 2008, Mantovani, 2004).

Chemokines

Inflammatory macrophage accumulation at sites of injury is mediated by chemokines. Four families have been identified: C, CC, CXC and CX3C. In addition to sequence variations, the number of amino acids between the first and second cysteines in the N-terminal end are used to identify various families of chemokines (Luster, 1998, Nomiyama, 2010]. Due to their similarities, several chemokine and chemokine receptors show certain degrees of redundancy. For example, chemokine (C-C motif) receptor 2 (CCR2) can bind to several pro-inflammatory chemokines such as CCL2, CCL7, CCL8, and CCL13 (Mantovani, 1999, Crown, 2006). This redundancy ensures a robust recruitment system that encompasses multiple types of immune cells. Monocytes express a wide range of chemokine receptors including CCR2, CCR5, and CX3CR1 [Xuan, 2015]. Furthermore, subpopulations of infiltrating monocytes often express distinct chemokine receptor combinations (Geissmann, 2003, Ransohoff, 2009). CCR2 is critical in monocyte emigration from bone marrow and recruitment of the pro-inflammatory subpopulation of monocytes toward inflamed sites. Studies have shown that CCR2 knockout mice are severely deficient in leukocyte migration, pathogen defense, and granuloma formation (Hokeness, 2005, Papadopoulou, 2008, Jinnouchi, 2003). CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), is the main ligand for CCR2. While CCR2 can also interact with other pro-inflammatory chemokines, CCR2 is the only target for CCL2. Studies using CCL2 knockout mice have demonstrated the non-redundant role of CCL2 in the recruitment of pro-inflammatory monocytes (Baeck, 2011, Huang, 2001). In contrast, CX3CR1 is critical in the

recruitment of anti-inflammatory monocytes. Anti-inflammatory monocyte subpopulations from mice deficient in CX3CR1 have exhibited depressed rolling and adhesion abilities [Auffray, 2007]. This results in a prominent accumulation of pro-inflammatory macrophages, exaggerated tissue damage and a prolonged inflammatory response [Karlmark, 2010]. Fractalkine, also known as chemokine CX3C ligand 1 (CX3CL1), is the only known ligand for CX3CR1. The lack of redundancies for CCL2-CCR2 and CX3CR1-CX3CL1 interactions allow circulating monocytes to be recruited to the inflamed site in a phenotype specific manner. Corresponding subpopulations of monocytes are recruited during different stages of inflammation, and the recruited macrophages along with resident macrophages, in turn, direct the progression of inflammation toward resolution.

Inflammation

Inflammation is the immune response to tissue injury or infection. The cardinal signs of inflammation include heat, redness, swelling, pain, and loss of function. A wide range of cytokines and inflammatory mediators secreted by multiple cell types are involved in the inflammatory response. The overall goal of the inflammatory response is to remove the deleterious stimuli and restore tissue homeostasis. The persistence of inflammatory cells and stimuli and dysregulated inflammation can also cause chronic inflammation resulting in tissue destruction, fibrosis, and tumor initiation.

Initiation of the inflammatory response

Inflammation is a non-specific immune response launched to remove injurious and infectious agents after warning signals are detected. Various agents derived from exogenous and endogenous materials have been identified as inflammatory triggers. A major source of exogenous stimuli is the cellular components of invading pathogens known as PAMPs. Surveilling macrophages and neutrophils recognize conserved PAMPs via PRRs such as TLRs. TLRs consist of families of transmembrane receptors that contain extracellular leucine-rich repeats region for PAMP recognition and

intracellular toll–IL-1 receptor (TIR) domains for downstream signal activation [Beutler, 2009]. Thus far 10 different functional TLRs have been identified in humans and 12 in mice, which recognize specific PAMPs and serves distinct functions in initiation of the inflammatory response. The recognition of PAMPs by TLR triggers a series of downstream signals including activation of the transcription factor, NF-κB; this leads to the production of inflammatory cytokines such as IFN-γ, and TNF-α [Kawai, 2010].

Non-pathogenic stimuli can also initiate inflammatory reactions. Following exposure to toxic chemicals and drugs, damaged cells and necrotic tissues release endogenous danger signals that activate the innate immune system and trigger an inflammatory response. This type of inflammation is referred to as “sterile inflammation” and the triggering dangers signals are called DAMPs. DAMPs are often pre-existing molecules such as functional proteins or cells components that are released through either non-canonical (endoplasmic-reticulum-Golgi-independent) pathway or when the plasma membrane is disrupted (Shi, 2000, Keller, 2008). Like PAMPs, DAMPs are recognized by PRR on surveying innate immune cells. DAMPs includes intracellular molecules such as high mobility group box 1 (HMGB1), a DNA binding protein that promotes transcription factors and other nuclear proteins binding to DNA (Agresti, 2003, Lotze, 2005), S100 proteins, which are a group of calcium binding proteins [Foell, 2007], mitochondrial N-formylmethionyl peptides (Carp, 1982, Zhang, 2010), heat shock protein (HSP), HSP chaperoned peptides (Suto, 1995, Wheeler, 2009), ATP [Mariathasan, 2006], and uric acid (Shi, 2003, Kono, 2010).

Progression of the inflammatory response

In order to neutralize invading pathogens or limit tissue injury, an acute inflammatory response is rapidly launched by innate immune cells including neutrophils and monocytes. In the acute inflammatory response, sentinel resident macrophages and mast cells are the first to recognize DAMPs and PAMPs. After PPR binding, these cells

become activated, releasing multiple inflammatory mediators that initiate signaling cascades. Mediators including vasoactive amines such as histamine and eicosanoids such as prostaglandin E_2 (PGE_2) interact with the local endothelium and induce vasodilation [Medzhitov, 2008]. Vasodilation induces vessel leakiness and allows greater blood flow, thus increasing exposure of inflamed site to immune cells. The dilated blood vessel and extravasation of plasma account for the heat, redness, and swelling, three of the cardinal signs of inflammation.

Activated resident macrophages also release inflammatory cytokines such as TNF-α, which upregulate expression of cell adhesion molecules including intercellular adhesion molecule 1 (ICAMs), P-selectin, and E-selectin on endothelial cells (Kunkel, 1996, Suárez, 2010). Endothelial cell surface selectins and adhesion molecules can bind to their respective receptors on leukocytes and cause leukocyte rolling, adhesion, and transmigration. Selectins interact with the P-selectin glycoprotein ligand 1 (PSGL1) and other glycosylated ligands located on leukocyte surface to capture or tether the circulating cells (Sperandio, 2003, An, 2008, Schmidt, 2013). Activated surface integrin binds to endothelial cell surface ligands, ICAMs, to further promote leukocyte rolling and provides firm leukocyte adhesion (Salas, 2004, Schmidt, 2013). Binding of integrin, selectin, and other adhesion proteins causes circulating immune cells to migrate toward inflamed tissues.

Activated endothelial cells and macrophages release chemotactic factors including lipid mediators and chemokines which attract immune cells toward the inflamed site. Once the ligands are detected, immune cells in the circulation displaying appropriate chemokine receptors migrate toward the inflammatory site based on a gradient of chemokines [Frevert, 2006]. The combination of vasodilation, increasing endothelial stickiness and the release of chemokines, causes infiltration and accumulation of leukocytes including neutrophils and macrophages at sites of injury.

Neutrophils are usually the first cells to appear during the early stage of the acute inflammatory response. The cytokines, TNF-α, IL-6 and IL-1β, released during acute inflammation induce the production of IL-8 or CXCL8, which is a major chemokine for neutrophils (Martich, 1991, de Oliveira, 2013). Once neutrophils arrive at the site of inflammation, they become activated through leukotriene B_4 receptor binding (Tager, 2003, Oyoshi, 2012), undergo a respiratory burst and release ROS, antimicrobial peptides such as defensins and bactericidal/permeability-increasing protein (BPI), proteinases, elastases and other cytotoxic molecules (Faurschou, 2003, Kolaczkowska, 2013). While infiltrating neutrophils are important at the initial stage of inflammation and pathogen clearance, they quickly undergo apoptosis (Kasahara, 1997, Kolaczkowska, 2013). Subsequently, circulating monocytes are recruited to injured tissues and mature into macrophages. Once activated, macrophages assist in the clearance of remaining microbes and damaged cells by producing cytotoxic mediators such as ROS and RNS (Cruz, 2007, Vallyathan, 1992). Macrophages are also critical in maintaining the inflammatory response by releasing large amounts of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, while also promoting leukocyte recruitment by secreting chemokines including CCL2 and CCL8 (Luster, 1994, Boswell, 1988, Romano, 1997, Dean, 2008).

Resolution of the Inflammatory Response

Although inflammatory cytokines and cytotoxic metabolites generated by macrophages and neutrophils are critical in the elimination of invading pathogens and damaged cells, the process can also contribute to tissue injury when it is unresolved. To initiate resolution, macrophages clear aged and apoptotic neutrophils to limit tissue injury (Savill, 1989, Kolaczkowska, 2013). The production of pro-inflammatory mediators by macrophages is suppressed and anti-inflammatory/wound repair molecules are released. Several anti-inflammatory mediators have been identified including haptoglobin,

apolipoprotein E (ApoE), transforming growth factor beta (TGFβ), and lipid mediators such as prostaglandin D_2 (PGD_2), and lipoxin A_4 (LXA_4), (Huynh, 2002, Bannenberg, 2005). With the inhibition of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines, wound repair is initiated.

Acetaminophen

Acetaminophen (APAP) is a commonly used over the counter analgesic. Although safe and effective at therapeutic doses, when ingested in excess, APAP can cause hepatotoxicity, which can lead to liver failure. APAP induced tissue necrosis is one of the most common causes of acute liver failure in United States (Bower, 2007, Li, 2011). Within therapeutic doses, APAP is safely metabolized through glucuronidation or sulfation in the liver and intestine. After conjugation, APAP derivatives can be safely excreted in urine. Once the glucuronidation and sulfation pathways are overwhelmed by overabundance of APAP, alternative metabolism via cytochrome P450 can result in hepatotoxicity. Early stages of APAP induced toxicity can be treated with N-acetylcysteine (NAC). However, treatment options are still limited in later stages of APAP intoxication. Patients often require a liver transplant in severe cases of APAP induced toxicity.

Mechanisms of APAP Toxicity

Normally, APAP is metabolized through phase II drug metabolism. The majority of APAP undergoes glucuronidation by the enzyme UDP-glucuronosyltransferases (UGT), while the remaining undergoes sulfation by sulfotransferases (SULT). Under these conditions, only a minor percentage of APAP is oxidized by cytochrome P450 isoform 2E1 (CYP2E1). However, following APAP overdose, normal metabolic pathways are overwhelmed, and metabolism through CYP2E1 results in generation of N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive metabolite. Metabolism of NAPQI depletes antioxidant defenses, including glutathione, and results in irreversible covalent

binding of NAPQI to cellular proteins and nucleic acids. This generates mitochondrial membrane instability, followed by disruption of ATP generation and release of toxic metabolites, resulting in centrilobular hepatocellular necrosis; tissue associated with inflammation and macrophage accumulation (Masubuchi, 2005, Laskin, 2011). Liver CYP2E1 activities are concentrated in the centrilobular regions; therefore, NAPQI production is maximal around hepatic central vein. In addition, glutathione and oxygen rich blood from the portal vein affords resistance to oxidative stress and hypoxia in the periportal zone [Malarkey, 2005]. The variations in enzyme and nutrient content across the liver zones cause the signature centrilobular necrosis pattern ascribed to APAP intoxication. Administration of NAC replenishes the antioxidant glutathione which neutralized NAPQI and reactive oxygen species, thereby decreasing the potential toxicity in the early intoxication stages. The treatment is most effective within 8 hours of APAP administration and effectiveness is lost with delay (Kerr, 2005, Woodhead, 2012).

Role of macrophages in APAP hepatotoxicity

After the initial reactive metabolite insult, accumulating evidence suggests that macrophages play an active role in APAP induced toxicity and repair. Studies have shown the influx of bone marrow derived macrophages into inflamed liver especially concentrating in the centrilobular necrotic regions (Laskin, 2007, Dambach, 2002). Distinct morphologies of these macrophages including larger size, increased vacuolization, greater cytoplasmic nuclear ratio, enhanced phagocytic ability, and up-regulated cytokine production suggest that these cells are activated (McCloskey, 1992, Laskin, 1988, Pilaro, 1986, Laskin, 1986). Further investigation suggests that these activated macrophages consist of both M1 pro-inflammatory and M2 anti-inflammatory subpopulations (Dragomir, 2012, Holt, 2008).

APAP injured or necrotic tissues release DAMPs such as HMGB1 and cause activation of M1 macrophages [Dragomir, 2011]. In addition, loss of hepatocytes

incapacitates the liver's ability to neutralize PAMPs such as endotoxin [Fukui, 2005]. Exposure of gut-derived PAMPs delivered from portal vein can also potentially activate macrophages. Activated M1 macrophages intensify oxidative stress by upregulating iNOS expression and producing cytotoxic molecules including ROS and RNS [Dragomir, 2012]. The pathogenic roles of ROS and RNS is supported by findings that iNOS knockout mice are protected against oxidative stress and APAP induced toxicity (Gardner, 2002, Jaeschke, 2011). Macrophages also play a key role directing and sustaining inflammation. APAP induced oxidative stress activates transcription factors such as NF-κB and up-regulates the production of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1α, IL-1β, and IL-6 by macrophages, as well as local tissues (Dragomir, 2012, Powell, 2006, Aggarwal, 2006, Antoniades, 2014). NF-κB knockout mice show decreased inflammation and down-regulated expression of TNF-α, IL-1β, and COX-2 [Dambach, 2006]. Inhibition of M1 macrophages by gadolinium chloride or dextran sulfate alleviates the toxicity induced by APAP (Laskin, 1995, Abdel-Zaher, 2007, Kinoshita, 2005). The pathogenic role of M1 macrophages is further confirmed by exacerbated APAP intoxication when mice pretreated are M1 activator such as LPS [Maddox, 2009].

Activated M2 macrophages also play an active role in the pathogenic response to APAP. The expression of M2 macrophage markers including Ym1, Fizz-1 and arginase is up-regulated following APAP toxicity [Holt, 2010]. These alternatively activated macrophages produce anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 that recruit additional M2 macrophages; It has been reported that IL-4, IL-10, or IL-13 knockout mice are more susceptible to APAP induced toxicity (Bourdi, 2007, Antoniades, 2006, Yee, 2007). Wound repair cytokines such as connective tissue growth factor (CTGF), TGFβ and vascular endothelial growth-factor (VEGF) are also secreted by M2 macrophages (Gardner, 2002, Donahower, 2006). Suppression of M2 macrophages with

encapsulated dichloromethylene diphosphonate (clodronate) liposomes increase APAP induced toxicity whereas a M2 inducer, such IL-13, protects against APAP induced injury (Holt, 2010, Campion, 2008, Yee, 2007). Interference with monocyte migration can also alter the outcome of APAP intoxication. CCR2 knockout mice which are unable to mobilized bone marrow monocytes exhibit delayed resolution compared to wild type mice after APAP administration [Holt, 2008].

Liver

The liver is one of the largest organs in the body second only to the skin. It carries out a wide range of biological functions including biosynthesis of blood proteins, metabolism, storage of glycogen, and immunity that are critical in maintaining physiological homeostasis. Mouse and rat liver can be divided into 5 lobes, left median, right median, left lateral, right lateral, and caudate lobes. In contrast, human livers have four lobes, the right, left, quadrate, and caudate [Malarkey, 2005]. The liver receives approximately 25% of the cardiac output. Blood enters the liver through the portal vein and hepatic artery, supplying 70% and 30% of blood flow respectively. The portal vein delivers nutrients rich blood from mesenteric, gastric, splenic, and pancreatic veins while hepatic artery supplies the liver with oxygenated blood [Malarkey, 2005]. In addition to nutrients from the gut, the portal vein delivers toxins, waste, and other materials such as hormones. Anatomically, the portal vein is accompanied by the hepatic artery and bile duct forming a structure called the portal triad. Liver blood is collected in the central vein before exiting the liver and merging with the vena cava.

At the histological level, the liver consists of numerous hexagonal lobules with 4 to 6 portal triads surrounding a central vein. Based on the proximity of blood vessels, hepatic lobules can be divided into centrilobular, midzonal, periportal zones. Blood flow from the portal triad into sinusoids toward central vein thus creating a gradient of

nutrients, glutathione, and oxygen between different zones. In addition, metabolic enzyme activities vary from periportal to centrilobular zones. The periportal region contains higher levels of enzymes involved in glycogenesis, urea cycle activity, and glucose-6-phosphatase activity, whereas cytochromes such as CYP2E1 and CYP1A2, and metabolic enzymes glucokinase, carboxylesterase and glutamine synthetase are concentrated in the centrilobular region (Malarkey, 2005, Braeuning, 2006). The liver is largely composed of four different types of specialized parenchymal and non-parenchymal cells. Hepatocytes are the major cell type in the liver. Lining the sinusoidal capillaries and surrounding hepatocytes are the sinusoidal endothelial cells. Kupffer cells patrol in the sinusoid for traces of pathogens, waste, and debris. Finally, stellate cells located in the space of Disse play a critical role in liver regeneration and fibrosis [Juza, 2014].

Hepatocytes

The major parenchymal cells, hepatocytes, make up 60% of all cells in liver and up to 80% of its total volume [Malarkey, 2005]. Hepatocytes are arranged in single cell layer plates that radiate from a central vein. Each plate is separated by a sinusoid allowing hepatocytes access to the blood flow. The sinusoidal surfaces of hepatocytes are covered with microvilli that increase surface area. While most hepatocytes contain a large single round nucleus, it is also common to find binucleated cells. Hepatocytes contain many mitochondria that contribute to substantial energy metabolism. Hepatocytes display extensive rough endoplasmic reticulum (ER) reflecting the high capacity of protein synthesis for molecules such as clotting factors, fibrinogen, and albumin in the liver; patients with liver damage often experience coagulation problem [Pluta, 2010]. Smooth ER is a major center for phase I and phase II metabolism containing biotransformation enzymes. Phase I enzymes include various isoforms of cytochrome P450 (CYP), flavin monooxygenase, and epoxide hydrolase, whereas

Phase II enzymes consist of glucuronosyl transferases, sulphotransferases, N-acetyltransferase, and glutathione-S-transferases [Sevior, 2012]. Uneven disruptions of various enzymes across different hepatic lobules zones contribute to the gradient of metabolizing processes [Malarkey, 2005].

Hepatocytes have remarkable regenerative abilities. Rodent liver is capable of rapid regeneration; a complete recovery can be achieved even after 2/3 of liver is surgically removed [Michalopoulos, 2010]. After hepatotoxicity or trauma, hepatocytes are the first to enter mitosis. Proliferation of hepatocytes is initialized from periportal regions and progresses toward centrilobular regions [Michalopoulos, 2007]. Non-parenchymal cells such as Kupffer cells and sinusoidal endothelial cells contribute to the matrix remodeling by releasing enzymes and growth factors such as insulin-like growth factor binding protein (IGFBP1), metalloproteinase 9 (MMP9), and hepatocyte growth factor (HGF) [Böhm, 2010]. Mice depleted of Kupffer cells experience difficulties in liver regeneration [Bilzer, 2006]. Unlike other cell types which require stem cells to replenish, mature hepatocytes are able to undergo mitosis and rapidly produce new hepatocytes. In severe injury where hepatocyte proliferation is suppressed, hepatic stem cells, or oval cells, which resemble cholangiocytes can undergo expansion and further mature into hepatocytes [Michalopoulos, 2007]. Finally, when all alternative methods of regeneration fail, it is possible that bone marrow derived stem cells migrate to severely injured liver and serve as hepatic progenitor cells. [Margini, 2014]

Kupffer Cells

Kupffer cells are the largest population of tissue macrophages in the body, constituting 80%- 90% of all macrophages and 35% of the non-parenchymal cells in mice [Bilzer, 2006]. Kupffer cells are mature tissue macrophages expressing epidermal growth factor (EGF)-like module-containing mucin-like hormone receptor-like 1 (F4/80), a surface glycoprotein which participates in immune tolerance and denotes macrophage

maturity [van den Berg, 2005]. In addition, Kupffer cells express a wide range of surface receptors including Fc receptors, complement receptors, scavenger receptors, and TLRs [Gardner, 2007]. Two populations of Kupffer cells are observed in liver, one subset is bone marrow derived and short lived whereas the second is a longer-lived radioresistant population which proliferates within the organ (Kinoshita, 2010, Klein, 2007). Kupffer cells specialized in the removal of cellular debris, pathogens, bacterial components, and foreign materials from the portal circulation. In addition, they have potent secretory capacity, releasing numerous cytokines, growth factors, lipid mediators, ROS, RNS, and proteolytic enzymes that are critical in maintaining liver homeostasis [Laskin 2011]. A functional gradient can also be observed in Kupffer cells across different regions of hepatic lobules. As the first line of contact with gut derived blood, Kupffer cells in periportal region are found to be more phagocytic and larger in size. They also produce more ROS, TNF-α, PGE_2 and IL-1 whereas centrilobular macrophages secrete higher level of nitro oxide (NO) [Bilzer, 2006].

Sinusoidal Endothelial Cells

Sinusoidal endothelial cells are specialized cells that form the lining of the sinusoids. These endothelial cells account for approximately 20% of liver cells and have specialized fenestrations approximately 125–175 nm in diameter that cluster forming sieve plates which allow hepatocytes access to the sinusoid [Baratta, 2009]. An extensive cytoskeleton and actin fibers allow sinusoidal endothelial cells to control fenestral dilation in response to various cytokines [Svistounov, 2012]. Regulation of fenestrae control hepatocyte exposure to blood and maintain the exchange of fluid, solutes, particles and metabolites (Braet, 2002, Pfeiffer, 2014). Furthermore, sinusoidal endothelial cells lack the basal lamina that is common to vascular endothelial cells. Sinusoidal endothelial cells are capable of endocytosis and pinocytosis of smaller particles and are active in transporting materials and scavenging debris [Elvevold, 2008].

They also play a significant role in the innate immune response expressing Fc-γ receptors, mannose receptors and scavenger receptors and promote antigen presentation by expressing MHC class I and II and the costimulatory molecules CD40, CD80, and CD86 (Elvevold, 2008, Gardner, 2007, Diehl, 2008). Furthermore, sinusoidal endothelial cells can produce cytokines such as IL-6 when stimulated with LPS and secrete pro-inflammatory mediators in liver fibrosis (Uhrig, 2005, Connolly, 2010).

Stellate Cells

Hepatic stellate cells represent roughly 10% of liver cells [Puche, 2013]. They are referred to as including perisinusoidal cells, parasinusoidal cells, hepatic pericytes, lipocytes, fat storing cells, and Ito cells. Stellate cells are star shaped cells that are located in the space between sinusoid endothelial cells and hepatocytes, also known as space of Disse. These cells are concentrated in the periportal region and have extensive subendothelial processes connecting various cells up to 140 μm away [Puche, 2013]. Numerous lipid droplets rich in vitamin A are stored in stellate cells while they are in a quiescent state. However inflammatory cytokines from hepatocytes, Kupffer cells, sinusoidal endothelial cells, and infiltrating monocytes, along with tissue injury and oxidative stress can cause stellate cell activation (Imamura, 2005, Kisseleva, 2006). Once activated, these cells lose stored lipid and mature into a fibroblast-like phenotype that actively participates in fibrogenesis through collagen deposition [Friedman, 2008]. In addition, activated stellate cells secrete several wound healing factors such as TGF-β1 and platelet-derived growth factor (PDGF) that induce tissue regeneration and cause autocrine stimulation [Wu, 2008]. The resolution of liver injury usually results in the deactivation or apoptosis of stellate cells, however, perpetuation of stellate cell activities can cause the development of fibrosis (Krizhanovsky, 2008, Troeger, 2012).

SPECIFIC AIM

Acetaminophen (APAP) is a commonly used analgesic that is also present as an active ingredient in many over the counter medicines. While APAP is safe and effective at therapeutic doses, overdose with APAP can lead to severe hepatotoxicity. Studies showed that different subpopulations of macrophage contribute to the pathogenesis of APAP-induced hepatotoxicity. However, the origin, phenotype, and development of the macrophage subpopulations are still poorly understood.

We hypothesize that bone marrow is the source of both pro- and anti-inflammatory infiltrating macrophage subpopulations and that chemokine receptor CX3CR1 is critical for the anti-inflammatory monocyte recruitment. To test this hypothesis, studies are designed to:

(1) Analyze the role of bone marrow as a source of pro- and anti-inflammatory macrophages that accumulate in the liver after APAP

(2) Assess the role of CX3CR1 chemokine receptor in macrophage subpopulation trafficking.

MATERIALS AND METHODS

Animals

Mice were housed in microisolation cages and allowed free access to food and water. All animals received humane care in compliance with the institution's guidelines, as outlined in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Male specific pathogen-free wild type mice (C57BL/6J) and GFP-transgenic mice (C57BL/6-Tg (CAG-EGFP) 131Osb/LeySopJ) were obtained from the Jackson Laboratory (Bar Harbor, ME). Wild type mice were subject to 10-12 Gy whole body radiation for 45 min in Faxitron cabinet X-ray system model 43855A (Faxitron X-ray Corporation, AZ). GFP^+ mice were euthanized with nembutal (200 mg/kg, i.p.) and bone marrow collected from left and right femurs and tibiae. Isolated bone marrow cells ($2x10^6$) were quantified then injected i.v. into irradiated mice thus creating bone marrow GFP^+ chimeric mice. Mice were used four weeks later (Klein, 2007, Lin, 2009).

Female breeder $CX3CR1^{tm1Litt}$ ($CX3CR1^{GFP/GFP}$) mice from the Jackson Laboratory (Bar Harbor, ME) were cross bred with C57BL/6J wild type mice to generate reporter mice ($CX3CR1^{+/GFP}$).

Liver nonparenchymal cell isolation and flow cytometry

Bone marrow GFP^+ chimeric mice or $CX3CR1^{+/GFP}$ reporter mice were fasted overnight prior to i.p administration of APAP (300 mg/kg) or pyrogen-free phosphate-buffered saline (PBS) control. After 24-96 h, mice were euthanized with nembutal (200 mg/kg) and the liver perfused with ice cold PBS, removed and teased through 70 μm nylon mesh cell strainer. The resultant cell suspension was layered onto a 2-step (40%/70% in PBS) discontinuous Percoll gradient and centrifuged at 720xg for 30 min at 24°C [Rahman, 2014]. Isolated non-parenchymal cells were then incubated with anti-mouse FcR II/III antibody (1:1000 dilution in staining buffer) to block nonspecific binding

followed by anti-mouse Alexafluor (AF)647-conjugated-CD11b (1:2000 dilution), Phycoerythrin (PE)-conjugated-Ly6C (1:2000 dilution in staining buffer), eFluor700-conjugated-Ly6G (1:2000 dilution in staining buffer), and PE/Cy7-conjugated-F4/80 antibodies (1:2000 dilution in staining buffer). After fixation with 2% paraformaldehyde, cells were analyzed on a Beckman Coulter Gallios flow cytometer (Beckman Coulter, Sharon Hill, PA). The resulting data were analyzed using Kaluza software (Beckman Coulter). All antibodies used for flow cytometry were purchased from Biolegend, (San Diego, CA). Cells were gated first based on forward and side scatter, followed by viability to identify live cells. Live cells were analyzed for Ly6G, CD11b, and Ly6C expressions for macrophages. The Ly6C expressing macrophage populations were then evaluated for F4/80 expressions.

Immunofluorescence

Livers samples from both APAP and PBS treated bone marrow GFP^+ chimeric mice and CX3CR1 reporter mice were collected from the left lateral lobes and immediately frozen in liquid nitrogen chilled isopentane then imbedded in optimal cutting temperature medium (Sakura Finetek, Torrance, CA). Six micron sections were prepared and fixed in 90% methanol/10% acetone. Slides were incubated with anti-mouse mannose receptor (MR, Abcam, Cambridge, MA, 1:1000 dilution in 5% PBS-serum), heme oxygenase (HO-1, Enzo Life Sciences, Farmingdale, NY, 1:500 dilution in 5% PBS-serum), F4/80 (AbD Serotec, Raleigh, NC, 1:50 dilution in 5% PBS-serum) or cyclooxygenase (COX)-2 (Abcam, Cambridge, MA, 1:750 dilution in 5% PBS-serum) antibody, followed by isotype-specific, AF555-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, California) or AF647-conjugated goat anti-rat secondary antibody (Invitrogen, Carlsbad, California). Images were acquired using a Leica SP5 confocal microscope (Leica, Wetzlar, Germany). Identical laser power, gain and offset settings were used to obtain all images to allow for quantitative comparisons; data were

analyzed using Leica AF co-localization software. Fluorescent image was analyzed for total colored pixel count; this was compared with the number of pixels that carried both GFP and AF555 or AF647 to obtain the percentage of colocalization.

Statistical analysis:

Statistical analysis was performed using GraphPad Prism 6 (La Jolia, CA). Analysis of multiple time points was performed using one-way ANOVA followed by Tukey's HSD test and Student's t test. A p-value of ≤ 0.05 was considered significant.

RESULTS

Characterization of Bone Marrow-Derived Myeloid Cells Accumulating in the Liver after APAP

In previous studies, we observed multiple populations of infiltrating inflammatory cells in the liver following APAP administration to mice [Dragomir, 2011]. In the present study, we determined whether these cells were derived from the bone marrow by using GFP^+ chimeric mice. In the bone marrow of chimeric mice, more than 80% of the cells were found to be GFP^+ (Fig. 1), demonstrating effective bone marrow transplantation. The bone marrow GFP^+ population remained high following APAP administration, which confirmed the establishment of a GFP^+ progenitor cell population.

In the livers of control mice, 33% of nonparenchymal cells were found to be GFP^+ (Fig. 1). A significant increase in GFP^+ cells was observed in the liver after APAP intoxication (Fig. 1). This peaked 48 h post APAP, returning to control levels by 96 h. To further characterize the cells, four different cell surface markers were used (Table 1): CD11b, the α_M-chain of the Mac-1 $\alpha_M\beta_2$ integrin, expressed on infiltrating myeloid cells (Shechter, 2009, Dziennis, 1995); Ly6G, a leukocyte marker that is expressed strongly on granulocytes; Ly6C, a macrophage marker which is highly expressed on pro-inflammatory macrophages and expressed weakly on anti-inflammatory subpopulations [Dragomir, 2012]; and F4/80, a macrophage marker expressed on mature cells (Rose, 2012, Ahn, 2010, Arnold, 2007, Gao, 2005). GFP^+ cells were initially analyzed for expression of CD11b and Ly6G. We found that there were two distinct populations of $CD11b^+$ infiltrating GFP+ myeloid cells in the liver which were either $Ly6G^+$ or $Ly6G^-$. The $GFP^+Ly6G^-CD11b^+$ subpopulation was increased after APAP administration reaching maximum levels by 48 h (Fig. 2). The GFP^+ $Ly6G^+$ $CD11b^+$ subpopulation was also increased, and similarly peaked 48 h post APAP treatment; by 72 h these cells were at control levels (Fig. 1).

To further characterize the macrophage populations responding to APAP, we focused on $Ly6G^-$ cells. Two subpopulations of Ly6C positive cells were identified in the liver. Based on the degree of expression, the cells were characterized as Ly6C high ($Ly6C^{hi}$) pro-inflammatory or Ly6C low ($Ly6C^{lo}$) anti-inflammatory subpopulations (Fig. 3). Both $Ly6C^{hi}$ and $Ly6C^{lo}$ subpopulations increased after APAP administration, following a similar pattern as GFP^+CD11b^+cells, peaking at 48 h (Fig. 3). Next, the $GFP^+Ly6G^-CD11b^+Ly6C^{hi}$ and $GFP^+Ly6G^-CD11b^+Ly6C^{lo}$ subpopulations were analyzed for F4/80 expression. $Ly6C^{hi}F4/80^-$ immature pro-inflammatory cells were increased after APAP, reaching a maximum at 48 h and returning to control by 96 h (Fig. 4). The $Ly6C^{hi}F4/80^+$ mature pro-inflammatory subpopulation increased at 24 h and remained high for at least 96 h (Fig. 4). $Ly6C^{lo}$ $F4/80^-$ immature anti-inflammatory cells were also increased at 24 h post APAP; however they returned to control levels by 48 h (Fig. 4). $Ly6C^{lo}F4/80^+$ mature anti-inflammatory cells accumulated in the liver more slowly after APAP reaching a peak at 48 h and then declined (Fig. 4).

Bone Marrow-Derived Myeloid Cells are Involved in Diverse Pro-inflammatory and Anti-inflammatory Activities

In further studies GFP^+ myeloid inflammatory cells responding to APAP were analyzed in histologic sections by confocal microscopy. Immunostaining with F4/80 antibody confirmed the presence of bone marrow-derived GFP^+ mature macrophages in the livers after APAP administration to mice (Fig. 5). The GFP^+ $F4/80^+$ macrophages decreased in necrotic areas surrounding central veins following APAP administration up to 24 h and then reappeared by 48 h. We next measured expressions of COX-2, the enzyme mediating pro-inflammatory eicosanoid production [Turini, 2002]. Low level of COX-2 expressions were observed in endothelial cells and some macrophages in livers of control and APAP treated mice (Fig 6). Following APAP administration, GFP^+ macrophages expressing higher levels of COX-2 appeared in the liver after 24 h in

centrilobular regions. Next we analyzed expression of HO-1, a marker of oxidative stress and anti-inflammatory macrophages (Immenschuh, 2010, Sierra, 2010, Orozco, 2007). HO-1 was expressed by both GFP^- and GFP^+ macrophages in livers of control and APAP treated mice. After APAP administration, HO-1 expression on GFP^+ macrophages increased in a time dependent manner, a response that plateaued by 96 h in areas surrounding central veins (Fig. 5). In further studies, we analyzed expression of MR, a scavenge receptor important in phagocytosis (Malovic, 2007, Auffray, 2007, Taylor, 2005). MR expressing endothelial cells and macrophages were observed in livers of control and APAP treated mice. Some MR^+ macrophages were also found to be GFP^+. Following APAP administration, a reduction in GFP^+ MR^+ macrophages was observed up to 72 h which was reversed by 96 h (Fig. 5).

Characterization of $CX3CR1^{+/GFP}$ Cells Accumulating in the Liver after APAP Intoxication

Fractalkine receptor, CX3CR1 is a chemokine receptor that is predominantly associated with the recruitment of circulating anti-inflammatory monocytes to sites of injury (Karlmark, 2010, Tacke, 2007). In our next series of studies we analyzed $CX3CR1^+$ macrophage migration into the liver following APAP administration using $CX3CR1^{+/GFP}$ reporter mice. We found that approximately 5% of nonparenchymal cells expressed CX3CR1 in control mice (Fig. 6). A significant influx of $CX3CR1^+$ cells was observed in the liver after APAP administration; however this was not evident until 96 h post treatment (Fig. 6).

Next, we characterized the $CX3CR1^+$ cells by analyzing CD11b, Ly6G, Ly6C and F4/80 expression by flow cytomertry. We found that the $CD11b^+$ cells account for roughly half of the $CX3CR1^+$ cells after APAP administration. $CX3CR1^+CD11b^+$ cells were observed in the liver 24 h following APAP and they increased rapidly for at least 96 h (Fig. 7). Ly6G staining demonstrated that the majority of the $CX3CR1^+$ cells were

$Ly6G^-$ in APAP treated mice. $CX3CR1^+Ly6G^+CD11b^+$ granulocytic cells also showed a trend toward increased number. This increase was biphasic with an initial peak at 48 h and then again by 96 h (Fig. 7). The majority of $CX3CR1^+$ cells in the liver were $Ly6G^-CD11b^+$ monocytic subpopulation in control and APAP treated livers which increased significantly 96 h after APAP administration (Fig. 7).

$CX3CR1^+Ly6G^-CD11b^+$ cells were then analyzed for Ly6C expression to characterize the monocytic population. Two populations of $Ly6C^+$ cells were identified: $Ly6C^{hi}$ and $Ly6C^{lo}$ (Fig. 8). The majority of $Ly6C^+$ cells were found to be $Ly6C^{hi}$ following APAP administration up to 72 h. By 96 h, the majority of the $Ly6C^+$ population was $Ly6C^{lo}$. While different percentages of $Ly6C^{hi}$ and $Ly6C^{lo}$ were found in the liver after APAP administration, due to the low total cell count at the early time points, differences between these subpopulations was not apparent, and increase in these cells did not become significant until 96 h (Fig. 8).

Both the $CX3CR1^+Ly6G^-CD11b^+Ly6C^{hi}$ and $CX3CR1^+Ly6G^-CD11b^+Ly6C^{lo}$ cell populations were further analyzed for F4/80 expression. The majority of the pro-inflammatory $CX3CR1^+Ly6G^-CD11b^+Ly6C^{hi}$ population was $F4/80^+$ mature macrophages; both mature and immature subpopulations increased significantly at 96 h post APAP (Fig. 9). There was also a trend toward increased numbers of $CX3CR1^+Ly6G^-CD11b^+Ly6C^{lo}F4/80^-$ immature anti-inflammatory cells at 24 and 48 h (Fig. 9). $CX3CR1^+Ly6G^-CD11b^+Ly6C^{lo}F4/80^+$ mature cells also exhibited a trend toward increased numbers at 24 h, followed by a more significant increase at 96 h after APAP administration (Fig. 9).

$CX3CR1^{+/GFP}$ Cells Consist of Pro-inflammatory and Anti-inflammatory Phenotypes

An influx of $CX3CR1^+$ cells was observed accumulating around centrilobular regions following APAP administration to mice which reached a maximum by 96 h. To

further characterize the CX3CR1^{+} cell subpopulations, liver sections were stained with phenotype markers. The majority of CX3XR1^{+} cells in control liver also expressed F4/80, indicating that these cells consisted of mature cells. However, this subpopulation was not evident until 72 h after APAP (Fig. 10). Cells coexpressing CX3CR1 and HO-1 were observed to increase in a time dependent manner, peaking at 72 h after APAP administration (Fig. 10). In contrast, a trend toward decreased COX-2 expression was noted in CX3CR1^{+} cells 24 to 48 h after APAP administration (Fig. 10). MR-1 was expressed at low levels in CX3CR1^{+} macrophages of control mice and its expression remained consistent after APAP administration (Fig. 10).

DISCUSSION

Evidence suggests that macrophages play important roles in the pathogenesis of APAP-induced hepatotoxicity. Following APAP intoxication, monocyte-derived inflammatory macrophages are observed in the livers that are distinct from resident liver macrophages (Kupffer cells) [Holt, 2008]. These subpopulations consist of classically activated Ly6C^{hi} pro-inflammatory M1 macrophages and alternatively activated Ly6C^{lo} anti-inflammatory M2 macrophages (Dragomir, 2012, Laskin, 2011, Holt, 2008). Whereas M1 macrophages appeared in the liver during early stages of injury, M2 macrophages were found during the resolution stages. Evidence suggests that macrophages are capable of switching phenotypes from M1 to M2 in response to signals in their microenvironment (Liu, 2013, Arnold, 2007, Porcheray, 2005). It is possible that the bone marrow-derived anti-inflammatory macrophages observed late during the pathology were first recruited to the liver as pro-inflammatory macrophages and later converted into anti-inflammatory phenotype. Conversely, reports of classical Ly6C^{hi} and non-classical Ly6C^{lo} monocytes subpopulations in the circulation suggest that monocytes are recruited and mature into macrophage in the liver with a predetermined pro- or anti-inflammatory phenotype (Varol, 2015, Hettinger, 2013, Ziegler-Heitbrock, 2010). The present studies were designed to track inflammatory macrophage accumulation in the liver from the bone marrow using GFP^{+} chimeric mice. We also analyzed migration of CX3CR1^{+} macrophages into the liver which are anti-inflammatory (Arnold, 2007, Nahrendorf, 2010, Zimmermann, 2012, Geissmann, 2003).

In order to identify the bone marrow-derived macrophages, GFP^{+} bone marrow cells were introduced into mice after myeloablation by radiation (Klein, 2007, Lin, 2009). The present studies demonstrate a consistently high level of GFP^{+} cells in bone marrow of control mice, as well as APAP treated mice. This indicates the establishment of donor hematopoietic progenitor cells. Following APAP administration, we observed an influx of

GFP expressing bone marrow-derived immune cells into the liver. This coincided with the accumulation of leukocytes reported in our previous studies in our wild type mouse model, and confirmed that the cells consist of both infiltrating and resident subpopulations [Dragomir, 2012]. While we found no observable tissue damage in PBS treated liver, approximately one third of liver immune cells expressed GFP. These findings are in agreement with reports that some bone marrow-derived leukocytes, including lymphocytes, are present in healthy livers (Mackay, 2002, Baratta, 2009). It has also been demonstrated that intense radiation causes liver inflammation and stimulates an unexpected leukocyte infiltration into the liver or Kupffer cell turnover, which is also consistent with our findings [Pan, 2010]. While Kupffer cells were thought to be long lived and radiation resistant, a small subpopulation of Kupffer cells is radiosensitive and derived from a bone marrow progenitor; this provides an additional explanation for our findings [Klein, 2007].

In the present studies, a significant increase in CX3CR1 expressing cells was observed in the liver during the later stages of APAP hepatotoxicity. This is consistent with the reports that CX3CR1 expressing cells are M2-like and important in the resolution phase of injury (Auffray, 2007, Ghassabeh, 2006). Interestingly, when we compared CX3CR1$^{+/GFP}$ mice to GFP bone marrow chimeric mice, higher total leukocyte cell counts were observed in the liver. This suggests a fundamental difference in the recruitment profile in these two models.

We also analyzed isolated GFP^{+} chimeric and CX3CR1$^{+/GFP}$ mouse liver leukocytes for expression of CD11b, an integrin expressed on the surface of myeloid cells, which is important for immune cell migration and infiltration into injury tissues (Stoneman, 2007, Kanda, 2006). Following APAP administration, two populations of GFP^{+} CD11b^{+} infiltrating myeloid cells were identified based on Ly6G expression. Ly6G, along with Ly6C, is part of antigen Gr-1 that is expressed by both monocytic and

granulocytic cells (Chiang, 2007, Rose, 2012). However, Ly6G expression is almost exclusively found on granulocytic cells [Daley, 2008]. By employing both CD11b and Ly6G in our gating strategy, infiltrating monocytic and granulocytic cells could be separated. Whereas the $CD11b^+$ $Ly6G^-$ subpopulation includes monocytes, macrophages, and monocytic myeloid derived suppressor cells (MDSCs) (Youn, 2010, Gabrilovich, 2009], $CD11b^+$ $Ly6G^+$ cells consists of neutrophils and granulocytic MDSC (Youn, 2010, Gabrilovich, 2009). In CX3CR1 reporter mice, our studies showed an accumulation of bone marrow-derived and CX3CR1 expressing granulocytic cells during early stages of APAP intoxication. The accumulation of neutrophils has been reported to contribute to early liver injury induced by APAP (Ishida, 2006, Liu, 2006). Findings of neutrophils in the liver suggest another possible mechanism by which bone marrow could participate in APAP-induced hepatotoxicity [Liu, 2006]. Interestingly, while our findings support reports that neutrophils express low level of CX3CR1, other investigators demonstrated that circulating neutrophils do not require CX3CR1 for recruitment and accumulation of $CX3CR1^+$ neutrophils (Ishida, 2008, Oh, 2008, Yang, 2007, Jung, 2000).

The current studies demonstrate that majority of bone marrow-derived GFP^+ leukocytes and CX3CR1 expressing cells detected in the liver are $CD11b^+$ and not $Ly6G^+$ indicating their monocytic phenotype. This finding further supports the importance of monocytic cells such as macrophages in APAP induced hepatotoxicity [Laskin, 2011].

Monocytes and macrophages were identified in the liver leukocyte population based upon their Ly6C expression [Dragomir, 2012]. Monocytes and macrophages expressing Ly6C were further divided into $Ly6C^{lo}$ anti-inflammatory and $Ly6C^{hi}$ pro-inflammatory subpopulations (Dragomir, 2012, Gordon, 2005). We observed similar increases in GFP^+ of bone marrow-derived and CX3CR1 expressing inflammatory infiltrating cells within 24 h of APAP administration. Our findings that both

subpopulations originated in the bone marrow suggest that bone marrow-derived monocytes can give rise to both pro-inflammatory and anti-inflammatory macrophage subpopulations (Varol, 2015, Hettinger, 2013, Ziegler-Heitbrock, 2010). This observation is in accord with other studies in which mice that were unable to recruit infiltrating monocytes were found to be resistant to inflammation, yet displayed delayed resolution of injury (Mitchell, 2009, Holt, 2008, Hokeness, 2005, Dambach, 2002). Previous studies suggested that anti-inflammatory macrophages express high levels of CX3CR1 when compared to pro-inflammatory macrophages; however, in the present studies, we did not find a clear distinction between the two subpopulations.

We also evaluated the maturity of cells responding to APAP induced injury based on F4/80 expression. It has been reported that as macrophages mature, F4/80 expression is upregulated, and that these cells acquire a more anti-inflammatory M2-like phenotype (Karlmark, 2009, Volarevic, 2012, Li, 2008, Hirsch, 1981). Our studies confirmed previous reports that F4/80 expression in the liver decreased during early inflammation, but returned during the resolution phase (Dragomir, 2012, Gardner, 2012). Both bone marrow-derived GFP^+ and CX3CR1 expressing $F4/80^+$ mature macrophages increased in the liver in a time dependent manner, consistent with previous reports [Dragomir, 2012]. This indicates that these cells are distinct from mature resident hepatic macrophages (Dey, 2014, Ginhoux, 2014). Our investigation found similar numbers of mature and immature pro-inflammatory GFP^+ bone marrow-derived macrophages in the liver immediately after APAP administration, which suggested that a subpopulation of bone marrow-derived macrophages mature prior to entering the liver. This is supported by reports of F4/80 expressing monocytes in the circulation (Tsou, 2007, Sunderkötter, 2003). Conversely, the continued influx of mature pro-inflammatory macrophages observed in our studies can be attributed to both recruitment from the bone marrow and maturation from immature cells as suggested by other reports (Lyamina, 2012, Stout,

2005). Our analysis of CX3CR1 expressing cells showed that the overwhelming majority of pro-inflammatory macrophages were a F4/80 positive mature subpopulation. The low number of immature proinflammatory cells suggests that the increase in mature subpopulation was likely the result of recruitment from the circulation as previously suggested [Sunderkötter, 2003]. In addition, these mature pro-inflammatory macrophages are likely responsible for the CX3CR1 expressing cell-induced inflammatory pathology described in previously (Oh, 2008, Li, 2008).

The present studies showed a switch toward a predominantly mature phenotype in the GFP^+ bone marrow-derived anti-inflammatory macrophage subpopulation. This is similar to the phenotypic shift reported in isolated liver macrophages [Holt, 2008]. An increase in mature macrophages observed via immunofluorescent staining also supports the notion that an immature anti-inflammatory subpopulation quickly matures after its arrival at the site of injury [Tsou, 2007]. Prior studies demonstrated that pro-inflammatory macrophages can also give rise to anti-inflammatory macrophages following exposure to anti-inflammatory stimuli such as IL-10 and IL-4/IL-13 (Liu, 2013, Arnold, 2007, Porcheray, 2005). This provides additional evidence that macrophages are capable of adapting to their microenvironment. This plasticity allowed the switch in macrophage phenotype during the resolution of APAP induced injury which was shown to be critical in resolving inflammation and initiating tissue repair (Dragomir, 2012, Arnold, 2007, Tacke, 2006). Interestingly, no notable differences in cell numbers between mature and immature anti-inflammatory CX3CR1 expressing cell were observed in our studies. This suggests that CX3CR1 associated recruitment of anti-inflammatory cells occurs regardless of the maturity of the monocytes. This idea challenges the notion that the expression of CX3CR1 receptor is associated exclusively with mature M2-like anti-inflammatory macrophages (Auffray, 2007, Ghassabeh, 2006).

We also found that the majority of CX3CR1 expressing macrophages observed in the liver consisted of anti-inflammatory and mature pro-inflammatory subpopulations. While the mature anti-inflammatory macrophages are thought to be associated with M2 phenotypes, both immature anti-inflammatory and mature pro-inflammatory subpopulation are thought to have the potential to switch into M2 mature anti-inflammatory subpopulations (Dey, 2014, Liu, 2013, Dragomir, 2012, Laskin, 2011). This further confirmed the association between the CX3CR1 receptor and M2 like macrophages (Auffray, 2007, Ghassabeh, 2006).

Immunofluorescent staining of APAP treated livers for COX-2, HO-1, and MR revealed additional information about the phenotypes of GFP expressing bone marrow-derived leukocytes. Coexpression of GFP and other markers was assessed by determining the percentage of overlapping fluorescent in cells localized in areas around central veins [Inman, 2005]. This approach provided data on the trend in response within the GFP expressing population; however, it might not accurately represent expression in the whole liver. Furthermore, while the colocalization percentages suggested levels of marker expression which overlapped with GFP, they do not represent the overall quantity of specific expression or cell numbers.

APAP-induced hepatic hemorrhagic necrosis results in a large quantity of red blood cells and debris in the area of necrosis. Debris such as hemoglobin and its degraded form, bilirubin were found to share similar emission and excitation wavelength as enhanced green fluorescent protein (EGFP) (Lamola, 2014, Glushko, 1982).This accumulation of cellular debris was likely responsible for the elevated background observed within necrotic area. In addition to the background, all cells originating from the bone marrow are likely to express GFP. To avoid misidentification, GFP expressing macrophages were identified by their shape, size and location.

Lipid mediators such as lipoxin, prostaglandins, and leukotrienes are critical in both pro- and anti-inflammatory responses (Harizi, 2008, Serhan, 2008, Stables, 2011). COX-2 is an enzyme critical for the production of major pro-inflammatory lipid mediators such as the prostaglandins E_2 [Turini, 2002]. Inhibition of COX-2 has been shown to decrease inflammation in various tissues (Horrillo, 2007, Pinheiro, 2002, Masferrer, 1994). We observed increased coexpression of COX-2 and GFP after APAP administration demonstrating the participation of infiltrating cells in the pro-inflammatory process. The finding that infiltrating cells contain a pro-inflammatory subpopulation supports reports that inhibition of monocyte infiltration decreases the severity of inflammatory pathology (Karlmark, 2009, King, 2009, Kulkarni, 2007). Interestingly, the low colocalization of COX-2 observed in CX3CR1 expressing macrophages during APAP intoxication suggests that these cells are not directly involved in COX-2 associated pro-inflammatory prostaglandin production.

APAP induced toxicity is associated with oxidative stress (Dragomir, 2011, Das, 2010). HO-1 is an antioxidant upregulated in response to oxidative stress. It is also a phenotypic marker of anti-inflammatory macrophages (Immenschuh, 2010, Sierra, 2010, Orozco, 2007). We found elevated HO-1 expression in GFP^+ and CX3CR1 expressing liver macrophages after APAP administration. This is in agreement with previous reports that macrophages participate in oxidative stress responses and suggest that this is mediated by the M2 subpopulation (Yang, 2014, Auffray, 2007, Inui, 2011). Mannose receptor (MR) is a surface marker associated with pattern recognition and debris clearance that is primarily expressed on macrophages and liver sinusoidal endothelial cells (Malovic, 2007, Auffray, 2007, Taylor, 2005). Previous investigations have demonstrated that increased MR expression is associated with anti-inflammatory activities (Gazi, 2009, Zhang, 2005, Chieppa, 2003). Findings that MR is increased in GFP^+ cells in GFP chimeric mice livers confirmed that a subpopulation of these cells

participates in debris removal and possibly mitigation of inflammation. The identification of M2 like subpopulation observed in GFP^+ cells in GFP chimeric mouse liver supports the idea that inhibition of monocytic infiltration causes a delay in wound healing in various tissues (Brancato, 2011, Ishida, 2008, Nahrendorf, 2007, Dewald, 2005). Interestingly, there was no change in MR expression in CX3CR1 positive cells. This suggests that CX3CR1 associated cells may have limited MR associated phagocytic ability (Martinez-Pomares, 2003]. Findings that some CX3CR1 positive macrophages express HO-1 but not MR suggests that HO-1 expressing CX3CR1 positive cells belong to a subset of M2 like macrophages (Naito, 2014, Colin, 2014).

The present studies demonstrate that during APAP induced toxicity, the bone marrow serves as a major source of infiltrating macrophages. These infiltrating cells consist of both pro-inflammatory and anti-inflammatory subpopulations. Further analysis suggests that pro-inflammatory macrophages exhibit both mature and immature phenotypes. Conversely, while the anti-inflammatory subpopulation of macrophages infiltrated into the liver as immature cells, they switched to a mature dominant phenotype with time.

In conclusion, the present studies demonstrate that bone marrow plays a critical role in APAP-induced toxicity by acting as a source of both pro- and anti-inflammatory monocytes and CX3CR1 associated recruitment is a possible mechanism by which infiltration of mature or anti-inflammatory monocytes occurs. Further understanding of the origin and development of different subpopulations of macrophages which participate in APAP-induced hepatotoxicity may shed light on these highly complex macrophage subpopulations.

FUTURE STUDIES

Unlike wild-type mice which have two functional alleles, only one CX3CR1 allele remains functional in CX3CR1$^{+/GFP}$ mice. It is possible that the CX3CR1 response of anti-inflammatory macrophages was reduced in this animal model. In our studies, a persistent accumulation of macrophages was observed after APAP intoxication. Studies have also shown that CX3CR1$^{-/-}$ mice sustain prolonged and more severe injury (Aoyama, 2010, Karlmark, 2010). It would be interesting to extend the time points in our APAP model to determine if there is a similar sustained response. Published studies also suggest that CX3CR1 expressing monocytes can mature into bone-marrow derived resident macrophages (Italiani, 2014, Klein, 2007). This allows us to investigate whether CX3CR1 expressing cells egress from the liver or remain, and subsequently mature into resident macrophages.

Another chemokine receptor, CCR2 is critical in monocyte egress from bone marrow (Tsou, 2007, Serbina, 2006). Inhibition of CCR2 has been shown to reduce monocyte recruitment during inflammatory responses [Fujimura, 2015]. Furthermore, CCR2$^{-/-}$ mice which are deficient in monocyte recruitment experienced delayed resolution of APAP toxicity, which was reversed by reintroducing wild type hematopoietic progenitor cells from bone marrow (Si, 2010, Dambach, 2002). In future studies, it would be interesting to analyze expression of CCR2 in conjunction with CX3CR1. A mouse model carrying both CX3CR1$^{+/GFP}$ and CCR2$^{+/\text{Red fluorescent protein (RFP)}}$ reporters would provide a unique opportunity to simultaneously track the CCR2^{+} and CX3CR1^{+} monocytes into the liver following APAP administration (Saederup , 2010, Mizutani, 2012).

Different subpopulations of monocytes and macrophages are known to participate in various roles during inflammation, ranging from initiation to resolution (Laskin, 2011, Geissmann, 2010, Gordon, 2005). With the addition of CCR2, further

categorization of macrophages in tandem with CX3CR1 is possible. By isolating different subpopulations of macrophages and examining their functional activities, it will be possible to assess their precise contribution to APAP induced injury and repairs. Analysis of their capacities such as phagocytosis and pinocytosis, responsiveness to oxidative stress, and secretory profile will also provide a detailed description of the phenotype associated with different subpopulations.

Intercepting monocytes directly in the circulation can also provide insight into CCR2 and CX3CR1 associated monocyte recruitment and turnover (Yona, 2013, Tacke, 2007). In the present study, we demonstrated that different subpopulations of macrophages accumulating in the liver display various levels of maturity. However, it remains unclear whether the macrophage subpopulations are the result of recruitment from the circulation or from maturation or phenotypic switching of the accumulated cells. By comparing subpopulations of monocytes found in the circulation to macrophage subpopulations in the liver, difference between recruitment and accumulation may be evaluated.

Figure 1. Effects of APAP on GFP^+ Cells in the Liver and Bone Marrow. Bone marrow cells and liver leukocytes, isolated 24-96h after treatment of GFP chimeric mice with APAP or PBS control, were analyzed by flow cytometry. Cells were gated on monocytes/macrophages based on forward and side scatter and then evaluated for GFP expression. Upper panel, the percentage of GFP^+ bone marrow and liver cells. Lower panel, the total number of liver GFP^+ cells. Data are means ± SE (n = 4-5 mice). [a]Significantly different ($p < 0.05$) from PBS.

Percentage GFP$^+$ Cells

	PBS	24 h	48 h	72 h	96 h
Bone Marrow	87.4 ± 3.8	88.5 ± 3.0	88.8 ± 1.5	82.7 ± 4.1	86.9 ± 1.8
Liver	33.7 ± 1.7	73.2 ± 2.5	56.8 ± 1.1	52.4 ± 4.5	53.5 ± 1.6

Total Number of GFP$^+$ Liver Leukocytes

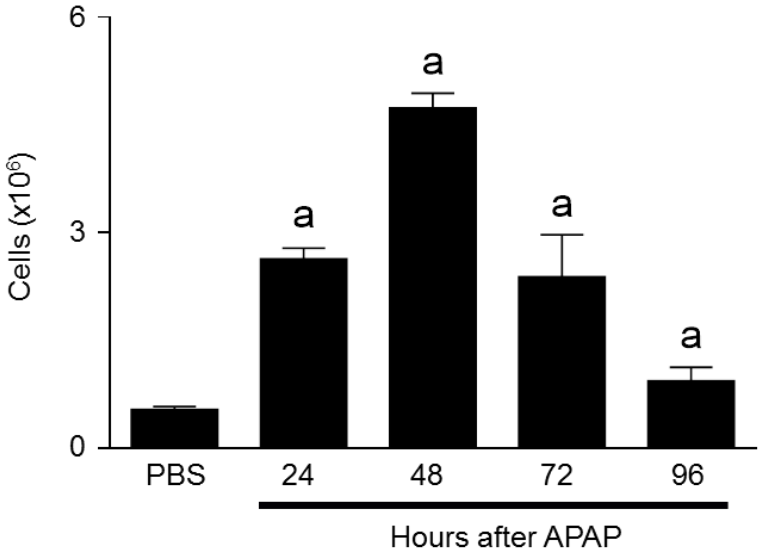


Figure 2. Effects of APAP on GFP^+ Subpopulations in the Liver. Hepatic nonparenchymal cells were isolated 24-96h after treatment of GFP chimeric mice with APAP or control. Cells were incubated with eFluor700-Ly6G and AF647-CD11b and analyzed by flow cytometry. GFP^+ cells were analyzed for expression of Ly6G, followed by CD11b. Bars are means ± SE (n = 4-5 mice). [a]Significantly different ($p \leq 0.05$) from PBS.

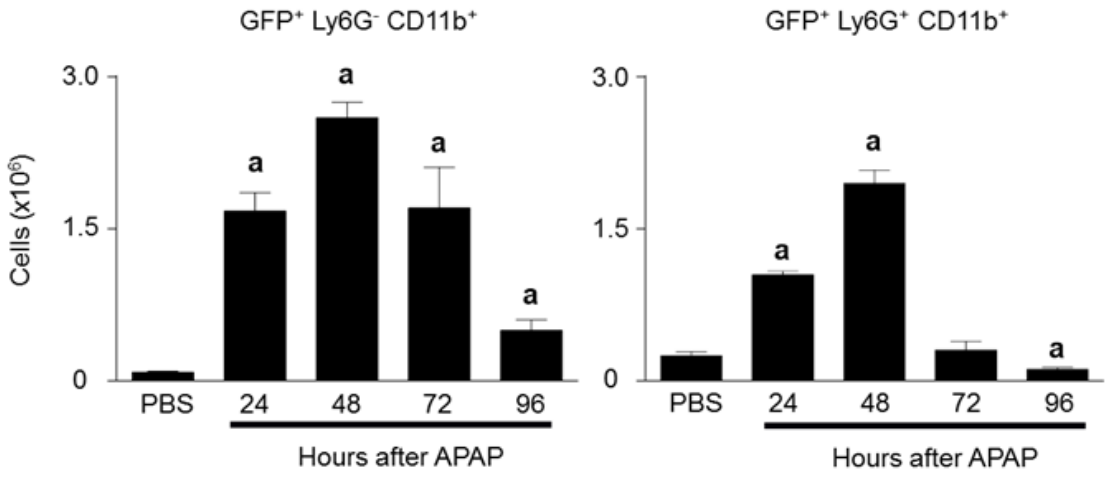

GFP+ Ly6G- CD11b+
GFP+ Ly6G+ CD11b+
Cells (x10^6)
3.0
1.5
0
a
PBS
24
48
72
96
Hours after APAP

Figure 3. Effects of APAP on Infiltrating Pro-inflammatory and Anti-inflammatory Macrophages in the Liver. Liver nonparenchymal cells, isolated 24-96h after treatment of GFP chimeric mice with APAP or PBS control, were incubated with AF647-CD11b, eFluor700-Ly6G, and PE-Ly6C and analyzed by flow cytometry. Upper panel, expression of Ly6C and cell size (forward scatter). Lower panel, average cell counts based on the percentage of $Ly6C^{hi}$ and $Ly6C^{lo}$ cells. Bars are means ± SE (n = 4-5 mice). [a]Significantly different ($p \leq 0.05$) from PBS.

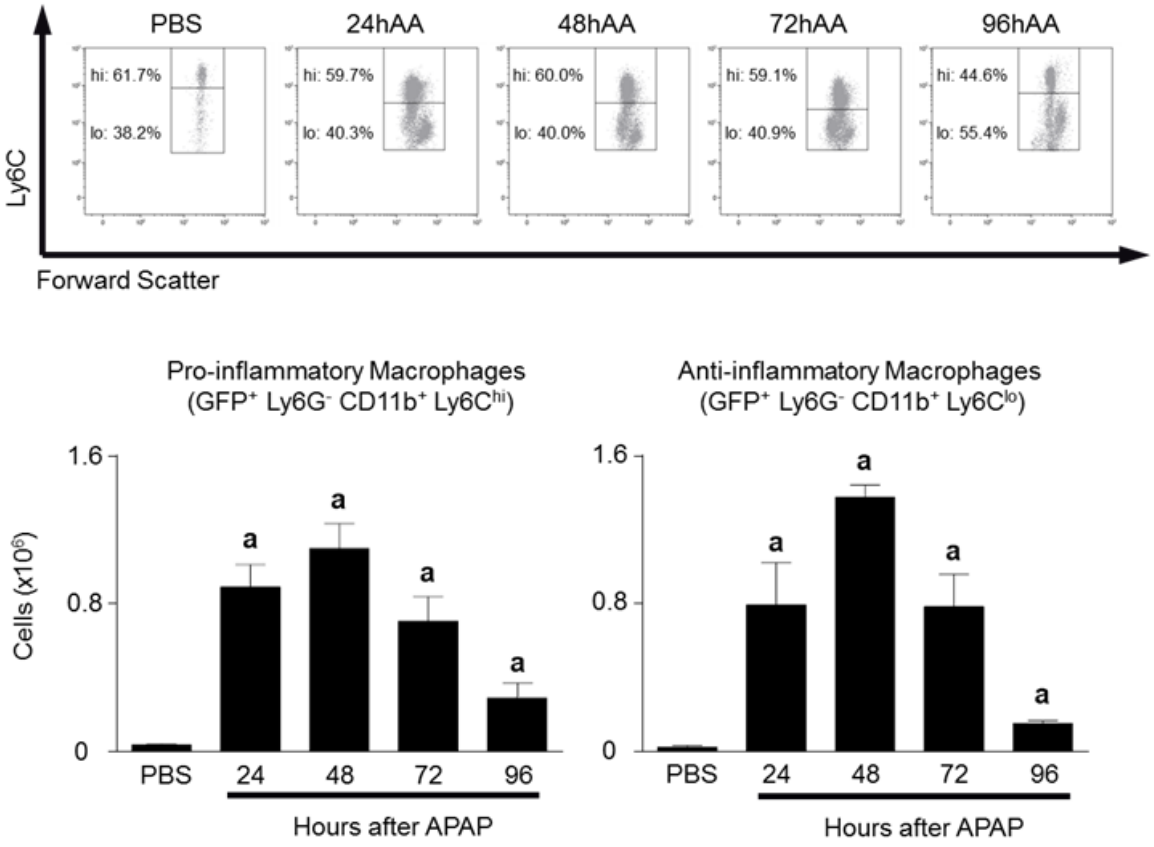

PBS
24hAA
48hAA
72hAA
96hAA
hi: 61.7%
lo: 38.2%
hi: 59.7%
lo: 40.3%
hi: 60.0%
lo: 40.0%
hi: 59.1%
lo: 40.9%
hi: 44.6%
lo: 55.4%
Ly6C
Forward Scatter
Pro-inflammatory Macrophages
(GFP+ Ly6G- CD11b+ Ly6Chi)
Anti-inflammatory Macrophages
(GFP+ Ly6G- CD11b+ Ly6Clo)
Cells (x10^6)
1.6
0.8
0
a
PBS
24
48
72
96
Hours after APAP

Figure 4. Identification of Pro-inflammatory and Anti-inflammatory Macrophages Infiltrating into the Liver after APAP. Liver nonparenchymal cells, isolated 24-96h after treatment of GFP chimeric mice with APAP or PBS control, were incubated with AF647-CD11b, eFluor700-Ly6G, PE-Ly6C and PE/Cy7-F4/80 and then analyzed by flow cytometry. Upper panel, the pro-inflammatory Ly6C^{hi} macrophage cell numbers. Lower panel, the anti-inflammatory Ly6C^{lo} macrophage cell numbers. Bars are means ± SE (n = 4-5 mice). [a]Significantly different ($p \leq 0.05$) from PBS.

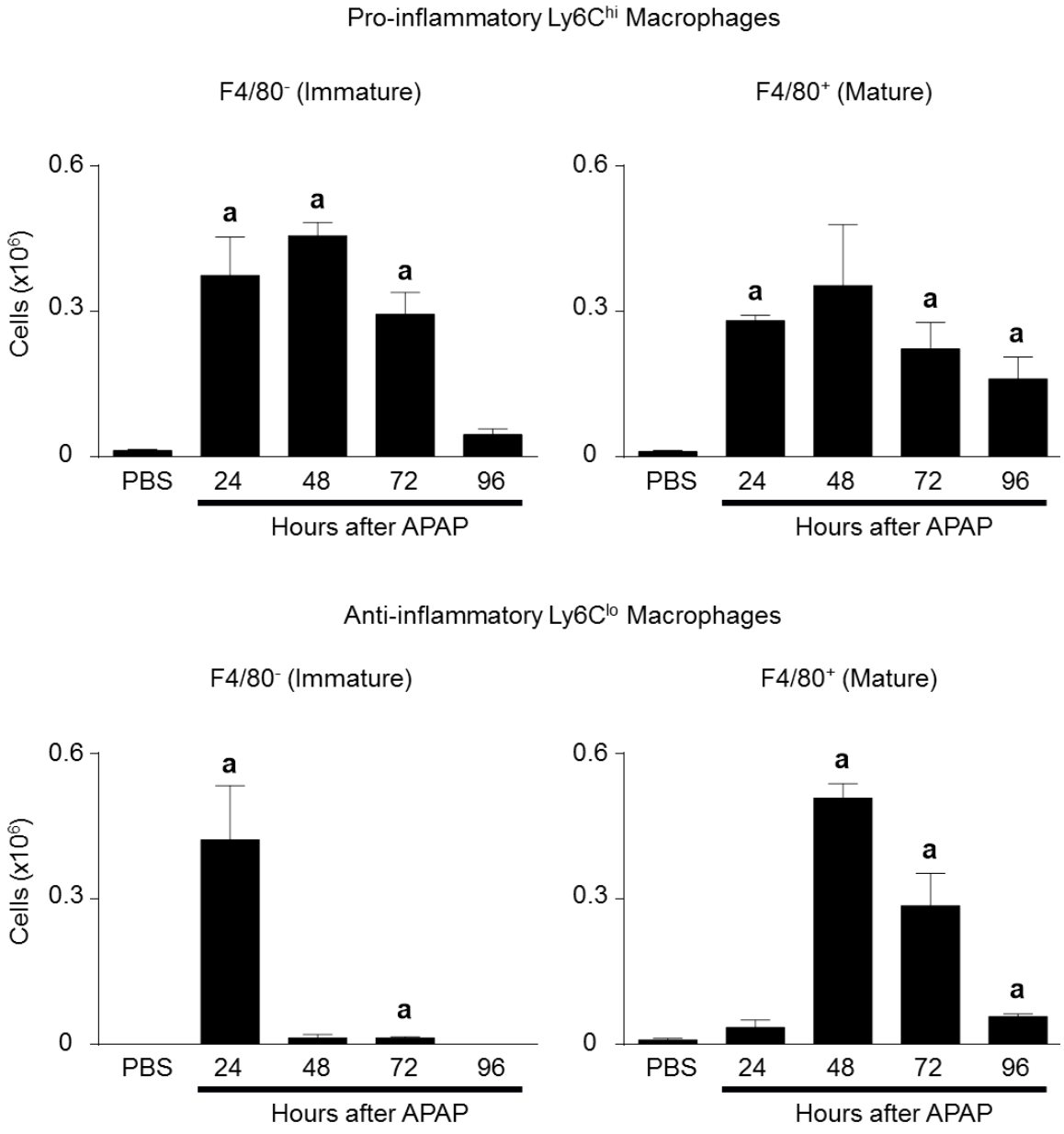
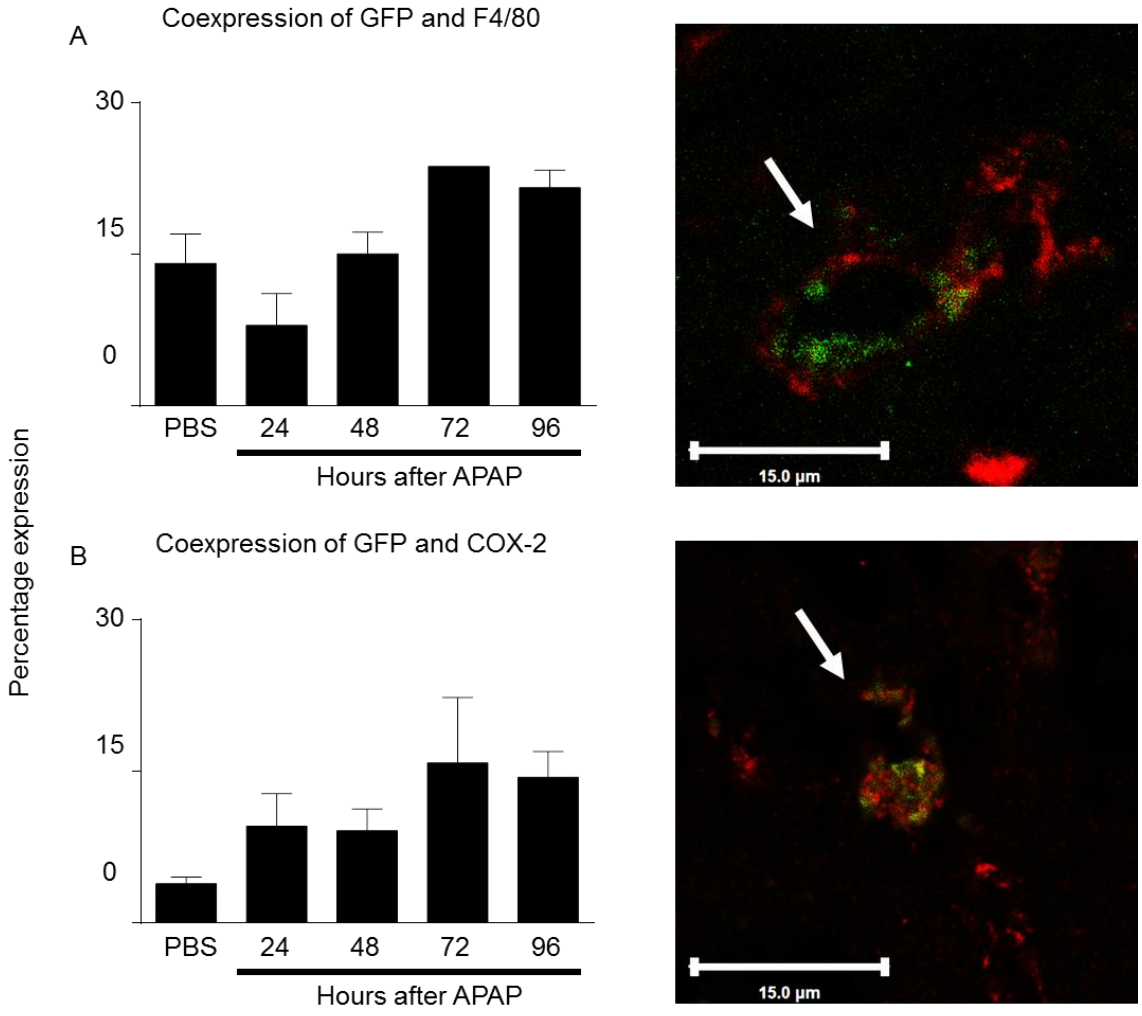

Pro-inflammatory Ly6C^{hi} Macrophages
F4/80^{-} (Immature)
F4/80^{+} (Mature)
Cells (x10^{6})
0.6
0.3
0
a
PBS
24
48
72
96
Hours after APAP
Anti-inflammatory Ly6C^{lo} Macrophages
F4/80^{-} (Immature)
F4/80^{+} (Mature)

Figure 5. Expression of Maturity and Macrophage Phenotype Markers in GFP Bone Marrow Chimeric Mice. Frozen liver sections, prepared 24-96h after treatment of GFP chimeric mice with APAP or PBS control, were incubated with antibodies to HO-1, F4/80 COX-2, or mannose receptor (MR) followed by AF555-anti-rabbit or AF647-anti-rat secondary antibodies. Sections were then analyzed by confocal microscopy. Five images were taken per section and the results were averaged. Panel A, F4/80. Panel B, COX-2. Panel C,HO-1. Panel D, MR. The percentage of co-expression of GFP and markers were quantified (left panels). Bars are means + SD (n = 2-4 mice). Representative liver sections prepared 96 h post APAP demonstrating GFP co-localization of markers (right panels). Arrows indicate cells colocalizing GFP and markers.


A
Coexpression of GFP and F4/80
30
15
0
PBS
24
48
72
96
Hours after APAP
15.0 µm
B
Coexpression of GFP and COX-2
Percentage expression
30
15
0
PBS
24
48
72
96
Hours after APAP
15.0 µm

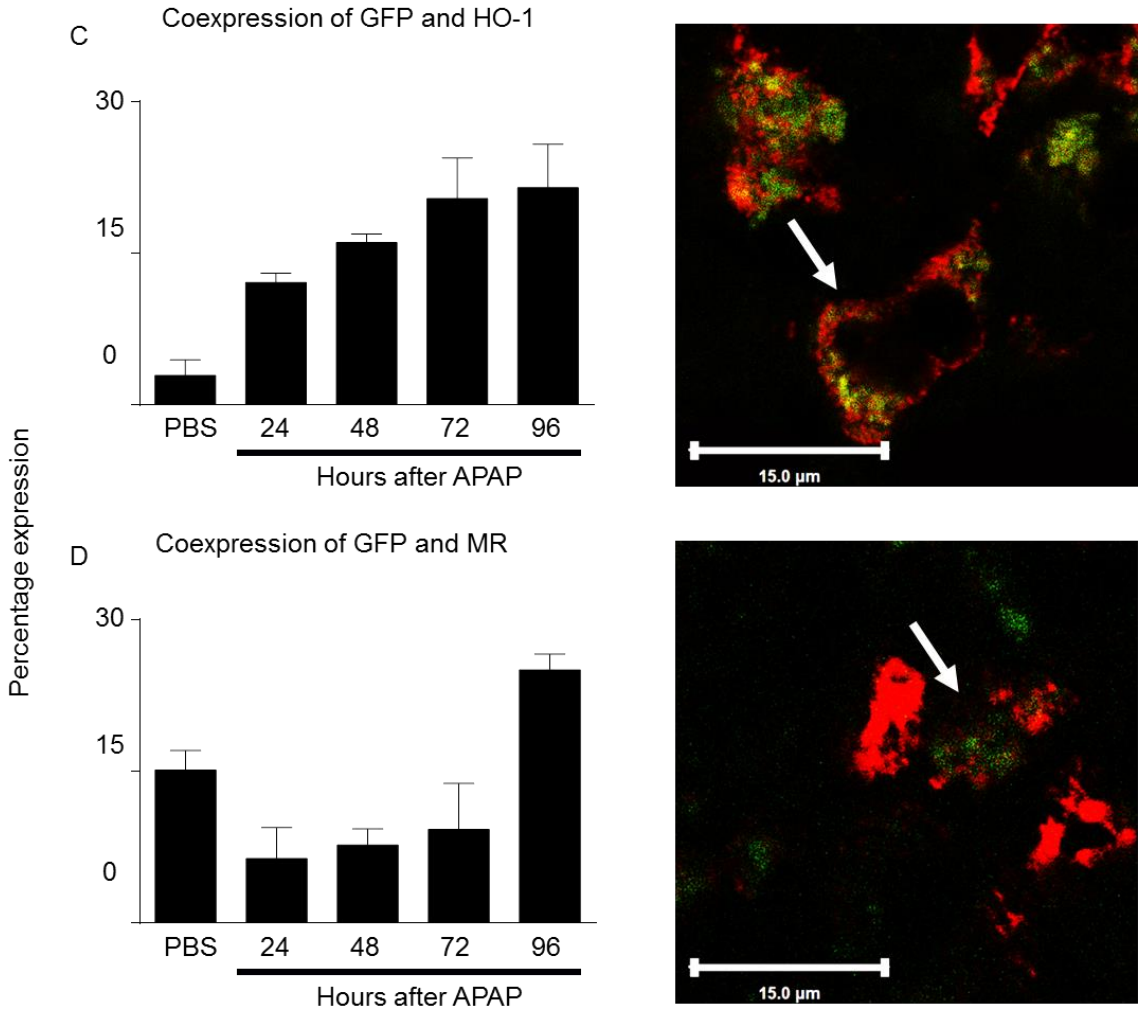

C
Coexpression of GFP and HO-1
30
15
0
PBS
24
48
72
96
Hours after APAP
15.0 µm
D
Coexpression of GFP and MR
Percentage expression
30
15
0
PBS
24
48
72
96
Hours after APAP
15.0 µm

Figure 6. Effects of APAP on CX3CR1$^{+/GFP}$ Cells in the Liver. Liver leukocytes, isolated 6-96h after treatment of CX3CR1$^{+/GFP}$ reporter mice with APAP or PBS control, were analyzed by flow cytometry. Cells were gated on monocyte/macrophages by forward and side scatter and then evaluated for GFP expression. Upper panel: Percentages of CX3CR1$^{+/GFP}$ cells in the liver following APAP or control. Lower panel, Total cell number of CX3CR1$^{+/GFP}$ cells in the liver. Data are means ± SE (n = 3-4 mice). [a]Significantly different ($p \leq 0.05$) from PBS.

Percentage CX3CR1$^{+/GFP}$ Cells

	PBS	6h	24 h	48 h	72 h	96 h
Liver	5.5 ± 0.4	5.0 ± 1.0	7.4 ± 1.6	3.6 ± 0.7	6.2 ± 0.6	9.7 ± 2.1

Total Number of CX3CR1$^{+/GFP}$ Liver Leukocytes

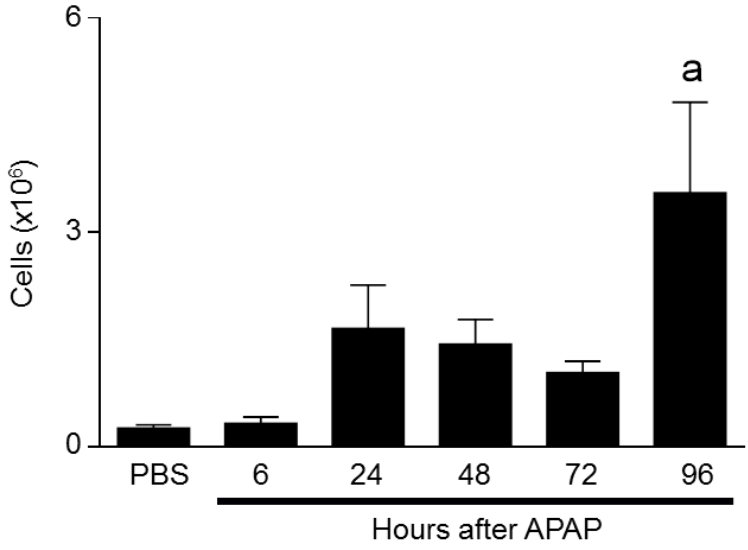


Figure 7. Effects of APAP on CX3CR1$^{+/GFP}$ Subpopulations in the Liver. Hepatic nonparenchymal cells were isolated 6-96h after treatment of CX3CR1 reporter mice with APAP or control. Cells were incubated with eFluor700-Ly6G and AF647-CD11b and analyzed by flow cytometry. GFP^{+} cells were analyzed for expression of Ly6G, followed by analysis for CD11b. Bars are means ± SE (n = 4-5 mice). ^{a}Significantly different ($p \leq 0.05$) from PBS.

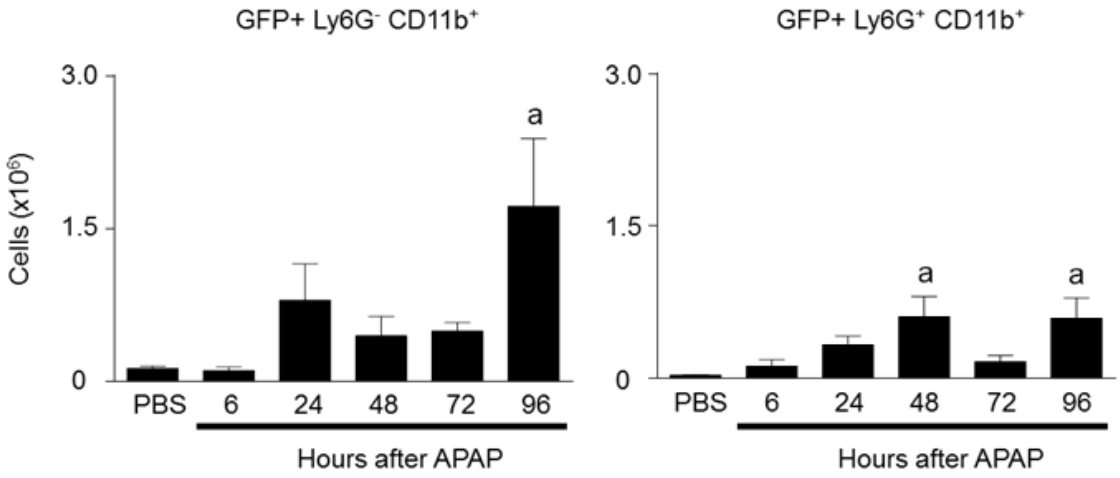

GFP+ Ly6G- CD11b+
GFP+ Ly6G+ CD11b+
Cells (x10^6)
3.0
1.5
0
a
a
a
PBS
6
24
48
72
96
Hours after APAP

Figure 8. Effects of APAP on Infiltrating Pro-inflammatory and Anti-inflammatory Macrophages in the Liver. Liver nonparenchymal cells, isolated 6-96h after treatment of $CX3CR1^{+/GFP}$ reporter mice with APAP or PBS control, were incubated with AF647-CD11b, eFluor700-Ly6G, and PE-Ly6C and analyzed by flow cytometry. GFP^{+} $Ly6C^{+}$ cells were found to consist of two subpopulations, expressing high and low levels of Ly6C. Upper panel, scatter plots of Ly6C expression and size (forward scatter). Lower panel, total cell number of pro- and anti-inflammatory macrophages. Bars are means ± SE (n = 4-5 mice). [a]Significantly different ($p \leq 0.05$) from PBS.

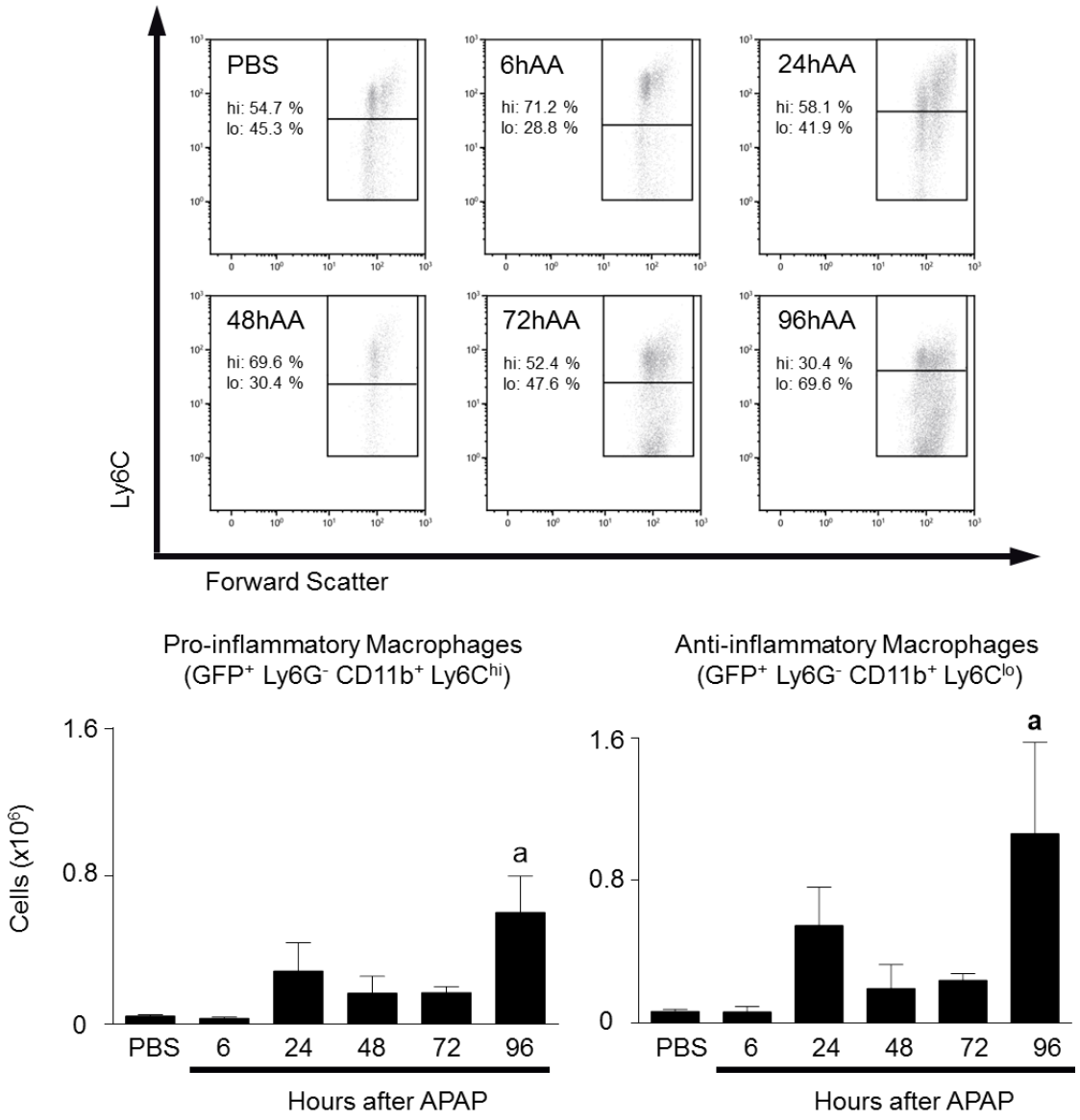

PBS
hi: 54.7 %
lo: 45.3 %
6hAA
hi: 71.2 %
lo: 28.8 %
24hAA
hi: 58.1 %
lo: 41.9 %
48hAA
hi: 69.6 %
lo: 30.4 %
72hAA
hi: 52.4 %
lo: 47.6 %
96hAA
hi: 30.4 %
lo: 69.6 %
Ly6C
Forward Scatter
Pro-inflammatory Macrophages
(GFP+ Ly6G- CD11b+ Ly6Chi)
Anti-inflammatory Macrophages
(GFP+ Ly6G- CD11b+ Ly6Clo)
Cells (x10^6)
1.6
0.8
0
a
PBS
6
24
48
72
96
Hours after APAP

Figure 9. Identification of Pro-inflammatory and Anti-inflammatory Macrophages Infiltrating into the Liver after APAP. Macrophages isolated 6-96h after treatment of CX3CR1$^{+/GFP}$ reporter mice with APAP or PBS control, were incubated with AF647-CD11b, eFluor700-Ly6G, PE-Ly6C and PE/Cy7-F4/80 and then analyzed by flow cytometry. Upper panel, numbers if pro-inflammatory Ly6C^{hi} macrophages. Lower panel, numbers of anti-inflammatory Ly6C^{lo} macrophages. Bars are the mean ± SE (n = 4-5 mice). [a]Significantly different ($p \leq 0.05$) from PBS.

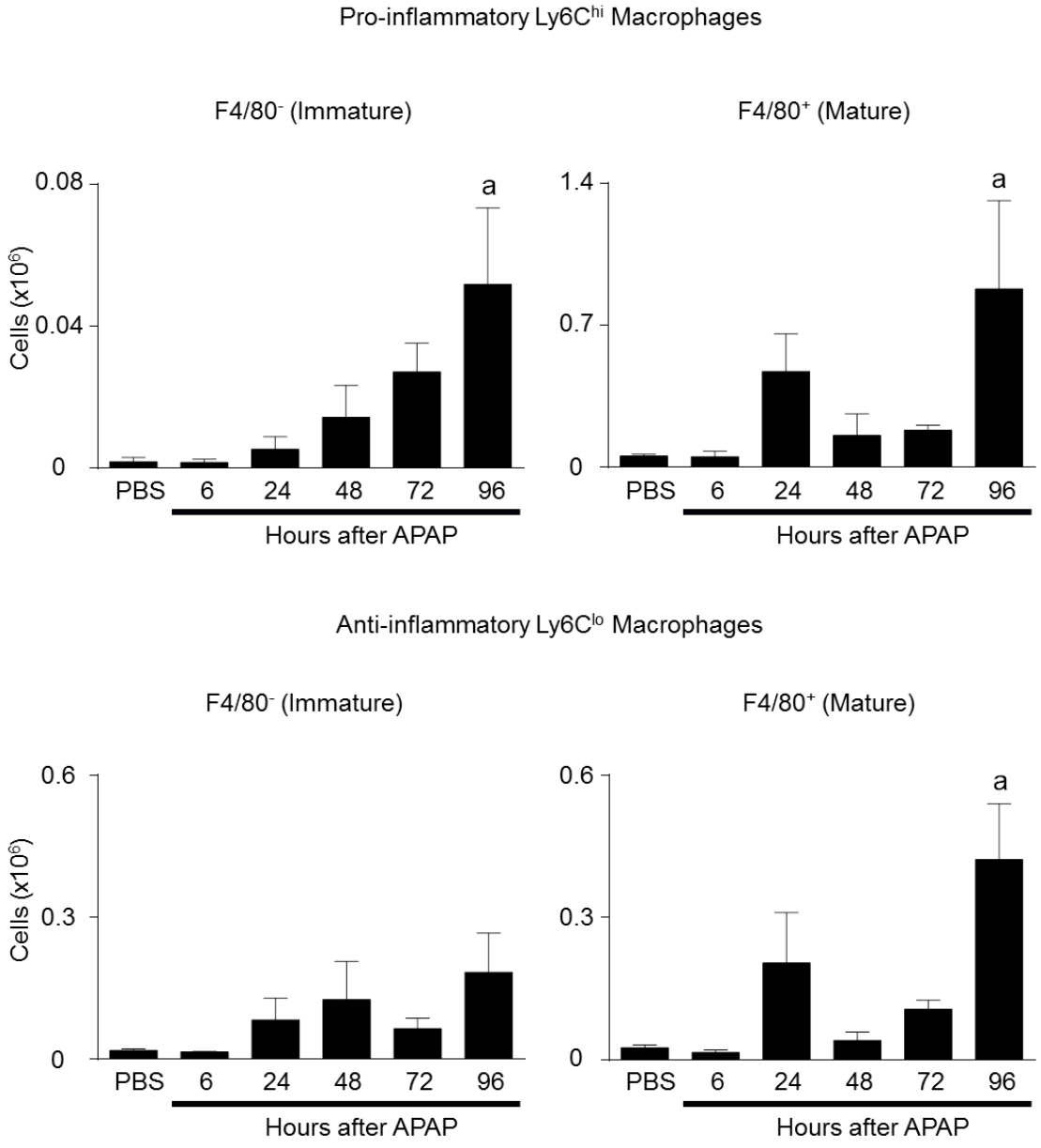
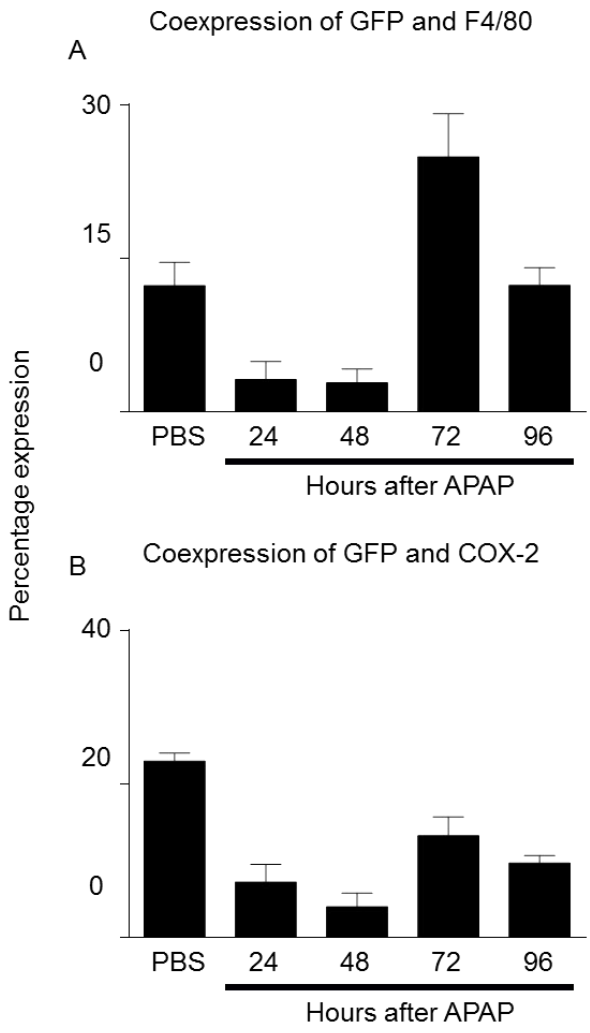
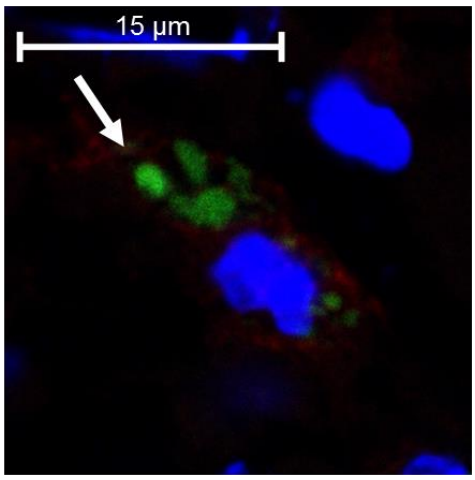
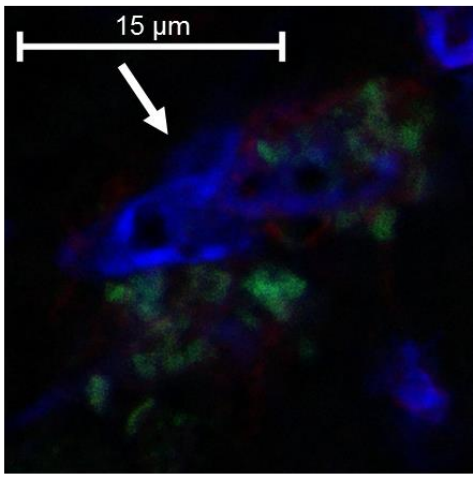
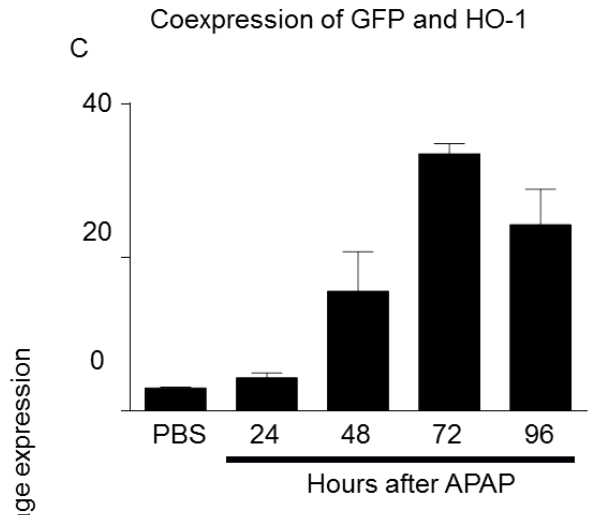

Pro-inflammatory Ly6C^hi Macrophages
F4/80- (Immature)
F4/80+ (Mature)
Cells (x10^6)
0.08
0.04
0
1.4
0.7
0
a
a
PBS
6
24
48
72
96
Hours after APAP
Anti-inflammatory Ly6C^lo Macrophages
F4/80- (Immature)
F4/80+ (Mature)
0.6
0.3
0
a
Hours after APAP

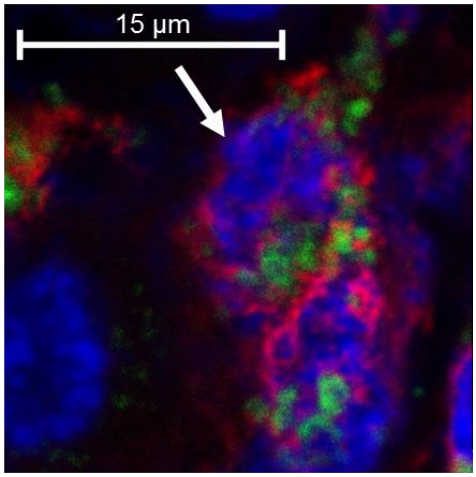
Figure 10. Expression of Maturity and Macrophage Phenotype Markers in CX3CR1$^{+/GFP}$ Reporter Mice. Frozen liver sections, prepared 24-96h after treatment of CX3CR1$^{+/GFP}$ reporter mice with APAP or PBS control, were then incubated with antibodies to HO-1, F4/80 COX-2, or mannose receptor (MR) followed by AF555-anti-rabbit or AF647-anti-rat secondary antibodies. Sections were analyzed by confocal microscopy. 5 images were taken per section Panel A, F4/80. Panel B, COX-2. Panel C, HO-1. Panel D, MR. The percentage of co-expression of GFP and markers were quantified (left panels). Bars are means ±SD (n = 2-4 mice). Representative liver sections prepared 96 h post APAP demonstrating GFP co-localization (right panels). Arrows indicate cells colocalizing GFP and markers.

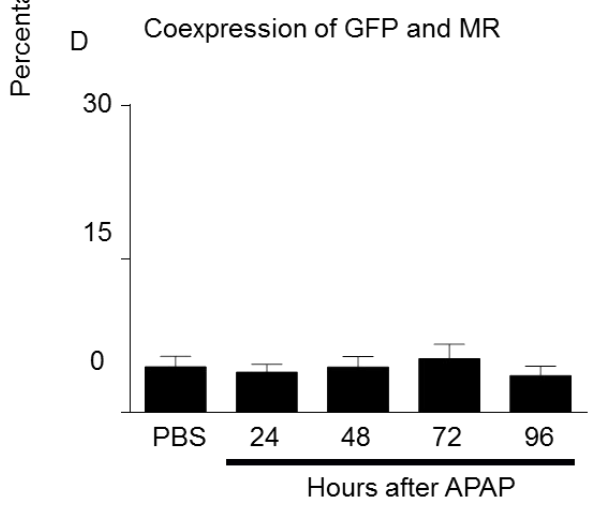

Coexpression of GFP and F4/80
A
30
15
0
Percentage expression
PBS
24
48
72
96
Hours after APAP
B
Coexpression of GFP and COX-2
40
20
0
PBS
24
48
72
96
Hours after APAP


15 µm


15 µm


Coexpression of GFP and HO-1
C
Percentage expression
40
20
0
PBS
24
48
72
96
Hours after APAP


15 µm


D
Coexpression of GFP and MR
30
15
0
PBS
24
48
72
96
Hours after APAP

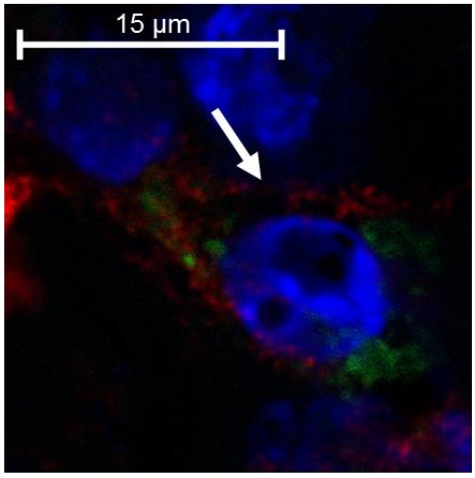
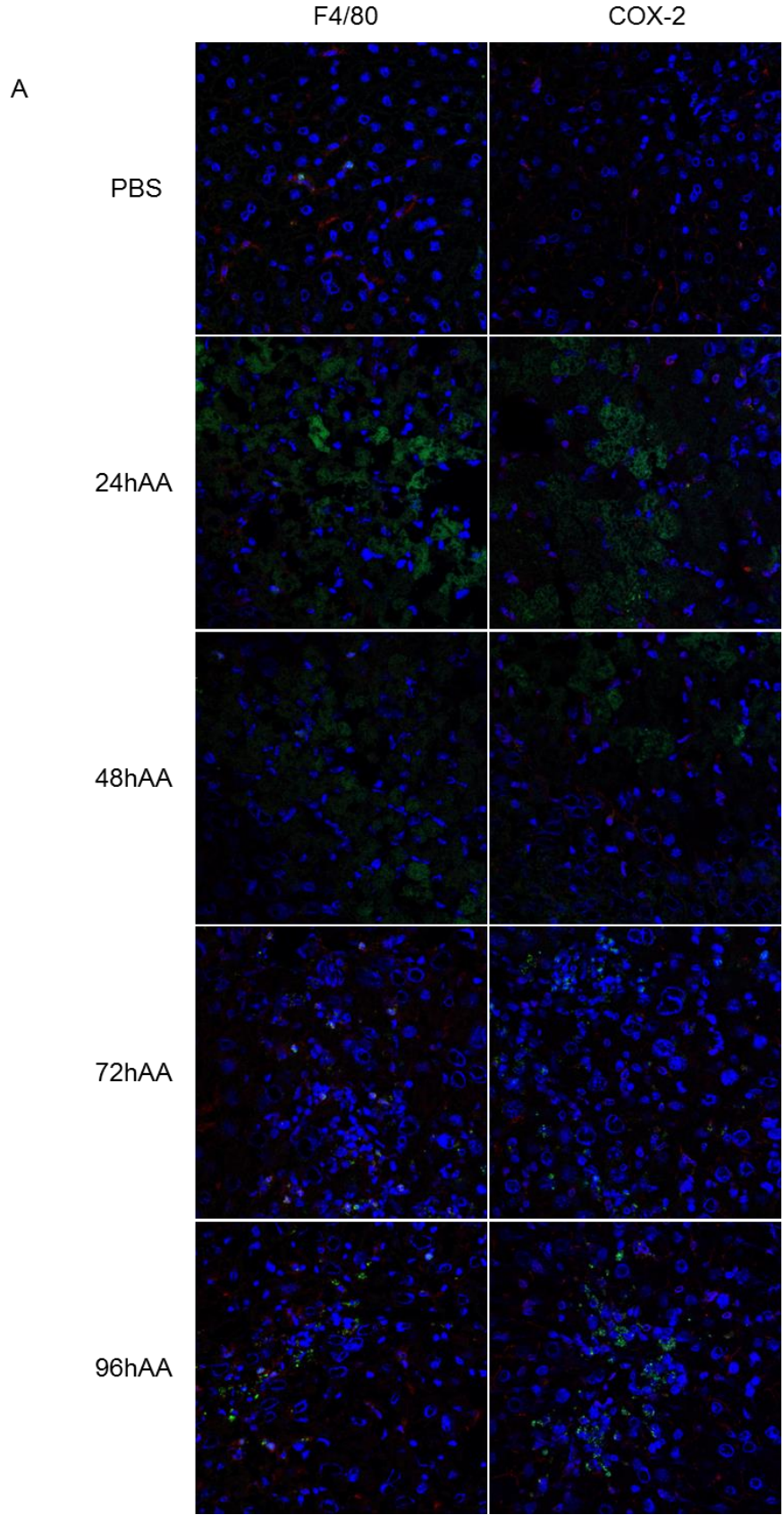

15 µm

Figure 11. Effects of APAP on the Expression of Maturity and Macrophage Phenotype Markers in CX3CR1$^{+/GFP}$ Reporter Mice. Frozen liver sections, prepared 24-96h after treatment of CX3CR1$^{+/GFP}$ reporter mice with APAP or PBS control, were then incubated with antibodies to HO-1, F4/80 COX-2, or mannose receptor (MR) followed by AF555-anti-rabbit or AF647-anti-rat secondary antibodies. Sections were analyzed by confocal microscopy. Five images were taken per section and the results were averaged. Panel A, expression of F4/80 and COX-2 in CX3CR1$^{+/GFP}$ reporter mice 24-96h after APAP or control. Panel B, expression of HO-1 and MR in CX3CR1$^{+/GFP}$ reporter mice and 24-48h after APAP or control.


A
F4/80
COX-2
PBS
24hAA
48hAA
72hAA
96hAA

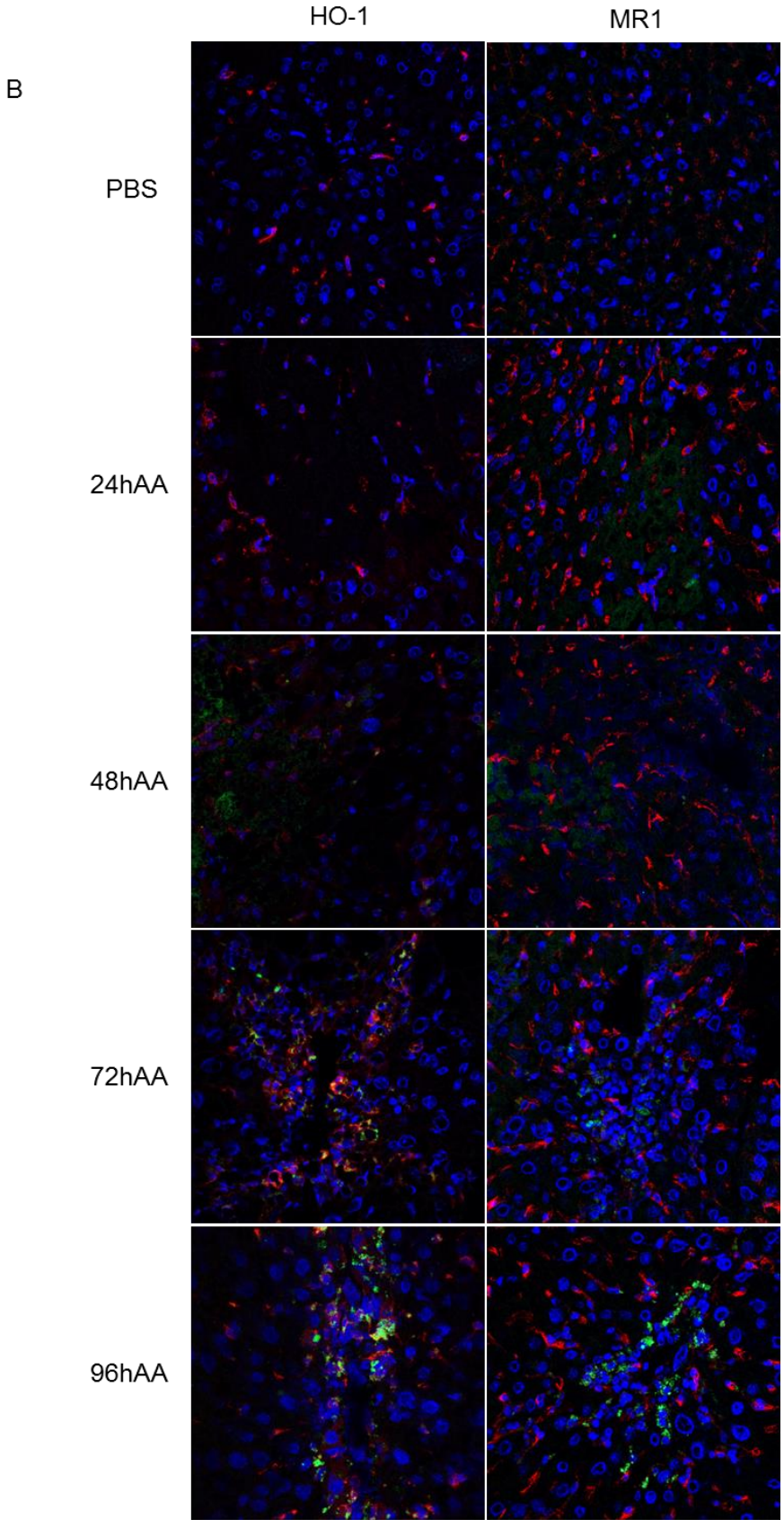

B
HO-1
MR1
PBS
24hAA
48hAA
72hAA
96hAA

Table 1. Markers Expressed by Monocyte/Macrophage Subpopulations. Ly6G is a leukocyte marker strongly expressed on granulocytes. CD11b is a marker expressed on infiltrating myeloid cells. Ly6C is expressed at low levels on anti-inflammatory macrophages and high levels on pro-inflammatory macrophages. F4/80 is a mature macrophage marker.

	Markers			
	Ly6G	CD11b	Ly6C	F4/80
Pro-inflammatory immature	-	+	+++	-
Pro-inflammatory mature	-	+	+++	+
Anti-inflammatory immature	-	+	+	-
Anti-inflammatory mature	-	+	+	+

Reference

Abdel-Zaher, A.O., Abdel-Rahman, M.M., Hafez, M.M., and Omran, F.M. (2007). Role of nitric oxide and reduced glutathione in the protective effects of aminoguanidine, gadolinium chloride and oleanolic acid against acetaminophen-induced hepatic and renal damage. Toxicology 234: 124-134.

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