MECHANISMS MEDIATING MACROPHAGE ACTIVATION DURING ACETAMINOPHEN-INDUCED HEPATOTOXICITY: ROLE OF HIGH MOBILITY GROUP BOX-1 AND GALECTIN-3

By

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ABSTRACT OF THE DISSERTATION

Mechanisms Mediating Macrophage Activation during Acetaminophen-Induced Hepatotoxicity: Role of High Mobility Group Box-1 and Galectin-3

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Classically and alternatively activated macrophages and inflammatory mediators they release play a key role in the pathogenesis of acetaminophen (APAP)-induced hepatotoxicity. In the present studies, we investigated the role of the DNA-binding protein, high mobility group box-1 (HMGB1), and the β-galactoside binding lectin, galectin-3 (Gal-3), in regulating the phenotype of activated macrophages in the liver following APAP intoxication. Culture medium collected from hepatocytes treated for 24 h with 5 mM APAP (CM-AA) stimulated macrophage production of reactive oxygen species and upregulated expression of the antioxidant enzymes catalase and heme oxygenase-1 (HO-1). CM-AA also upregulated expression of the proinflammatory chemokines MIP-1α (CCL3) and MIP-2 (CXCL2), and the eicosanoid biosynthetic enzymes, COX-2 and 12/15-LOX, effects dependent on the p44/42 MAP kinase. Treatment of hepatocytes with ethyl pyruvate (EP) prior to APAP blunted the effects of CM-AA on macrophage ROS production, expression of antioxidant proteins, and COX-2, suggesting that part of the activity in CM-AA was HMGB1. Further studies demonstrated that APAP-induced hepatotoxicity was associated with upregulation of Gal-3 mRNA and

protein expression. Loss of Gal-3 resulted in reduced hepatotoxicity in response to APAP. This correlated with decreases in APAP-induced expression of the inflammatory proteins lipocalin 2 (24p3), inducible nitric oxide synthase (iNOS), interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-3α (MIP-3α), CD98, the macrophage Gal-3 receptor, and TNF-α receptor 1 (TNFR1). In contrast, APAP-induced expression of the alternative activation markers Ym1 and Fizz-1, as well as tissue repair, was increased in Gal-3$^{-/-}$ mice. These results suggest that Gal-3 contributes to inflammatory mediator production and hepatotoxicity after APAP. We next investigated the role of Gal-3 in regulating macrophage proinflammatory phenotype. Following APAP intoxication, increased numbers of proinflammatory CD11b^{+}/Ly6C^{hi} macrophages accumulated in necrotic regions of the liver. These cells were distinct from resident Kupffer cells and expressed Gal-3. Loss of Gal-3 resulted in reduced accumulation of CD11b^{+}/Ly6C^{hi} macrophages in response to APAP and increased accumulation of anti-inflammatory CD11b^{+}/Ly6C^{lo} macrophages. These results suggest that Gal-3 promotes a proinflammatory, classically activated macrophage phenotype in the liver during APAP-induced hepatotoxicity. Taken together, these studies indicate that multiple mechanisms contribute to proinflammatory macrophage activation following APAP intoxication.

DEDICATION

This work is dedicated to Ciprian. I could not have done this without your permanent support and encouragement, and I am thankful every day for having you in my life.

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ABBREVIATIONS

24p3	lipocalin 2
ANOVA	analysis of variance
APAP	acetaminophen
ASC	apoptosis-associated speck-like protein
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CCL20	chemokine (C-C motif) ligand 20, macrophage inflammatory protein-3α (MIP-3α)
CCL3	chemokine (C-C motif) ligand 3, macrophage inflammatory protein-1α (MIP-1α)
CCL4	chemokine (C-C motif) ligand 4, macrophage inflammatory protein-1β (MIP-1β)
CCL5	chemokine (C-C motif) ligand 5
CCR1	chemokine (C-C motif) receptor 1
CCR5	chemokine (C-C motif) receptor 5
cDNA	complementary deoxyribonucleic acid
CM-H_2DCFDA	5-(and-6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate acetyl ester
COX	cyclooxygenase
CRD	carbohydrate-recognition domain
CTGF	connective tissue growth factor
CX3CL1	chemokine (C-X3-C motif) ligand 1, fractalkine
CX3CR1	chemokine (C-X3-C motif) receptor 1
CXCL1	chemokine (C-X-C motif) ligand 1

CXCL12	chemokine (C-X-C motif) ligand 12
CXCL13	chemokine (C-X-C motif) ligand 13
CXCL2	chemokine (C-X-C motif) ligand 2, macrophage inflammatory protein-2 (MIP-2)
CXCR2	chemokine (C-X-C motif) receptor 2
DAMP	danger (or damage) associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DT	diphtheria toxin
DTPA	diethylene triamine pentaacetic acid
DTR	diphtheria toxin receptor
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
EP	ethyl pyruvate
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
EYFP	enhanced yellow fluorescent protein
FADD	Fas-associated death domain protein
FITC	fluorescein isothiocyanate
Fizz1	resistin-like α
Flt3L	FMS-like tyrosine kinase 3 ligand
g	gram
Gal-1	galectin-1
Gal-3	galectin-3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein

gp91	glycoprotein 91
GSH	glutathione
h	hour
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HMGB1	high-mobility group box1
HO-1	heme oxygenase-1
HRP	horseradish peroxidase
IFN-γ	interferon-γ
IL-10	interleukin 10
IL-12	interleukin-12
IL-13	interleukin-13
IL-17	interleukin-17
IL-1β	interleukin 1β
IL-23	interleukin-23
IL-4	interleukin-4
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IP-10	chemokine (C-X-C motif) ligand 10
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factor
JAK	Janus-activated kinase
JNK	c-Jun N-terminal kinase
kD	kilodalton
kg	kilogram
LOX	lipoxygenase
LPS	lipopolysaccharide

Ly6C	lymphocyte antigen 6 complex, locus C
MAPK	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor, CSF-1
mg	milligram
MHC	major histocompatibility complex
min	minute
MIP	macrophage inflammatory protein
ml	milliliter
mM	millimolar
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTT	methylthiazolyldiphenyl-tetrazolium bromide
MyD88	myeloid differentiation primary response gene (88)
NADPH	nicotinamide adenine dinucleotide phosphate
NAPQI	N-acetyl-para-benzoquinoneimine
NF-κB	nuclear factor *kappa*-light-chain-enhancer of activated *B* cells
NK	natural killer
NLRP3	nucleotide-binding domain and leucine-rich repeat containing protein 3
nm	nanometer
NOS	nitric oxide synthase
p22	protein 22
p47	protein 47
p55	protein 55
p67	protein 67
p75	protein 75

PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PE	phycoerythrin
PGE2	prostaglandin E2
PPAR	peroxisome proliferator activated receptor
PRR	pattern recognition receptor
RAGE	receptor for advanced glycation end-products
RIP-1	receptor interacting protein-1
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	transforming growth factor β
TIMP	tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNFR1	tumor necrosis factor-α receptor 1
TNFR2	tumor necrosis factor-α receptor 2
TNF-α	tumor necrosis factor-α
TRADD	TNFR-associated death domain protein
TRAF2	TNFR-associated factor 2
U	unit

VEGF	vascular endothelial growth factor
Ym1	Chi3l3 chitinase 3-like 3
μM	micromolar

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INTRODUCTION

INFLAMMATION

Inflammation is a complex adaptive response triggered by noxious stimuli, primarily infections or tissue injury. The purpose of an inflammatory response is generally containment of the injurious stimulus and restoration of homeostasis. To this end, a complex network of mediators generated by multiple cell types is mobilized and regulates the progression and resolution of inflammation (Medzhitov, 2008). Proper response to infection or injury is critical for survival. For example, an inability to kill microbes due to genetic deficiency in NADPH oxidase, the enzyme responsible for reactive oxygen species (ROS) production by macrophages, significantly increases the risk for bacterial and fungal infection and mortality; this condition is known as chronic granulomatous disease (Nathan, 2002). Conversely, chronic or dysregulated inflammation has considerable pathological consequences, including inflammatory tissue injury, autoimmunity, fibrosis, and cancer.

Inflammatory triggers

An inflammatory response is initiated when the appropriate stimulus is detected by cells of the innate immune system. These stimuli can be exogenous or endogenous. The best characterized exogenous triggers of inflammation are microbial components known as pathogen associated molecular patterns (PAMPs). These are conserved molecular structures present on the surface of microorganisms that are recognized by innate immune cell receptors termed pattern recognition receptors (PRRs). Several classes of PRRs have been identified, including toll-like receptors (TLRs). These are comprised of a group of 11 highly conserved cell surface and intracellular receptors

containing leucine rich-repeats (Chen *et al.*, 2007a). Each member of the family recognizes a specific category of microbial products. Thus, cell surface TLR2 binds peptidoglycan and lipotechoic acid from Gram-positive bacteria, while TLR4 recognizes lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria. TLR3, 7, and 9 are localized intracellularly in endosomes, and sense viral double stranded RNA, single stranded RNA, and CpG containing DNA, respectively (Warren, 2005; Chen *et al.*, 2007a). Recognition of PAMPs by specific receptors on innate immune cells engages signaling pathways resulting in activation for proinflammatory and cytotoxic activity critical for destruction of pathogens.

In addition to microbial products, exogenous inducers of inflammation include foreign bodies and toxic compounds. Asbestos and silica particles are well known examples of causative agents of inflammatory reactions. These large particles are thought to trigger a phagocytic response from macrophages; however, due to their large size, they cannot be efficiently phagocytized and macrophages form a granuloma around these particles (Medzhitov, 2008). Moreover, macrophage recognition of foreign particles is associated with activation of a sensor system, the NALP3 inflammasome, which ultimately leads to the production of inflammatory cytokines and initiation of an inflammatory response.

Inflammation also occurs in response to endogenous triggers. The observation that tissue injury, in the absence of infection, causes what has been termed “sterile” inflammation has led to the identification of molecules released from stressed or damaged cells that activate innate immune cells, initiating an inflammatory response. These molecules, known as danger or damage-associated molecular patterns (DAMPs), are normally found inside cells; often, they perform important physiologic functions. For example, HMGB1 (high mobility group box 1) is a DNA-binding nuclear protein with roles in DNA recombination and gene transcription (Ulloa and Messmer, 2006). Cellular death

results in breakdown of membranes and leakage of cellular contents into the extracellular environment, making DAMPs accessible to sensing by innate immune cells. A number of molecules have been identified as DAMPs, including ATP, uric acid, oligonucleotides, HMGB1, the small nuclear riboprotein component SIN3A-associated protein 130 (SAP130), and members of the S100 calcium binding family of proteins (S100A8, S100A9, S100A12) (Green *et al.*, 2009; Medzhitov, 2008).

The nature of cell death determines the type of response initiated by the immune system. For example, necrotic cell death is associated with tissue damage and macrophage recognition of signals released by dying cells elicits an inflammatory response (Green *et al.*, 2009). Apoptotic cells are also recognized by macrophages; however, apoptosis can occur under physiological conditions, therefore macrophage responses depend on the context. Apoptotic cells present during development are ingested by macrophages without immunological consequences. Tissue stress may also induce apoptosis in some cells, and macrophages that phagocytose these cells initiate repair processes (Medzhitov, 2008). In contrast, apoptosis that occurs as a result of infection poses a significant threat to the host and triggers a macrophage immune response leading to the induction of Th17 cells (Torchinsky *et al.*, 2009).

In addition to cellular constituents released from damaged cells, other endogenous inflammatory stimuli include products of extracellular matrix breakdown that are formed during tissue injury processes. For example, hyaluronan, a large molecular weight glycosaminoglycan, is degraded into smaller fragments that can activate TLR4 (Medzhitov, 2008). Products of altered metabolic pathways can also cause inflammation. Representative examples include crystals of monosodium urate, responsible for the chronic inflammatory joint disease known as gout, and oxidized lipoproteins, which contribute to inflammatory responses in the progression of atherosclerosis (Medzhitov, 2008).

Cascade of events in the inflammatory response

An acute inflammatory response is characterized by redness, heat, pain, and swelling, features that were first described by Celsus in the first century AD (Nathan, 2002). Since this process is initiated in response to danger signals, either from invading pathogens or from tissue injury, it is critical for survival that the immune system mounts a rapid and effective response. It becomes apparent then that cells of the innate immune system, primarily neutrophils, monocytes and macrophages, are key players in this process. Resident tissue macrophages and mast cells are essential in the early recognition of infection or injury. Rapid activation of these cells results in the release of a wide variety of mediators which contribute to the formation of a local inflammatory exudate and attract more immune cells to the inflammatory site. The release of vasoactive amines, including histamine, and eicosanoids such as prostaglandin E2 (PGE2) produced via cycloxygenase-2 (COX-2), causes vasodilation and fluid extravasation, responsible for heat, redness, and swelling (Nathan, 2002); (Medzhitov, 2008). Pre-formed tumor necrosis factor-a (TNF-α) released from activated tissue macrophages and mast cells activates the capillary endothelium, leading to the upregulation of selectins and adhesion molecules on the endothelial cell surface (Petri *et al.*, 2008). These molecules interact with integrins on blood leukocytes, facilitating their emigration from the blood into the site of injury or infection. Interaction of selectins with leukocytes results in tethering and rolling of leukocytes on the surface of endothelium, whereas adhesion molecules facilitate stronger adhesive interactions. Interestingly, in some vascular beds, such as the hepatic sinusoids, leukocyte recruitment relies exclusively on adhesion molecules, without selectin-dependent rolling (Petri *et al.*, 2008). Tissue macrophages also release chemokines, which contribute to the influx of immune cells. Neutrophils are the first immune cells to arrive at the site of inflammation. These

cells are activated by proinflammatory mediators, including TNF-α, interleukin 1β (IL-1β), and leukotrienes, and undergo a respiratory burst, releasing proteases, hydrolases, cytotoxic reactive oxygen species, antimicrobial peptides such as defensins and azurocidin, mediators that promote destruction of pathogens during infection (Nathan, 2002). Neutrophils also produce neutrophil extracellular traps (NETs), loose extracellular networks of nuclear or mitochondrial DNA covered in proteases that entrap pathogens to facilitate their destruction (Phillipson and Kubes, 2011). Further release of proinflammatory cytokines and chemokines perpetuates the infiltration and activation of leukocytes. Monocytes and macrophages are recruited into inflamed sites at later times after the initiation of injury. However, in contrast to neutrophils, which undergo apoptosis after activation, mononuclear cells are long-lived. They play a complex role during inflammatory responses, contributing to injury, as well as tissue repair. Mechanisms of macrophage activation and their role in inflammation are discussed in greater detail below.

With time, the profile of mediators at the inflammatory site shifts from pro- to anti-inflammatory, and as a result, the resolution phase is initiated. Several mechanisms have been described to control this transition. Thus, whereas in the early stages of inflammation, the eicosanoids produced by immune cells are proinflammatory COX-2-derived prostaglandins and 5-lipoxygenase (LOX)-derived leukotrienes, as inflammation progresses, 15-LOX-derived antiinflammatory lipoxins become predominant (Serhan *et al.*, 2008). Macrophages themselves release factors that act in an autocrine and paracrine manner to downregulate inflammatory mediator production. The secretory leukocyte protease inhibitor (SLPI) is an example of a protease inhibitor that is secreted by macrophages in the later stages of stimulation with microbial products. SLPI acts on neutrophils to suppress protease and ROS production and has wound healing effects (Nathan, 2002). As levels of inflammatory cytokines and chemokines decrease,

neutrophil activation is no longer sustained, and these cells undergo apoptosis. Macrophage phagocytosis of apoptotic neutrophils results in the release of transforming growth factor β (TGF-β), a growth factor important for wound repair (Nathan, 2002). Another important mechanism promoting tissue repair is the alternative activation of macrophages, which leads to the release of anti-inflammatory mediators and growth factors. The parasympathetic arm of the autonomic nervous system also has a role in the inhibition of inflammation, by releasing acetylcholine, which acts on nicotinic receptors on macrophages to block TNF-α release (Tracey, 2002).

Thus, under normal circumstances, acute inflammation is a tightly orchestrated, self-limiting event, with numerous levels of control that ensure containment of infection, while limiting injury to the host. However, exaggerated inflammatory responses are detrimental, and inflammation plays an important pathogenic role in acute clinical conditions such as anaphylaxis, ischemia-reperfusion injury, myocardial infarction, and sepsis syndrome (Nathan, 2002).

In cases of persisting stimulation, such as the presence of foreign bodies, acute inflammation does not subside, and perpetuates into a chronic state. Chronic inflammation is characterized by the persistence of activated macrophages, and sometimes lymphocytes at sites of injury or infection. These cells can form granulomas (Nathan, 2002; Laskin, 2010a). Some chronic inflammatory pathologies are also associated with activated neutrophils. A number of chronic diseases have a significant inflammatory component. Moreover, inflammation is a contributor to the pathogenesis of asthma, atherosclerosis, obesity, chronic obstructive pulmonary disease, gout, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, hepatitis C, tuberculosis, idiopathic pulmonary fibrosis, and liver cirrhosis (Nathan, 2002); (Nathan and Ding, 2010).

INFLAMMATORY MEDIATORS

Reactive oxygen species

Free radicals are defined as molecular species containing one or more unpaired electrons, a property which imparts a high degree of reactivity. Reactive oxygen species (ROS) represent a group of molecules that includes oxygen-derived radicals, including superoxide anion ($O_2^{\cdot}$), hydroxyl, hydroperoxy, as well as nonradical molecules such as hydrogen peroxide (H_2O_2) and hypochlorous acid (Bayir, 2005). ROS are formed by the sequential addition of one electron to molecular oxygen. This results in the formation of the superoxide anion, which has limited reactivity and cannot cross cell membranes. Further reduction of superoxide anion by the enzyme superoxide dismutase leads to formation of H_2O_2, an oxidizing agent with the ability to diffuse through membranes. H_2O_2 is inactivated by catalase or peroxidases which convert it to water. While superoxide anion and hydrogen peroxide have limited reactivity, in the presence of transitional metals such as Fe (II) or Cu (I) they have the potential to form highly reactive hydroxyl radicals via the Fenton reaction. Hydroxyl radicals are highly reactive with a very short half-life, thus limiting their reactivity to the proximity of the site where they are produced (Valko *et al.*, 2007). Other oxygen radicals that can be formed in cells include peroxyl radicals and hydroperoxyl, which initiate lipid peroxidation.

Due to their reactivity, ROS have the potential to cause damage to cellular components; therefore, cells possess several mechanisms for antioxidant defense. These include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, as well as small molecules such as tocopherol (vitamin E), ascorbic acid (vitamin C), and glutathione (GSH). Glutathione is the main soluble antioxidant in the cytosol and mitochondria, where it is present in mM concentrations. The reaction of GSH with ROS leads to the formation of oxidized glutathione, which accumulates inside the

cell. GSH acts as an antioxidant directly, by reacting with ROS, or indirectly, as a cofactor for glutathione peroxidase; it also contributes to regenerating vitamins C and E to their active forms (Valko *et al.*, 2007). ROS are generated in low amounts during normal metabolic processes and play physiological roles in signal transduction, activation of transcription factors, mitochondrial electron transport. When ROS are produced in excess, or when protective mechanisms are inadequate, damage to proteins, lipids or DNA ensues, and contributes to the pathogenesis of disease, including sepsis, cardiovascular disease, ischemia-reperfusion injury, cancer, and diabetes (Valko *et al.*, 2007).

ROS are key for the microbicidal activities of neutrophils and macrophages. The enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the main source of ROS during phagocyte respiratory burst. NADPH oxidase consists of two membrane subunits, gp91 and p22, and three cytoplasmic subunits, p47, p67, and the G-protein Rac2. In unstimulated phagocytes, the enzyme is inactive. Following stimulation of neutrophils and macrophages with proinflammatory cytokines TNF-α, IL-1β, interleukin-6 (IL-6), or with LPS, the cytoplasmic subunits translocate to the membrane and form the active enzyme, which generates large amounts of superoxide (Bayir, 2005; Valko *et al.*, 2007). The enzyme myeloperoxidase is another source of ROS primarily found in neutrophils. This enzyme catalyzes the oxidation of chlorine to hypochlorous acid in the presence of hydrogen peroxyde (Bayir, 2005).

Reactive nitrogen species

Nitric oxide (NO) is a small highly reactive gaseous molecule with one unpaired electron. NO is produced enzymatically by nitric oxide synthases (NOS), which catalyze the reaction of arginine with oxygen to form citrulline and nitric oxide. Three NOS isoforms have been identified: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or

NOS-2), and endothelial NOS (eNOS or NOS-3). Two isoforms, nNOS and eNOS, are calcium-dependent, constitutively present enzymes responsible for generating low amounts of NO for short periods of time. In contrast, iNOS is calcium-independent and not present in physiological conditions in most tissues, with the exception of epithelial cells of the intestine, bronchi, and kidney tubules (Levy *et al.*, 2005). Expression of iNOS is upregulated in response to proinflammatory cytokines, LPS, hypoxia, or oxidative stress. Moreover, unlike constitutive isoforms, iNOS can generate large quantities of NO for longer periods of time.

NO is soluble in both aqueous and lipid environments, and diffuses across membranes and between cells. In water, NO has a half-life of a few seconds, but its stability is significantly increased in environments with low oxygen concentrations (Valko *et al.*, 2007). These properties make NO an important oxidative and signaling molecule involved in a wide variety of physiological and pathological processes. Originally identified as an endothelium derived-relaxing factor, NO has also been implicated in neutrotransmission, regulation of blood pressure and vascular tone, and maintenance of perfusion in the microcirculation. These roles of NO appear to be mediated by low concentrations of this molecule. In high amounts, NO is detrimental, and promotes altered vascular permeability, increased vasodilation, and causes cytotoxicity due to nitrosative stress (Thomas *et al.*, 2008). This process is characterized by increased production of NO and NO-derived reactive nitrogen species (RNS) which react with protein thiols, amine, or hydroxy groups (Thomas *et al.*, 2008). In situations where ROS are present, such as the phagocyte respiratory burst, NO can react with superoxide to form peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO2), which are potent oxidants (Valko *et al.*, 2007). It is important to note that the stimuli which induce ROS production by activation of the NADPH oxidase also upregulate iNOS expression; thus, phagocytes

activated by proinflammatory stimuli release high amounts of both ROS and RNS, which are critical for killing of pathogens.

Tumor necrosis factor-α

TNF-α was first described in the 1970s as a glycoprotein induced by LPS that induces necrosis of tumor cells. A large body of work accumulating in the past decades indicates the critical role of this cytokine in a wide range of conditions, including inflammation, infection, and cancer. While TNF-α is not detectable under homeostatic conditions, its expression is increased during inflammatory reactions. Macrophages and monocytes represent the predominant cellular source of TNF-α; T lymphocytes, as well as natural killer cells, neutrophils, mast cells, and endothelial cells can also produce this cytokine during inflammation. TNF-α is synthesized as a 26 kD cell-surface associated protein; cleavage by matrix metalloproteinases results in the release of 17 kD soluble TNF-α (Bradley, 2008).

The effects of TNF-α are mediated by two cell-surface receptors, TNFR1 (p55) and TNFR2 (p75), which have distinct cellular expression patterns and activate complex signaling pathways to perform multiple TNF-α-induced biologic effects. TNFR1 is ubiquitously expressed, and mediates the majority of proinflammatory and programmed cell death effects of TNF-α during injury. TNF-α binding to TNFR1 leads to the recruitment of the adaptor protein TNFR-associated death domain protein (TRADD), which initiates signaling by recruiting two cytoplasmic proteins, receptor interacting protein-1 (RIP-1) and TNFR-associated factor 2 (TRAF2). The most important downstream pathway activated by TNFR1 activation is the nuclear factor *kappa*-light-chain-enhancer of activated *B* cells (NF-κB), which regulates gene expression of numerous proinflammatory mediators (Bradley, 2008). In some cases, signaling through TNFR1 may initiate apoptotic cell death; thus, TRADD can bind the Fas-associated

death domain protein (FADD), leading to recruitment of pro-caspase-8 and autocatalytic activation to caspase-8, which cleaves caspase 3, an important executor of apoptosis. TNFR2 is expressed on immune and endothelial cells. TNFR2-dependent signaling is incompletely characterized, but has been suggested to promote cell migration, proliferation, and tissue repair (Aggarwal, 2003). Production of TNF-α is an important consequence of activation by proinflammatory stimuli. TNF-α further promotes inflammatory responses, through endothelial cell activation for leukocyte recruitment, sustained macrophage activation and subsequent release of other mediators of inflammation. TNF-α plays a key role in the containment of infections, but excessive production is an important contributor to the pathogenesis of inflammatory diseases, including sepsis, and rheumatoid arthritis (Bradley, 2008). In the liver, TNF-α appears to play a dual role during tissue injury, initially promoting inflammation and subsequently upregulating antioxidants and inducing hepatocyte proliferation and tissue repair (Laskin, 2009).

Interleukin-1β

IL-1β is a 15 kD cytokine with proinflammatory activity whose expression is induced following macrophage activation by bacterial products such as LPS. IL-1β is secreted via a non-classical pathway. Cell activation results in the transcription of a 30 kD inactive IL-1β precursor, which is subsequently cleaved into the active form by a complex known as the inflammasome. This complex consists of cryopyrin (nucleotide-binding domain and leucine-rich repeat contain protein 3, or NLRP3), apoptosis-associated speck-like protein (ASC), and caspase 1. Assembly of this complex leads to the conversion of pro-caspase 1 to active caspase 1, the enzyme responsible for cleavage of the inactive IL-1β precursor to the active form (Dinarello, 2011b). IL-1β acts on the corresponding cell surface receptor, IL-1R, which shares common cytoplasmic

adapter and signaling proteins with the TLR pathway (Dinarello, 2011b). A naturally occuring IL-1R antagonist, IL-1Ra prevents binding of the cytokine to its receptor and antagonizes its biological effects (Dinarello, 2011a). IL-1β is one of the first cytokines identified, and it was originally characterized as a fever-inducing agent. The effects of IL-1β also include upregulation of proinflammatory mediators by activated macrophages and neutrophils; these mediators include proinflammatory cytokines TNF-α, IL-6, chemokines, COX-2, iNOS, adhesion molecules and matrix metalloproteinases (Dinarello, 2005; Dinarello, 2011a). Additionally, IL-1β is a neutrophil chemoattractant and activator, and induces hepatic acute phase protein synthesis (Dinarello, 2011a).

Interleukin-6

IL-6 is a 22-27 kD cytokine with a structure characterized by a four-helix bundle. IL-6 is produced by immune and non-immune cells and acts on receptors with an immunoglobulin structure. The IL-6 receptor complex consists of the IL-6R chain, which has a short intracytoplasmic domain, and binds two glycoprotein 130 (gp130) molecules to initiate signal transduction (Song and Kellum, 2005). gp130 is expressed on a wide variety of cells, but IL-6 binding to gp130 alone is insufficient for activation. In contrast, IL-6R has a more restricted expression pattern, limited to hepatocytes, macrophages, monocytes, neutrophils, B and T lymphocytes, and determines the responsiveness of a particular cell to IL-6 (Garbers *et al.*, 2012). IL-6 is produced by LPS-activated macrophages and has proinflammatory activity and upregulates expression of monocyte and macrophage-attractant chemokines and of cell adhesion molecules; it also induces the synthesis of acute phase proteins. IL-6 is also important in regulation of insulin sensitivity and glucose tolerance, and contributes to bone homeostasis through its stimulatory effects on osteoclasts (Scheller *et al.*, 2011).

Interleukin-10

Interleukin-10 (IL-10) was first characterized as a product of Th2 lymphocytes which inhibits the production of interferon-γ from Th1 cells. IL-10 is a 35 kD homodimer composed of two non-covalently bound subunits, with 75% identity in the amino acid sequence between mice and humans (Sabat *et al.*, 2010). Macrophages and monocytes, as well as T helper lymphocytes, are the main sources of IL-10 *in vivo*. IL-10 binds to a specific cell surface receptor, the IL-10 receptor (IL-10R), which consists of two chains, IL-10R1 and IL-10R2. The cell and tissue distribution of these two subunits varies; thus, IL-10R1 is primarily expressed on immune cells, especially on macrophages, while IL-10R2 is more widely expressed. IL-10 binds with much higher affinity to the IL-10R complex than to each of the individual subunits; moreover, IL-10R1 appears to be critical for IL-10 binding and downstream signaling, while IL-10R2 by itself does not bind IL-10 directly (Sabat *et al.*, 2010). Receptor engagement by IL-10 leads to the activation of JAK-STAT pathways, and signaling through STAT3 is primarily responsible for the biological effects of IL-10. Macrophages represent the main target cell for the biological functions of IL-10, which consist of inhibition of proinflammatory mediator release, including LPS and IFN-γ-induced production of TNF-α, IL-1β, IL-6, IL-12; IL-10 also induces the release of antiinflammatory mediators through alternative activation of macrophages (Martinez *et al.*, 2009). Together, these properties indicate a prominent role for IL-10 as an antiinflammatory cytokine; this is further supported by studies in IL-$10^{-/-}$ mice, which develop spontaneous inflammatory bowel disease, and exhibit exaggerated proinflammatory cytokine release in response to LPS (Sabat *et al.*, 2010).

Interleukin-4 and interleukin-13

IL-4 and IL-13 are cytokines characterized by a short amino acid sequence with a four-helix structure. IL-4 and IL-13 are produced by Th2 lymphocytes, natural killer T cells, mast cells, basophils, and eosinophils, and act on receptors present on macrophages, fibroblasts, B and T lymphocytes (Kelly-Welch *et al.*, 2005). Despite their limited sequence homology (approximately 30%), IL-4 and IL-13 signal through common receptor complexes and share some biological properties (Martinez *et al.*, 2009); (McKenzie, 2000). IL-4 is recognized by type I and type II receptors, while IL-13 binds to the type II receptor and to IL-13Rα2. The IL-4Rα chain binds IL-4, which leads to dimerization with another protein to form the type I or type II receptor. Thus, binding of IL-4Rα to the gamma chain result in the formation of the type I receptor, and dimerization of IL-4Rα with IL-13Rα1 forms the type II receptor. The type II receptor is the major receptor responsible for the biological effects of IL-13. In contrast, IL-13Rα2 has a short intracytoplasmic tail, and functions as a decoy receptor, promoting internalization after ligand binding, with no downstream signal transduction (Kelly-Welch *et al.*, 2005). IL-4 and IL-13 are produced during parasitic infections and are important inducers of alternatively activated macrophages. These cytokines also play pathogenic roles in allergy and asthma (Martinez *et al.*, 2009).

Chemokines

Chemokines consist of a group of cytokines that induce directional migration of immune cells. Almost all chemokines are secreted from the cells that produce them, with the exception of fractalkine (CX3CL1). Structurally, chemokines are small proteins, 8-12 kD, and contain one to three disulfides. Although the amino acid sequences vary, all chemokines share a similar tertiary structure, known as the chemokine scaffold, organized around critical cysteine residues whose relative position enables classification

of chemokines into four main groups. Thus, CC chemokines contain two adjacently positioned cysteine residues, whereas in CXC chemokines the two cysteines are separated by a single amino acid; these two groups account for the majority of known members of the chemokine family. The third and fourth groups consist of C and CX3C chemokines and contain one member each (Allen *et al.*, 2007; Mantovani *et al.*, 2010).

Chemokines exert their effects on immune cells by binding to specific seven-transmembrane-domain, G-protein coupled receptors. There is considerable redundancy and promiscuity in ligand-receptor interactions within the chemokine family, such that one chemokine can bind to multiple receptors, and one receptor can be activated by multiple chemokines. However, receptors are limited in their interaction to a specific subgroup of cytokines, such that CCRs only bind CC chemokines and CXCRs only recognize CXC chemokines. For example, the receptor CCR1 can interact with CCL3, CCL5, CCL23, CCL14, CCL15, CCL16; the ligand CCL3 can bind to both CCR1 and CCR5. This redundancy ensures a significant degree of flexibility in directing leukocyte trafficking.

Chemokines play important roles in physiological and pathological conditions and are functionally classified into “homeostatic” and “inflammatory” chemokines. Homeostatic chemokines control lymphocyte homing and circulation in physiologic conditions, and include CXCL12, CXCL13, CCL18, and CCL19. In contrast, inflammatory chemokines are produced during inflammation and are important in amplifying the immune response by stimulating the influx of leukocytes to sites of injury. These chemokines can be further classified according to the cell type(s) on which they act. Thus, CXC chemokines such as CXCL1 and CXCL2 act on their receptors CXCR1 and CXCR2 expressed on neutrophils, whereas CC chemokines, including CCL2, CCL3, CCL5, CCL7, and CCL8, act on their corresponding receptors present on monocytes and macrophages (Mantovani *et al.*, 2004).

Eicosanoids

Eicosanoids represent a class of lipid mediators derived from arachidonic acid that are active in nanomolar concentrations and act in an autocrine and paracrine manner on target cells. Cells of the innate immune system are important sources of eicosanoids, which are involved in cytokine production, cell proliferation, differentiation, and migration. Arachidonic acid is a 20-carbon polyunsaturated fatty acid normally presents in cell membranes that can be released from membrane phospholipids by the action of phospholipases. Once localized in the cytoplasm, arachidonic acid becomes available for enzymatic conversion to various classes of eicosanoids. Thus, cyclooxygenases (COXs) convert arachidonic acid to prostaglandins (PGs) and thromboxane, collectively known as prostanoids. Leukotrienes and lipoxins are formed by lipoxygenases (LOXs), and P450 epoxygenases produce hydroxyeicosatetraenoic acids (HETEs) (Stables and Gilroy, 2011; Harizi *et al.*, 2008).

Two major isoforms of cyclooxygenase have been identified: COX-1 and COX-2. Whereas COX-1 is constitutively expressed in many cells and tissues, COX-2 expression is induced during inflammation in innate immune cells. Cyclooxygenases catalyze the cyclization and oxidation of arachidonic acid to PGG2, and its conversion to PGH2. PGH2 is the common precursor for other biologically active compounds, including PGE2, formed via prostaglandin E synthase, PGI2 or prostacyclin, formed via prostaglandin I synthase, and thromboxane A2, synthesized via thromboxane A2 synthase (Stables and Gilroy, 2011). The pattern of expression for these synthases determines the overall prostanoid profile. For example, macrophages activated by proinflammatory stimuli, including LPS and cytokines, produce primarily PGE2. Several cell surface G-protein coupled receptors mediate the effects of PGE2, including EP1, EP2, EP3, and EP4. PGE2 is released during inflammation and promotes vasodilation,

increases vascular permeability, and is a potent pyretic agent (Harizi *et al.*, 2008). Interestingly, PGE2 can also indirectly have anti-inflammatory and pro-resolution effects by inducing the expression of enzymes required for synthesis of anti-inflammatory eicosanoids (Nathan, 2002).

Several lipoxygenases convert arachidonic acid to different lipid mediators. Thus, 5-lipoxygenase catalyzes the formation of leukotrienes LTA4, and LTB4, which are further converted to LTC4, LTD4, and LTE4. Activation of the 12/15-lipoxygenase pathway results in synthesis of 12- or 15-hydroxyeicosatetraenoic acid (12-HETE or 15-HETE), and further transformation of 15-HETE produced lipoxins A4 and B4 (Cook, 2005). Leukotrienes are generated by activated macrophages and perform distinct functions during inflammation. For example, LTB4 induces migration and activation of neutrophils and monocytes to sites of inflammation; LTC4, LTD4, and LTE4 increase vascular permeability and promote formation of edema (Cook, 2005). In contrast, lipoxins generated via 12/15-lipoxygenase-dependent mechanisms are anti-inflammatory and play an important role in the resolution of inflammation (Stables and Gilroy, 2011).

MACROPHAGES

Macrophages are mononuclear phagocytes present in numerous lymphoid and non-lymphoid tissues. They play a key role in innate immune defense, including phagocytic removal of debris, worn-out cells and pathogens, initiation of an inflammatory response to infection or injury, antigen presentation, and initiation of the adaptive immune response. Resident tissue macrophages are found throughout the body and perform specialized functions depending on their anatomical location. Thus, while osteoclasts are involved in bone resorption, alveolar macrophages in the lung function to remove inhaled particles and pathogens, and Kupffer cells in the liver, to destroy

pathogens derived from the intestine and to remove toxins and debris from the portal circulation. In the intestine, macrophages are important in removal of gut pathogens and in establishing tolerance to normal microbial flora and food antigens; in the spleen, red pulp macrophages clear aged erythrocytes, whereas marginal zone macrophages ingest particulates from the circulation. Langerhans cells are skin macrophages involved in phagocytosis and antigen presentation; lymph node macrophages perform important antigen-presenting roles; brain microglia are resident macrophages of the brain important in local immune functions (Murray and Wynn, 2011; Gordon and Taylor, 2005; Laskin *et al.*, 2011).

Macrophage receptors

In order to perform their numerous immune functions, macrophages express a wide variety of cell surface and intracellular receptors. These include pattern recognition receptors (PRRs), scavenger receptors, phagocytosis-related receptors, which allow macrophages to sample their environment; cytokine and chemokine receptors, which convey signals from other cells in the form of soluble mediators, and allow macrophages to mount an appropriate response, including activation, mediator release, and chemotaxis. Macrophages also possess integrins, selectins, and adhesion molecules, which regulate macrophage motility (Taylor *et al.*, 2005). The most prominent PRRs expressed by macrophages are TLRs; other PRRs expressed by macrophages includine Dectin-1, which recognizes fungal β-glucan oligosaccharides, and mannose receptor, which binds mannose and fucose-containing structures on bacteria and fungi (Verschoor *et al.*, 2012). Macrophages also express scavenger receptors, such as scavenger receptor-A and CD36, which mediate recognition and phagocytosis of bacteria, apoptotic cells, and also endogenous oxidized low-density lipoproteins. Other receptors involved in phagocytosis include Fc receptors gamma (CD16, CD32, CD64) and epsilon (CD23),

which enable recognition of antibody-coated bacteria and subsequent phagocytosis (Taylor *et al.*, 2005; Verschoor *et al.*, 2012). It is important to note that receptor expression pattern is carefully regulated in macrophages according to the developmental stage, tissue localization, and activation state.

Origin and development of macrophages

The origin of macrophages varies according to developmental stage. During early fetal development in rodents, macrophages are first observed in the yolk sac; these embryonic macrophages arise directly from mesenchymal progenitor cells, without going through an intermediate monocytic stage (Pollard, 2009). Later in the process of embryonic development, myeloid precursors populate the liver, which becomes the primary hematopoietic site (Gordon and Taylor, 2005). Postnatally, the bone marrow becomes the main source of macrophage precursors. Hematopoietic stem cells in the bone marrow undergo several commitment stages, including myeloid progenitors, followed by macrophage/dendritic cell progenitor (MDPs). This precursor cell gives rise to monocytes, which are released into the blood and migrate into tissues to mature into resident macrophages (Geissmann *et al.*, 2010). Local proliferation appears to be an important and previously unrecognized mechanism of maintenance for some tissue macrophage subpopulations, including microglia, Langerhans cells, and Kupffer cells (Gordon and Taylor, 2005; Naito *et al.*, 2004). The development and survival of monocytes and macrophages is controlled by the growth factor receptor Csf1r (colony stimulating factor-1 receptor, c-fms, M-CSFR, CD115), which is expressed on all macrophages and their precursors, and has two ligands, csf1 (M-CSF), and IL-34 (Chitu and Stanley, 2006; Lin *et al.*, 2008). The importance of csf1r-dependent signals in macrophage physiology is best illustrated by the severe phenotype of the op/op mouse strain, which has a spontaneous mutation in the *csf1* gene and lacks detectable levels of

this growth factor. These mice have a severe deficiency of most tissue macrophages, along with multiple abnormalities, including growth retardation, lack of teeth, osteopetrosis, skeletal deformities, reduced lifespan and poor breeding (Wiktor-Jedrzejczak *et al.*, 1990; Ryan *et al.*, 2001).

Classical and alternative macrophage activation

Macrophages are characterized by remarkable plasticity, and respond to inflammatory stimuli and pathogens by becoming activated. Macrophage activation involves a series of biochemical, phenotypical, and functional changes that occur in response to cues from the microenvironment. Accumulating evidence suggests that this leads to the appearance of distinct populations of activated macrophages that express specific markers and play divergent roles in immunity and inflammation.

Classically activated macrophages, referred to as M1 macrophages, develop in response to interferon-γ (IFN-γ) and bacterial-derived LPS (Mosser and Edwards, 2008). These macrophages produce high levels of reactive oxygen and nitrogen species, along with proinflammatory mediators including TNF-α, IL-1β, IL-6, IL-12, IL-23, and chemokines CCL3, CCL4, CCL5, CCL20, CXCL2, CXCL9, CXCL10, CXCL11 (Martinez *et al.*, 2009; Laskin *et al.*, 2011; Mantovani *et al.*, 2004). During infection, IFN-γ is produced by innate and adaptive immune cells, including natural killer cells and Th1 lymphocytes (Mosser and Edwards, 2008). Upon stimulation with IFN-γ, macrophages are “primed” for classical activation, but expression of the complete set of genes associated with this phenotype requires presence of bacterial-derived products such as LPS (Martinez *et al.*, 2009). IFN-γ signalling through IFNγ-receptor results in activation and nuclear translocation of the transcription factor STAT1, which induces gene expression of iNOS, CCL5, CXCL9, and CXCL10. LPS recognition by TLR4 leads to activation of the NF-κB pathway, a transcription factor responsible for upregulating

expression of TNF-α, IL-12, and proinflammatory CC and CXC chemokines. Other transcription factors, including interferon regulatory factor 3 (IRF3) and IRF5, have been shown to play an important role in promoting classical macrophage activation. IRF3 is activated through a TLR4-dependent mechanism and induces expression of IFN-β, which acts on the IFNα/β-receptor to induce IRF5, leading to increased production of proinflammatory cytokines IL-12 and IL-23 (Sica and Mantovani, 2012; Krausgruber *et al.*, 2011).

Another pathway of macrophage activation leads to the generation of alternatively activated or M2 macrophages. This is the result of stimulation of macrophages with IL-4, IL-13, or with IL-10 (Mosser and Edwards, 2008). Subpopulations of alternatively activated macrophages also develop in response to glucocorticoids or immune complexes in combination with LPS (Edwards *et al.*, 2006); (Laskin, 2009). These macrophages are characterized by increased expression of arginase, mannose receptor, Ym1, Fizz1, chemokines such as CCL17, CCL22, CCL24, and growth factors including TGF-β, vascular endothelial growth factor (VEGF) and anti-inflammatory mediators (IL-10) (Martinez *et al.*, 2009; Mantovani *et al.*, 2004). IL-10 signals through a specific cell surface receptor (IL-10R) (Sica and Mantovani, 2012). Receptors for IL-4 and IL-13 share a common subunit, the IL-4Rα. This subunit can dimerize with a gamma subunit to form the type I receptor, or with the IL-13Rα1 to form the type II receptor. IL-4 can bind to both receptor types, while IL-13 only recognizes the type II receptor. A second IL-13 receptor chain, IL-13Rα2, has been identified, and is thought to represent a decoy receptor for this cytokine (Martinez *et al.*, 2009). Binding of IL-4 and IL-13 to their respective receptors results in activation and nuclear translocation of the transcription factor STAT6, while IL-10R engagement signals through STAT3, leading to induction of gene expression of markers for alternatively activated macrophages. Peroxisome proliferator activated receptor-γ (PPARγ) and PPARδ are a

family of transcription factors that control expression of enzymes mediating the synthesis of antiinflammatory lipids associated with alternative macrophage activation. Interestingly, activation of these pathways also leads to expression of molecules important in negative regulation of classical activation-specific genes. Thus, STAT6 activation by IL-4/IL-13 results in upregulation of suppressor of cytokine signaling 1 (SOCS1), which inhibits STAT1-dependent gene expression; IL-4 induces the expression of the transcription factor IRF4, which prevents IRF5-dependent induction of proinflammatory cytokines. Moreover, during classical activation, one of the genes induced via STAT1 is SOCS3, which prevents STAT3-mediated expression of alternative activation genes (Sica and Mantovani, 2012).

Activated macrophages in health and disease

Classically and alternatively activated macrophages perform distinct functions in nonspecific immunity; hyperactivity of these cells has also been implicated in numerous pathologies. Following injury or infection, classical macrophage activation usually occurs early in the pathogenic response, and the cytokines and chemokines they release serve to recruit and activate other immune cells (Mantovani *et al.*, 2004). Due to their significant microbicidal activity, classically activated macrophages are key for host defense, and have been shown to play a critical role in resistance against intracellular bacteria, such as *Listeria monocytogenes* (Shaughnessy and Swanson, 2007). Deficiency in IFN-γ or IL-12, cytokines important in inducing classical macrophage activation, or mutations in their signalling pathways are associated with increased susceptibility to bacterial or viral infection (Filipe-Santos *et al.*, 2006). Classically activated macrophages are also important in tumor surveillance as they can directly destroy cancer cells and induce Th1 cytotoxic responses (Sica and Mantovani, 2012). However, persistent or excessive production of cytotoxic and proinflammatory mediators

by these macrophages can lead to damage to host cells, either directly, or by promoting activation of the Th1 and Th17 proinflammatory T cells. Thus, IL-1, IL-6 and IL-23 generated by classically activated macrophages contribute to the development of Th17 cells, which in turn produce IL-17, a proinflammatory cytokine (Mosser and Edwards, 2008). Classically activated macrophages and their products have been implicated in the pathogenesis of rheumatoid arthritis, autoimmune diseases such as lupus, experimental colitis, and experimental autoimmune encephalitis (Szekanecz and Koch, 2007; Sica and Mantovani, 2012; Nathan and Ding, 2010; Martinez *et al.*, 2009; Murray and Wynn, 2011).

Alternative macrophage activation typically occurs later in the inflammatory response to injury and infection. The antiinflammatory mediators released by these cells function to downregulate inflammatory responses mediated by classically activated macrophages. Moreover, subpopulations of alternatively activated macrophages are critical for tissue repair. In this regard, it has been shown that prolonged classical macrophage activation due to an inability to switch to an alternatively activated phenotype underlies persistent inflammation and defective wound healing associated with chronic venous ulcers (Sindrilaru *et al.*, 2011). Alternatively activated macrophages contribute to tissue repair by producing growth factors that stimulate fibroblasts to proliferate and differentiate into collagen-producing myofibroblasts (Wynn and Barron, 2010). Additionally, alternatively activated macrophages remove dead cells and debris via phagocytosis, release matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) which control matrix turnover, and activate Th2 and regulatory T cells (Murray and Wynn, 2011). However, excessive production of extracellular matrix due to the prolonged presence of alternatively activated macrophages is a key factor in the development of fibrosis. The critical role of these macrophages in liver fibrosis has been demonstrated using diphtheria toxin receptor

(DTR)-mediated conditional ablation of macrophages (Duffield *et al.*, 2005). Unlike the human DTR, the mouse DTR has very low affinity for the diphtheria toxin (DT); thus, transgenic mice were engineered to express the human DTR under the control of the CD11b promoter, which confers sensitivity to DT-induced cell death to cells that express CD11b, such as macrophages. Depletion of macrophages during the progression of carbon tetrachloride-induced chronic injury resulted in decreased fibrosis; this was associated with reduced myofibroblast activation and decreased TGF-β production, suggesting that the macrophage populations contributing to fibrosis exhibited an alternatively activated phenotype (Duffield *et al.*, 2005).

Alternatively activated macrophages accumulate during with parasitic infections and are important components of granulomas. Since large, extracellular pathogens such as helminths cannot be efficiently phagocytosed and killed by classically activated macrophages, it has been suggested that IL-4 and/or IL-13-dependent responses contribute to parasite containment and expulsion (Martinez *et al.*, 2009). Consistent with this, IL-13$^{-/-}$, IL-4/IL-13$^{-/-}$ and IL-4Rα$^{-/-}$ mice exhibit reduced granuloma formation and increased parasite burden during infection with *Nippostrongylus brasiliensis* or *Schistosoma mansoni* (Martinez *et al.*, 2009). In contrast to their protective role in helminthic infections, alternatively activated macrophages contribute to increased susceptibility to infection with the unicellular parasite *Leishmania major* (Martinez *et al.*, 2009). Alternatively activated macrophages are also involved in allergy and asthma, chronic inflammatory diseases associated with increased production of IL-4 and IL-13, and the accumulation of alternatively activated macrophages in the lung (Martinez *et al.*, 2009). However, while some studies show a role for these cells in promoting airway inflammation, others suggest increased injury in the absence of chitinase-like proteins (Ym1, Ym2), major products of alternatively activated macrophages (Murray and Wynn, 2011; Nair *et al.*, 2009). It is likely that these diverse and sometimes opposing functions

are mediated by distinct subpopulations of alternatively activated macrophages responding to disease-specific stimuli.

Tumor-associated macrophages have been suggested to exhibit a phenotype similar to that of alternatively activated macrophages (Martinez *et al.*, 2009). However, their phenotype and functions appear to be controlled by cytokines other than IL-4 and IL-13, and they express markers of alternative activation (arginase, IL-10, CCL22, VEGF), as well as some proinflammatory cytokines such as CXCL9 and CXCL10; moreover, considerable heterogeneity exists within macrophage populations in different tumor models (Martinez *et al.*, 2009; Gordon and Martinez, 2010). Tumor-associated macrophages contribute to tumor progression, angiogenesis and metastasis (Gordon and Martinez, 2010; Qian and Pollard, 2010).

Adipose tissue macrophages represent an interesting example of a physiological role for the alternatively activated phenotype. In lean mice, adipose tissue macrophages release antiinflammatory mediators, produced via PPARγ dependent mechanisms that promote insulin sensitivity and glucose tolerance in adipocytes. Moreover, alternatively activated macrophages in adipose tissue regulate thermogenesis by inducing secretion of catecholamines (Nguyen *et al.*, 2011b). During the progression of obesity, these macrophages switch from an alternatively to a classically activated phenotype with proinflammatory activity which contributes to insulin resistance (Lumeng *et al.*, 2007; Lumeng *et al.*, 2008).

It is important to note that macrophages exhibit significant plasticity, and alter their phenotype in the course of inflammation in response to changing signals in their environment. Moreover, M1/classically activated and M2/alternatively activated macrophages are extremes of a continuous spectrum of phenotypes and it is likely that multiple subpopulations co-exist during inflammation *in vivo*. The complexity of activated macrophage subpopulations is further underscored by findings that they possess a

hybrid phenotype in some situations. For example, activated macrophages present in skin wounds have been shown to express markers of both classical and alternative activation. Thus, these cells express high levels of arginase, Ym1, and mannose receptor, but also TNF-α and IL-6; moreover, their phenotype is independent of IL-4 or IL-13-dependent pathways (Daley *et al.*, 2009). In a model of zymosan-induced peritoneal inflammation, macrophages present during the resolution phase exhibited a mixed phenotype, with high levels of IL-10 and mannose receptor, but also iNOS and COX-2 (Bystrom *et al.*, 2008). Taken together, these data suggest that complex and heterogeneous subpopulations of activated macrophages arise in response to different injurious stimuli and a detailed understanding of these subpopulations in specific models is key to developing adequate therapeutic strategies for modulating their responses.

Monocyte-derived macrophages in inflammation

Evidence suggests that bone marrow-derived monocytes are an important source of newly recruited macrophages during inflammatory reactions (Robbins and Swirski, 2010). Like macrophages, monocytes consist of a heterogenenous group of cells. In mice, two main subpopulations of monocytes have been identified in the blood, based on their expression levels for the surface marker Ly6C, a 12-20 kD, phosphatidyl inositol anchored, cell surface glycoprotein (Gumley *et al.*, 1995). The exact function of Ly6C in monocytes is unclear, but it has been proposed to play a role in T lymphocyte adhesion and homing of memory $CD8^+$ T cells to lymph nodes (Hanninen *et al.*, 2011; Jaakkola *et al.*, 2003). $Ly6C^{high}$ monocytes represent about 60% of all blood monocytes in mice and express high levels of the chemokine receptor CCR2, as well as L-selectin (CD62L); in contrast, they express low levels of the chemokine receptor CX3CR1. $Ly6C^{hi}$ monocytes are not detected in tissues following adoptive transfer, indicating that they have a short lifespan under physiological conditions; however, during acute

inflammation, they are preferentially recruited into tissues (Geissmann *et al.*, 2003; Geissmann *et al.*, 2010). In contrast to Ly6C^{hi} monocytes, Ly6C^{low} monocytes, which account for approximately 40% of mouse blood monocytes, express low levels of CCR2 and CD62L, but high levels of CX3CR1. They also have a longer lifespan, and migrate into tissue under homeostatic conditions to give rise to resident macrophages (Geissmann *et al.*, 2003; Sunderkotter *et al.*, 2004; Geissmann *et al.*, 2010; Robbins and Swirski, 2010). The relationship between Ly6C^{hi} and Ly6C^{lo} monocytes is unclear. It has been suggested that Ly6C^{hi} monocytes are more immature precursors that are released from the bone marrow; as they mature, Ly6C expression is downregulated (Sunderkotter *et al.*, 2004). Recent reports that the nuclear receptor NR4A1 controls survival of Ly6C^{lo} monocytes, but has no effect on Ly6C^{hi} monocytes raise the possibility that these cells may develop independently (Hanna *et al.*, 2011).

Monocyte heterogeneity has also been described in humans. Thus, about 90% of all blood monocytes exhibit what has been termed a “classical” phenotype characterized by high levels of CD14 and lack of CD16 expression. These classical monocytes are similar to Ly6C^{hi} mouse monocytes in that they also express high levels of CCR2, but low levels of CX3CR1; in contrast to their murine counterparts, CD14^{hi}/CD16^{-} human monocytes do not produce proinflammatory cytokines (Auffray *et al.*, 2009). The remaining 10% of monocytes in the human blood are “non-classical” monocytes that express high levels of CD16; these cells can be further grouped based on the levels of CD14 expression into CD16^{+}/CD14^{lo} monocytes, which release proinflammatory mediators in response to LPS, and CD16^{+}/CD14^{-} monocytes, whose exact role is poorly characterized (Auffray *et al.*, 2009).

In mice, Ly6C^{hi} monocytes are preferentially recruited into tissues during acute and chronic inflammation (Robbins and Swirski, 2010; Yona and Jung, 2010). The bone marrow is an important source of Ly6C^{hi} monocytes during inflammation, and migration

of these cells into the blood is critically dependent on CCR2 (Serbina and Pamer, 2006). However, the spleen has recently been shown to contain a monocyte reserve; these monocytes appear to be produced in the bone marrow, but reside in the splenic red pulp and are mobilized to sites of injury (Swirski *et al.*, 2009). Once localized in the inflamed tissue, Ly6C^{hi} monocytes from the bone marrow or spleen develop into classically activated macrophages characterized by increased production of proinflammatory mediators. The pathogenic role of this subpopulation of macrophage precursors differs according to the injury model. Thus, in acute pancreatitis, Ly6C^{hi} monocytes infiltrate into the pancreas and contribute to tissue injury through a TNF-α-dependent mechanism (Perides *et al.*, 2011). During experimental autoimmune encephalitis, Ly6C^{hi} monocytes migrating into the brain upregulate TNF-α, iNOS, IL-6 and IL-12 and contribute to exacerbated disease (King *et al.*, 2008). CCR2-dependent influx of Ly6C^{hi} monocytes into the brain is associated with increased mortality during West Nile virus-induced encephalitis (Getts *et al.*, 2008). Adoptive transfer experiments in a model of chronic liver inflammation induced by high-fat diet, have shown that Ly6C^{hi} cells contribute to hepatocellular injury by producing increased levels of TNF-α and nitric oxide (Deng *et al.*, 2009). Hepatic inflammation and fibrosis induced by chronic carbon tetrachloride administration is mediated by a subpopulation of Ly6C^{hi} monocytes that migrate into the liver using a CCR2/CCR6-dependent mechanism, and mature into proinflammatory macrophages that promote hepatic stellate cell activation and collagen deposition (Karlmark *et al.*, 2009). Ly6C^{hi} monocytes are recruited to atherosclerotic lesions and have been suggested to contribute to the progression of diet-induced atherosclerosis (Swirski *et al.*, 2009). Proinflammatory mediators produced by accumulating Ly6C^{hi} monocytes/macrophages, including TNF-α and IL-12, are critical for defense against *Toxoplasma gondii* (Mordue and Sibley, 2003; Dunay *et al.*, 2008).

A few studies have analyzed the fate of both Ly6C^{hi} and Ly6C^{lo} monocyte subpopulations during inflammation. Acute myocardial infarction represents an interesting example of time-dependent recruitment of both Ly6C^{hi} and Ly6C^{lo} monocytes. Early in the pathogenic process, Ly6C^{hi} monocytes/macrophages accumulate in the injured myocardium and contribute to tissue damage through release of proteases and TNF-α; subsequently, tissue repair is mediated by Ly6C^{lo} monocytes which release VEGF and are recruited independent of Ly6C^{hi} cells (Nahrendorf *et al.*, 2007). In acute skeletal muscle necrosis, Ly6C^{hi} monocytes are exclusively recruited from the circulation and exhibit a proinflammatory phenotype upon tissue extravasation, with high expression of TNF-α and IL-1β; phagocytosis of muscle debris induces a phenotype switch from Ly6C^{hi} to Ly6C^{lo} cells, which are critical for repair through release of TGF-β and IL-10 (Arnold *et al.*, 2007). A similar preferential recruitment of Ly6C^{hi} monocytes is observed during kidney fibrosis (Lin *et al.*, 2009). However, in this situation Ly6C^{hi} cells are the precursors for three distinct subpopulations of kidney macrophages, including an M1-like subset and an M2-subset with profibrotic activity.

Taken together, these data suggest that monocyte phenotypic and functional heterogeneity represents a key contributor to the diversity of macrophage subpopulations observed during *in vivo* inflammatory injury.

LIVER

Anatomy and physiology

The liver is one of the largest organs in the body and performs vital physiological functions pertaining to metabolism, biosynthesis, and innate and adaptive immunity. The essential role of the liver is well illustrated by the fact that no procedure, such as dialysis, or machine, such as a pump, can replace the functions of a failing liver.

Anatomically, the liver is composed of 4 lobes: right, left, median and caudate, in rodents, and right, left, quadrate, and caudate in humans. The liver is strategically located between the intestinal tract and the circulatory system and contains approximately 25% of the total cardiac output (Malarkey *et al.*, 2005). Blood supply to the liver is provided by the portal vein, which drains blood from the mesenteric, gastric, splenic, and pancreatic veins, and the hepatic artery. These vessels contribute 70% and 30%, respectively, of the blood flow into the liver. Blood arriving in the liver via the portal vein and hepatic artery flows through smaller branches called preterminal and terminal portal venules, then continues into the sinusoids, and collects into central veins, which empty into larger hepatic veins followed by the vena cava (Malarkey *et al.*, 2005). Blood flow through the sinusoids has a reduced speed relative to other vascular beds, and is tightly regulated by endothelial cells lining the hepatic sinusoids; this serves to facilitate sampling of blood components by hepatic cells (Shetty *et al.*, 2008).

Structurally, the liver consists of chords of hepatocytes organized into functional units including the lobule and the acinus. Hepatic lobules are organized around a central vein and have a hexagonal shape, with portal triads at its corners. Portal triads are structures composed of branches of the portal vein, the hepatic artery, and bile ducts. The portal triads are surrounded in connective tissue, which also includes lymphatic vessels, and nerves. The acinus consists of hepatocytes between two adjacent central veins. Depending on the distance from the portal to the central vein, lobules and acini are divided into the periportal, midzonal, and centrilobular regions of the liver (Malarkey *et al.*, 2005).

Several different cell types are found in the liver. Hepatocytes represent the predominant cell type (about 60-80% of all cells in a normal liver). The remaining 20-40% consists of nonparenchymal cells, which include sinusoidal endothelial cells (about 50% of all nonparenchymal cells), resident macrophages called Kupffer cells (20%),

lymphocytes, mostly T cells and natural killer cells (25%), biliary epithelial cells (5%), and a small number of hepatic stellate cells (Racanelli and Rehermann, 2006).

Hepatocytes

Hepatocytes are the most abundant cells in the liver, representing 60% of all cells and 80% of the total volume (Malarkey *et al.*, 2005). They are large cells, approximately polyhedral in shape, with each cell surface contacting the perisinusoidal space through microvilli, neighbouring hepatocytes, or bordering bile canaliculi. Hepatocytes are separated from the endothelial cells lining the sinusoids by the space of Disse. They possess an elaborate biosynthetic machinery. Thus hepatocytes contain one, sometimes two large nuclei, numerous mitochondria, an extensive endoplasmic reticulum, peroxisomes and lysosomes, free ribosomes, Golgi complex, and abundant cytoplasm with varying amounts of lipid and glycogen. They also contain numerous enzymes involved in bioactivation; phase I enzymes present in hepatocytes include cytochrome P450 isoforms, flavin monooxygenases, and epoxide hydrolase; among phase II enzymes, glucuronosyl transferases, sulphotransferases, N-acetyltransferase, and glutathione-S-transferases are more prominent (Sevior *et al.*, 2012). The distribution of various enzymes and processes follows a zonal pattern. Thus, periportal hepatocytes contain higher levels of glutathione, glycogen synthesis, urea cycle activity, and glucose-6-phosphatase activity than centrilobular hepatocytes; in contrast, cytochrome P4502E1, glucokinase, and carboxylesterase are present in higher levels in hepatocytes around central veins, and glutamine synthetase is exclusively expressed in centrilobular hepatocytes (Malarkey *et al.*, 2005).

Hepatocytes are also characterized by long lifespan and very low rates of proliferation under physiological conditions, such that they are replaced approximately once a year; in contrast, skin cells are replaced every 12-30 days (Sell, 2003). However,

hepatocytes can divide in response to cell loss due to toxicant-induced injury or surgical resection. Importantly, hepatocytes require signals from nonparenchymal cells, such as Kupffer cells, to re-enter the cell cycle and proliferate. It should be noted that, unlike most other organs, tissue regeneration in the liver occurs primarily by division of mature hepatocytes. Intra-hepatic hepatocyte precursors, known as oval cells, become a source of new hepatocytes only in situations when hepatocyte proliferation is prevented (Fausto and Campbell, 2003). Recent studies using *in vivo* fate tracing of EYFP-labeled hepatocytes have confirmed that the majority of newly formed hepatocytes arise from other hepatocytes; moreover, only a small percentage of newly formed hepatocytes are derived from progenitor cells in particular situations, such as partial hepatectomy and chronic carbon tetrachloride injury (Malato *et al.*, 2011).

Hepatocytes perform a wide array of metabolic and biosynthetic functions. These include glucose synthesis and storage, cholesterol uptake, lipid and aminoacid metabolism, protein synthesis (albumin, fibrinogen, clotting factors, lipoproteins, and transferrin), bioactivation and detoxification of endogenous and exogenous compounds, formation and secretion of bile. Hepatocytes are also a source of acute phase proteins during inflammation (Parker and Picut, 2005).

Endothelial cells

Sinusoidal endothelial cells are the second most numerous cell type in the liver. They line the sinusoids and provide a barrier between hepatocytes and the blood. Hepatic sinusoidal endothelial cells are unique, both structurally and functionally. Unlike vascular endothelial cells, they have fenestrae, large pores of 100-200 nm diameter, which lack a basal lamina and are grouped in clusters called sieve plates (Shetty *et al.*, 2008). Moreover, sinusoidal endothelial cells have extensive cytoskeleton and actin fibers and participate in local control of blood flow. These features allow sinusoidal cells

to act as a filter between blood-derived fluids and colloids and hepatocyte microvilli extending into the space of Disse. They also have the ability to engulf materials and fluids by endocytosis and pinocytosis (Malarkey *et al.*, 2005; Racanelli and Rehermann, 2006). Another unique characteristic of sinusoidal endothelial cells is their significant involvement in immune responses. In response to inflammatory stimuli such as LPS, they undergo a process of activation and release cytokines, chemokines, and other mediators which alter the phenotype of other nonparenchymal cells. They also express costimulatory molecules such as CD80, CD86, MHC I and II, and can function as antigen-presenting cells (Gardner and Laskin, 2007).

Kupffer cells

Kupffer cells represent the largest resident macrophage population in the body and account for 25-30% of all nonparenchymal cells (Racanelli and Rehermann, 2006). They are mainly derived from blood monocytes, which migrate into the liver and differentiate into macrophages (Crofton *et al.*, 1978). Recent studies have shown that local proliferation is also important in maintenance of the Kupffer cell population (Klein *et al.*, 2007). Morphologically, Kupffer cells have an ameboid appearance, with an enlarged kidney-shaped nucleus, numerous mitochondria, lysosomes, and cytoplasmic vacuoles, and extensive endoplasmic reticulum (Laskin *et al.*, 2001; Naito *et al.*, 2004). Kupffer cells are highly phagocytic and play a key role in the removal of aged eryhtrocytes, particulates, bacteria and bacterial components such as endotoxin, and foreign materials from the portal circulation. They express a diverse array of cell surface receptors involved in phagocytosis, including Fc receptors, complement receptors, scavenger receptors, and TLRs (Gardner and Laskin, 2007). Additionally, they are potent secretory cells, releasing a large number of mediators which participate in homeostasis and inflammation, including cytokines and chemokines, growth factors, eicosanoids, reactive

oxygen and nitrogen intermediates, and proteolytic enzymes (Laskin, 2009). Similar to hepatocytes, a morphologic and functional gradient has been established for Kupffer cells. Thus, periportal Kupffer cells are more numerous, larger, produce more TNF-α and IL-1β, and exhibit higher phagocytic and lysosomal enzyme activity when compared to centrilobular Kupffer cells; these cells produce more reactive oxygen and nitrogen species, and prostaglandin E2, and are more responsive to proinflammatory stimuli such as LPS (Bouwens *et al.*, 1986; Sleyster and Knook, 1982; Bykov *et al.*, 2003; Mochida *et al.*, 1989).

Liver lymphocytes

The liver contains a significant number of lymphocytes (about 25% of liver nonparenchymal cells), including T, NK (natural killer), NKT, and B cells. Hepatic T cells represent almost two thirds of the lymphocyte population and include $CD3^+/CD8^+$ and $CD3^+/CD4^+$ subpopulations, albeit at a different CD4:CD8 ratio than in the circulation (1:3.5 in the liver versus 2:1 in the blood). The liver also contains a higher percentage of "double positive" and "double negative" T cells ($CD3^+/CD4^+/CD8^+$, and $CD3^+/CD4^-/CD8^-$, respectively), and a significant population of unconventional T lymphocytes which express TCRγδ, rather than TCRαβ (Parker and Picut, 2005). The slow rate of blood flow through the hepatic sinusoids facilitates extensive interaction of activated T cells in the circulation with antigen-presenting cells. In the liver, Kupffer cells, sinusoidal endothelial cells, and in some cases hepatocytes can present antigens to T lymphocytes (Thomson and Knolle, 2010). The liver is an important site for removal of T cells activated during inflammatory reactions at extrahepatic sites, and for induction of tolerance to ingested antigens (Racanelli and Rehermann, 2006; Parker and Picut, 2005). NK and NKT cells are present in the liver in higher numbers than in other organs – up to 30% of the entire hepatic lymphocyte population (Racanelli and Rehermann,

2006). They play an important role in killing of virus-infected and tumor cells due to their potent cytotoxic activity. The liver also contains a small population of B cells (approximately 6% of all lymphocytes), and their functions are less well studied to their scarcity. Recent reports have suggested that the liver contains a subpopulation of B cells with phagocytic activity, which may be involved in bacterial clearance (Nakashima *et al.*, 2012).

Stellate cells

Hepatic stellate cells are located in the space of Disse, between sinusoidal endothelial cells and hepatocytes (Atzori *et al.*, 2009). These cells are also referred to as hepatic pericytes, lipocytes, fat storing cells, and Ito cells. They have a star-like appearance, with extensive cytoplasmic processses extending between the sinusoids, and are more abundant in periportal than in the centrilobular regions. In the normal liver, stellate cells store vitamin A in cytoplasmic lipid droplets. However, following exposure to fibrogenic hepatotoxic chemicals such as carbon tetrachloride, stellate cells undergo activation, lose lipid droplets, and acquire a myofibroblast-like phenotype characterized by increased collagen production (Friedman, 2008). This process is regulated by macrophages, which accumulate during hepatic fibrogenesis in response to chemokines and M-CSF produced by activated stellate cells, and are often found in close contact with these cells (Wynn and Barron, 2010). Activated macrophages produce TGF-β, an important pro-fibrogenic cytokine which stimulates collagen deposition by activated myofibroblasts, and platelet-derived growth factor (PDGF), a growth factor that promotes proliferation of myofibroblasts (Wynn and Barron, 2010). Recent studies have identified a subpopulation of monocye-derived liver macrophages as the main source of fibrogenic factors during chronic carbon tetrachloride-induced injury. These monocyte-derived macrophages express high levels of Ly6C and accumulate in a CCR2 and CCR6-

dependent manner, mature into classically activated, iNOS-expressing macrophages, which contribute to the perpetuation of injury and the development of fibrosis (Karlmark *et al.*, 2009).

ACETAMINOPHEN

Acetaminophen (APAP) is a widely used analgesic and antipyretic. Its therapeutic indications include the relief of mild to moderate pain and fever reduction; APAP has minimal antiinflammatory activity, therefore is not effective in reducing inflammatory symptoms associated with arthritis, such as redness, stiffness or swelling (USP Drug Information, 2007). The maximal daily dose recommended is 4 g for adults (USP Drug Information, 2007). At higher doses, APAP is hepatotoxic and accounts for approximately 50% of all cases of acute liver failure in the United States due to accidental or intentional overdose (Lee, 2012). Clinical signs and symptoms of APAP intoxication include gastrointestinal upset (nausea, vomiting, stomach cramps, and pain), abdominal pain and tenderness, and alteration of liver function tests. An important feature of APAP intoxication is the delayed presentation: the first signs and symptoms can occur up to 24 h after ingestion, and maximal alterations in functional tests are usually detected after 3 to 5 days (USP Drug Information, 2007). These signs and symptoms can progress to overt hepatotoxicity and acute liver failure, which occurs 4 to 6 days after overdose, and is characterized by hepatic encephalopathy (with mental alterations ranging from agitation to confusion and stupor), along with one or more of the following: seizures, coma, cerebral edema, impaired coagulation, gastrointestinal hemorrhage, hypoglycemia, metabolic acidosis, and cardiovascular collapse (USP Drug Information, 2007).

APAP is rapidly absorbed from the gastrointestinal tract, within 30 min, and has a normal metabolic half-life of approximately 2 h (Rumack, 2004). When administered in therapeutic doses, a large percentage of the parent drug is metabolized in the liver by glucuronidation and sulfation into inactive metabolites that are excreted in the bile and urine. A small proportion of the ingested dose is metabolized by cytochrome P4502E1, and to a lesser extent by P4503A4, to a reactive intermediate, N-acetyl-para-benzoquinoneimine (NAPQI). Glutathione plays a critical role in eliminating NAPQI by forming an inactive adduct that is excreted in the bile (Jaeschke and Bajt, 2006). When APAP is ingested in excess, a larger proportion of the drug is available for P450-dependent conversion to the reactive metabolite, and, when cellular glutathione stores are depleted, NAPQI is available to bind covalently to cellular proteins (Mitchell *et al.*, 1973a; Jollow *et al.*, 1973; Potter *et al.*, 1973). The end result is hepatocellular death, which occurs primarily by necrosis (Gujral *et al.*, 2002).

While covalent binding is a critical step in the initiation of hepatotoxicity, oxidative stress is an important mechanism in propagating the injury. APAP intoxication is associated with increased mitochondrial levels of oxidized glutathione and increased formation of peroxynitrite (Knight *et al.*, 2001; Knight *et al.*, 2002). Moreover, administration of antioxidants such as glutathione, N-acetylcysteine, or superoxide dismutase mimics is protective against APAP hepatotoxicity both *in vivo* and *in vitro* (Mitchell *et al.*, 1973b; Bajt *et al.*, 2003; Ferret *et al.*, 2001). Oxidative stress has been suggested to promote the formation of the mitochondrial permeability transition (MPT), a process characterized by a sudden increase in inner membrane permeability to ions and small molecules. Interestingly, MPT leads to altered ATP production, and uncoupling of oxidative phosphorylation, thus facilitating the generation of reactive intermediates (Hinson *et al.*, 2010). Intracellular signaling pathways activated by reactive oxygen species are thought to contribute to APAP-induced hepatocellular injury. These include

activation of c-Jun N-terminal kinases, Ca^{2+}-dependent endonuclease, and translocation of endonuclease G and AIF (apoptosis-inducing factor) from mitochondria to the nucleus (Gunawan *et al.*, 2006; Hanawa *et al.*, 2008; Corcoran *et al.*, 1987; Ray *et al.*, 1990; Bajt *et al.*, 2006). An important characteristic of APAP hepatotoxicity is that the mechanisms of cell death appear to be comparable *in vivo* and in cultured hepatocytes (Bajt *et al.*, 2004). Moreover, *in vitro* sensitivity of primary hepatocytes from different species to APAP is comparable to *in vivo* susceptibility of the same species (Jemnitz *et al.*, 2008). These data suggest that isolated primary hepatocytes represent a relevant model for studying APAP-induced injury.

Macrophages and macrophage-derived mediators in APAP-induced hepatotoxicity

Accumulating data indicate that activated macrophages play an important role in the pathogenesis of APAP-induced toxicity. Early studies described an influx of macrophages into centrilobular areas of the liver in rodents treated with a toxic dose of APAP (Laskin and Pilaro, 1986; Laskin *et al.*, 1986). These cells exhibited morphological features of activated macrophages, including larger size, vacuolated cytoplasm, increased cytoplasmic: nuclear ratio, enhanced phagocytic and migratory abilities, and increased production of reactive oxygen species (Laskin and Pilaro, 1986). These studies also suggested that factors released by APAP-injured hepatocytes contribute to macrophage accumulation and activation (Laskin *et al.*, 1986). Consistent with a classically activated phenotype, macrophages in the livers of APAP-treated animals were found to release and/or express reactive oxygen and nitrogen species, and proinflammatory cytokines and chemokines, including TNF-α, IFN-γ, IL-1α, IL-1β, IL-6, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL7 (MCP-3), CXCL1 (KC), CXCL2 (MIP-2) (Dambach *et al.*, 2002; Blazka *et al.*, 1995; Hogaboam *et al.*, 1999; Gardner *et al.*, 2002; Bourdi *et al.*, 2007; Liu *et al.*, 2006; Laskin, 2009). Findings that depletion of classically

activated macrophages with gadolinium chloride blunts APAP-induced hepatotoxicity provide strong support for a role of these cells in the pathogenic process (Laskin *et al.*, 1995; Michael *et al.*, 1999).

The specific classically activated macrophage-derived mediators responsible for promoting APAP-induced hepatotoxicity have not been established. Classically activated macrophages are known to generate reactive oxygen and nitrogen species, and these have been linked to hepatotoxicity following APAP intoxication. Thus, APAP intoxication is associated with production of increased levels of reactive oxygen and nitrogen species and upregulation of iNOS. Moreover, pharmacological inhibition or genetic deletion of inducible nitric oxide synthase is protective during APAP intoxication; this is associated with increased levels of the alternative macrophage activation marker connective tissue growth factor (CTGF) (Gardner *et al.*, 1998; Gardner *et al.*, 2002). However, sinusoidal endothelial cells and hepatocytes also express iNOS and generate reactive nitrogen species, suggesting that they may also contribute to the pathogenesis of tissue injury. In contrast, it appears that reactive oxygen species play a minimal role in APAP-induced hepatotoxicity. Thus, loss of gp91phox, the main subunit of NADPH oxidase, a critical enzyme in macrophage production of superoxide anion, has been shown to have no significant effects on hepatotoxicity caused by APAP (James *et al.*, 2003).

Another product of classically activated macrophages implicated in APAP hepatotoxicity is TNF-α. APAP administration is associated with a rapid increase in TNF-α in the liver; moreover, neutralization of TNF-α using antibodies results in decreased toxicity, suggesting a detrimental role for TNF-α at early times following APAP administration (Blazka *et al.*, 1995). In contrast, TNF-α produced later in the pathogenic process appears to have a protective role. Thus, mice lacking TNFR1, the main receptor responsible for the inflammatory effects of TNF-α, exhibit exaggerated hepatotoxicity after APAP. This response was associated with delayed upregulation of antioxidant

proteins and impaired liver regeneration (Gardner *et al.*, 2003; Chiu *et al.*, 2003a; Chiu *et al.*, 2003b).

Evidence suggests that alternatively activated macrophages also play a role in the pathogenesis of APAP-induced liver injury. However, their activity is distinct from classically activated macrophages. These cells release antiinflammatory mediators and growth factors that contribute to tissue repair (Laskin *et al.*, 2011). Following APAP administration, expression levels of Ym1, Fizz-1 and arginase, markers of alternatively activated macrophages, are upregulated in liver macrophages isolated from APAP-treated mice (Holt *et al.*, 2008). This is associated with increases in expression of CCL2 and its receptor CCR2, which have been shown to play an important role in recruiting alternatively activated macrophages into the liver (Dambach *et al.*, 2002; Holt *et al.*, 2008; Gardner *et al.*, 2012). Expression of IL-4, IL-10, and IL-13, inducers of alternatively activated macrophages, is also upregulated during APAP intoxication (Gardner *et al.*, 2002; Reilly *et al.*, 2001; Dambach *et al.*, 2006; Yee *et al.*, 2007), as well as expression of growth factors known to be released by alternatively activated macrophages, including VEGF, TGF-β, and CTGF (Gardner *et al.*, 2002; Dambach *et al.*, 2006; Donahower *et al.*, 2006).

The importance of alternatively activated macrophages in liver repair after APAP-induced injury is supported by macrophage depletion studies using clodronate liposomes. These studies show increased hepatotoxicity in mice treated with liposomes containing clodronate, a response accompanied by a significant decrease in APAP-induced expression of IL-10, a product of alternatively activated macrophages (Ju *et al.*, 2002). Further evidence that alternatively activated macrophages are key to repair of APAP-injured liver comes from studies demonstrating that loss of these cells due to deficiencies in chemokines or chemokine receptors (e.g., CCL2, CCR2), inducing stimuli (IL-4, IL-10. IL-13), or products they release (VEGF) results in exaggerated

hepatotoxicity (Hogaboam *et al.*, 2000; Dambach *et al.*, 2002; Holt *et al.*, 2008; Si *et al.*, 2010; Bourdi *et al.*, 2002; Bourdi *et al.*, 2007; Yee *et al.*, 2007; Kato *et al.*, 2011). It is important to note that increased liver injury in the absence of these protective cytokines is associated with high levels of markers of classically activated macrophages, including iNOS, TNF-α, IL-1, IL-6, CCL3, and CXCL2 (Bourdi *et al.*, 2002; Bourdi *et al.*, 2007; Yee *et al.*, 2007). These results suggest that persistent classical macrophage activation in the absence of alternatively activated macrophages is a key factor in enhancing susceptibility to hepatotoxicity caused by APAP.

Taken together, these data suggest that a balance exists in the APAP-injured liver between proinflammatory, classically activated macrophages and antiinflammatory alternatively activated macrophages. Thus, early in the pathogenic process, classically activated macrophages contribute to tissue damage by releasing cytotoxic and proinflammatory mediators, whereas subsequent alternative macrophage activation is important for tissue repair. Moreover, the outcome of toxicity is dependent on which population is predominant: increased hepatotoxicity results from persistent classical activation, whereas protection from injury is associated with increased alternative macrophage activation.

Role of other innate immune cells in APAP-induced hepatotoxicity

Experimental evidence suggests that other cells of the innate immune system, including neutrophils, NK, NKT, and dendritic cells, play a role in the pathogenesis of APAP-induced hepatotoxicity. Thus, following APAP administration, an accumulation of neutrophils is evident in the liver (Lawson *et al.*, 2000; Cover *et al.*, 2006; Ishida *et al.*, 2006). This is correlated with upregulation of cytokines and chemokines important in neutrophil recruitment, including IL-1β and MIP-2 (Dambach *et al.*, 2006; Imaeda *et al.*, 2009; Liu *et al.*, 2004). However, the role of neutrophils in APAP-induced hepatotoxicity

is unclear. Some studies have suggested that neutrophils contribute to the pathogenesis of APAP-induced injury. This is based on findings that neutrophil depletion is protective against APAP toxicity, and that mice deficient in CXCR2, the main receptor for CXCL2, exhibit reduced hepatotoxicity in response to APAP (Liu *et al.*, 2006, Ishida *et al.*, 2006). In contrast, other groups have found no significant role of neutrophils in this model using different genetic and pharmacologic approaches. Thus, neutrophils accumulating into APAP-injured livers did not release reactive oxygen species, and neutrophil depletion did not alter the extent of necrosis (Lawson *et al.*, 2000; Cover *et al.*, 2006). Anti-CD18 antibodies or genetic deletion of CD18, an integrin critical for neutrophil extravasation, similarly had no significant effect on the progression of injury (Lawson *et al.*, 2000; Williams *et al.*, 2010; Williams *et al.*, 2011).

Imaeda *et al* (2009) have described a central role for the Nalp3 inflammasome in IL-1β production in the APAP-injured liver. In these studies, DNA released from necrotic hepatocytes was found to activate TLR9, resulting in Nalp3-dependent processing of pro-IL-1β and pro-IL-18 into active forms, which contribute to APAP-induced injury and inflammation. Subsequent studies using mice deficient in inflammasome components caspase-1, ASC and Nalp3 could not confirm these findings (Williams *et al.*, 2011). Furthermore, APAP-induced hepatotoxicity was not altered in mice deficient in IL-1-receptor-1 or by administration of recombinant IL-1β (Williams *et al.*, 2010). Taken together, these studies indicate a minimal role for neutrophils or IL-1β in the pathogenesis of tissue injury after toxic doses of APAP.

Natural killer and NKT cells have been identified in the liver after APAP and it has been suggested that they play a detrimental role in the pathogenesis of APAP hepatotoxicity (Liu *et al.*, 2004). Using antibody-mediated depletion of NK and NKT cells, it was reported that these cells release proinflammatory mediators including IFN-γ, IP-10, and KC, which contribute to injury. These studies have been challenged by reports

that the solvent used for APAP, DMSO, may have confounded the results by activating NK and NKT cells in the liver (Masson *et al.*, 2008).

Dendritic cells have recently been reported to play a protective role during APAP intoxication (Connolly *et al.*, 2011). APAP administration to mice resulted in dendritic cell maturation, with increased expression of costimulatory molecules MHC II and CD86. Selective depletion of these cells using transgenic mice that expressed the human DTR under the control of the CD11c promoter resulted in exacerbated toxicity after APAP and increased levels of proinflammatory cytokines TNF-α, IL-6, and the chemokine CCL2, whereas dendritic cell expansion with Flt3L was protective against APAP hepatotoxicity.

HMGB1

High-mobility group box 1 (HMGB1) is a non-histone DNA-binding protein consisting of 215 amino acids. It contains two rigid, L-shaped DNA-binding domains of approximately 80 amino acids each, referred to as box A and box B, and a negatively charged C-terminal region with 30 amino acids, connected by short linkers (Yamada and Maruyama, 2007). Both the sequence and structure of HMGB1 proteins are highly conserved among mammalian species, with 99% identity (Bianchi and Manfredi, 2007). HMGB1 is essential for survival, as evidenced by findings of reduced survival in mice lacking *Hmgb1*. These mice are characterized by reduced size, abnormal gait, long hindpaws, lack of fat, and they die soon after birth due to severe hypoglycemia (Calogero *et al.*, 1999). HMGB1 is expressed in the nuclei of most eukaryotic cells in relatively high levels (estimated at about 1 million copies/cell), and is especially abundant in neonatal livers, testes, thymus, and lymphoid tissues (Bianchi and Manfredi, 2007). Within the nucleus, HMGB1 plays a role in the regulation of gene transcription, DNA recombination, and repair, and maintenance of nucleosome structure. These

activities are facilitated by the ability of HMGB1 to bind to the minor groove of DNA, without sequence specificity, and induce bending of the DNA helix. After binding to DNA, HMGB1 can interact with various nuclear proteins, including transcription factors such as p53, NF-κB, homeobox-containing proteins, recombination activating gene 1/2 (RAG 1/2), and steroid hormone receptors (Ulloa and Messmer, 2006; Dintilhac and Bernues, 2002; Agresti *et al.*, 2003).

HMGB1 is actively secreted by innate immune cells, including macrophages, monocytes, dendritic cells, neutrophils, and NK cells (Yang and Tracey, 2010). Monocytes and macrophages secrete HMGB1 following stimulation with proinflammatory stimuli such as LPS, TNF-α, or IL-1 (Wang *et al.*, 1999; Ulloa and Messmer, 2006). Since HMGB1 lacks a secretory signal peptide, the mechanism of secretion does not involve the ER-Golgi system. In LPS-stimulated monocytes, HMGB1 becomes acetylated and translocates from the nucleus to the cytoplasm, where it localizes in secretory vesicles; secretion requires a second proinflammatory signal, such as lysophosphatidylcholine (Gardella *et al.*, 2002). Methylation of HMGB1 on Lys-42 and phosphorylation have also been demonstrated to result in cytoplasmic relocation (Ito *et al.*, 2007; Youn and Shin, 2006). HMGB1 is also passively released from necrotic cells, whereas in apoptotic cells it remains bound to chromatin (Scaffidi *et al.*, 2002).

Extracellular HMGB1 functions as a cytokine, inducing classical activation of macrophages and promoting inflammation (Yang *et al.*, 2005). The biologic actions of HMGB1 are mediated by multiple receptors, including receptor for advanced glycation end-products (RAGE), TLR4, and TLR2 (Kokkola *et al.*, 2005; Park *et al.*, 2004). Stimulation of leukocytes with HMGB1 leads to upregulation of TNF-α, IL-1β, IL-6, CCL3, CCL4, CXCL2, iNOS, and COX-2, and increased production of reactive oxygen and nitrogen species (Andersson *et al.*, 2000; Fan *et al.*, 2007; Faraco *et al.*, 2007; Kokkola *et al.*, 2005; Pedrazzi *et al.*, 2007). The signaling pathways involved in the

biologic actions of HMGB1 appear to depend on the cell type and injury model. Thus, in rat macrophages, HMGB1-induced expression of proinflammatory cytokines is partly dependent on RAGE (Kokkola *et al.*, 2005). In contrast, in mouse neutrophils and macrophages, TLR4, TLR2, and the downstream signaling proteins MyD88, IRAK-1, and IRAK-2 appear to play a predominant role in HMGB1-induced activation of NF-κB pathway, with RAGE playing only a minor role (Park *et al.*, 2004). The ability of HMGB1 to signal through TLR4 is critically dependent on the oxidation status of the cysteine 106 within the B-box domain (Yang and Tracey, 2010). In eosinophils, HMGB1 induced ROS production occurs partly via RAGE, while in neutrophils this effect is due to activation of TLR4 and downstream MAP kinases (Fan *et al.*, 2007). RAGE and downstream activation of ERK1/2 signaling mediate HMGB1-dependent induction of COX-2 and chemokines in astrocytes (Pedrazzi *et al.*, 2007).

The proinflammatory role of HMGB1 was initially described in mouse models of sepsis, where HMGB1 was hypothesized to play a pathogenic role and contribute to delayed mortality (Wang *et al.*, 1999). Thus, LPS administration to mice resulted in increased HMGB1 serum levels, and neutralizing anti-HMGB1 antibodies reduced mortality. Interestingly, antibody effectiveness was maintained even if treatment was delayed for several hours after LPS (Wang *et al.*, 1999). Activation of HMGB1 and downstream signaling pathways are contributing factors to the pathogenesis of rheumatoid arthritis, acute lung injury, colitis, ischemic brain injury, hepatic and cardiac ischemia-reperfusion injury (Taniguchi *et al.*, 2003; Abraham *et al.*, 2000; Maeda *et al.*, 2007; Tsung *et al.*, 2005; Muhammad *et al.*, 2008; Andrassy *et al.*, 2008). In these models, neutralization of HMGB1 results in protection from injury and reduced inflammatory mediator production. Moreover, administration of purified HMGB1 to mice causes inflammation. Intraperitoneal administration results in sepsis symptoms, including lethargy, diarrhea, and piloerection; high doses are lethal (Wang *et al.*, 1999).

Intra-tracheal instillation of HMGB1 causes acute lung injury, with increased production of TNF-α, IL-1β, and CXCL2, whereas intra-articular injection promotes inflammatory changes and the development of arthritis (Abraham *et al.*, 2000; Pullerits *et al.*, 2003). HMGB1 has been detected in the sera of mice following APAP intoxication (Antoine *et al.*, 2009); moreover, hepatocyte cell lines treated with APAP release HMGB1 (Martin-Murphy *et al.*, 2010). The biologic activity of HMGB1 appears to be significantly increased upon binding to cytokines or bacterial-derived components (Sha *et al.*, 2008; Hreggvidsdottir *et al.*, 2009).

GALECTIN-3

Glycosylation is an important post-translational modification of proteins which leads to the formation of a large number of glycan structures inside cells and on their surface. In vertebrates, the process of glycosylation involves the addition of a monosaccharide (glucose, mannose, fucose, galactose, N-acetylgalactosamine, N-acetylglucosamine, sialic acid, xylose) to various protein residues through an anomeric linkage. Covalent binding to asparagine residues leads to the formation of N-glycans, whereas binding to hydroxyl groups of serine or threonine results in O-glycans. In addition to decorating the surface of host cells, glycoconjugates are expressed on the surface of many prokaryotic and eukaryotic pathogens. The immune system recognizes these endogenous and exogenous carbohydrate structures via a large class of glycan-binding proteins, or lectins, the most important of which are C-type lectins, siglecs, and galectins (van Kooyk and Rabinovich, 2008). Galectins comprise a family of soluble lectins with affinity for β-galactoside-containing glycans. The minimal structure recognized by galectins is the disaccharide N-acetylgalactosamine. In mammals, 15 galectins have been identified; they all share a common structural fold and a conserved

carbohydrate-recognition domain (CRD) of approximately 130 amino acids. Structurally, galectins are classified as “prototype” (galectins 1, 2, 5, 7, 10, 11, 13, 14, and 15), with one CRD that can dimerize, and as “tandem-repeat” (galectins 4, 6, 8, 9, and 12), with two identical CRDs in a single polypeptide chain connected by a linker of approximately 70 amino acids. Galectin-3 (Gal-3) has a unique structure with one CRD and a non-lectin N-terminal domain of about 120 amino acids, with a high frequency of proline and glycine, which mediates oligomerization and cross-linking of ligands. In all galectins, the CRD exhibits a tight fold, with two anti-parallel β-sheets forming a sandwich-like structure that binds sugar residues (Liu *et al.*, 2002; van Kooyk and Rabinovich, 2008).

Gal-3 is localized both intra- and extracellularly and performs distinct functions depending on the cell type, the activation state of the target cell, and the intra- or extracellular location. Inside the cell, Gal-3 is present both in the cytoplasm and nucleus; in fibroblasts, Gal-3 is found in the cytoplasm of quiescent cells, but translocates to the nuclear compartment in proliferating fibroblasts. Gal-3 can undergo phosphorylation at Ser-6 and Ser-12, a process important for intracellular localization: non-phosphorylated Gal-3 is cytoplasmic, whereas phosphorylated Gal-3 is both cytoplasmic and nuclear (Dumic *et al.*, 2006). Several intracellular proteins have been found to interact with Gal-3, most notably apoptosis-related Bcl-2, AIP-1, and Nucling; interestingly, these binding partners interact with Gal-3 through protein-protein interactions (Liu *et al.*, 2002). The intracellular functions described for Gal-3 include involvement in pre-mRNA splicing, regulation of growth, and inhibition of apoptosis (Dumic *et al.*, 2006). As such, Gal-3 expression is increased in numerous cancers, its localization shifts from nuclear to cytoplasm with progression from adenoma to carcinoma, and correlates with metastatic potential (Newlaczyl and Yu, 2011). Gal-3 can be secreted outside the cell through a nonclassical exocytosis mechanism and forms dimers in the absence of ligands; ligand binding triggers oligomerization of Gal-3, which enables cross-linking into a lattice-like

structure. This is thought to result in activation of cell signaling pathways (Henderson and Sethi, 2009). Extracellularly, Gal-3 functions in cell activation, cell adhesion, and chemoattraction (Dumic *et al.*, 2006).

The mechanisms that regulate Gal-3 expression are incompletely characterized. Gal-3 is encoded by a single gene, *lgals3*, which has six exons and five introns in mice and humans. The promoter region contains multiple regulatory elements, including potential Sp1 and NF-κB-like binding sites, cAMP-dependent response element motifs, AP-1 sites, a sis-inducible element (SIE), and a consensus basic helix-loop-helix (bHLH) sequence (Kadrofske *et al.*, 1998). Both mRNA and protein expression of Gal-3 can be induced in quiescent fibroblasts by serum, a feature of immediate early genes (Moutsatsos *et al.*, 1987; Agrwal *et al.*, 1989).

Gal-3 is expressed by numerous cell types in a developmental stage and activation-dependent manner. During mouse embryonic development, Gal-3 is first detected at E4, and by 8.5-11.5 days, it is exclusively expressed in notochord cells. At later stages of fetal development, Gal-3 is expressed in osteoclasts, liver and lung macrophages. In humans, Gal-3 is primarily found in epithelial cells during embryogenesis. In adults, Gal-3 is broadly expressed in epithelial cells in the intestine, cornea, kidney, lung, thymus, breast, and prostate; fibroblasts, chondrocytes, keratinocytes, and Schwann cells also express Gal-3 (Dumic *et al.*, 2006). Some cells of the immune system express Gal-3, including eosinophils, mast cells, human, but not mouse neutrophils, dendritic cells, monocytes, and macrophages. While Gal-3 is not detectable in resting lymphocytes, it is upregulated following activation (Dumic *et al.*, 2006).

Gal-3 is expressed at low levels in monocytes, but its cell surface expression is upregulated during activation or differentiation into macrophages. Macrophage activation by proinflammatory stimuli, including TNF-α, IFN-γ, LPS, and oxidized low density

lipoproteins, further upregulates Gal-3 and promotes its release into the extracellular environment (Liu *et al.*, 1995; Le Marer, 2000; Nishi *et al.*, 2007; Li *et al.*, 2008; Shanmugam *et al.*, 2003). In obese mice, proinflammatory adipose tissue macrophages express high levels of Gal-3 (Li *et al.*, 2010). Gal-3 binding to macrophages and other cells of the innate immune system results in activation to a proinflammatory profile characterized by increased production of cytokines, including TNF-α, IL-1β, IL-8, IL-12, of CC chemokines CCL2, CCL4, CCL5, CCL8, and CCL20, and reactive nitrogen species via iNOS (Papaspyridonos *et al.*, 2008; Nishi *et al.*, 2007; Jeon *et al.*, 2010). Gal-3 has also been suggested to exhibit chemotactic activity for monocytes and macrophages, and to stimulate macrophage phagocytosis and superoxide production by monocytes (Sano *et al.*, 2000; Papaspyridonos *et al.*, 2008; Liu *et al.*, 1995; Jeon *et al.*, 2010). Only a few studies have characterized the signaling pathways mediating the effects of Gal-3 on monocytes and macrophages. For example, the proinflammatory effects of Gal-3 in rat microglia have been shown to be mediated by the IFNγR1 and downstream activation of STAT1, STAT3, and STAT5. In these cells, Gal-3 also increases NF-κB nuclear binding (Jeon *et al.*, 2010). A role for NF-κB in Gal-3-dependent responses is further supported by findings that reduced peritoneal inflammation in Gal-$3^{-/-}$ mice after administration of thioglycollate is associated with decreased NF-κB activation (Hsu *et al.*, 2000). The transmembrane protein CD98 has been suggested to act as the macrophage receptor for Gal-3 (Dong and Hughes, 1997). CD98 is a glycoprotein initially identified as a component of a cell surface aminoacid transporter. CD98 has been reported to be induced *in vitro* upon macrophage stimulation with LPS, and *in vivo* during colitis (Sato *et al.*, 1999; Yan *et al.*, 2007). Moreover, CD98 appears to play an important role in intestinal inflammation and inflammation-related carcinogenesis by promoting release of TNF-α, IL-1β, and proinflammatory chemokines (Nguyen *et al.*, 2011a).

Gal-3 plays a proinflammatory role during acute inflammatory injury *in vivo*. Intra-articular administration of Gal-3 promotes inflammation and contributes to cartilage destruction by inducing extracellular matrix-degrading enzymes (Janelle-Montcalm *et al.*, 2007). Mice with a targeted mutation for Gal-3 exhibit an impaired ability to mount an acute inflammatory response to thioglycollate, as a result of reduced accumulation of macrophages and increased apoptosis in these cells (Hsu *et al.*, 2000). Moreover, Gal-3$^{-/-}$ mice are less susceptible to renal ischemia-reperfusion and brain hypoxic-ischemic injury, antigen-induced arthritis, and concanavalin A-induced hepatitis (Fernandes Bertocchi *et al.*, 2008; Doverhag *et al.*, 2010; Forsman *et al.*, 2011; Volarevic *et al.*, 2012). Decreased susceptibility in these mice is associated with reduced expression of proinflammatory mediators TNF-α, IL-1β, IFN-γ, IL-6, IL-12, CCL2, MMP-9, decreased production of reactive oxygen and nitrogen species, and increased numbers of alternatively activated macrophages. A role for Gal-3 has also been demonstrated in chronic inflammation. Loss of Gal-3 results in reduced injury and inflammation after ovalbumin challenge, following streptozotocin-induced diabetes, and diet-induced steatohepatitis (Zuberi *et al.*, 2004; Mensah-Brown *et al.*, 2009; Iacobini *et al.*, 2011). Gal-3$^{-/-}$ mice are also less susceptible to experimental autoimmune encephalitis, and develop reduced liver and kidney fibrosis following carbon tetrachloride administration, and unilateral ureteric obstruction, respectively (Henderson *et al.*, 2006; Henderson *et al.*, 2008). The involvement of Gal-3 in infection appears to be dependent on the pathogen and mainly related to the modulation of Th1-Th2 balance. Thus, Gal-3$^{-/-}$ mice exhibit reduced macrophage infiltration and hepatic granuloma formation during *Schistosoma mansoni* infection (Oliveira *et al.*, 2007). These mice also develop a diminished hepatic inflammatory response to *Toxoplasma gondii* oral infection with increased Th1 polarization, but they are more susceptible to peritoneal infection with this pathogen (Bernardes *et al.*, 2006). In contrast, lack of Gal-3 is associated with increased

sensitivity to the fungal organism *Paracoccidioides brasiliensis* due to Th2 skewing (Ruas *et al.*, 2009). Additionally, mortality after high doses of LPS is increased in Gal-3$^{-/-}$ mice; they are however resistant to Salmonella infection (Li *et al.*, 2008).

SPECIFIC AIMS

Acetaminophen (APAP) is a widely used over-the counter analgesic. While safe and effective at therapeutic doses, when ingested in excess, APAP is hepatotoxic and represents the main cause of drug-induced acute liver failure in the United States. Activated macrophages and inflammatory mediators they release have been shown to play a key role in the pathogenesis of APAP-induced hepatotoxicity. However, the mechanisms that regulate macrophage activation and inflammatory mediator production are poorly understood. High mobility group box-1 (HMGB1) is a DNA-binding protein passively released from necrotic cells that activates macrophages to a proinflammatory phenotype. Galectin-3 (Gal-3) is a β-galactoside-binding lectin expressed by activated macrophages that promotes inflammatory mediator production by these cells. We speculate that HMGB1 and Gal-3 are important in regulating macrophage activation following APAP intoxication. To test this hypothesis, studies are designed to:

1. Analyze the effects of APAP on release of HMGB1 by hepatocytes and its role in macrophage activation.
2. Analyze the effects of APAP on hepatic expression of Gal-3; assess the role of Gal-3 in inflammatory mediator production during APAP-induced hepatotoxicity.
3. Analyze the role of Gal-3 in classical macrophage activation following APAP intoxication.

MATERIALS AND METHODS

Animals

Male specific pathogen-free C57Bl/6J wild type mice, Gal3$^{-/-}$ mice, and TNFR1$^{-/-}$ mice (8-12 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). 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. Mice were fasted overnight prior to i.p administration of APAP (300 mg/kg) or pyrogen-free phosphate-buffered saline (PBS) control. After 3-72 h, mice were euthanized with nembutal (200 mg/kg) and blood collected from the abdominal vena cava for determination of aspartate and alanine transaminases using diagnostic assay kits (Thermo Fisher Scientific, Waltham, MA). Liver samples (100 mg aliquots) were collected and stored at -20°C in RNA*later* until RNA isolation. The remaining tissue was snap frozen in liquid nitrogen.

Hepatocyte isolation and preparation of conditioned medium (CM)

Mice were fasted overnight prior to hepatocyte isolation. Animals were euthanized with Nembutal (200 mg/kg). Hepatocyte isolation was performed as previously described with some modifications (Gardner *et al.*, 1998). Briefly, the liver was perfused through the portal vein with warm Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (pH 7.3) containing 25 mM HEPES and 0.5 mM EGTA, followed by Leibowitz L-15 medium containing HEPES and 0.2 U/ml Liberase 3 Blendzyme. The liver was excised, disaggregated, and the resulting cell suspension filtered through a 220 μm nylon mesh. Hepatocytes were recovered by centrifugation at 50 g, and viability (>90%) assessed by trypan blue dye exclusion. Hepatocytes were plated on type I collagen-coated plates at a

density of 7.5 x 10^6 cells/dish in William's Medium E supplemented with 10% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, 1% insulin-transferrin-selenium, and 2 mM L-glutamine. Non-adherent cells were removed by washing the plates 2-3 h later. After overnight incubation at 37°C, the cells were washed and refed with medium control, or medium containing 5 mM APAP. In some experiments, hepatocytes were treated with ethyl pyruvate (30 mM) or with medium control for 1 h, and then with medium containing APAP and ethyl pyruvate, or ethyl pyruvate alone. After 24 h, hepatocyte CM was collected in pyrogen-free tubes, centrifuged at 300 g (4°C, 5 min) to remove cell debris, diluted in medium to 30% and assayed immediately.

Hepatic nonparenchymal cell isolation

Nonparenchymal cells were isolated from the liver as previously described, with some modifications (Chen *et al.*, 2007b). The liver was perfused through the portal vein with warm Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (pH 7.3) containing 25 mM HEPES and 0.5 mM EGTA, followed by Leibowitz L-15 medium containing HEPES, 0.2 U/ml Liberase 3 Blendzyme, and 0.5 mg/ml protease type XIV. The liver was excised, disaggregated, and incubated with 2 mg/ml protease type XIV for 15 min at 37°C. The resulting cell suspension was filtered through a 220 μm nylon mesh. Hepatocytes were separated from nonparenchymal cells by four succesive washes (50xg, 3 min). Supernatants containing nonparenchymal cells were centrifuged (300xg, 7 min), and then purified by density gradient centrifugation using Optiprep medium (Sigma-Aldrich, St Louis, MO). Viability was assessed by trypan blue staining and was >95%. Cells were analyzed immediately after isolation by flow cytometry/cell sorting.

Macrophage cell line

RAW 264.7 murine macrophages (ATCC, Manassas, VA) were cultured in

DMEM-GlutaMax with 10% FBS, 0.1% penicillin/streptomycin, and 1 mM sodium pyruvate. Twenty four h prior to initiating experiments, confluent cells were washed in Hank's balanced salt solution, trypsinized, resuspended in DMEM supplemented with 1% FBS, and plated onto 6-well (1 x 10^6 cells/well), or 12-well (0.3 x 10^6 cells/well) dishes. Pyrogen-free reagents, tubes, dishes, pipets, and pipet tips were used in all experiments.

MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay

Hepatocytes were plated onto collagen-coated 96 well dishes at a density of 1.5 x 10^5 cells/well. Non-adherent cells were removed by washing 2-3 h later. After overnight incubation at 37°C, cells were washed and refed with medium control, or medium containing 1-10 mM APAP. At the indicated times, medium was removed and fresh medium containing 0.5 mg/ml MTT added. After an additional 2 h incubation, dimethyl sulfoxide was added, and absorbance measured at 550 nm using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). A wavelength of 600 nm was used as reference.

Measurement of reactive oxygen species production

5-(and-6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H_2DCFDA) was used to assess ROS production by macrophages (Gomes *et al.*, 2005). Macrophages were incubated in polypropylene tubes with 1 μM CM-H_2DCFDA for 15 min at 37°C in a shaking water bath and then plated onto 96-well dishes (5 x 10^4 cells/well). Cells were treated with control or APAP (1.5 mM), or with CM from control or APAP-treated hepatocytes, and then analyzed for fluorescence using a SpectraMax M5 fluorescent spectrophotometer (Molecular Devices, Sunnyvale, CA) every 2 min for 30 min. The excitation and emission wavelengths were 495 nm and 520 nm, respectively.

Western blotting

Macrophages or liver samples (50 mg) were lysed in buffer containing 20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM diethylene triamine pentaaacetic acid (DTPA), 1 mM phenylmethylsulfonylfluoride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitor cocktail. Protein concentrations were measured using the Bradford Assay (Bio-Rad, Hercules, CA). Proteins were separated on 10.5-14% Tris-glycine polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes. Non-specific binding was blocked by incubation of the blots for 1 h at room temperature with buffer containing 5% non-fat milk or bovine serum albumin (BSA) for phosphorylated proteins, 10 mM Tris-base, 200 mM sodium chloride, and 0.1% polysorbate 20. Membranes were then incubated overnight at 4°C with anti-HMGB1 (1:2000, Abcam, Cambridge, MA), anti-heme oxygenase-1 (1:1000, Stressgen, Ann Arbor, MI), anti-cyclooxygenase-2 (1:2000, Abcam, Cambridge, MA), anti-phospho-p44/42 (1:2000, Cell Signaling, Beverly, MA), anti-p44/42 (1:2000, Cell Signaling, Beverly, MA), anti-actin (1:1000), anti-inducible nitric oxide synthase (iNOS, 1:4000, BD Biosciences, San Jose, CA), or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies, followed by incubation with isotype-specific HRP-conjugated secondary antibodies (1:10,000) for 1 h at room temperature. Binding was visualized using an ECL Plus chemiluminescence kit (GE Healthcare, Piscataway, NJ).

Real-time PCR

Total RNA was isolated from macrophages using an RNeasy kit (Qiagen, Valencia, CA). RNA purity and concentration were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA was converted into

cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's directions. Standard curves were generated using serial dilutions from pooled randomly selected cDNA samples. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT thermocycler (Applied Biosystems, Foster City, CA). All PCR primer pairs were generated using Primer Express 2.0 (Applied Biosystems), and synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are presented in Table 1. For each sample, gene expression changes were normalized relative to 18s RNA. Data are expressed as fold change relative to control.

Preparation of liver microsomes and measurement of cytochrome P450 2e1 (Cyp2e1) activity

Frozen liver samples (1-2 g) were homogenized at 4°C in 4 volumes of buffer (50 mM Tris-hydrochloride, 1.15% potassium chloride, and 0.5 mM phenylmethylsulfonylfluoride, pH 7.4), and then centrifuged at 12,000 g for 20 min. Supernatants were collected and centrifuged at 105,000 g for 90 min. Microsomes were then washed in buffer containing 1.15% potassium chloride and 10 mM EDTA (pH 7.4), resuspended in 10 mM potassium phosphate buffer containing 0.25 M sucrose, and stored at -80°C until analysis. Cyp2e1 activity was measured by the generation of para-nitrocatechol from para-nitrophenol (Chang, 1998). Microsomes were incubated with 100 μM para-nitrophenol and 500 μM NADPH at 37°C for 20 min. The reaction was stopped by the addition of trichloroacetic acid. The mixture was then centrifuged (13,000 g, 5 min, 4°C), supernatants collected and mixed with 2 M NaOH. Changes in absorbance were measured spectrophotometrically at 535 nm. Concentrations of para-nitrocatechol in the samples were determined based on a standard curve generated with authentic para-nitrocatechol.

Measurement of hepatic glutathione

Frozen livers (50 mg) were homogenized in ice cold 5% metaphosphoric acid (1:10) and centrifuged at 3000 g for 10 min. Supernatants were collected and reduced glutathione determined using a colorimetric assay kit (OxisResearch, Portland, OR). Glutathione concentrations in the samples were calculated based on a standard curve and expressed as μmol/g wet liver.

Histology and immunohistochemistry

Livers were collected, and 5 mm samples of the left lobes immediately fixed overnight at 4°C in 3% paraformaldehyde/2% sucrose. Tissue was washed three times in PBS/ 2% sucrose, and then transferred to 50% ethanol. After embedding in paraffin, 5 μm sections were prepared and stained with hematoxylin and eosin (Goode Histolabs, New Brunswick, NJ). Histopathological evaluation was performed by a board certified veterinary pathologist (L.B. Hall). Findings were graded on a scale of 1 to 4, where 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe changes. For immunohistochemistry, sections were rehydrated and stained with antibody to Gal-3 (1:25,000, R&D Systems, Minneapolis, MN), PCNA (1:800, Abcam, Cambridge, MA), Ym1 (1:450, StemCell, Vancouver, BC) or IgG control (ProSci, Poway, CA). Binding was visualized using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Three to five random sections of each liver were examined.

Immunofluorescence

Livers were collected and and samples from the left lobes were immediately snap frozen in liquid nitrogen-cooled isopentane and embedded in OCT medium (Sakura Finetek, Torrance, CA). Six μm sections were prepared and fixed in 90% acetone/10% methanol. Sections were stained with anti-myeloperoxidase antibody (1:100) (Dako,

Carpinteria, CA), followed by isotype-specific, AlexaFluor488-conjugated secondary antibody (Molecular Probes, Carlsbad, CA). For double immunofluorescence, a sequential staining procedure was used (Lloyd *et al.*, 2008). Sections were stained with anti-Ly6C antibody (1:50, AbD Serotec, Kidlington, UK), followed by isotype-specific, AlexaFluor488-conjugated secondary antibody (Molecular Probes, Carlsbad, CA). After blocking with 5% rat serum, sections were stained with FITC-conjugated anti-F4/80 antibody (1:50, AbD Serotec, Kidlington, UK), followed by anti-FITC AlexaFluor488-conjugated secondary antibody. Images were acquired using a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Identical laser power, gain and offset settings were used for all analyses.

Flow cytometry and cell sorting

Non-specific binding was blocked by incubation of the cells with anti-mouse-FcRII/III antibody (BD Biosciences, Franklin Lakes, NJ) for 5 min at 4°C. The cells were then incubated with FITC-conjugated anti-CD11b and PE-conjugated anti-Ly6C antibodies or isotype controls (BioLegend, San Diego, CA). After 30 min, the cells were fixed in 3% paraformaldehyde, permeabilized in buffer containing 0.1% saponin, 0.1% sodium azide, and 1% fetal bovine serum in PBS, and stained with anti-Gal-3 antibody or goat IgG (R&D Systems, Minneapolis, MN), followed by isotype-specific AlexaFluor633-conjugated secondary antibody (Molecular Probes, Carlsbad, CA). Cells were analyzed on a FC500 flow cytometer (Beckman Coulter, Brea, CA). For sorting, cells were incubated with anti-mouse-FcRII/III antibody, followed by FITC-conjugated anti-CD45, AlexaFluor647-conjugated CD11b, and PE-conjugated anti-Ly6C antibodies (BioLegend, San Diego, CA) for 30 min. DAPI was added to the cell suspension immediately before analysis to exclude dead cells. Cells were sorted into $DAPI^-/CD45^+/CD11b^+/Ly6C^{hi}$ and $DAPI^-/CD45^+/CD11b^+/Ly6C^{lo}$ using a MoFlo XDP Cell Sorter

(Beckman Coulter, Brea, CA), and immediately processed for RNA isolation.

Statistical analysis

All experiments were repeated two to three times. Data were analyzed using the Student's *t* test or one-way ANOVA followed by Dunn's post hoc analysis. A *p* value of $\leq$ 0.05 was considered statistically significant.

TABLE 1. Primer sequences used for RT-PCR.

Gene Target	Primer Forward	Primer Reverse
12/15-LOX	ACCAGCAAGGACGACGTGAC	ATCAGGTAGCGACCCCATCA
18sRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
24p3	AGGAACGTTTCACCCGCTTT	TGTTGTCGTCCTTGAGGCC
CAT	CCAGGGCATCAAAAACTTG	GCCCTGAAGCTTTTTGTCAG
CCL2	TTGAATGTGAAGTTGACCCGTAA	GCTTGAGGTTGTGGAAAAG
CCL3	TCTTCTCAGCGCCATATGGA	TCCGGCTGTAGGAGAAGCA
CCL3	TCTTCTCAGCGCCATATGGA	TCCGGCTGTAGGAGAAGCA
CCL4	AGGGTTCTCAGCACCAATGG	CCGGGAGGTGTAAGAGAAACAG
CCL4	CAGCACCAATGGGCTCTGA	GCCGGGAGGTGTAAGAGAAAC
CCL20	TGGCCGATGAAGCTTGTGA	AGCGCACACAGATTTTCTTTTCT
CCR1	CTGAGGGCCCGAACTGTTAC	GGCTAGGGCCCAGGTGAT
CCR2	TCCACGGCATACTATCAACATCTC	GGCCCCTTCATCAAGCTCTT
CCR5	TGATAAGCTGCAAAAAGCTGAAGA	GTCAGAGATGGCCAGGTTGAG
CD98	GAAGCTCTGAGTTCTTGGTTGCA	CTTTCCCACATCCCGGAAT
COX-2	GCTTCGGGAGCACAACAGA	GCTCATCACCCCACTCAGGAT
CX3CL1	GCACAGGATGCAGGGCTTAC	TGTCAGCCGCCTCAAAACT
CX3CR1	TCGGTCTGGTGGGAAATCTG	GGCTTCCGGCTGTTGGT
CXCL2	ACTGACCTGGAAAGGAGGAGC	TGGTTCTTCCGTTGAGGGAC
CXCL2	AGGCTTCCCGATGAAGAG	CAGGATAAGAGCGAGAGCCTACA

Fizz-1	CAGCTGATGGTCCCAGTGAA	TTCCTTGACCTTATTCTCCACGAT
Gal-1	CGGACGCCAAGAGCTTTGT	TGAAGTGTAGGCACAGGTTGTTG
Gal-3	CACAATCATGGGCACAGTGAA	TTCCCTCTCCTGAAATCTAGAACAA
HO-1	CCTCACTGGCAGGAATCATC	CCTCGTGGAGACGCTTTACATA
IL-10	AGGCAGCCTTGAGAAAAGA	AGTAAGAGCAGGCAGCATAGCA
IL-12	CCTGGAGCACTC CCCATTC	TGCGCTGGATTCGAACAA
IL-1β	CCAAAAGATGAAGGGCTGCT	TCATCTGGACAGCCCAGGTC
IL-1β	AGTTGACGGACCCCAAAAGAT	GGACAGCCCAGGTCAAAGG
iNOS	GGCAGCCTGTGAGACCTTTG	TGAAGCGTTTCGGGATCTG
MMP-9	CAAGTGGGACCATCATAACATCA	CTCGCGGCAAGTCTTCAGA
RAGE	TCCCGATGGCAAAGAAACA	GAGTCCCGTCTCAGGGTGTCT
TLR4	AGCCATTGCTGCCAACATC	ACCTTCCGGCTCTTGTGGA
TNFR1	CAGACTTGCATGGTGAGCTCTT	AGCCCAGTTACCCAACAGACA
TNF-α	AGGGATGAGAAGTTCCCAAATG	TGTGAGGGTCTGGGCCATA
TNF-α	AAATTCGAGTGACAAGCCGTA	CCCTTGAAGAGAACCTGGGAGTAG

PART I. MACROPHAGE ACTIVATION BY FACTORS RELEASED FROM APAP-INJURED HEPATOCYTES. POTENTIAL ROLE OF HMGB1

Accumulating evidence suggests that activated macrophages and inflammatory mediators contribute to APAP-induced hepatotoxicity; however, their role in the pathogenic process depends on the timing of their appearance in the liver and mediators they encounter in the hepatic microenvironment, which direct their phenotype and function (Laskin, 2009). Thus, whereas early in the pathogenic process, macrophages are classically activated to release cytotoxic and proinflammatory mediators which contribute to APAP-induced liver injury, subsequent alternative activation of these cells leads to the release of cytokines and growth factors important in downregulating the inflammatory response and initiating wound repair. Previous studies have shown that hepatocytes injured by APAP release factors that stimulate chemotaxis and production of ROS by Kupffer cells (Laskin and Pilaro, 1986; Horbach *et al.*, 1997). These findings suggest that hepatocytes may be a source of mediators that induce early macrophage activation in the liver. However, the identity of these mediators and their effects on other markers of macrophage activation are unknown.

HMGB1 is a DNA-binding protein passively released from necrotic cells (Scaffidi *et al.*, 2002). Extracellular HMGB1 functions as a cytokine, inducing classical activation of macrophages and promoting inflammation (Yang *et al.*, 2005). The biologic actions of HMGB1 are mediated by multiple receptors, including RAGE and Toll-like receptor 4 TLR4 (Kokkola *et al.*, 2005; Park *et al.*, 2004). Subsequent activation of downstream signaling pathways including p44/42 mitogen-activated protein (MAP) kinase leads to the production of proinflammatory and cytotoxic mediators, such as chemokines, ROS, and eicosanoids (Lotfi *et al.*, 2009; Faraco *et al.*, 2007; Pedrazzi *et al.*, 2007). Activation of HMGB1 and downstream signaling pathways are contributing factors in the

pathogenesis of sepsis, rheumatoid arthritis, acute lung injury, and hepatic ischemia-reperfusion injury, each of which involves inflammatory macrophages (reviewed in Andersson and Tracey, 2011). We speculate that early macrophage activation in the liver is mediated, in part, by factors such as HMGB1, released from injured hepatocytes and this was investigated in the present studies.

RESULTS

Effects of hepatocyte-derived mediators on macrophages

Initially, we determined if APAP-induced cytotoxicity in primary cultures of mouse hepatocytes was associated with the release of mediators that activate macrophages for cytotoxic/ proinflammatory activity. For these studies, we used conditioned medium (CM) collected from hepatocytes 24 h after treatment with 5 mM APAP, a dose which caused approximately 50% reduction in viability (Fig. 1). CM from APAP-treated hepatocytes (CM-APAP) caused a time-dependent increase in ROS production by macrophages (Fig. 2, upper panel). This activity was not observed in cells incubated with CM from hepatocytes treated with control (CM-CTL). A similar lack of biological activity was noted after treatment of macrophages with APAP by itself (not shown). Treatment of macrophages with CM-APAP also resulted in a 2.5 fold increase in mRNA expression of the antioxidant catalase, which was evident within 6 h, and persisted for at least 24 h (Fig. 3, upper panel). HO-1 mRNA expression was also upregulated in macrophages following treatment with CM-APAP; however, this response was transient, reaching a maximum after 6 h. Upregulation of HO-1 mRNA in response to CM-AA was correlated with increases in HO-1 protein at both 6 h and 24 h post-treatment (Fig. 3, lower panel).

We next analyzed the effects of hepatocyte CM on enzymes mediating eicosanoid biosynthesis. Incubation of macrophages with CM from APAP-treated, but not from control hepatocytes or APAP alone, upregulated COX-2 protein and mRNA expression (Figs. 3 and 4). CM-APAP also increased 12/15-LOX mRNA expression. In contrast, CM-APAP caused a small, but reproducible decrease in expression of 5-LOX (Fig. 4).

We also assessed macrophage expression of proinflammatory mediators including CC and CXC chemokines in response to CM from APAP-treated hepatocytes.

Expression of MIP-1α (CCL3) and MIP-2 (CXCL2) mRNA was upregulated in macrophages by CM-APAP (Fig. 5). Interestingly, APAP by itself was also found to upregulate MIP-2 expression, although not as effectively as CM-APAP. In contrast, basal levels of MCP-1 (CCL2) mRNA expression were downregulated by CM-APAP, while no changes were noted in MIP-1β (CCL4) expression. CM-APAP also had no significant effects on macrophage expression of the proinflammatory cytokines TNF-α and IL-1 (Table 2).

Role of p44/42 MAP kinase in CM-APAP-induced macrophage activation

In our next series of studies, we analyzed the role of p44/42 MAP kinase in regulating macrophage responses to factors released from APAP-injured hepatocytes. Treatment of macrophages with CM-APAP resulted in a rapid increase in expression of activated p44/42 MAP kinase, which was evident within 30 min. In contrast, no significant changes were noted in expression of total p44/42 protein (Fig. 6, middle panel), or in expression of total or phospho-p38 MAP kinase or JNK (Fig. 6 upper panel). Pretreatment of macrophages with the MEK1/2 inhibitor U0126, completely blocked CM-induced expression of phospho-p44/42, with no effect on total p44/42. U0126 also blocked expression of COX-2 protein induced by CM-APAP with no effect on HO-1 (Fig. 4, lower panel). Expression of MIP-1α and MIP-2 mRNA was also significantly reduced by the MEK1/2 inhibitor (Table 3). In contrast, U0126 had no effect on CM-APAP-induced expression of catalase or 12/15-LOX mRNA.

Potential role of hepatocyte-derived HMGB1 in macrophage activation

HMGB1 is a proinflammatory protein released by necrotic cells implicated in macrophage activation and tissue injury (Yang *et al.*, 2005). In further experiments, we determined if APAP-injured hepatocytes release HMGB1 and if this protein is involved in

macrophage activation. Western blot analysis of hepatocyte culture supernatants showed that treatment with 5 and 10 mM APAP, but not control or 2.5 mM APAP, was associated with the release of HMGB1 (Fig. 1, middle panel). HMGB1 was not detectable in CM-APAP after immunoprecipitation with anti-HMGB1 antibody. Additionally, pretreatment of hepatocytes with ethyl pyruvate, which has been reported to block HMGB1 release (Tang *et al.*, 2010), prior to APAP, resulted in a loss of detectable HMGB1 in CM-APAP (Fig. 1, lower panel). Ethyl pyruvate by itself had no effect on control hepatocytes, and did not alter the survival of hepatocytes treated with AA (Fig. 1).

To assess the potential role of HMGB1 in macrophage activation induced by hepatocyte- derived mediators, we pretreated hepatocytes with ethyl pyruvate and then with APAP or CTL. The effects of hepatocyte CM on macrophages were then analyzed. Whereas ethyl pyruvate pretreatment had no effect on the activity of CM-CTL, the ability of CM-APAP to stimulate macrophage ROS production was completely suppressed (Fig. 2). A similar loss of ROS-generating activity was observed when CM-APAP was subjected to immunoprecipitation with anti-HMGB1 antibodies. We also found that CM-APAP-induced expression of catalase and HO-1 mRNA, as well as HO-1 and COX-2 protein in macrophages was blunted when hepatocytes were pretreated with ethyl pyruvate (Table 4 and Fig. 3). In contrast, CM-APAP induced expression of MIP-1α and MIP-2 in macrophages was not significantly altered by ethyl pyruvate pretreatment of hepatocytes (Table 4).

RAGE is a macrophage receptor for HMGB1 (Kokkola *et al.*, 2005). Treatment of macrophages with CM from APAP-injured hepatocytes resulted in a marked upregulation of RAGE mRNA expression (Fig. 5). Conversely, no major effects were observed on expression of TLR4, which has also been identified as an HMGB1 receptor (Park *et al.*, 2004).

DISCUSSION

Macrophages have been implicated as key cellular effectors in the pathogenesis of APAP-induced hepatotoxicity. These cells, which consist of resident and infiltrating macrophages, are rapidly activated following APAP intoxication to release cytotoxic and proinflammatory mediators which contribute to liver damage (Gardner and Laskin, 2007). Previous studies have suggested that macrophage activation is mediated in part, by factors released from APAP-injured hepatocytes (Laskin *et al.*, 1986). Characterization of these factors and their biological effects may provide a better mechanistic understanding of pathways leading to hepatotoxicity.

The present studies demonstrate that APAP-induced cytotoxicity in primary mouse hepatocytes was associated with the release of macrophage activating factors. Thus, in response to CM from APAP-injured hepatocytes, macrophage production of ROS and expression of COX-2, 12/15-LOX, MIP-1α, MIP-2 and RAGE were increased, indicating macrophage activation to a proinflammatory phenotype. Surprisingly, CM-APAP had no effect on expression of TNF-α or IL-1, two proinflammatory mediators generated by classically activated cytotoxic/ proinflammatory macrophages (Mosser, 2003). These findings suggest that there are multiple pathways regulating macrophage activation in the liver during the early stages of APAP-induced hepatotoxicity.

Evidence suggests that ROS including superoxide anion, hydrogen peroxide and hydroxyl radicals derived from inflammatory phagocytes contribute to hepatic injury induced by diverse xenobiotics (reviewed in Laskin *et al.*, 2011). ROS can induce membrane, protein and DNA damage leading to cytotoxicity and this may be important in the pathogenesis of APAP-induced hepatotoxicity (Jaeschke *et al.*, 2003). ROS generation is a characteristic feature of classically activated macrophages (Mosser and Edwards, 2008). We found that CM from APAP-injured mouse hepatocytes stimulated macrophage ROS production. These results are in accord with previous reports that

APAP-treated rat hepatocytes release factors that induce a respiratory burst in Kupffer cells (Laskin and Pilaro, 1986). These data support the idea that hepatocytes contribute to the proinflammatory environment in the liver following APAP intoxication. CM from APAP-injured hepatocytes was also found to upregulate catalase and HO-1 expression in macrophages. These antioxidants play an important role in protecting against APAP-induced hepatotoxicity (Ahmad *et al.*, 2002; Ferret *et al.*, 2001; Chiu *et al.*, 2002). Increased expression of antioxidants by macrophages may be a compensatory response to oxidative stress induced by hepatocyte-derived proinflammatory mediators.

COX-2 is the major enzyme mediating macrophage biosynthesis of proinflammatory prostaglandins, including PGE2. It is induced in macrophages by inflammatory stimuli and activation is associated with tissue injury (Stables and Gilroy, 2011). The present studies show that CM from APAP-injured hepatocytes upregulated COX-2 expression in macrophages, which is consistent with a cytotoxic/proinflammatory phenotype of these cells (Laskin *et al.*, 2011). Treatment of animals with APAP results in increased expression of COX-2 in the liver (Reilly *et al.*, 2001; Oz and Chen, 2008; Gardner *et al.*, 2010). Surprisingly, loss of COX-2 is associated with increased susceptibility of mice to AA-induced liver injury (Reilly *et al.*, 2001). This may be due to reduced generation of the anti-inflammatory prostanoids PGD2 and 15d-PGJ2 by COX-2. Recent studies suggest that activation of COX-2 and the generation of PGE2 during the onset phase of inflammation may also indirectly elicit pro-resolution effects by inducing the transcription of enzymes involved in the generation of anti-inflammatory lipoxins (Serhan *et al.*, 2008). It is possible that early activation of COX-2 is important in converting macrophages into anti-inflammatory/wound repair cells and initiating the resolution of the inflammatory response to APAP.

Macrophages activated by inflammatory stimuli are also known to synthesize leukotrienes via a family of LOX enzymes including 5-LOX, 12-LOX, and 15-LOX.

Whereas 5-LOX induces the generation of proinflammatory leukotrienes such as leukotriene B4, and is thought to be a pathogenic factor in acute liver injury, 12-LOX and 15-LOX are mainly involved in the production of anti-inflammatory lipoxins which serve a protective function, initiating the resolution of inflammation (Stables and Gilroy, 2011). Interestingly, CM from APAP-treated hepatocytes was found to upregulate expression of 12/15-LOX, but to downregulate expression of 5-LOX. These data suggest a potential mechanistic pathway, in addition to COX-2, involved in the transition of classically activated proinflammatory macrophages into alternatively activated immunosuppressive cells, an important step in wound repair.

Expression of the proinflammatory chemokines MIP-1α and MIP-2 is increased in the liver after APAP administration to rodents (Lawson *et al.*, 2000; Liu *et al.*, 2004; Bourdi *et al.*, 2007). These chemokines are important in macrophage and neutrophil trafficking into inflamed tissues and have been implicated in hepatotoxicity (Hogaboam *et al.*, 1999). Moreover, increased expression of chemokines, including MIP-1α and MIP-2, is a feature of classically activated macrophages (Mantovani *et al.*, 2004). We found that hepatocytes injured by APAP release factors that upregulate macrophage expression of both MIP-1α and MIP-2. These data are in accord with reports that hepatocytes treated with cytotoxic doses of APAP or with ethanol release chemotactic factors for rat Kupffer cells, as well as neutrophils, and provide additional support for the idea that injured hepatocytes aggravate hepatotoxicity via activation of phagocytic leukocytes (Laskin *et al.*, 1986; Horbach *et al.*, 1997). Interestingly, APAP by itself upregulated expression of MIP-2 in macrophages. These results were unexpected since previous studies showed that APAP has no effect on macrophage functional responses (Laskin *et al.*, 1986). It may be that gene expression is a more sensitive marker of macrophage activation than functional analyses. In contrast to its effects on MIP-1α and MIP-2, CM from APAP-injured hepatocytes had no effect on expression of MIP-1β.

These data suggest the presence of multiple factors in hepatocyte CM that differentially modulate macrophage accumulation in the liver. We also found that mRNA expression of MCP-1 was downregulated by CM-APAP in macrophages. MCP-1 expression is increased in the liver after APAP administration and it has been shown to be required for emigration of anti-inflammatory/repair macrophages into the tissue (Dambach *et al.*, 2002). The fact that CM-APAP suppresses MCP-1 supports the idea that hepatocytes damaged by APAP release factors that predominantly induce proinflammatory macrophage accumulation and activation.

In further studies we assessed potential biochemical mechanisms mediating the effects of hepatocyte-derived activating factors on macrophages. MAP kinases comprise a family of protein-serine/threonine kinases that transduce extracellular signals into a variety of cellular activities including proliferation, differentiation, survival and inflammation (Dong *et al.*, 2002). Following treatment of macrophages with CM from APAP-injured hepatocytes, activation of the p44/42 MAP kinase pathway was noted within 30 min. Additionally, CM-APAP induced expression of COX-2, MIP-1α, and MIP-2 was dependent on p44/42. Thus, pretreatment of macrophages with the MEK1/2 inhibitor U0126, blocked the effects of CM-APAP on expression of these genes. Evidence suggests that the biological actions of a number of classical macrophage activators, including lipopolysaccharide (LPS) and HMGB1, involve activation of the p44/42 MAP kinase signaling pathway (Kokkola *et al.*, 2005; Pedrazzi *et al.*, 2007). These data suggest a common biochemical mechanism mediating macrophage proinflammatory activity.

The precise identity of the hepatocyte-derived factors that activate macrophages for proinflammatory activity in the liver during the pathogenesis of APAP-induced toxicity is unknown. The present studies suggest that HMGB1 may be responsible, at least in part, for this activity. This is based on our findings that APAP-induced hepatocyte

cytotoxicity was associated with the release of HMGB1, a response that was blocked by pretreatment of hepatocytes with ethyl pyruvate, which prevents HMGB1 release (Tang *et al.*, 2010). Additionally, both CM-APAP and purified HMGB1 upregulate expression of COX-2, MIP-1α, MIP-2, RAGE, and phospho-p44/42 (Pedrazzi *et al.*; Li *et al.*, 1998; Kokkola *et al.*, 2005). Recent studies have shown that APAP treatment of immortalized hepatocytes leads to HMGB1 release (Martin-Murphy *et al.*, 2010). However, higher doses of APAP were required to induce this response, which may reflect the reduced sensitivity of transformed cells, relative to primary cells, to the cytotoxic effects of APAP, potentially as a result of diminished metabolic capacity.

Ethyl pyruvate pretreatment of hepatocytes also blocked CM-APAP-induced macrophage ROS production, a response that was also suppressed by immunoprecipitation of CM-APAP with anti-HMGB1 antibody; these data suggest that the ROS-inducing activity in CM-APAP is due to HMGB1. These findings are in accord with previous reports that HMGB1 released from necrotic cells induces a respiratory burst in eosinophils, and that purified HMGB1 stimulates neutrophil ROS production (Lotfi *et al.*, 2009; Fan *et al.*, 2007). Pretreatment of hepatocytes with ethyl pyruvate was also found to suppress CM-APAP-induced expression of COX-2, as well as HO-1. These results are consistent with reports that HO-1 deficiency is associated with enhanced release of HMGB1 and decreased survival during endotoxemia The fact that inhibition of HO-1 and COX-2 was not complete suggests that CM-APAP contains factors, in addition to HMGB1, that contribute to macrophage activation. This is supported by our findings that ethyl pyruvate pretreatment of hepatocytes had no effect on CM-APAP-induced macrophage chemokine expression. The mechanisms underlying the ability of ethyl pyruvate to block HMGB1 release have not been established. In lung epithelial cells, ethyl pyruvate-mediated inhibition of HMGB1 release appears to be due to a switch from necrotic to apoptotic cell death (Lim *et al.*, 2007). It remains to be determined if a similar

mechanism is involved in the inhibitory actions of ethyl pyruvate on APAP-injured hepatocytes.

A major macrophage receptor for HMGB1 is RAGE, which is reported to be activated during inflammation and in response to oxidative stress (Sims *et al.*, 2010). RAGE activation is detrimental in various models of inflammatory injury including sepsis, LPS-induced lung injury, arthritis, partial hepatectomy, and liver ischemia-reperfusion injury (Herold *et al.*, 2007; Zhang *et al.*, 2008; Zeng *et al.*, 2004). Blockade of RAGE has also been reported to protect against lethal doses of APAP (Ekong *et al.*, 2006). We found that macrophage expression of RAGE is upregulated in response to CM-APAP. Consequences of RAGE activation include the generation of ROS, and increased expression of COX-2, HO-1 and chemokines, such as MIP-2 (Lotfi *et al.*, 2009; Wautier *et al.*, 2001; Shanmugam *et al.*, 2003; Zeng *et al.*, 2009). Our findings that CM-APAP induced similar responses in macrophages, and that this correlated with increased RAGE expression, provide additional support for a role of HMGB1 in inducing these activities.

A question arises on potential differences in responsiveness of RAW 264.7 macrophages and primary liver macrophages to CM-APAP. RAW 264.7 macrophages exhibit many functional characteristics of primary mouse macrophages, including phagocytosis and pinocytosis, antibody-dependent killing, responsiveness to bacterial products, and high secretory profile (Raschke *et al.*, 1978). Moreover, in previous studies we demonstrated that primary cultures of Kupffer cells respond to hepatocyte CM with increased chemotaxis and oxidative metabolism (Laskin and Pilaro, 1986). These findings suggest that RAW 264.7 macrophages responses are indeed reflective of Kupffer cells.

In conclusion, the present studies suggest that factors released from APAP-injured hepatocytes, including HMGB1, activate macrophages to produce cytotoxic and

proinflammatory mediators known to be involved in hepatotoxicity (Laskin, 2009). Inhibition of HMGB1 release or neutralization of its biological activity may represent a potential approach to downregulating activated macrophages, a key step in mitigating APAP-induced hepatotoxicity.

FIGURE 1. APAP-induced cytotoxicity in hepatocytes and the release of HMGB1. Upper panel. Hepatocytes were treated with 1-10 mM APAP or medium control (CTL). After 24 h, cells were washed and refed with medium containing 0.5 mg/ml MTT. DMSO was added 2 h later and absorbance measured at 550 nm. Each bar represents the mean ± SE (n = 8-12). [a]Significantly different ($p \leq 0.05$) from CTL. Middle Panel. Hepatocytes were treated with 1-10 mM APAP or CTL. Equal volumes of culture supernatants were collected 24 h later, concentrated using Microcon centrifugal filter devices (Millipore, Billerica, MA), and analyzed for HMGB1 by western blotting. One representative blot from three independent experiments is shown. Lower Left Panel. Hepatocytes were treated with medium or with 30 mM ethyl pyruvate (EP) for 1 h, followed by APAP (5 mM) or CTL. Culture supernatants were collected 24 h later, concentrated, and analyzed for HMGB1 by western blotting. One representative blot from three independent experiments is shown. Lower Right Panel. Culture supernatants from hepatocytes treated with APAP (5 mM) or CTL for 24 h were incubated overnight with anti-HMGB1 antibody followed by immobilized protein G. Samples were then centrifuged and supernatants analyzed for HMGB1 by western blotting.

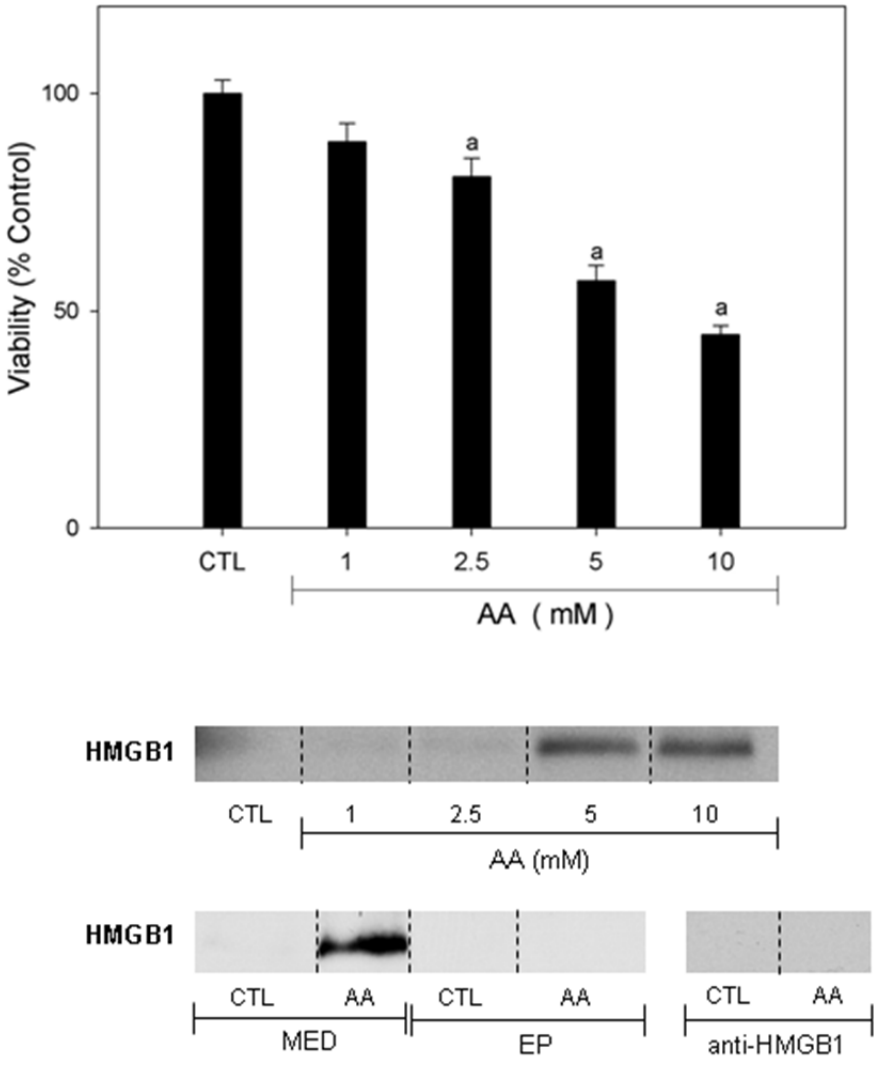

Viability (% Control)
100
50
0
a
a
a
CTL
1
2.5
5
10
AA (mM)
HMGB1
CTL
1
2.5
5
10
AA (mM)
HMGB1
CTL
AA
CTL
AA
CTL
AA
MED
EP
anti-HMGB1

FIGURE 2. Effects of hepatocyte conditioned medium (CM) on macrophage ROS production. Macrophages were preincubated with CM-H_2DCFDA for 15 min, and then with 30% CM from control (CTL) hepatocytes (CM-CTL), APAP-treated hepatocytes (CM-AA), CM-AA immunoprecipitated with anti-HMGB1 antibody, or with CM from hepatocytes pretreated with ethyl pyruvate (EP) for 1 h, and then with APAP for 24 h. Fluorescence was measured spectrophotometrically every 2 min for 30 min. Each point represents the mean ± SE (n = 3-5). Note that at all times greater than 6 min, CM-AA was significantly different ($p \leq 0.05$) from CM-CTL, CM-AA + anti-HMGB1, and CM-AA + EP.

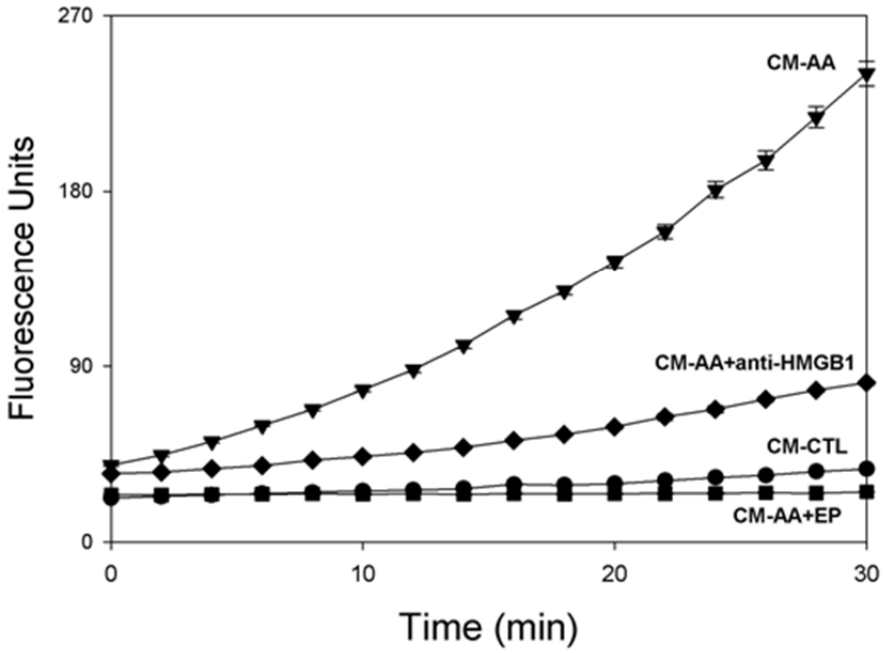
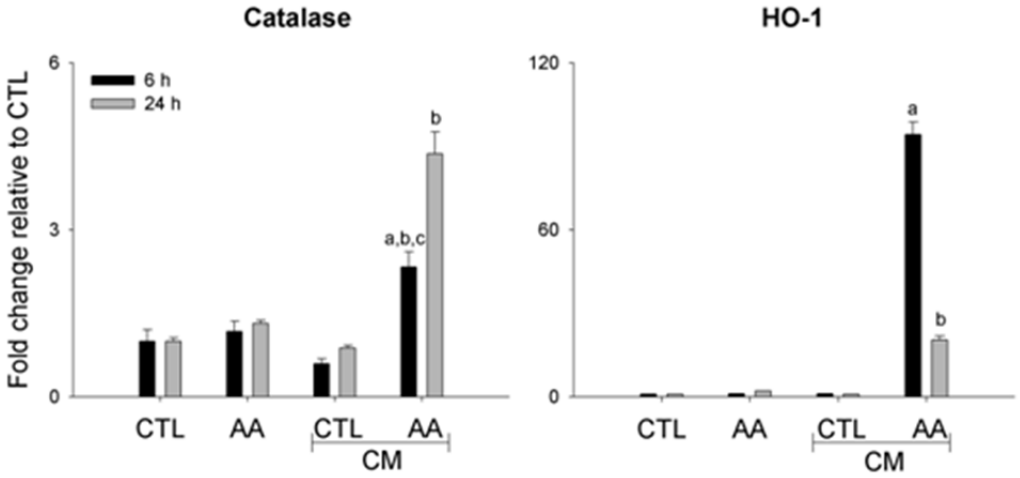

270
180
90
0
Fluorescence Units
0
10
20
30
Time (min)
CM-AA
CM-AA+anti-HMGB1
CM-CTL
CM-AA+EP

FIGURE 3. Effects of hepatocyte conditioned medium (CM) on macrophage expression of catalase, HO-1, and COX-2. Upper panel. Macrophages were treated with medium control (CTL), 1.5 mM APAP, or 30% CM from CTL or APAP-treated hepatocytes. After 6 h or 24 h, macrophage mRNA expression for catalase and HO-1 was analyzed by RT-PCR. Data were normalized to 18s RNA and expressed as fold change relative to time-matched CTL. Each bar represents the mean ± SE (n = 3). [a]Significantly different ($p \leq 0.05$) from CTL. [b]Significantly different ($p < 0.05$) from CM-CTL. [c]Significantly different ($p \leq 0.05$) from APAP. Lower panel. Macrophages were treated with CTL, APAP (1.5 mM), or 30% CM from CTL or APAP-treated hepatocytes, or with CM from hepatocytes pretreated for 1 h with medium (MED) or with ethyl pyruvate (EP) and then with APAP or CTL. Macrophage COX-2 and HO-1 protein expression was analyzed by western blotting 6 h and/or 24 h later. One representative blot from three independent experiments is shown.


Catalase
HO-1
Fold change relative to CTL
6 h
24 h
6
3
0
120
60
0
a,b,c
b
a
b
CTL
AA
CTL
AA
CM
CTL
AA
CTL
AA
CM

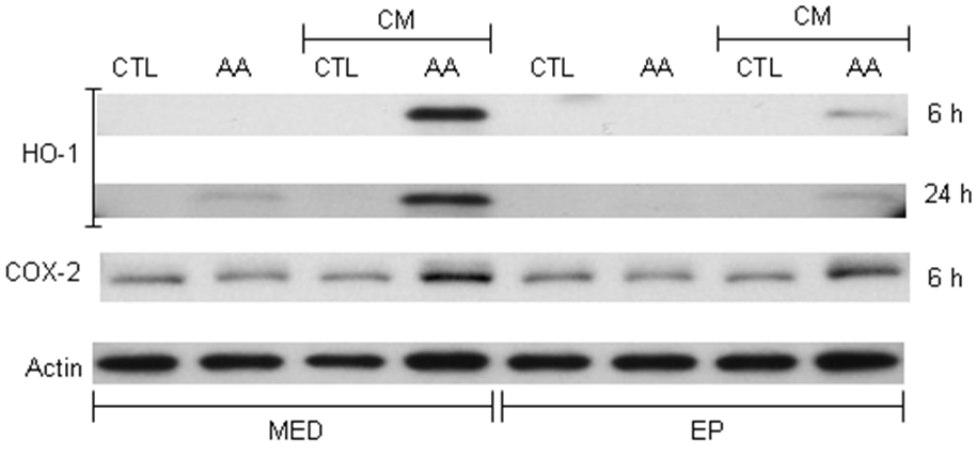

CM
CM
CTL
AA
CTL
AA
CTL
AA
CTL
AA
HO-1
6 h
24 h
COX-2
6 h
Actin
MED
EP

FIGURE 4. Effects of hepatocyte conditioned medium (CM) on expression of macrophage eicosanoid biosynthetic enzymes. Macrophages were treated with medium control (CTL), 1.5 mM APAP, or 30% CM from CTL or APAP-treated hepatocytes. After 6 h, macrophage mRNA expression of eicosanoid biosynthetic enzymes was analyzed by RT-PCR. Data were normalized to 18s RNA and expressed as fold change relative to time-matched CTL. Each bar represents the mean ± SE (n = 3). [a]Significantly different ($p \leq 0.05$) from CTL. [b]Significantly different ($p < 0.05$) from CM-CTL. [c]Significantly different ($p \leq 0.05$) from APAP.

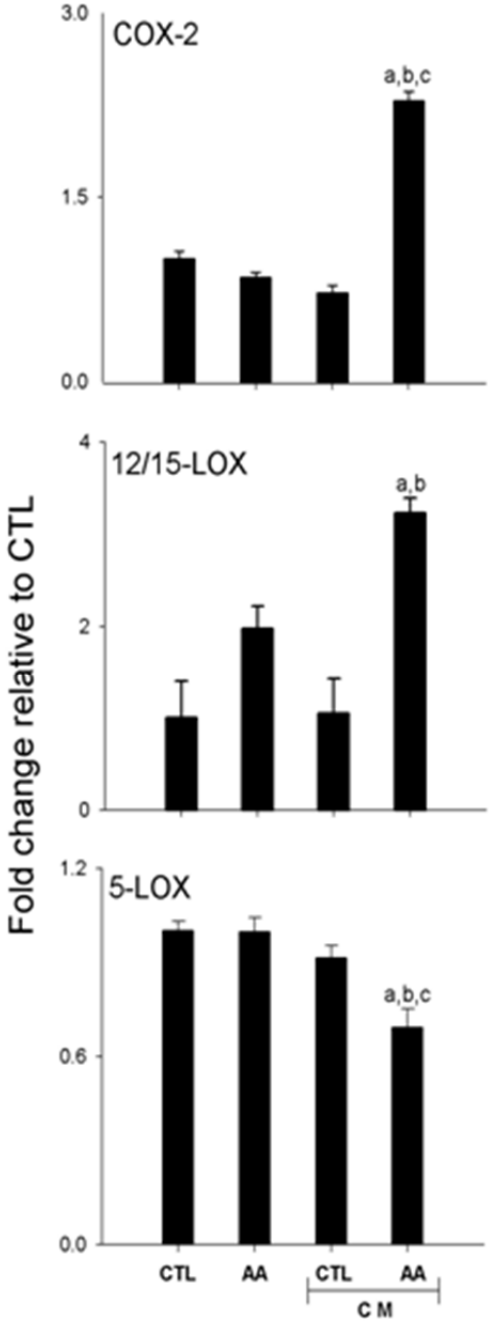

COX-2
3.0
1.5
0.0
a,b,c
12/15-LOX
4
2
0
a,b
5-LOX
1.2
0.6
0.0
a,b,c
Fold change relative to CTL
CTL
AA
CTL
AA
C M

FIGURE 5. Effects of hepatocyte conditioned medium (CM) on macrophage expression of chemokines and HMGB1 receptors. Macrophages were treated with medium control (CTL), 1.5 mM APAP, or 30% CM from CTL or APAP-treated hepatocytes. After 6 h, macrophage mRNA expression for chemokines and HMGB1 receptors was analyzed by RT-PCR. Data were normalized to 18s RNA and expressed as fold change relative to CTL. Each bar represents the mean ± SE (n = 3). [a]Significantly different ($p \leq 0.05$) from CTL. [b]Significantly different ($p \leq 0.05$) from CM CTL. [c]Significantly different ($p \leq 0.05$) from APAP.

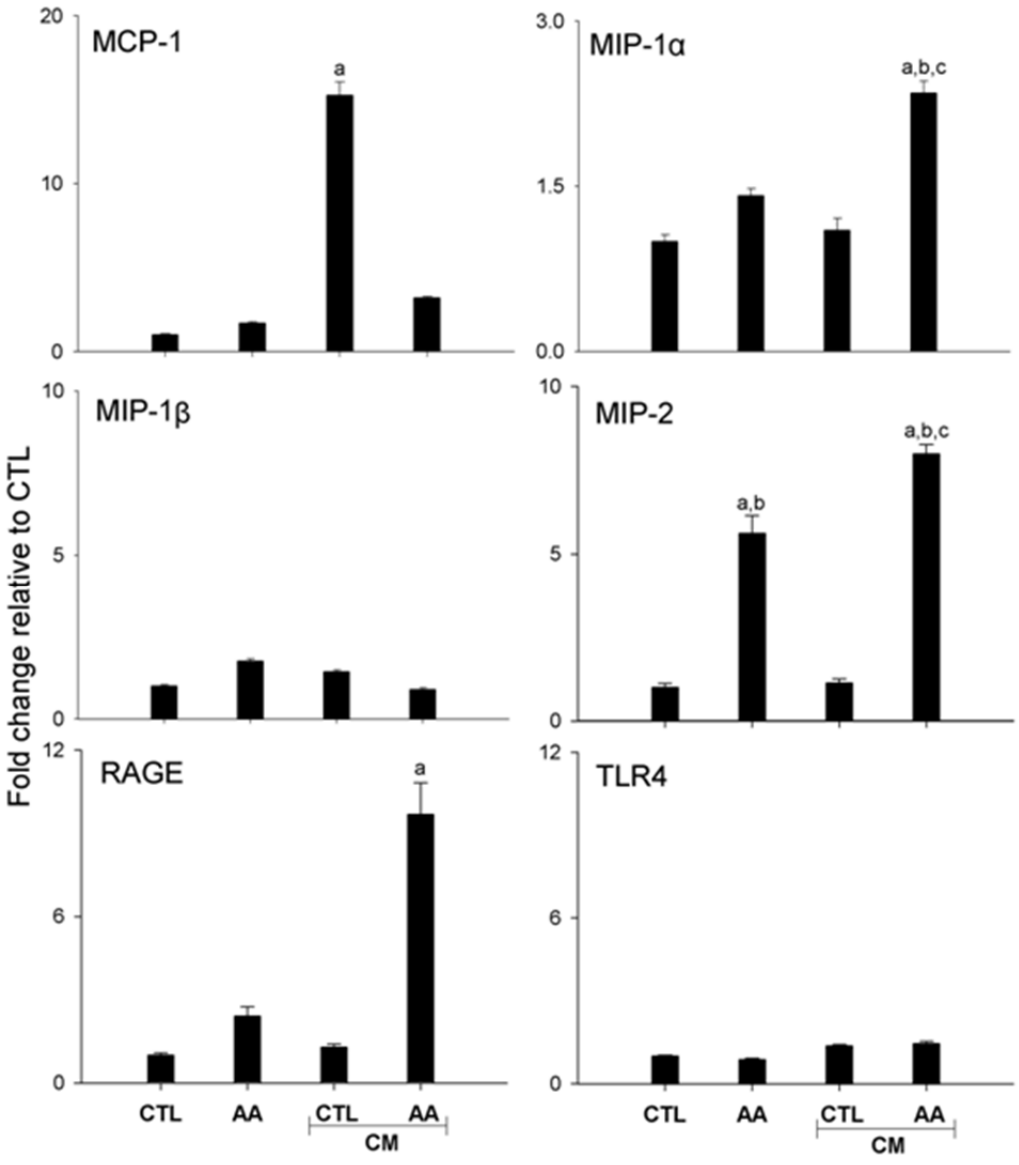

MCP-1
MIP-1α
MIP-1β
MIP-2
RAGE
TLR4
Fold change relative to CTL
a
a,b,c
a,b
CTL
AA
CTL
AA
CM

FIGURE 6. Role of p44/42 MAP kinase in hepatocyte CM-induced COX-2 and HO-1 expression. Upper Panel. Macrophages were treated with medium control (CTL), 1.5 mM APAP, or 30% CM from CTL or APAP-treated hepatocytes for 30 min. Expression of phosphorylated and total MAP kinase p38 and JNK was analyzed by western blotting. Middle panel. Macrophages were pretreated for 2 h with 10 uM U0126 or control, and then with medium control (CTL), 1.5 mM APAP, or 30% CM from CTL or APAP-treated hepatocytes for 30 min. Expression of phosphorylated and total MAP kinase p44/42 was analyzed by western blotting. Lower Panel. Macrophages were pretreated for 2 h with 10 μM U0126 or DMSO, and then with medium CTL or APAP, or with CM from control (CTL) or APAP-treated hepatocytes for 6 h. COX-2 and HO-1 protein expression were analyzed by western blotting. One representative blot from three independent experiments is shown.

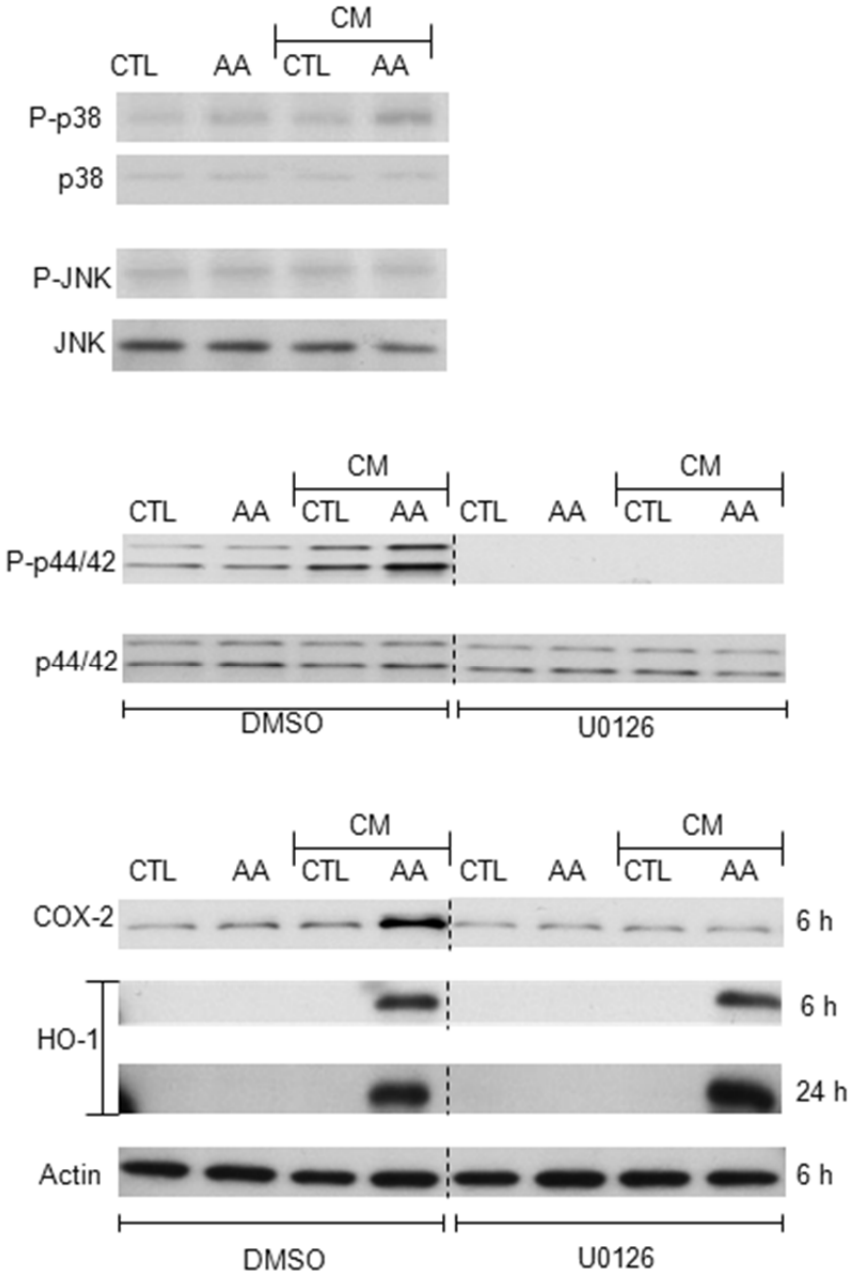

CM
CTL
AA
CTL
AA
P-p38
p38
P-JNK
JNK
CM
CM
CTL
AA
CTL
AA
CTL
AA
CTL
AA
P-p44/42
p44/42
DMSO
U0126
CM
CM
CTL
AA
CTL
AA
CTL
AA
CTL
AA
COX-2
6 h
HO-1
6 h
24 h
Actin
6 h
DMSO
U0126

TABLE 2. Effects of hepatocyte conditioned medium (CM) on macrophage expression of TNF-α and IL-1β. Macrophages were treated with medium control (CTL), 1.5 mM APAP, or 30% CM from CTL or APAP-treated hepatocytes. After 6 h, macrophage mRNA expression for cytokines was analyzed by RT-PCR. Data were normalized to 18s RNA and expressed as fold change relative to CTL. Each bar represents the mean ± SE (n = 3).

	CTL	AA	CM CTL	CM AA
TNF-α	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.8 ± 0.1
IL-1β	1.0 ± 0.0	1.3 ± 0.1	1.0 ± 0.1	1.0 ± 0.4

TABLE 3. Effects of p44/42 MAP kinase inhibition on macrophage gene expression. Macrophages were treated with U0126 or DMSO for 2 h, followed by conditioned medium (CM) from control (CTL) or APAP-treated hepatocytes. Six h later, mRNA expression was analyzed by RT-PCR. Data are expressed as fold change relative to CTL (mean ± SE, n=3). [a]Significantly different ($p \leq 0.05$) from CTL; [b]Significantly different ($p \leq 0.05$) from CM-CTL; [c]Significantly different ($p \leq 0.05$) from DMSO.

	CM-CTL		CM-AA	
	DMSO	U0126	DMSO	U0126
Catalase	0.7 ± 0.2	0.9 ± 0.1	2.3 ± 0.7 [a,b]	2.5 ± 0.1
12/15-LOX	1.0 ± 0.4	1.1 ± 0.2	3.2 ± 0.2 [a,b]	2.0 ± 0.2
MIP-1α	1.1 ± 0.1	1.2 ± 0.3	2.3 ± 0.1 [a,b]	0.7 ± 0.1[c]
MIP-2	1.1 ± 0.1	1.2 ± 0.3	8.0 ± 0.3 [a,b]	4.3 ± 0.4[c]

TABLE 4. Effects of ethyl pyruvate pretreatment of hepatocytes on macrophage gene expression. Macrophages were treated with conditioned medium (CM) from hepatocytes pretreated for 1 h with medium control (Med) or ethyl pyruvate (EP), and then with APAP or control (CTL). mRNA expression was analyzed 6 and 24 h later by RT-PCR. Data are expressed as fold change relative to CTL (mean ± SE, n = 3). [a]Significantly different ($p \leq 0.05$) from CM-CTL. [b]Significantly different ($p \leq 0.05$) from CM-AA without pretreatment.

	CM-CTL		CM-AA	
	Med	EP	Med	EP
Catalase				
6 h	0.7 ± 0.1	1.4 ± 0.1	3.7 ± 0.6[a]	1.0 ± 0.1[b]
24 h	0.9 ± 0.2	2.1 ± 0.3	4.4 ± 0.4[a]	0.9 ± 0.1[b]
HO-1				
6 h	0.7 ± 0.1	4.2 ± 0.3	64.3 ± 6.5[a]	2.7 ± 0.2[b]
24 h	0.8 ± 0.1	3.4 ± 0.2	39.6 ± 2.0[a]	5.4 ± 0.2[b]
MIP-1α				
6 h	0.9 ± 0.1	0.7 ± 0.0	2.1 ± 0.2	1.4 ± 0.1
MIP-2				
6 h	1.2 ± 0.2	6.3 ± 0.7	1.1 ± 0.1	3.8 ± 0.4

PART II. ROLE OF GALECTIN-3 IN APAP-INDUCED HEPATOTOXICITY AND INFLAMMATORY MEDIATOR PRODUCTION

Evidence suggests that proinflammatory/cytotoxic mediators released by activated macrophages play a role in promoting APAP-induced hepatotoxicity (reviewed in (Laskin, 2009). The factors that induce macrophage activation and inflammatory mediator production in the liver after APAP intoxication have not been clearly established. In previous studies we demonstrated that mediators released from injured hepatocytes, including HMGB1, are important in the activation process (Dragomir *et al.*, 2011; Laskin *et al.*, 1986). A question arises, however, about the role of macrophages themselves as a source of activating factors. Macrophages are potent secretory cells, releasing a myriad of mediators known to be important in nonspecific host defense and adaptive immunity, as well as inflammation and wound repair. These diverse activities are mediated by distinct subpopulations that develop in response to mediators macrophages encounter in their microenvironment (reviewed in (Laskin *et al.*, 2011). Two major phenotypically distinct subpopulations have been identified: classically activated proinflammatory macrophages and alternatively activated anti-inflammatory/wound repair macrophages. The ability of macrophages to release activating factors that act in an autocrine and paracrine manner to induce classical and alternative activation represents an important mechanism regulating inflammatory responses to tissue injury.

Galectins comprise a family of lectins with affinity for β-galactoside-containing carbohydrates. Gal-3 is the only member of this family with a chimeric structure consisting of a conserved carbohydrate recognition domain and a non-lectin domain (Henderson and Sethi, 2009). Whereas Gal-3 is expressed at low levels in monocytes, it is upregulated during their maturation into macrophages (Liu *et al.*, 1995).

Proinflammatory cytokines, including TNF-α, further upregulate Gal-3 in macrophages and also stimulate its release into the extracellular environment (Nishi *et al.*, 2007). Gal-3 binds to macrophages via CD98, stimulating the production of additional proinflammatory cytokines and chemokines (Norling *et al.*, 2009). Intra-articular administration of Gal-3 has been reported to induce inflammation in mice (Janelle-Montcalm *et al.*, 2007). Moreover, mice with a targeted mutation of Gal-3 exhibit an impaired ability to mount an acute inflammatory response to thioglycollate (Hsu *et al.*, 2000) or ovalbumin (Zuberi *et al.*, 2004). These data suggest that Gal-3 is important in promoting inflammation. Since macrophage-derived inflammatory mediators contribute to liver injury following APAP intoxication (Laskin *et al.*, 1995); (Laskin, 2009), we speculated that Gal-3 may be involved in the pathogenesis of hepatotoxicity and this was investigated.

RESULTS

Effects of APAP on expression of Gal-3 in the liver

In initial studies we analyzed the effects of APAP intoxication on Gal-3 expression in the liver. Treatment of wild type mice with APAP resulted in a time-dependent increase in hepatic Gal-3 mRNA expression which was evident at 24 h, becoming more pronounced at 48 and 72 h (Fig. 7, upper panel). This was correlated with an increase in hepatic Gal-3 protein expression, which was predominantly localized in inflammatory macrophages infiltrating into necrotic areas (Fig. 7, middle panel). No Gal-3 expression was observed in neutrophils (Fig. 7, middle panel inset). In contrast, a transient decline in serum Gal-3 levels was noted 6-24 h after APAP administration; subsequently serum levels began to increase, and by 72 h were at or above control levels (Fig. 7, lower panel).

Role of Gal-3 in APAP-induced hepatotoxicity

To investigate the role of Gal-3 in the pathogenesis of APAP-induced hepatotoxicity, we used mice with a targeted deletion of the *lgals3* gene. In wild type mice, APAP administration resulted in centrilobular hepatic necrosis, which was evident within 3 h (Fig. 8 and Gardner *et al.*, 2010). At 6 h post-APAP, mild degeneration, necrosis, and hemorrhage were noted in centrilobular areas, with no evidence of neutrophils (Table 5). By 24 h, moderate coagulative centrilobular necrosis was present, as well as minimal to mild hemorrhage within the necrotic areas; minimal infiltration of neutrophils was also noted in centrilobular areas. Moderate coagulative necrosis persisted in centrilobular regions of the liver for 48 h, and was accompanied by mild neutrophil infiltration. At 72 h post-APAP, mild necrosis and moderate neutrophil infiltration were evident (Figs. 8 and 9, Table 5). Mitotic figures were also observed in surviving hepatocytes surrounding necrotic areas at 48-72 h, indicating liver

regeneration (Fig. 8, inset). Histopathologic changes in the liver were associated with a time-related increase in serum transaminases, which peaked 6-24 h post APAP treatment (Table 6). APAP-induced hepatotoxicity was significantly blunted in Gal-3$^{-/-}$ mice when compared to wild type mice, as evidenced by more rapid decreases in serum transaminases and attenuated histologic alterations. Thus, by 72 h post-APAP, only minimal necrosis was observed in livers of Gal-3$^{-/-}$ mice relative to mild/moderate necrosis in wild type mice (Fig. 8, Tables 5 and 6). While the extent of neutrophil infiltration into the liver was similar in Gal-3$^{-/-}$ and wild type mice 24-48 h post-APAP, as determined histologically and by myeloperoxidase immunostaining (Table 5 and Fig. 9), by 72 h there were significantly fewer neutrophils in livers of Gal-3$^{-/-}$ mice when compared to wild type mice.

24p3 is an acute phase protein and a marker of oxidative stress and inflammation released by neutrophils, macrophages, and epithelial cells (Borkham-Kamphorst *et al.*, 2011; Roudkenar *et al.*, 2007; Sunil *et al.*, 2007). In wild type mice, APAP intoxication was characterized by a dramatic increase in expression of 24p3 mRNA, which was most prominent after 24 h (Fig. 10). Subsequently, levels began to decline towards control. Loss of Gal-3 resulted in a significant attenuation of this response.

Effects of loss of Gal-3 on APAP-induced expression of inflammatory proteins

In further studies we analyzed the effects of loss of Gal-3 on APAP-induced expression of pro- and anti-inflammatory proteins implicated in hepatotoxicity (Laskin, 2009). APAP administration to wild type mice was associated with a time-related increase in mRNA expression for the pro-inflammatory mediators MIP-2, MIP-3α, and IL-1β. Whereas MIP-2 expression increased within 6 h and persisted for 48 h after APAP, MIP-3α upregulation was delayed until 24 h and remained elevated for 72 h. In

contrast, IL-1β expression was transiently increased at 24 h post-APAP. MMP-9 mRNA expression also increased in the liver 24 h after APAP, remaining elevated for at least 72 h (Fig. 10). Protein expression of iNOS, the enzyme mediating macrophage production of nitric oxide (Laskin, 2010b), also increased 48-72 h after APAP administration to wild type mice (Fig. 11). Loss of Gal-3 blunted the effects of APAP on MIP-3α, MMP-9, and iNOS, but had no effect on expression of IL-1β. MIP-2 expression was also reduced in Gal-$3^{-/-}$ mice, relative to wild type mice, at 48-72 h post-APAP. In contrast, expression of this chemokine was significantly increased at 6 h in Gal-$3^{-/-}$ mice. Expression of the anti-inflammatory cytokine IL-10 was also upregulated in wild type mice following APAP intoxication (Fig. 10). This was observed within 6 h and remained elevated for 48 h, although at reduced levels. Loss of Gal-3 resulted in decreased IL-10 expression at 48 h post APAP. COX-2 is a key enzyme regulating the biosynthesis of both pro- and anti-inflammatory eicosanoids (Cook, 2005). Constitutive COX-2 protein was detected in the livers of both wild type and Gal-$3^{-/-}$ mice; however, expression of this protein was significantly reduced in Gal-$3^{-/-}$ mice (Fig. 11). Whereas in wild type mice APAP had no major effect on COX-2 protein expression, a significant decrease was noted in Gal-$3^{-/-}$ mice 48 h after treatment.

CD98 has been proposed as a macrophage receptor for Gal-3 (Dong and Hughes, 1997). In wild type mice, APAP administration resulted in a time-dependent increase in CD98 mRNA expression, which was maximal after 24 h (Fig. 10). Loss of Gal-3 blunted the effects of APAP on CD98 expression.

Reciprocal regulation of Gal-3 and TNFR1 expression in the liver following APAP intoxication

TNF-α signaling via TNFR1 has been shown to be important in the production of mediators involved in tissue repair and antioxidant defense during APAP-induced hepatotoxicity (Chiu *et al.*, 2003b; Chiu *et al.*, 2003a). In agreement with earlier studies (Ishida *et al.*, 2004), we found that APAP administration to wild type mice resulted in a significant increase in hepatic TNFR1 mRNA expression which was maximal after 24-48 h (Fig. 10). This was reduced in APAP-treated Gal-3$^{-/-}$ mice. To investigate the role of TNF-α signaling via TNFR1 in APAP-induced Gal-3 expression, we used mice with a targeted deletion of the gene encoding TNFR1, which we have previously reported to be more susceptible than wild type mice to APAP-induced hepatotoxicity (Chiu *et al.*, 2003a; Gardner *et al.*, 2003). Whereas loss of TNFR1 had minimal effects on APAP-induced CD98 expression, Gal-3 mRNA levels were significantly greater in TNFR1$^{-/-}$ mice relative to wild type mice 72 h post-APAP (Fig. 12). Gal-3 protein expression was also increased in livers of TNFR1$^{-/-}$ mice when compared to wild type mice (Fig. 13). This was noted within 6 h, and became more prominent after 48-72 h.

Effects of loss of Gal-3 on hepatic glutathione and Cyp2e1 activity

To determine if reduced hepatotoxicity in Gal-3$^{-/-}$ mice was due to altered APAP metabolism, we measured the activity of hepatic Cyp2e1, the major enzyme mediating the generation of the reactive APAP metabolite NAPQI (Lee *et al.*, 1996). No significant differences were noted in the activity of Cyp2e1 between wild type and Gal-3$^{-/-}$ mice (0.91 ± 0.05 versus 0.97 ± 0.05 nmol/min/mg protein, respectively). We also measured hepatic glutathione levels. APAP administration to mice resulted in a rapid and transient decline in hepatic reduced glutathione levels in wild type, which was evident within 3 h;

subsequently, glutathione levels increased and by 72 h were above control levels (Fig. 14). Loss of Gal-3 had no significant effect on APAP-induced alterations in hepatic glutathione levels.

DISCUSSION

The present studies demonstrate that Gal-3 plays a role in promoting late proinflammatory responses and perpetuating injury in the liver following APAP intoxication. This is based on our findings that Gal-3 is markedly upregulated in macrophages infiltrating into the liver 48-72 h after APAP administration, and that loss of Gal-3 results in reduced hepatotoxicity at these times and decreased expression of the proinflammatory proteins, 24p3, MMP-9, MIP-3α, iNOS, and CD98. Neutrophil influx into the liver is also suppressed. These findings are novel and may have therapeutic implications for developing new approaches to treating APAP overdose.

APAP administration resulted in a dramatic increase in Gal-3 expression in the liver, which was most prominent after 48-72 h. Moreover, the major cell population expressing Gal-3 consisted of mononuclear cells accumulating in necrotic areas, which have previously been shown to display features of classically activated macrophages (Laskin and Pilaro, 1986). These findings are in agreement with previous studies showing that Gal-3 is upregulated in leukocytes infiltrating the liver during carbon tetrachloride-induced fibrosis and *Toxoplasma gondii* infection (Henderson *et al.*, 2006; Bernardes *et al.*, 2006). Our observation that serum Gal-3 levels declined rapidly following APAP administration is consistent with increased Gal-3 accumulation in the liver and suggests a local, intra-hepatic role for Gal-3 in this model of injury. Blood monocytes have been shown to synthesize Gal-3 (Weber *et al.*, 2009); reduced serum levels after APAP may also be due to increased emigration of these cells into the liver.

Gal-3 has been reported to be increased in various models of tissue injury where it functions to stimulate macrophage production of proinflammatory mediators (Norling *et al.*, 2009). Consistent with this activity are findings that loss of Gal-3 is protective in antigen-induced arthritis (Forsman *et al.*, 2011), renal ischemia-reperfusion injury (Fernandes Bertocchi *et al.*, 2008), neonatal hypoxic-ischemic brain injury (Doverhag *et*

al., 2010), streptozotocin-induced diabetes (Mensah-Brown *et al.*, 2009), diet-induced steatohepatitis (Iacobini *et al.*, 2011), and concanavalin A-induced liver injury (Volarevic *et al.*, 2012). Moreover, protection in each of these models correlates with decreased levels of proinflammatory mediators. Similarly, we found that APAP-induced hepatotoxicity, as assessed histologically, by serum transaminases, and by expression of 24p3, was significantly reduced in Gal-3$^{-/-}$ mice at 48-72 h, a time coordinate with the accumulation of Gal-3-positive macrophages in livers of wild type mice. These findings, together with the observation that APAP-induced expression of iNOS, MIP-3α, and MMP-9, proinflammatory proteins implicated in tissue injury (Gardner *et al.*, 2002; Schutyser *et al.*, 2003; Ito *et al.*, 2005), was reduced at these times in Gal-3$^{-/-}$ mice, suggest that Gal-3 plays a role in promoting late inflammatory responses and the persistence of hepatic injury. This is supported by our finding that hepatotoxicity resolves more rapidly in Gal-3$^{-/-}$ mice relative to wild type mice. However, the observation that APAP-induced hepatotoxicity is not completely prevented by loss of Gal-3 indicates that factors released early after injury, including DAMPs such as HMGB1 and HSP70, contribute to the pathogenic response to APAP (Martin-Murphy *et al.*, 2010; Dragomir *et al.*, 2011).

Reactive nitrogen species generated via iNOS have been shown to contribute to APAP-induced oxidative stress and tissue injury (Laskin, 2009). APAP intoxication resulted in increased iNOS expression in the liver at 24-72 h. Findings that decreased hepatotoxicity in Gal-3$^{-/-}$ mice correlated with reduced iNOS expression suggest that Gal-3-positive macrophages may be a source of reactive nitrogen species. This is supported by reports that Gal-3 upregulates iNOS expression in brain macrophages (Jeon *et al.*, 2010).

MMP-9 is an extracellular matrix-degrading enzyme that plays a role in microvascular injury induced by APAP (Ito *et al.*, 2005). As previously reported (Gardner

et al., 2003), MMP-9 expression increased in the liver following APAP administration, a response most notable at 24 h. This was significantly attenuated in Gal-3$^{-/-}$ mice, and may contribute to reduced toxicity in these animals. These data are in agreement with reports that protection from hypoxic-ischemic brain injury in the absence of Gal-3 was associated with lower MMP-9 levels (Doverhag *et al.*, 2010).

MIP-3α (CCL20) is a chemokine produced by macrophages and epithelial cells in response to proinflammatory stimuli such as TNF-α, IFN-γ, and LPS, as well as Gal-3 (Schutyser *et al.*, 2003; Papaspyridonos *et al.*, 2008). Expression of MIP-3α has also been reported to increase in vivo during liver and brain inflammation (Utans-Schneitz *et al.*, 1998; Sugita *et al.*, 2002). Similarly, we found that expression of MIP-3α increased in the liver following APAP intoxication and that this correlated with increased numbers of Gal-3-positive macrophages. The fact that loss of Gal-3 blunted the effects of APAP on MIP-3α expression provides additional support for a role of Gal-3 in promoting inflammation in the liver (Iacobini *et al.*, 2011; Volarevic *et al.*, 2012).

In agreement with previous studies, we found that APAP administration to wild type mice resulted in increased expression of MIP-2 and IL-1β in the liver (Dambach *et al.*, 2006; Imaeda *et al.*, 2009; Liu *et al.*, 2004). MIP-2 is a potent neutrophil chemokine (Clarke *et al.*, 2009). Whereas in wild type mice, increases in MIP-2 expression correlated with increased numbers of neutrophils in the liver, this was not observed in Gal-3$^{-/-}$ mice; in these mice, a marked increase in MIP-2 expression was noted 6 h after APAP intoxication, with no neutrophil emigration into the liver at this time. These data suggest that MIP-2 does not contribute significantly to early neutrophilic responses to APAP. It has been suggested that MIP-2 may play a protective role in the liver following APAP intoxication by promoting hepatocyte regeneration (Hogaboam *et al.*, 1999). Our findings that MIP-2 expression is upregulated in Gal-3$^{-/-}$ mice relative to wild type mice, and that this was associated with reduced hepatotoxicity, are in accord with this idea.

The observation that MIP-2 levels decline more rapidly in Gal-3$^{-/-}$ mice is most likely due to reduced need for tissue repair processes. In contrast to MIP-2, APAP-induced IL-1β expression was unaffected by the loss of Gal-3. These results are consistent with recent studies suggesting that IL-1β does not play a role in the pathogenic response to APAP (Williams *et al.*, 2010). Gal-3 has been reported to upregulate IL-1β expression in microglia (Jeon *et al.*, 2010); moreover, tissue IL-1β levels are reduced in Gal-3$^{-/-}$ mice after renal ischemia reperfusion injury (Fernandes Bertocchi *et al.*, 2008). It may be that IL-1β plays distinct roles in different models of tissue injury.

The anti-inflammatory cytokine IL-10 has previously been shown to be upregulated in the liver following APAP intoxication and to play a hepatoprotective role in the pathogenic response (Bourdi *et al.*, 2007; Dambach *et al.*, 2006; Gardner *et al.*, 2002). Increases in IL-10 mRNA levels were observed in both wild type and Gal-3$^{-/-}$ mice 24 h after APAP administration. Surprisingly, by 48 h, IL-10 levels were reduced in Gal-3$^{-/-}$ mice relative to wild type mice. This may be a consequence of decreased inflammation and hepatotoxicity in these mice, resulting in a reduced requirement for IL-10.

COX-2 catalyzes the biosynthesis of both pro- and anti-inflammatory eicosanoids (Stables and Gilroy, 2011). We found that COX-2 was constitutively expressed in livers of wild type and Gal-3$^{-/-}$ mice. This is likely due to continuous exposure to endotoxin in the portal circulation (Ahmad *et al.*, 2002). Previous studies have shown that mice lacking COX-2 are hypersensitive to APAP, suggesting that COX-2 is important in limiting hepatotoxicity (Reilly *et al.*, 2001). This is thought to be due to increased generation of anti-inflammatory prostaglandins. Interestingly, hepatic COX-2 expression was reduced in Gal-3$^{-/-}$ mice relative to wild type mice, suggesting a positive regulatory role for Gal-3 in hepatic prostanoid production. This is supported by our findings that constitutive COX-2 levels declined in the livers of Gal-3$^{-/-}$ mice treated with APAP. In

contrast, Gal-3 appears to be negatively regulated by COX-2, as evident from reports that Gal-3 is downregulated in mice overexpressing COX-2 (Shen *et al.*, 2007). COX-2 mRNA has been reported to be upregulated in the liver following APAP intoxication (Reilly *et al.*, 2001). Conversely, we found no major effects of APAP on COX-2 protein expression in wild type mice. These differences may be due to strain-specific responses and/or analysis of protein expression in our studies versus mRNA expression in earlier reports.

CD98 is a transmembrane glycosylated protein thought to function as a receptor for Gal-3 on macrophages (Dong and Hughes, 1997). Like Gal-3, CD98 is upregulated during tissue injury and inflammation and contributes to disease pathogenesis (Nguyen *et al.*, 2011a). Following APAP intoxication, CD98 increased in livers of wild type mice; moreover, loss of Gal-3 blunted this response. This may contribute to the decreased sensitivity of Gal-3$^{-/-}$ mice to APAP.

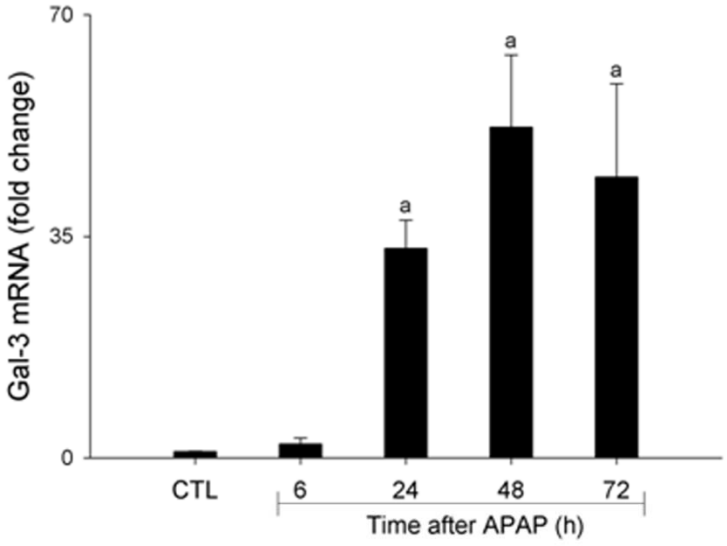
TNF-α signaling via TNFR1 plays a key role in limiting the production of proinflammatory mediators and promoting antioxidant generation and tissue repair in the liver following APAP-induced toxicity (Chiu *et al.*, 2003a; Chiu *et al.*, 2003b; Gardner *et al.*, 2003). Consistent with this activity is our finding that APAP administration to wild type mice resulted in increased expression of TNFR1, which paralleled the development of hepatotoxicity. Surprisingly, reduced hepatotoxicity in Gal-3$^{-/-}$ mice was correlated with decreased levels of TNFR1, potentially reflecting the reduced need for activation of protective signaling pathways in these mice. We previously reported that loss of TNFR1 results in an exaggerated hepatotoxic response to APAP (Chiu *et al.*, 2003a; Chiu *et al.*, 2003b; Gardner *et al.*, 2003). The present studies show that this is associated with increased Gal-3 expression in the liver; these data suggest that Gal-3 contributes to the increased susceptibility of TNFR1$^{-/-}$ mice to APAP. This is likely due to increased production of cytotoxic and proinflammatory mediators by Gal-3-positive activated

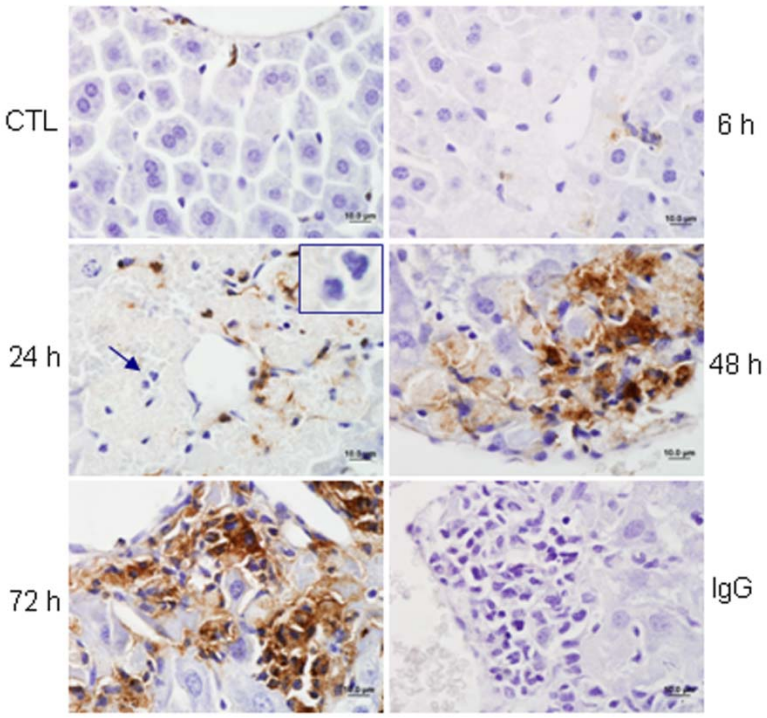
macrophages. In contrast to loss of Gal-3, loss of TNFR1 had no effect on APAP-induced expression of CD98. These results indicate that Gal-3 availability, and not receptor expression levels, is critical for the development of toxicity. It may also be that a different receptor mediates Gal-3-dependent responses in these mice.

Cyp2e1-dependent metabolism of APAP to NAPQI is a critical step in the onset of hepatotoxicity (Lee *et al.*, 1996; Potter *et al.*, 1973). Glutathione plays an important role in limiting toxicity by forming a non-reactive conjugate with NAPQI (Mitchell *et al.*, 1973b). Our data demonstrate that loss of Gal-3 did not alter hepatic Cyp2e1 activity or result in changes in hepatic glutathione levels in response to APAP. Taken together, these findings indicate that reduced susceptibility of Gal-3$^{-/-}$ mice to APAP-induced hepatotoxicity is not due to effects on NAPQI formation or inactivation.

In summary, the present studies identify Gal-3 as a novel regulator of late inflammatory responses in the liver following APAP intoxication and an important contributor to hepatotoxicity. Further studies are needed to identify the mechanisms involved in Gal-3-dependent inflammatory responses during the pathogenesis of APAP-induced liver injury.

FIGURE 7. Effects of APAP intoxication on Gal-3 expression. Livers were collected 6-72 h after treatment of wild type mice with APAP (300 mg/kg, i.p.) or control (CTL). Upper panel. Gal-3 expression was analyzed by RT-PCR. Data were normalized to 18s RNA. Each bar represents the mean ± SE (n = 3-10 mice). [a]Significantly different ($p<0.05$) from CTL. Middle panel. Sections were stained with anti-Gal-3 antibody or IgG control, as described in Materials and Methods. One representative section from three independent experiments is shown. Original magnification, 100x. Inset, neutrophils. Lower panel. Serum was collected 6-72 h after treatment of wild type mice with APAP or control (CTL). Gal-3 expression was analyzed by western blotting. One representative blot from five independent experiments is shown.


70
35
0
Gal-3 mRNA (fold change)
a
a
a
CTL
6
24
48
72
Time after APAP (h)


CTL
6 h
24 h
48 h
72 h
IgG

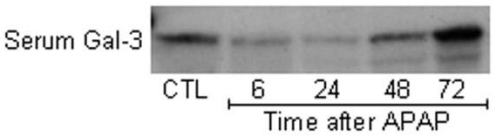

Serum Gal-3
CTL
6
24
48
72
Time after APAP

FIGURE 8. Effects of loss of Gal-3 on APAP-induced structural alterations in the liver. Livers were collected 6-72 h after treatment of wild-type and Gal-$3^{-/-}$ mice with APAP or control (CTL). Sections were stained with hematoxylin and eosin. One representative section from three independent experiments is shown. Original magnification, 20x. Insets, 60x.

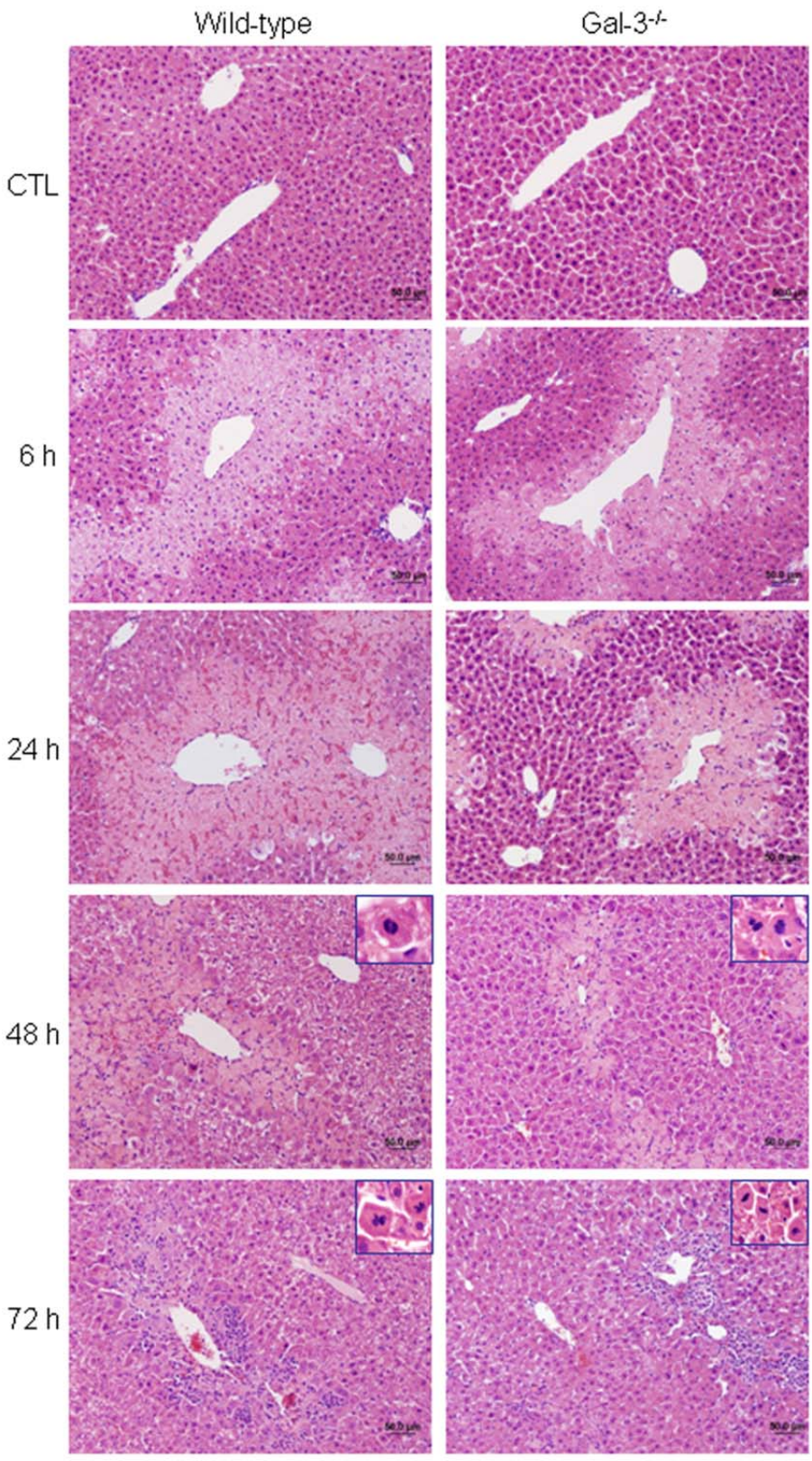

Wild-type
Gal-3-/-
CTL
6 h
24 h
48 h
72 h

FIGURE 9. Effects of loss of Gal-3 on APAP-induced neutrophil emigration into the liver. Livers were collected 24-72 h after treatment of wild-type and Gal-$3^{-/-}$ mice with APAP or control (CTL). Sections were stained with anti-myeloperoxidase antibody. One representative section from two independent experiments is shown.

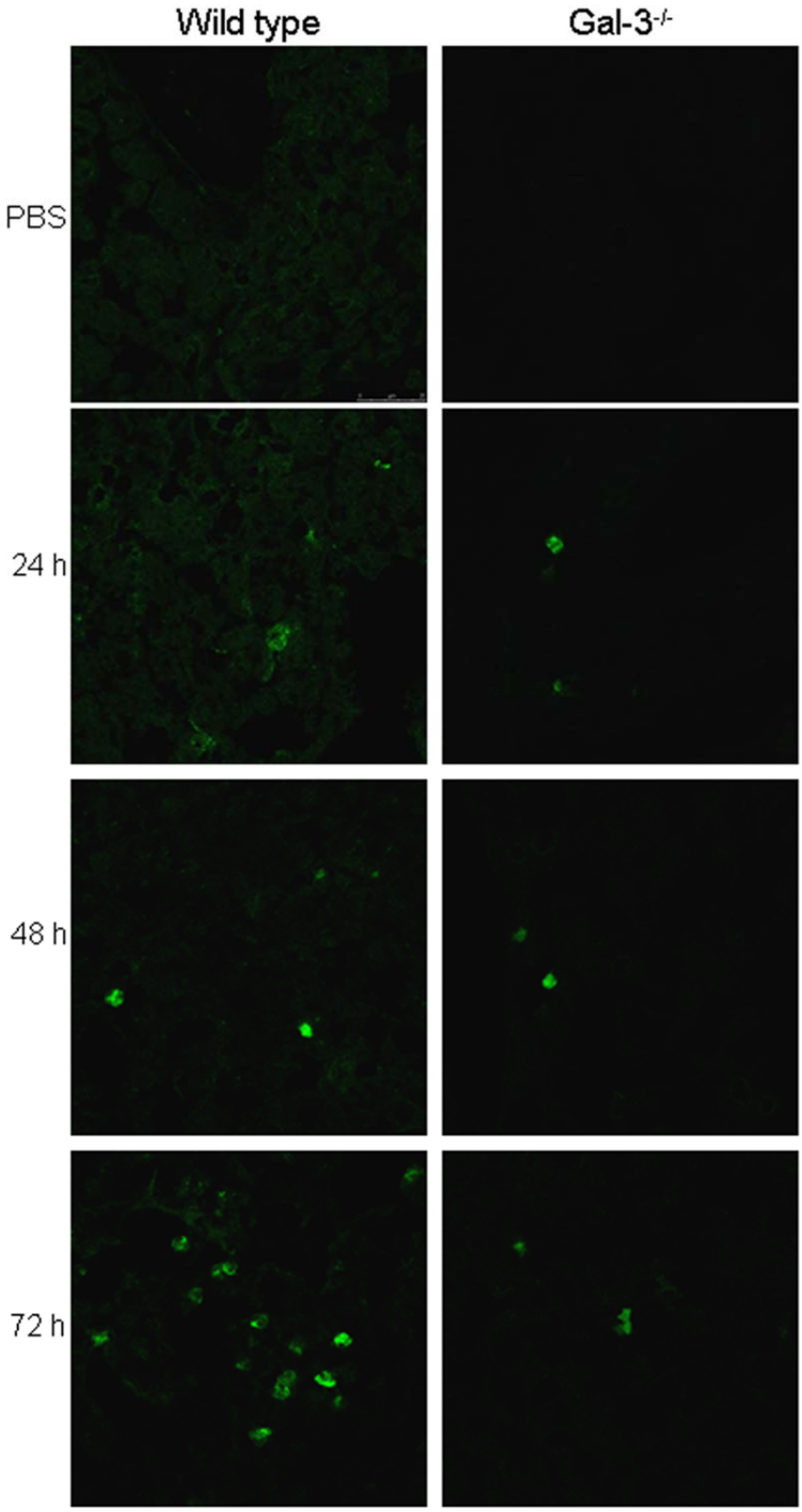

Wild type
Gal-3-/-
PBS
24 h
48 h
72 h

FIGURE 10. Effects of loss of Gal-3 on APAP-induced expression of inflammatory markers. Livers were collected from wild type and Gal-$3^{-/-}$ mice 6-72 h after treatment with APAP or control (CTL). Samples were analyzed by RT-PCR. Each bar represents the mean ± SE (n = 3-8 mice). [a]Significantly different ($p<0.05$) from CTL. [b]Significantly different ($p<0.05$) from wild type mice.

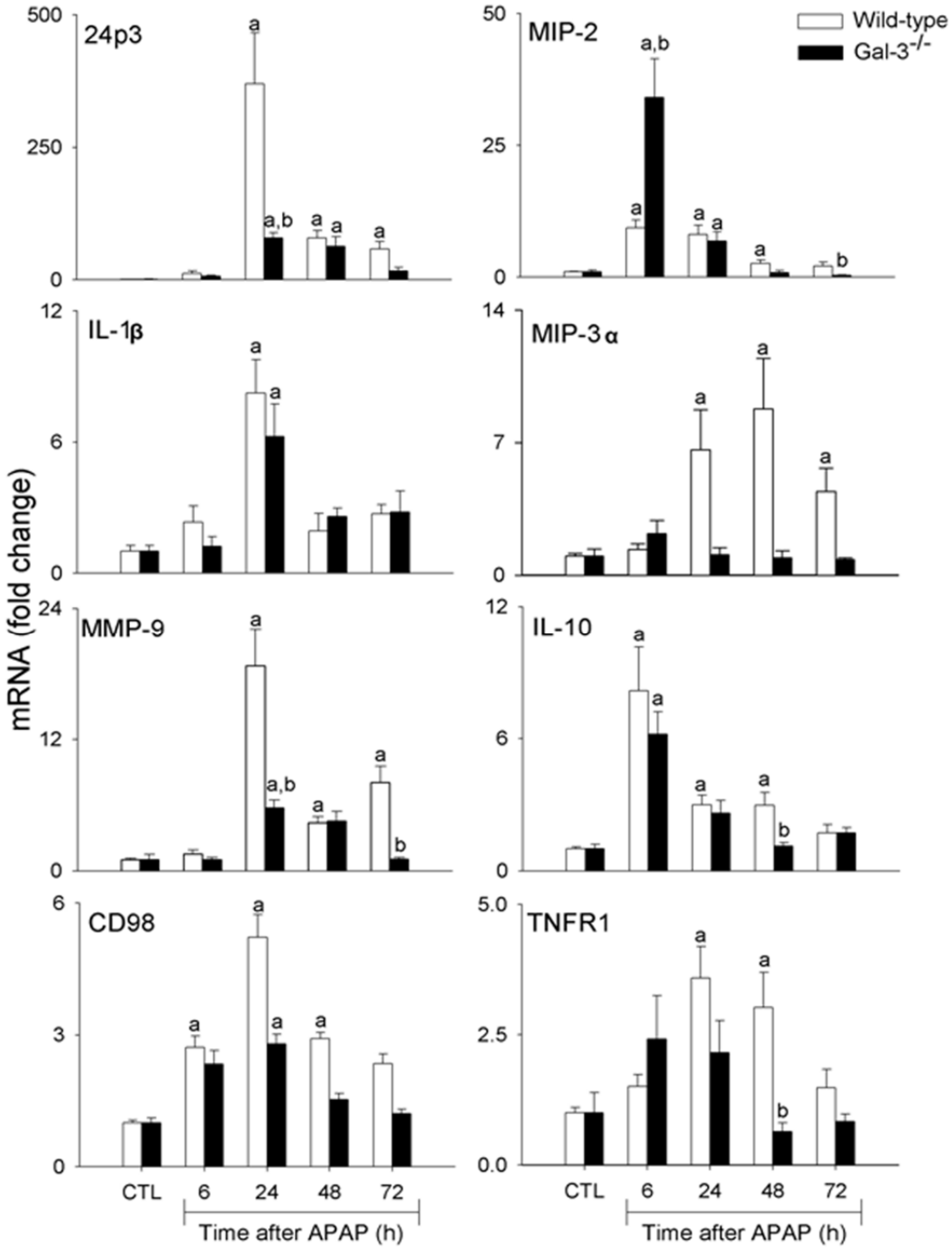

24p3
MIP-2
IL-1β
MIP-3α
MMP-9
IL-10
CD98
TNFR1
Wild-type
Gal-3-/-
mRNA (fold change)
CTL
6
24
48
72
Time after APAP (h)

FIGURE 11. Effects of loss of Gal-3 on APAP-induced expression of iNOS and COX-2. Livers were collected from wild type and Gal-$3^{-/-}$ mice 6-72 h after treatment with APAP or control (CTL). iNOS (*upper panel*) and COX-2 (*lower panel*) expression were analyzed by western blotting. Densitometric analysis was performed using ImageJ. Each bar represents the mean ± SE (n = 3-5 mice). [a]Significantly different ($p<0.05$) from CTL. [b]Significantly different ($p<0.05$) from wild type mice.

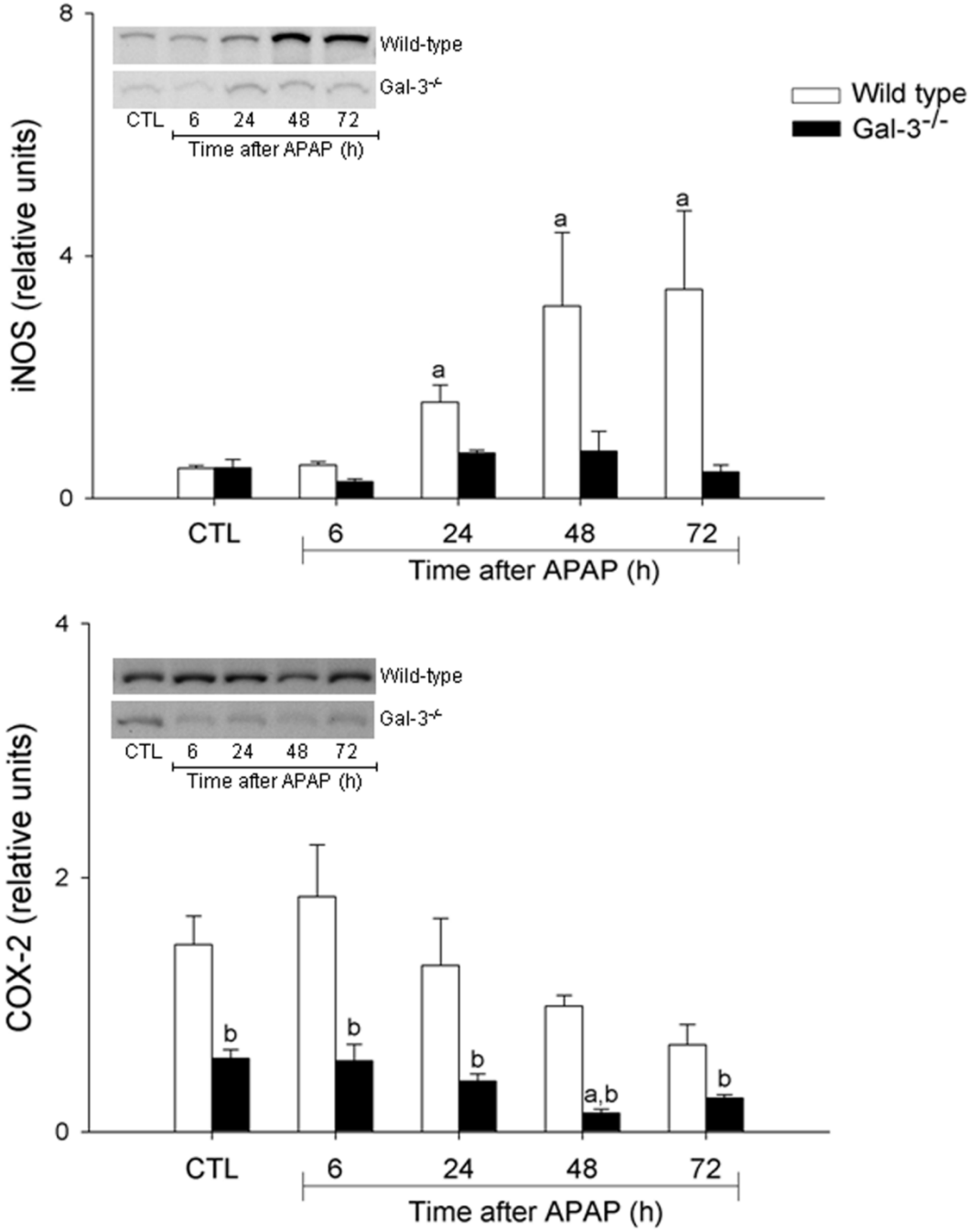

8
4
0
iNOS (relative units)
Wild-type
Gal-3-/-
CTL 6 24 48 72
Time after APAP (h)
Wild type
Gal-3-/-
a
a
a
CTL
6
24
48
72
Time after APAP (h)
4
2
0
COX-2 (relative units)
Wild-type
Gal-3-/-
CTL 6 24 48 72
Time after APAP (h)
b
b
b
a,b
b
CTL
6
24
48
72
Time after APAP (h)

FIGURE 12. Effects of loss of TNFR1 on APAP-induced Gal-3 and CD98 mRNA expression. Livers were collected from wild type and TNFR1$^{-/-}$ mice 6-72 h after treatment with APAP or control (CTL). Samples were analyzed by RT-PCR. Each bar represents the mean ± SE (n = 3-5 mice). [a]Significantly different ($p<0.05$) from CTL. [b]Significantly different ($p<0.05$) from wild type mice.

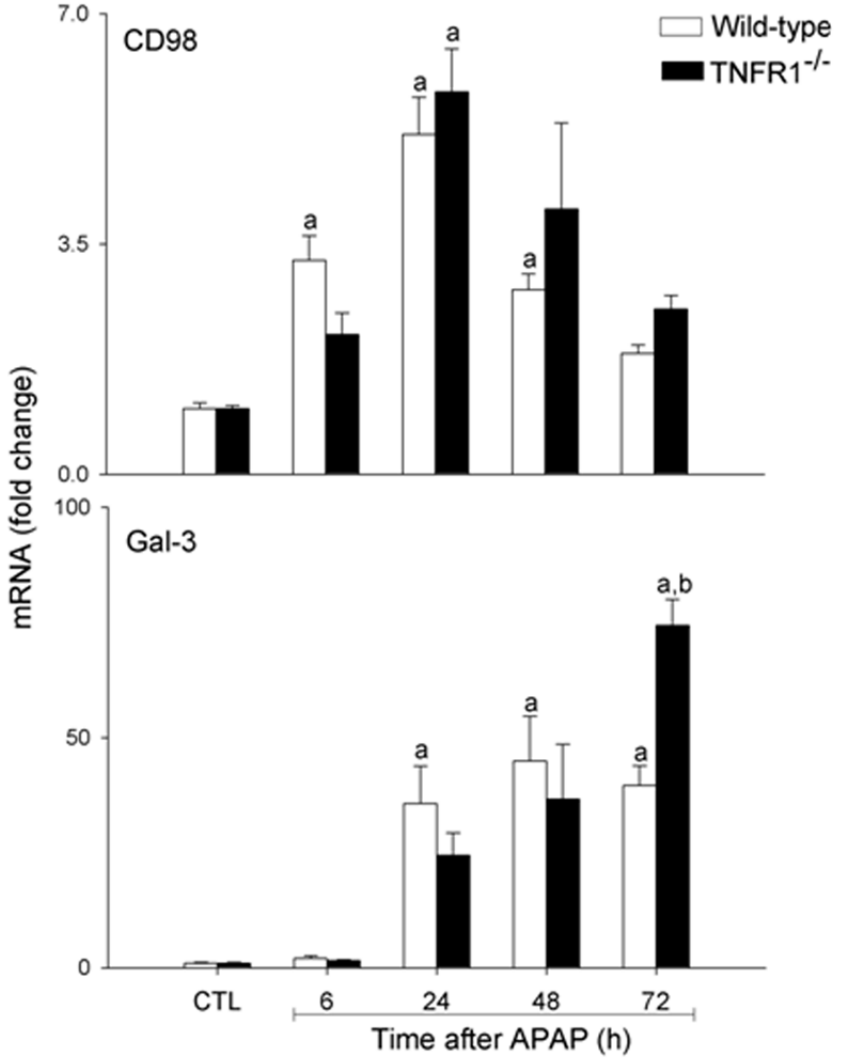

CD98
Wild-type
TNFR1-/-
7.0
3.5
0.0
a
a
a
a
Gal-3
100
50
0
a
a
a
a,b
mRNA (fold change)
CTL
6
24
48
72
Time after APAP (h)

FIGURE 13. Effects of loss of TNFR1 on APAP-induced Gal-3 protein expression. Livers were collected 6-72 h after treatment of wild type and TNFR1$^{-/-}$ mice with APAP or control (CTL). Sections were stained with anti-Gal-3 antibody as described in Materials and Methods. One representative section from three independent experiments is shown. Original magnification, 20x.

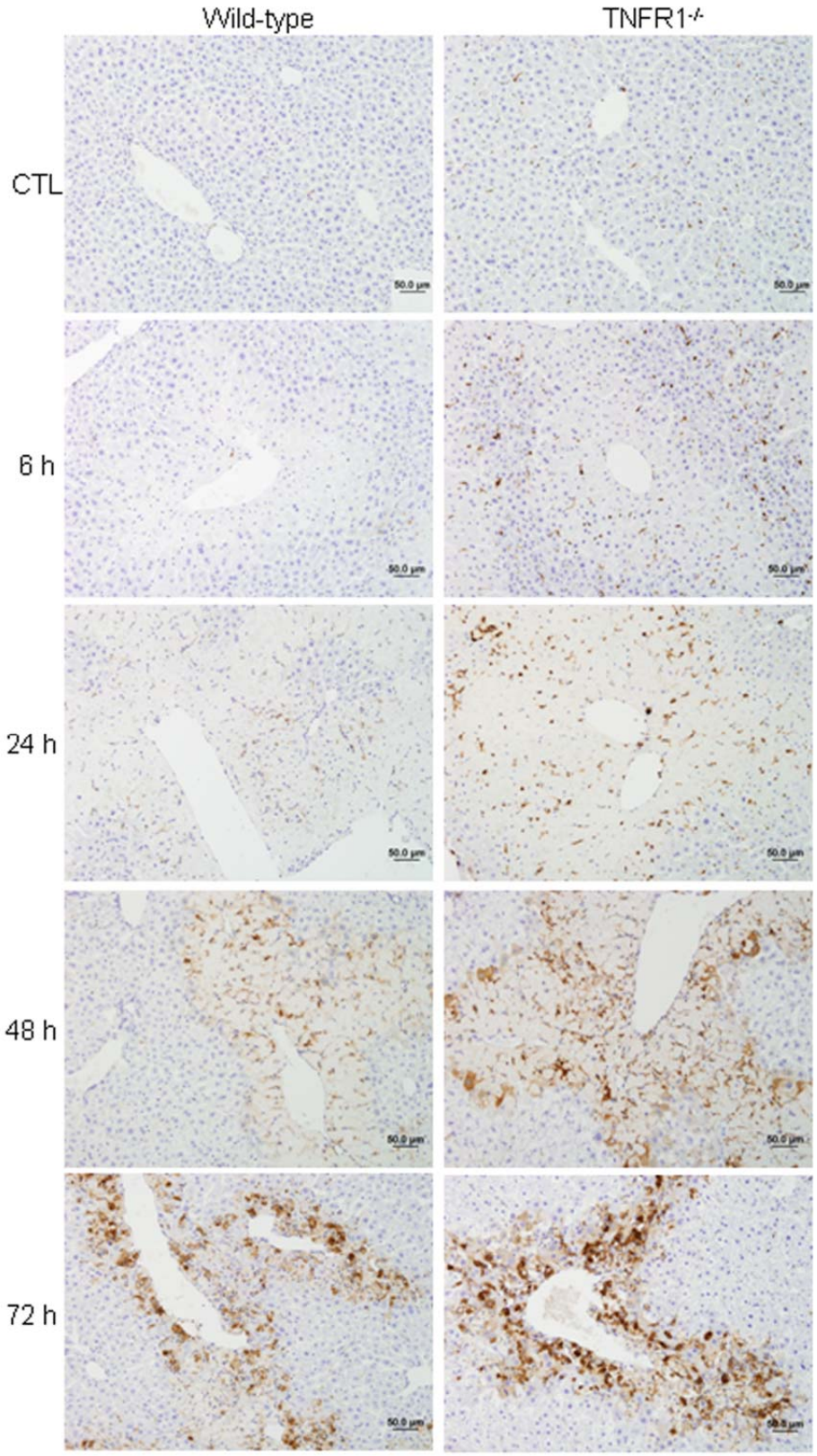

Wild-type
TNFR1-/-
CTL
6 h
24 h
48 h
72 h

FIGURE 14. Effects of APAP on hepatic glutathione levels. Livers were collected from wild type and Gal-3$^{-/-}$ mice 3-72 h after treatment with APAP or control (CTL) and total glutathione levels assayed. Each bar represents the mean ± SE (n = 3-6 mice). [a]Significantly different ($p<0.05$) from CTL.

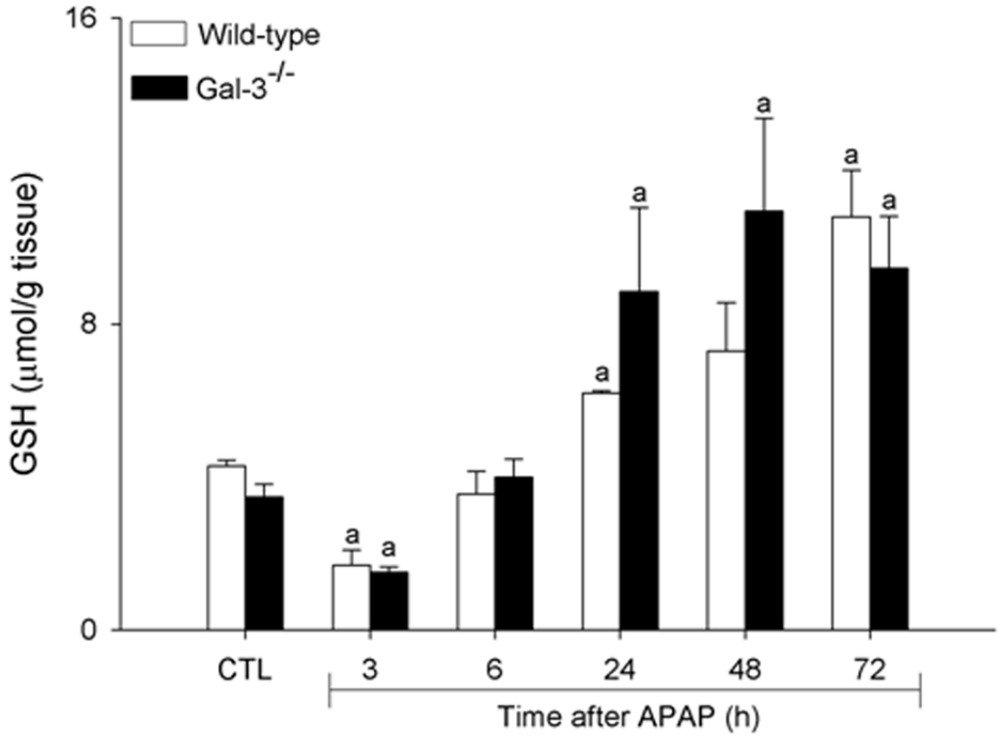

16
8
0
GSH (μmol/g tissue)
Wild-type
Gal-3-/-
a
CTL
3
6
24
48
72
Time after APAP (h)

TABLE 5. Histopathological evaluation of hepatic necrosis and neutrophilic infiltrates in wild type and Gal-3$^{-/-}$ mice after APAP administration. Wild type and Gal-3$^{-/-}$ mice were treated with 300 mg/kg APAP or PBS control. Liver sections were prepared 6 to 72 h later for histopathological analysis. Findings were graded on a scale of 0 to 4, where 0 = absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe changes. Data are expressed as mean $\pm$ SE (n = 3 mice).

Time (h)	**Necrosis**		**Neutrophils**	
	Wild type	Gal-3$^{-/-}$	Wild type	Gal-3$^{-/-}$
CTL	0	0	0	0
6	2.0 ± 0.0	2.0 ± 0.0	0	0
24	3.0 ± 0.0	2.7 ± 0.3	1.0 ± 0.0	1.3 ± 0.3
48	3.0 ± 0.0	2.0 ± 0.6	2.0 ± 0.0	2.0 ± 0.6
72	2.3 ± 0.3	0.3 ± 0.3	3.3 ± 0.3	0.7 ± 0.3

TABLE 6. Effects of APAP on serum transaminases in wild type and Gal-3$^{-/-}$ mice. Wild type and Gal-3$^{-/-}$ mice were treated with 300 mg/kg APAP or PBS control. Sera were collected 6 to 72 h later and analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Data are expressed as mean $\pm$ SE (n = 5-12 mice). [a]Significantly different ($p<0.05$) from PBS. [b]Significantly different from wild type.

Time (h)	**ALT (U/L)**		**AST (U/L)**	
	Wild type	Gal-3$^{-/-}$	Wild type	Gal-3$^{-/-}$
CTL	32.1 ± 2.6	45.4 ± 7.2	60.6 ± 5.6	82.5 ± 10.6
3	3087.3 ± 440.2	3336.8 ± 768.0	4290.0 ± 759.0	3711.4 ± 513.5
6	7797.0 ± 508.1[a]	6363.1 ± 1266.9	7473.0 ± 481.8[a]	7711.1 ±1917.4[a]
24	10905.8 ± 825.5[a]	7673.6 ± 1022.9[a,b]	6391.1 ± 911.4[a]	3908.2 ± 723.6[a]
48	1194.0 ± 126.1[a]	454.2 ± 53.5[b]	522.9 ± 51.8[a]	265.7 ± 26.5[b]
72	247.6 ± 41.4	116.6 ± 15.1[b]	261.4 ± 29.3	93.8 ± 15.4[b]

PART III. ROLE OF GALECTIN-3 IN CLASSICAL AND ALTERNATIVE MACROPHAGE ACTIVATION DURING APAP-INDUCED HEPATOTOXICITY

Activated macrophages contribute to the pathogenic response to APAP. However, the role of these cells in APAP hepatotoxicity depends on their origin, the timing of their appearance in the liver, and the inflammatory mediators they encounter, which control their phenotype and function. Based on studies using macrophage inhibitors and transgenic mice, two subpopulations of macrophages have been identified in the liver after APAP intoxication that play distinct roles in hepatotoxicity: classically activated proinflammatory/cytotoxic macrophages, and alternatively activated anti-inflammatory/wound repair macrophages (Laskin *et al.*, 1995; Ju *et al.*, 2002; Dambach *et al.*, 2002; Holt *et al.*, 2008; Gardner *et al.*, 2012). It appears that the outcome of tissue injury depends on which macrophage subpopulation predominates. Thus, hepatotoxicity results from exaggerated or persistent responses of classically activated macrophages, whereas hepatoprotection is associated with increases in numbers of alternatively activated macrophages (reviewed in Laskin *et al.*, 2011). The mechanisms regulating classical and alternative macrophage activation in the liver after APAP intoxication have not been established.

Gal-3 is a β-galactoside binding lectin secreted by macrophages in response to LPS, TNF-α, or IFN-γ (Liu *et al.*, 1995; Nishi *et al.*, 2007). Gal-3 acts in an autocrine and paracrine manner to promote macrophage release of proinflammatory mediators, including TNF-α, IL-12, CCL3, and CCL4, as well as reactive nitrogen species generated via iNOS (Liu *et al.*, 1995; Nishi *et al.*, 2007; Papaspyridonos *et al.*, 2008; Jeon *et al.*, 2010). Loss of Gal-3 has been reported to result in reduced susceptibility to antigen-induced arthritis, renal ischemia-reperfusion injury, hypoxic-ischemic brain injury, and

concanavalin A-induced hepatotoxicity, pathologies associated with exaggerated proinflammatory mediator activity (Forsman *et al.*, 2011; Fernandes Bertocchi *et al.*, 2008; Doverhag *et al.*, 2010; Volarevic *et al.*, 2012). These findings led us to hypothesize that Gal-3 plays a role in promoting classical macrophage activation and inflammatory mediator production in the liver following APAP intoxication. This is supported by our findings that loss of Gal-3 results in reduced hepatotoxicity and inflammatory mediator production in response to APAP (Dragomir *et al.*, 2012). In the present studies, we extended these observations and characterized the role of Gal-3 in regulating the phenotype of hepatic macrophage subpopulations accumulating in the liver during APAP-induced hepatotoxicity.

RESULTS

Effects of loss of Gal-3 on expression of classical and alternative activation markers in the liver following APAP intoxication

Treatment of mice with APAP resulted in a time-dependent increase in Gal-3 protein expression in the liver, which was evident within 24 h and persisted for at least 72 h (Fig. 15, upper and middle panel). Immunohistochemical analysis revealed that Gal-3 was localized in macrophages infiltrating into the necrotic areas of the liver (Fig. 15, lower panel and Dragomir *et al.*, 2012). Consistent with the proinflammatory activity of these cells (Laskin, 2009), we found that loss of Gal-3 blunted the effects of APAP on hepatic expression of iNOS, IL-12, and TNF-α (Fig. 16), mediators known to be generated by classically activated macrophages (Laskin, 2009). Whereas IL-12 suppression was evident at 24 h and 48 h post-APAP, iNOS and TNFα were reduced at 48 h and 72 h. In contrast, expression of the anti-inflammatory lectin, Gal-1, which increased 48-72 h after APAP intoxication, was not altered by loss of Gal-3 (Fig. 16). The effects of loss of Gal-3 on expression of Ym1 and Fizz-1, markers of alternatively activated anti-inflammatory macrophages (Raes *et al.*, 2002), were also analyzed. In wild type mice, APAP administration resulted in increased mRNA and protein expression of Ym1, which peaked after 24-48 h (Figs. 16 and 17). Whereas in control mice Ym1 was expressed in hepatic sinusoidal endothelial cells, after APAP administration Ym1 was upregulated in macrophages (Fig. 17). This was first evident 48 h post-APAP, and was correlated with decreased Ym1 expression in endothelial cells. Loss of Gal-3 resulted in a more rapid and abundant increase in Ym1-positive macrophages in the liver, which was apparent within 24 h, and remained elevated for at least 72 h. There was also a trend towards increased Ym1 mRNA expression in Gal-3$^{-/-}$ mice (Fig. 16). APAP administration also resulted in upregulation of Fizz-1 mRNA expression in Gal-3$^{-/-}$ mice after 48-72 h, with no effect on wild type mice (Fig. 16).

Effects of loss of Gal-3 on APAP-induced expression of chemokines and chemokine receptors

In further studies we determined if Gal-3 plays a role in recruiting macrophages into the liver by analyzing APAP-induced expression of chemokines and chemokine receptors. In wild type mice, increases in mRNA expression of CCL2/CCR2, CCL3/CCR1, CCL4/CCR5, and CX3CL1/CX3CR1 were noted 24-72 h after APAP intoxication (Fig. 18). Loss of Gal-3 reduced the effects of APAP on expression of CCL2/CCR2 and CCL3/CCR1 at 72 h post-treatment, while CCL4 expression increased. CX3CL1/CX3CR1 expression was also significantly increased in Gal-$3^{-/-}$ mice relative to wild type 24 h post-APAP; however, by 72 h, CX3CR1 was decreased in these mice (Fig. 18).

Distinct macrophage subpopulations accumulate in the liver following APAP intoxication

In our next series of studies we characterized the phenotype of macrophages accumulating in the liver after APAP intoxication and the role of gal-3 in this response using techniques in flow cytometry/cell sorting. CD11b is the alpha chain of the Mac-1 integrin expressed on myeloid cells (Dziennis *et al.*, 1995). APAP administration resulted in a time-related increase in $CD11b^+$ cells in the liver (Fig. 19, panels A and D). This was evident within 24 h and persisted for at least 72 h after APAP. To further characterize these cells, we analyzed their expression of Ly6C, a surface antigen present at high levels on proinflammatory monocytes/macrophages (Robbins and Swirski, 2010). In livers of both control and APAP-treated mice, two distinct subpopulations of $CD11b^+$ cells were identified based on their expression of Ly6C. These consisted of $Ly6C^{lo}$ cells and $Ly6C^{hi}$ cells (Fig. 19, panel A). In wild type mice, the majority of $CD11b^+$ cells

expressed low levels of Ly6C. Following APAP administration, time-related increases in both $Ly6C^{lo}$ and $Ly6C^{hi}$ subpopulations were observed. Similar time-related increases in cells expressing Ly6C were observed in histological sections following APAP administration (Fig. 20). These cells were mainly noted in centrilobular regions of the liver and were distinct from $F4/80^{+}$ resident Kupffer cells. In contrast to $Ly6C^{+}$ cells, $F4/80^{+}$ macrophages decreased in areas surrounding the central veins following APAP administration, a response which persisted for 48 h; subsequently, they began to reappear.

Characterization of $Ly6C^{hi}$ and $Ly6C^{lo}$ macrophages accumulating in the liver following APAP intoxication

In further studies, $Ly6C^{lo}$ and $Ly6C^{hi}$ cells were sorted and examined microscopically. Giemsa staining confirmed that these cells were mononuclear phagocytes. $Ly6C^{lo}$ cells were enlarged relative to $Ly6C^{hi}$ cells, and more irregularly shaped. These cells were also characterized by highly vacuolated cytoplasm and an increased cytoplasmic:nuclear ratio (Fig. 21, upper panel). By contrast, $Ly6C^{hi}$ cells were smaller and rounder than $Ly6C^{lo}$ cells. RT-PCR analysis of sorted cells revealed that $Ly6C^{hi}$ cells expressed increased levels of the proinflammatory proteins, TNF-α and iNOS, and the chemokine receptor CCR2, when compared to $Ly6C^{lo}$ cells (Fig. 21, lower panel). In contrast, mRNA expression of the anti-inflammatory cytokine IL-10 was reduced in $Ly6C^{hi}$ cells when compared to $Ly6C^{lo}$ cells.

Role of Gal-3 in activated macrophage phenotype following APAP intoxication

We next analyzed expression of Gal-3 in $Ly6C^{lo}$ and $Ly6C^{hi}$ subpopulations of liver macrophages. In control mice, the majority of Gal-3 expressing macrophages were $Ly6C^{lo}$ (Fig. 19, panel B). APAP administration resulted in an increase in the percentage

of Ly6C^{hi} cells expressing Gal-3. This response was time related, reaching a maximum 48 h post-APAP. In contrast, the percentage of Ly6C^{lo}/Gal-3^{+} cells declined 24-48 h after APAP, and began to recover by 72 h (Fig. 19, panel B).

We have previously reported that loss of Gal-3 results in reduced hepatotoxicity and inflammation following APAP intoxication (Dragomir *et al.*, 2012). In further studies we determined if this was associated with alterations in the macrophage subpopulations that accumulated in the liver in response to APAP. Whereas loss of Gal-3 had no effects on the total number of CD11b^{+} cells in the liver following APAP intoxication (Fig. 19, panels C and D), a significant decrease in CD11b^{+}/Ly6C^{hi} macrophages was observed. This was associated with an increase in CD11b^{+}/Ly6C^{lo} macrophages. Increased numbers of CD11b^{+}/Ly6C^{lo} cells were also noted in Gal-3$^{-/-}$ mice treated with control. Changes in the liver macrophage subpopulations in Gal-3$^{-/-}$ mice were correlated with increased repair of APAP-induced tissue injury. Thus, significant increases in hepatocyte proliferation, as indicated by mitotic index and the number of PCNA-positive hepatocytes, were observed in the livers of Gal-3$^{-/-}$ mice when compared to wild type mice (Figs. 22 and 23).

DISCUSSION

Classically and alternatively activated macrophage subpopulations are thought to play distinct roles in the pathogenesis of APAP-induced hepatotoxicity. Thus, while classically activated macrophages release cytotoxic/proinflammatory mediators which contribute to injury, alternatively activated macrophages promote tissue repair (reviewed in Laskin, 2009). The present studies demonstrate that Gal-3 contributes to the recruitment of proinflammatory/cytotoxic macrophages into the liver following APAP intoxication. These findings are important as they suggest a novel mechanism for macrophage activation during APAP-induced liver injury and may provide insight into new therapeutic approaches for treating APAP overdose.

In accord with our previous studies (Dragomir *et al.*, 2012), following APAP intoxication we observed an increase in Gal-3^{+} macrophages in the liver. The present studies demonstrate that these cells exhibited a classically activated proinflammatory phenotype. This is based on our findings that loss of Gal-3 results in reduced expression of the classical macrophage activation markers iNOS, TNF-α and IL-12 in the livers of mice treated with APAP, and decreased accumulation of proinflammatory $Ly6C^{hi}$ macrophages. The fact that this was correlated with an increase in anti-inflammatory $Ly6C^{lo}$ macrophages in the liver, and upregulation of Fizz-1 and Ym1, markers of alternatively activated macrophages, suggests a shift in the balance of macrophage subpopulations leading to accelerated tissue repair. This is supported by our observation that hepatocyte proliferation was increased in Gal-$3^{-/-}$ mice relative to wild type mice following APAP intoxication, and was associated with reduced hepatotoxicity (Dragomir *et al.*, 2012). These data provide additional evidence for the idea that there are multiple subpopulations of macrophages that play distinct roles in APAP-induced hepatotoxicity.

Consistent with previous reports, we found that APAP treatment of mice resulted in increased expression of TNF-α and iNOS (Gardner *et al.*, 1998; Gardner *et al.*, 2002).

These mediators, which are generated in large part by proinflammatory macrophages, have been shown to be important in the pathogenesis of APAP-induced hepatotoxicity (Blazka *et al.*, 1995; Gardner *et al.*, 2002; Bourdi *et al.*, 2002). IL-12, a potent inducer of IFN-γ, was also increased in the liver. Gal-3 has been reported to upregulate expression of TNF-α, iNOS, and IL-12 in primary microglia and human monocytes (Jeon *et al.*, 2010; Nishi *et al.*, 2007). Our findings that expression of these proinflammatory proteins is reduced in Gal-$3^{-/-}$ mice suggest that Gal-3 is important in regulating their activity. However, it remains to be determined if this is due to reduced secretion of Gal-3 by inflammatory macrophages or to decreased numbers of these cells in the liver. The observation that suppression of TNF-α and iNOS in Gal-$3^{-/-}$ mice is delayed for 48-72 h, a time coordinate with reduced Gal-3^{+} macrophage accumulation, suggests that infiltrating cells are a source of these mediators. Previous studies have demonstrated that reduced susceptibility to injury induced by streptozotocin, concanavalin A, or antigen-induced arthritis in Gal-$3^{-/-}$ mice is similarly associated with decreased expression of TNF-α, IL-12, and iNOS (Mensah-Brown *et al.*, 2009; Forsman *et al.*, 2011; Volarevic *et al.*, 2012). These data provide additional support for the idea that Gal-3 contributes to classical macrophage activation in this model.

Classically activated macrophages also produce chemokines such as CCL3 and CCL4, which recruit monocytes and macrophages to sites of damage via CCR1 and/or CCR5 (Mantovani *et al.*, 2004; Soehnlein and Lindbom, 2010). We found that CCL3 expression was increased following APAP administration to wild type mice, which is in accord with previous studies (Liu *et al.*, 2006; Bourdi *et al.*, 2007). CCL4, CCR1, and CCR5 expression also increased in the liver after APAP. Loss of Gal-3 resulted in decreased expression of CCL3, while CCL4 expression was delayed. These findings indicate that multiple chemokines and their receptors contribute to macrophage

recruitment to the injured liver after APAP administration and that Gal-3 is involved in regulating the expression of CCL3/CCR1 and CCL4. These results are consistent with reports that Gal-3 induces CCL3 and CCL4 expression in monocytes (Papaspyridonos *et al.*, 2008). Loss of Gal-3 also blunted the effects of APAP on expression of CCR1; in contrast, there were no effects on CCR5 expression. These data are consistent with the proinflammatory phenotype of macrophages accumulating in the liver following APAP intoxication. CCL2 and its receptor CCR2 have been demonstrated to be upregulated during APAP-induced hepatotoxicity and to play critical roles in hepatic recruitment of a subpopulation of macrophages which are key for liver repair (Hogaboam *et al.*, 2000; Dambach *et al.*, 2002; Holt *et al.*, 2008; Si *et al.*, 2010). As previously shown (Dambach *et al.*, 2002), we found that expression of CCL2 and CCR2 increased in the liver following APAP administration to wild type mice. The observation that this response was attenuated in Gal-$3^{-/-}$ mice 72 h after APAP may reflect a reduced need for tissue repair. A similar decrease in CCL2 expression has been described in Gal-$3^{-/-}$ mice following renal ischemia-reperfusion injury (Fernandes Bertocchi *et al.*, 2008).

The chemokine receptor CX3CR1 is highly expressed on antiinflammatory/wound repair monocytes and macrophages (Robbins and Swirski, 2010); the only known ligand, CX3CL1 (fractalkine) is produced primarily by hepatocytes and stellate cells following liver injury (Aoyama *et al.*, 2010; Karlmark *et al.*, 2010). A marked increase in expression of CX3CL1 and CX3CR1 was noted in Gal-$3^{-/-}$ mice treated with APAP, suggesting a potential mechanism underlying the recruitment of antiinflammatory/wound repair macrophages into the liver and decreased hepatotoxicity in these mice. This is supported by reports that CX3CL1 and CX3CR1 play a protective role during toxin A-induced enteritis and carbon tetrachloride-induced liver inflammation and fibrosis, an effect thought to be due to the recruitment of antiinflammatory, alternatively activated macrophages to site of infection/injury (Inui *et al.*, 2011; Aoyama *et al.*, 2010; Karlmark

et al., 2010). The fact that CX3CR1 expression was reduced in Gal-3$^{-/-}$ mice relative to wild type mice by 72 h after APAP may reflect their reduced need for antiinflammatory mediators.

Alternatively activated macrophages have been implicated in tissue repair following APAP intoxication (Ju *et al.*, 2002; Holt *et al.*, 2008; Si *et al.*, 2010; Gardner *et al.*, 2012). Consistent with earlier reports (Holt *et al.*, 2008), we found that APAP administration to wild type mice resulted in increased numbers of Ym1-positive macrophages in the liver. Expression of the antiinflammatory lectin, Gal-1, which has been suggested to promote alternative macrophage activation (Correa *et al.*, 2003; Cooper *et al.*, 2012; Liu and Rabinovich, 2010), also increased in wild type mice after APAP administration. In contrast, APAP had minimal effects on Fizz-1 expression in wild type mice. Differences in the kinetics of Ym1, Gal-1, and Fizz-1 expression likely reflect the accumulation of distinct subpopulations of alternatively activated macrophages in the liver in response to APAP. Loss of Gal-3 resulted in a more rapid appearance of Ym1-positive macrophages in the liver and a marked increase in Fizz-1 expression. These data, together with our findings of accelerated tissue repair in Gal-3$^{-/-}$ mice, suggest that Gal-3 is a negative regulator of alternative macrophage activation and provide further evidence for the proinflammatory role of Gal-3 during APAP-induced hepatotoxicity. This is supported by studies indicating that hepatoprotection in Gal-3$^{-/-}$ mice treated with concanavalin A is associated with increased numbers of alternatively activated macrophages in the liver (Volarevic *et al.*, 2012). APAP-induced Gal-1 expression was not altered by loss of Gal-3; this may be due to the compensatory upregulation of Gal-1 in sinusoidal endothelial cells, which are known to express Gal-1 (Lotan *et al.*, 1994).

Evidence suggests that classically and alternatively activated macrophages accumulating in the liver after APAP-induced injury arise from distinct precursors (Dambach *et al.*, 2002; Ju *et al.*, 2002; Holt *et al.*, 2008; Si *et al.*, 2010; Gardner *et al.*,

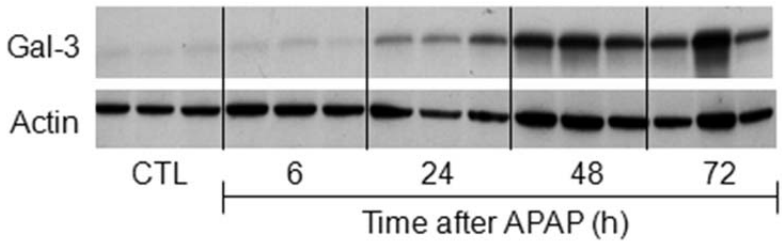
2012). The present studies provide additional support for this idea. Thus, we confirmed previous reports that APAP intoxication results in an accumulation of $CD11b^+$ macrophages in the liver that are distinct from resident Kupffer cells (Holt *et al.*, 2008). Moreover, we demonstrated that the $CD11b^+$ cells are heterogeneous with respect to their expression levels of Ly6C. Whereas the $CD11b^+/Ly6C^{hi}$ macrophages exhibit a proinflammatory phenotype, characterized by increased expression of TNF-α, iNOS, and CCR2, $CD11b^+/Ly6C^{lo}$ macrophages expressed increased levels of the anti-inflammatory cytokine, IL-10, suggesting that they are alternatively activated. Depletion of alternatively activated macrophages with clodronate liposomes is associated with a reduction in APAP-induced IL-10 expression in the liver and exaggerated hepatotoxicity (Ju *et al.*, 2002). These data, along with reports that IL-10 plays an important protective role following APAP intoxication (Bourdi *et al.*, 2002; Bourdi *et al.*, 2007), suggest that $CD11b^+/Ly6C^{lo}$ cells are key to tissue repair in this model. Both $Ly6C^{hi}$ and $Ly6C^{lo}$ macrophages were found to accumulate in the liver following APAP intoxication; the response of $Ly6C^{hi}$ was more rapid than $Ly6C^{lo}$ macrophages. Similar increases in $Ly6C^{hi}$ monocytes/macrophages have been reported to play a pathogenic role in injury and inflammation induced by high-fat diet and carbon tetrachloride, acute pancreatitis, and myocardial infarction (Deng *et al.*, 2009; Karlmark *et al.*, 2009; Perides *et al.*, 2011; Nahrendorf *et al.*, 2007).

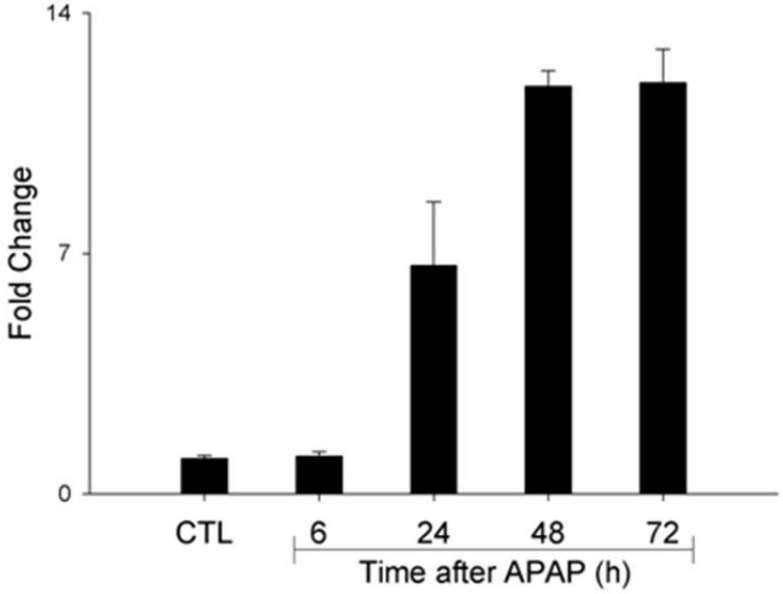
The present studies also show that $Ly6C^{hi}$ macrophages express Gal-3. Moreover, $Ly6C^{hi}$ influx into the liver is significantly reduced in Gal-$3^{-/-}$ mice treated with APAP. These findings are in accord with the proinflammatory role of Gal-3 in this model (Dragomir *et al.*, 2012). The fact that in control mice Gal-3 is expressed on $CD11b^+/Ly6C^{lo}$ cells is in agreement with reports that some liver resident macrophages express this protein (Nibbering *et al.*, 1987; Dragomir *et al.*, 2012). The persistence of Gal-3 expression on $Ly6C^{lo}$ cells following APAP intoxication may reflect the presence of

multiple subpopulations of activated macrophages in the liver. Of note is our observation that decreases in $CD11b^{+}/Ly6C^{hi}$ macrophages in Gal-$3^{-/-}$ mice are accompanied by increases in $Ly6C^{lo}$ antiinflammatory macrophages in both control and APAP-treated mice. These findings suggest that Gal-3 promotes the persistence of a proinflammatory classically activated macrophage phenotype in the liver. Gal-3 has been reported to regulate alternative macrophage activation in culture (MacKinnon *et al.*, 2008). Differences between our results may be due to the use of bone marrow-derived macrophages stimulated *in vitro* with cytokines versus primary cells isolated from treated mice. In this regard, Gal-3 expression and intracellular distribution have been shown to be altered when primary macrophages are cultured *in vitro* (Dumic *et al.*, 2000).

In summary, these studies identified and characterized multiple macrophage subpopulations present in the liver during APAP intoxication, and demonstrate a role for Gal-3 in promoting a persistent classical macrophage activation which contributes to hepatotoxicity. A more detailed understanding of the mechanisms regulating the phenotype of activated macrophages during APAP-induced liver injury may suggest novel approaches to mitigating toxicity caused by this analgesic.

FIGURE 15. Effects of APAP on Gal-3 expression in the liver. Livers were collected 6-72 h after treatment of wild type mice with APAP or control (CTL). *Upper panel.* Gal-3 expression was analyzed by western blotting. Actin was used as loading control. Each lane represents a different animal. *Middle panel.* Densitometric analysis was performed using ImageJ. Each bar represents the mean ± SE (n = 3 mice). *Lower panel.* Livers were collected 72 h after treatment of wild type mice with APAP or control. Gal-3 expression was analyzed by immunohistochemistry. One representative of three independent experiments is shown. Original magnification, 100x.


Gal-3
Actin
CTL
6
24
48
72
Time after APAP (h)


14
7
0
Fold Change
CTL
6
24
48
72
Time after APAP (h)

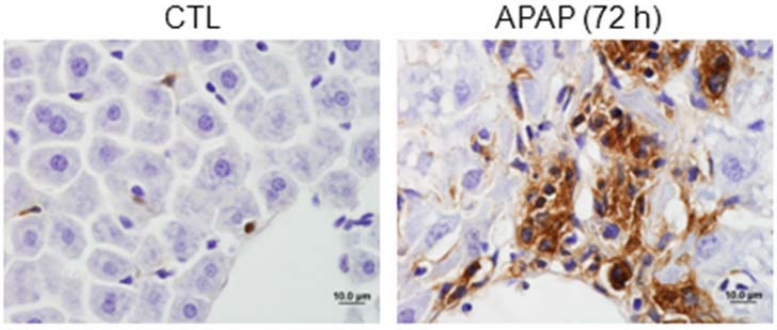

CTL
APAP (72 h)

FIGURE 16. Effects of APAP intoxication on expression of markers of classical and alternative macrophage activation. Liver samples, collected 24-72 h after treatment of wild type and Gal-3$^{-/-}$ mice with APAP or PBS control, were analyzed by RT-PCR. Data were normalized to 18s RNA and presented as fold change relative to control. Each bar represents the mean ± SE (n=3-8 mice). [a]Significantly different ($p<0.05$) from CTL. [b]Significantly different ($p<0.05$) from wild type.

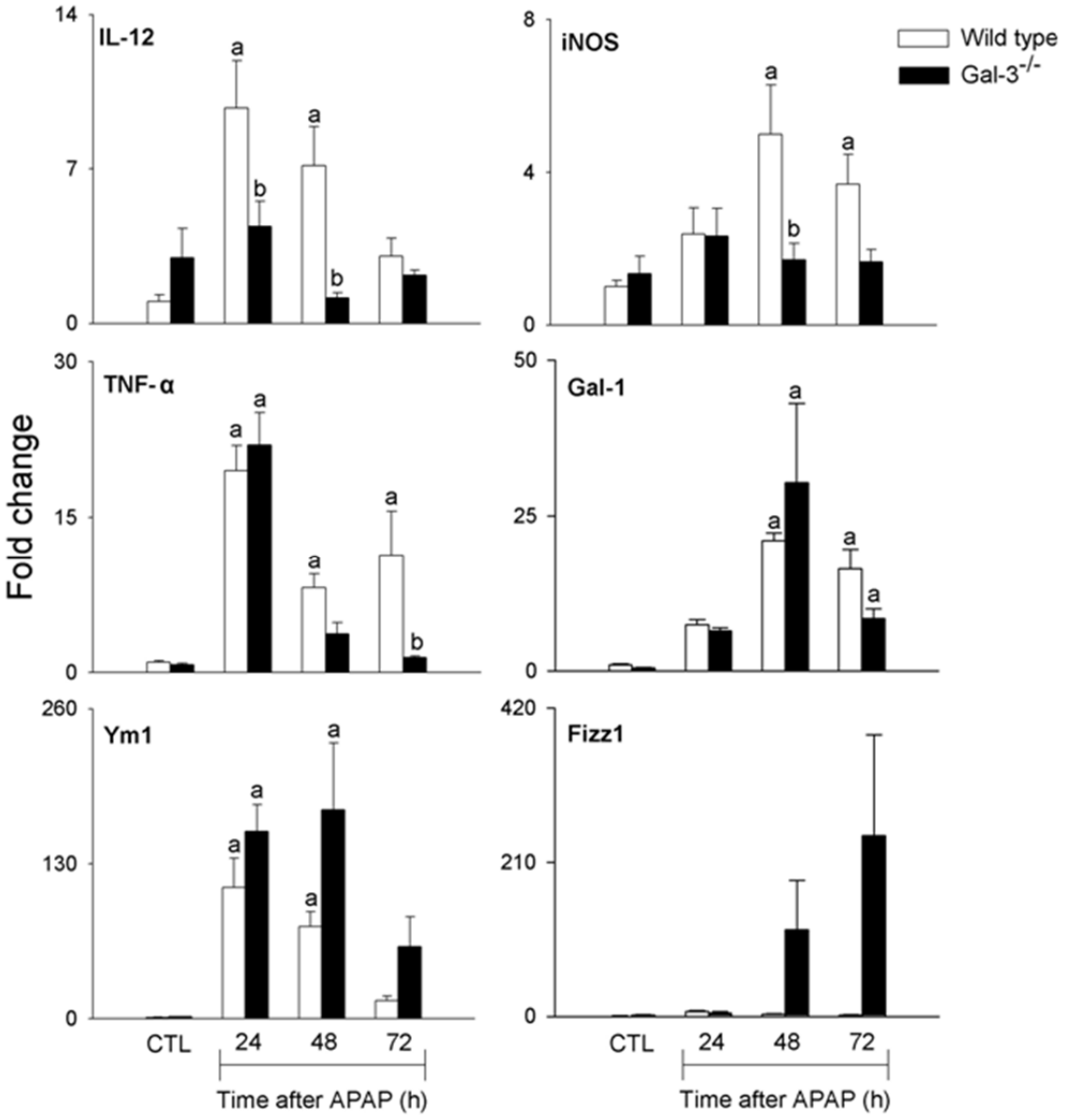

IL-12
iNOS
TNF-α
Gal-1
Ym1
Fizz1
Wild type
Gal-3-/-
Fold change
CTL
24
48
72
Time after APAP (h)

FIGURE 17. Effects of APAP intoxication on Ym1 expression. Liver sections, prepared 24 to 72 h after treatment of wild type (WT) and Gal-3$^{-/-}$ mice with APAP or PBS control, were stained with anti-Ym1 antibody or IgG control. Binding was visualized using a Vectastain Elite ABC kit, with 3-3'-diaminobenzidine as substrate. One representative section from three mice is shown. Original magnification, 40x. Insets, 100x.

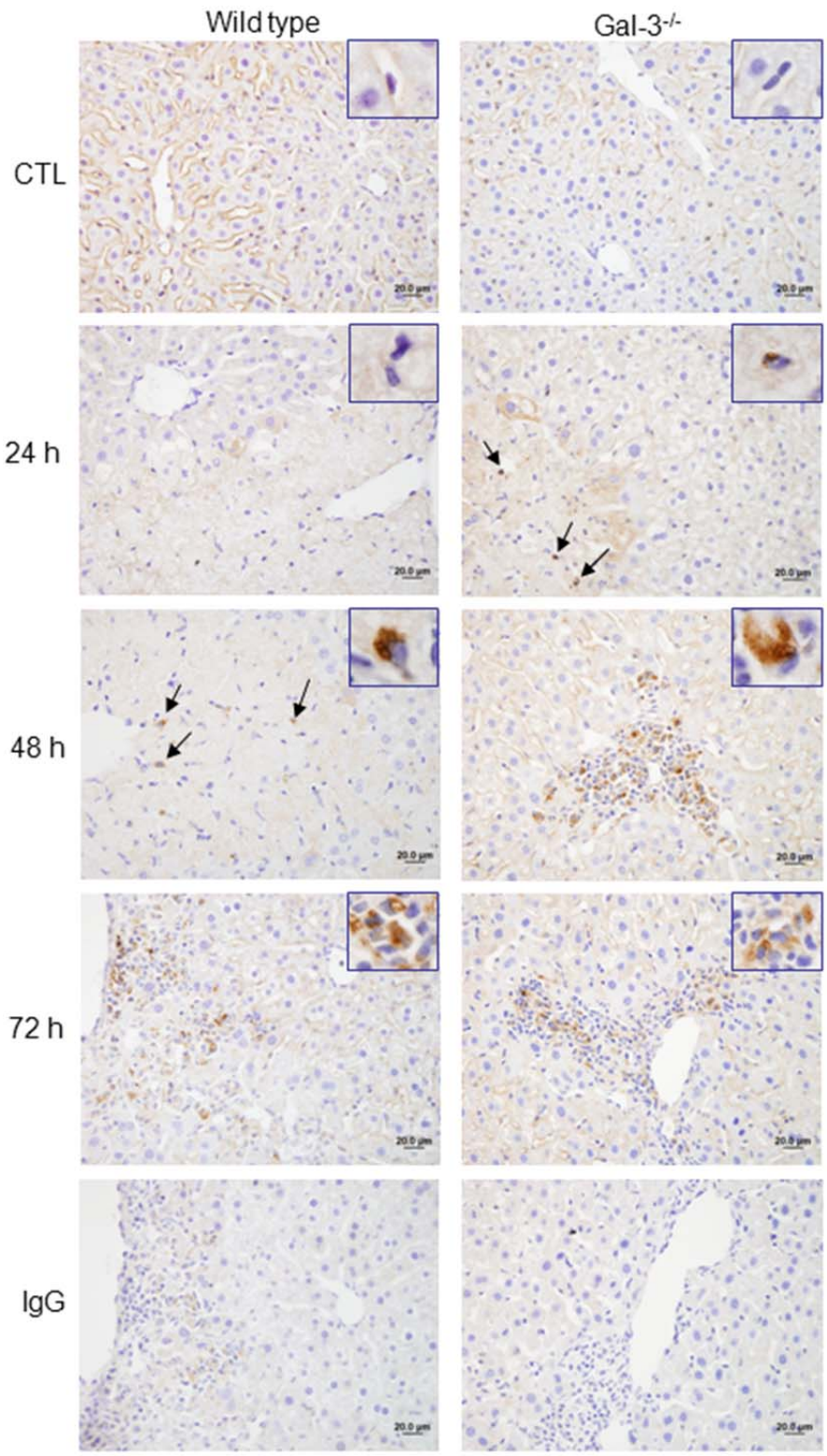

Wild type
Gal-3-/-
CTL
24 h
48 h
72 h
IgG

FIGURE 18. Effects of APAP intoxication on expression of chemokines and chemokine receptors. Liver samples, collected 24-72 h after treatment of wild type and Gal-3$^{-/-}$ mice with APAP or PBS control, were analyzed by RT-PCR. Data were normalized to 18s RNA and presented as fold change relative to PBS control. Each bar represents the mean ± SE (n=3-8 mice). [a]Significantly different ($p<0.05$) from CTL. [b]Significantly different ($p<0.05$) from wild type.

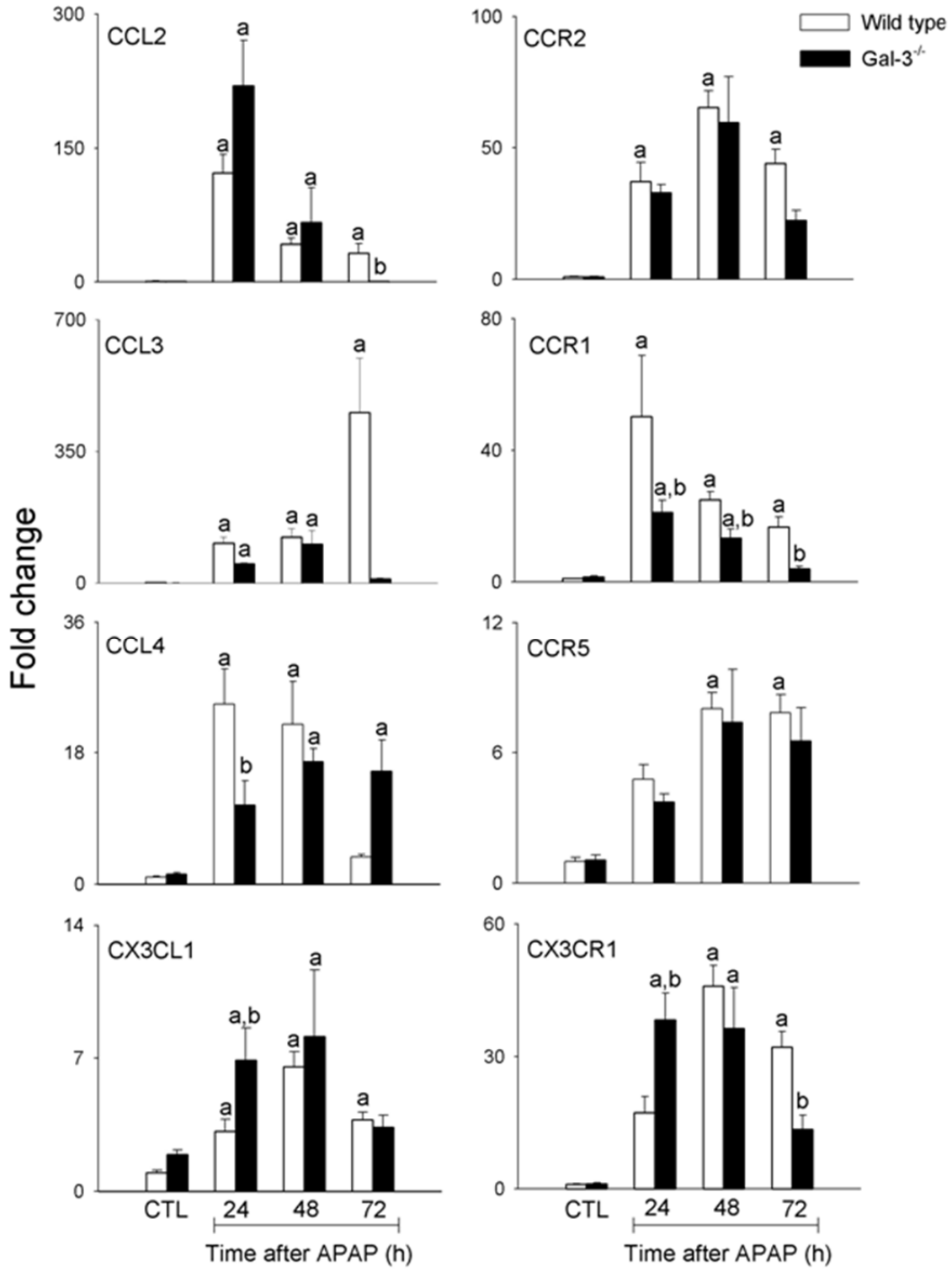

Wild type
Gal-3-/-
Fold change
CCL2
CCR2
CCL3
CCR1
CCL4
CCR5
CX3CL1
CX3CR1
CTL
24
48
72
Time after APAP (h)

FIGURE 19. Distinct macrophage subpopulations accumulate in the liver following APAP intoxication. Liver nonparenchymal cells were isolated 24-72 h after treatment of wild type mice with APAP (300 mg/kg, i.p.) or PBS control. *Panel A.* CD11b and Ly6C expression was analyzed by flow cytometry. *Panel B.* CD11b-positive cells were analyzed for expression of Ly6C and Gal-3 by flow cytometry. *Panel C.* Liver nonparenchymal cells were isolated 24-72 h after treatment of Gal-$3^{-/-}$ mice with APAP or CTL. CD11b and Ly6C expression was analyzed by flow cytometry. The average percentages of each cell population are indicated and are expressed as mean ± SE. One representative plot from 4-12 mice is shown. *Panel D.*The number of total $CD11b^{+}$ cells, $CD11b^{+}/Ly6C^{hi}$ cells, and $CD11b^{+}/Ly6C^{lo}$ cells isolated were calculated from the percentage-positive cells relative to the total number of liver nonparenchymal cells recovered. Bars represent the mean ± S.E. (n=4-12 mice). [a]Significantly different ($p<0.05$) from CTL. [b]Significantly different ($p<0.05$) from wild type.

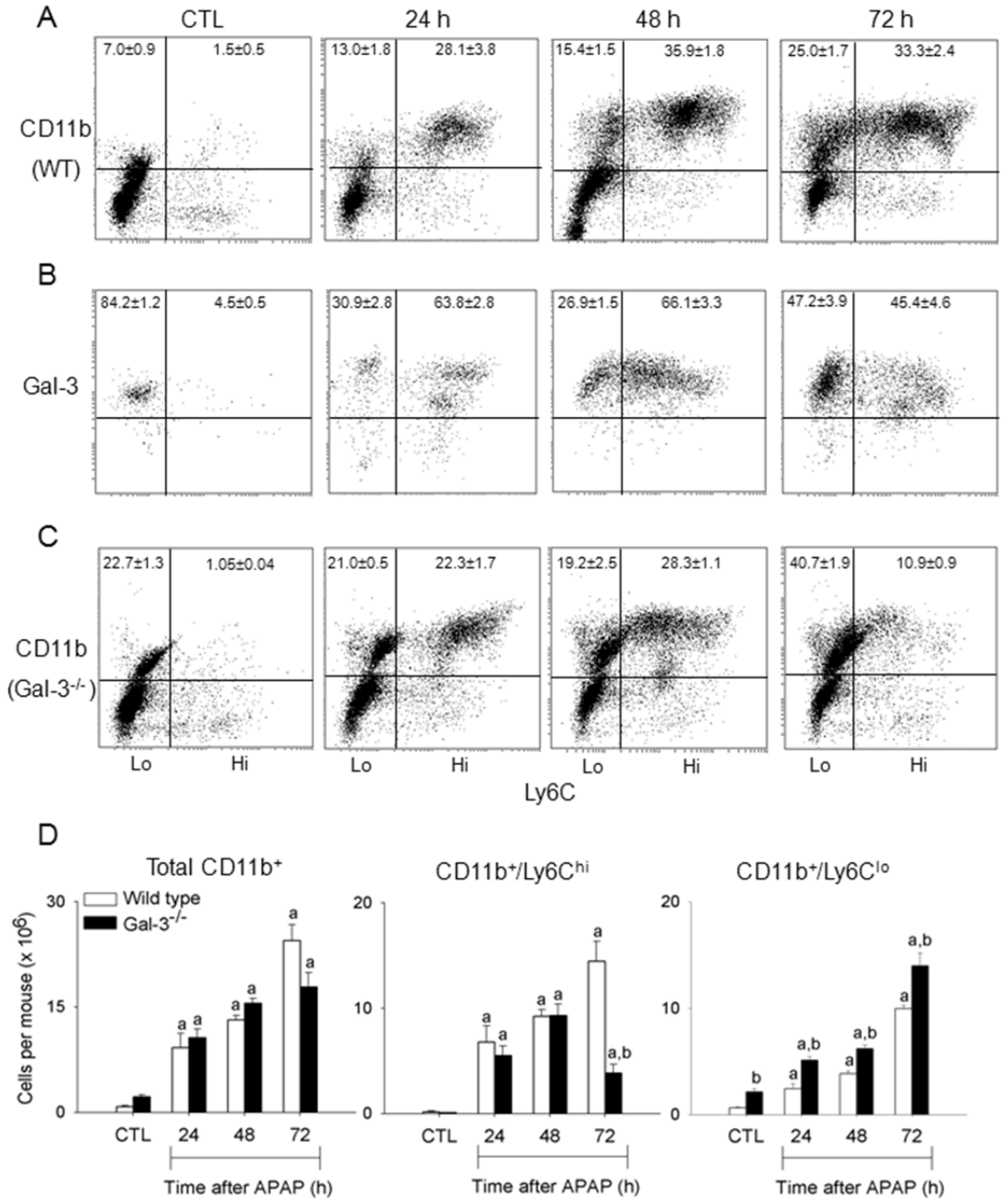

A
CTL
24 h
48 h
72 h
CD11b
(WT)
7.0±0.9
1.5±0.5
13.0±1.8
28.1±3.8
15.4±1.5
35.9±1.8
25.0±1.7
33.3±2.4
B
Gal-3
84.2±1.2
4.5±0.5
30.9±2.8
63.8±2.8
26.9±1.5
66.1±3.3
47.2±3.9
45.4±4.6
C
CD11b
(Gal-3-/-)
22.7±1.3
1.05±0.04
21.0±0.5
22.3±1.7
19.2±2.5
28.3±1.1
40.7±1.9
10.9±0.9
Lo
Hi
Ly6C
D
Total CD11b+
CD11b+/Ly6Chi
CD11b+/Ly6Clo
Wild type
Gal-3-/-
Cells per mouse (x 10^6)
CTL
24
48
72
Time after APAP (h)

FIGURE 20. Ly6C^{hi} macrophages accumulating in the liver after APAP intoxication are distinct from F4/80-positive resident macrophages. Liver sections were prepared 24-72 h after treatment of wild type mice with APAP or PBS control and stained with anti-Ly6C and anti-F4/80 antibodies, as described in Materials and Methods. Scale bar, 25 μm. One representative section from 3-4 mice is shown.

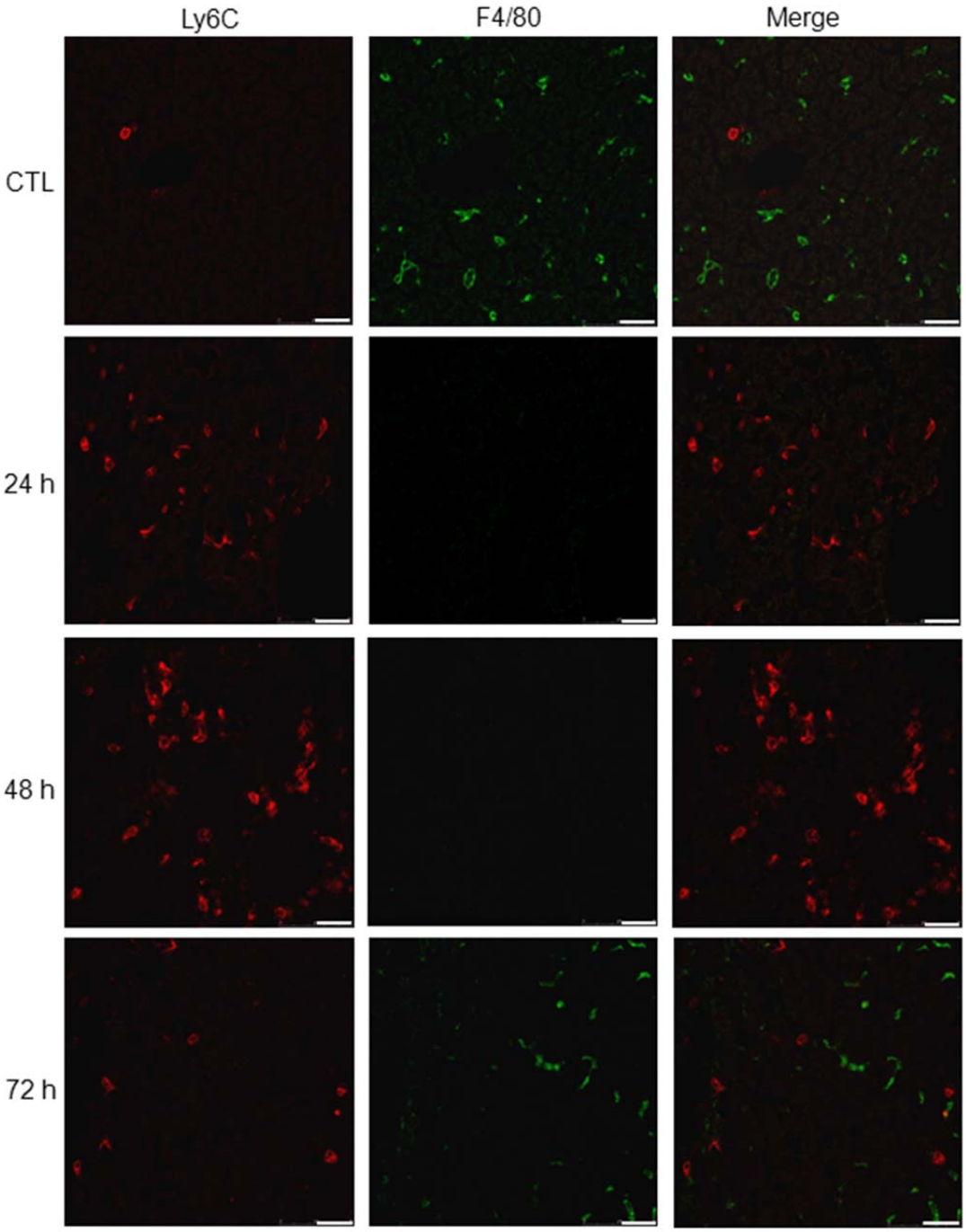

Ly6C
F4/80
Merge
CTL
24 h
48 h
72 h

FIGURE 21. Phenotype of Ly6C^{hi} and Ly6C^{lo} macrophages accumulating in the liver following APAP intoxication. Liver nonparenchymal cells were isolated 48 h after treatment of wild type mice with APAP or PBS control. CD11b^{+} were sorted based on expression of Ly6C into CD11b^{+}/Ly6C^{hi} and CD11b^{+}/Ly6C^{lo} subpopulations. *Upper panel.* Cytospin preparations were stained with Giemsa. (data pending). *Lower panel.* mRNA expression was analyzed by RT-PCR. Data were normalized relative to GAPDH; bars represent mean ± SE (n=4-6).

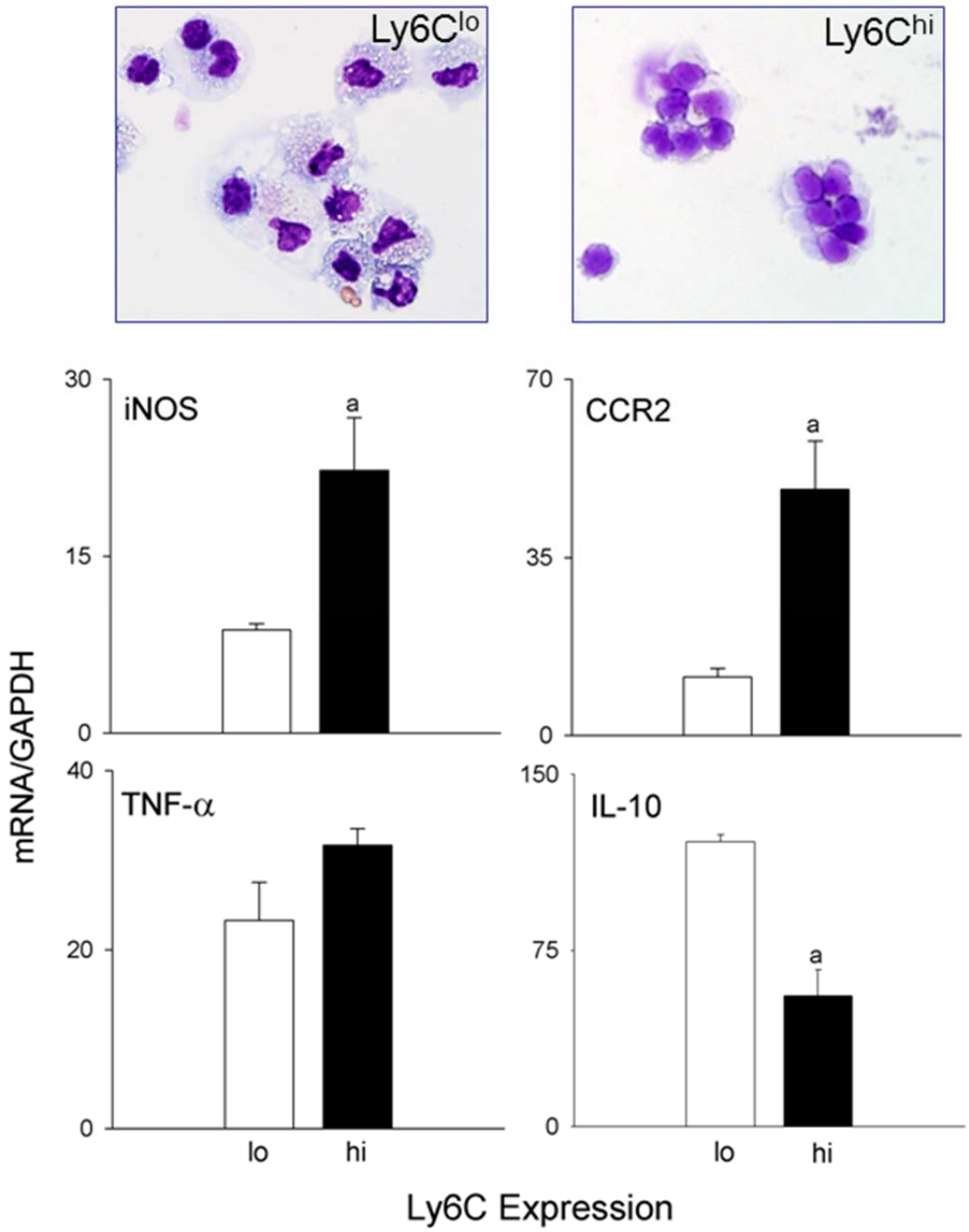

Ly6C^lo
Ly6C^hi
iNOS
CCR2
TNF-α
IL-10
a
mRNA/GAPDH
30
15
0
70
35
40
20
150
75
lo
hi
Ly6C Expression

FIGURE 22. Effects of loss of Gal-3 on liver repair following APAP intoxication. Liver sections were prepared 48-72 h after treatment of wild type and Gal-$3^{-/-}$ mice with APAP or PBS control. *Left panel.* Sections were stained with hematoxyllin and eosin and mitotic figure were enumerated in 10-12 random fields. *Right panel.* Sections were stained with anti-PCNA antibody or IgG control. Binding was visualized using a Vectastain Elite ABC kit, with 3-3'-diaminobenzidine as substrate. PCNA-positive hepatocyte nuclei were counted in six random fields per section (magnification 200x). Bars represent the mean $\pm$ SE (n=3-6 mice). [b]Significantly different ($p<0.05$) from wild type.

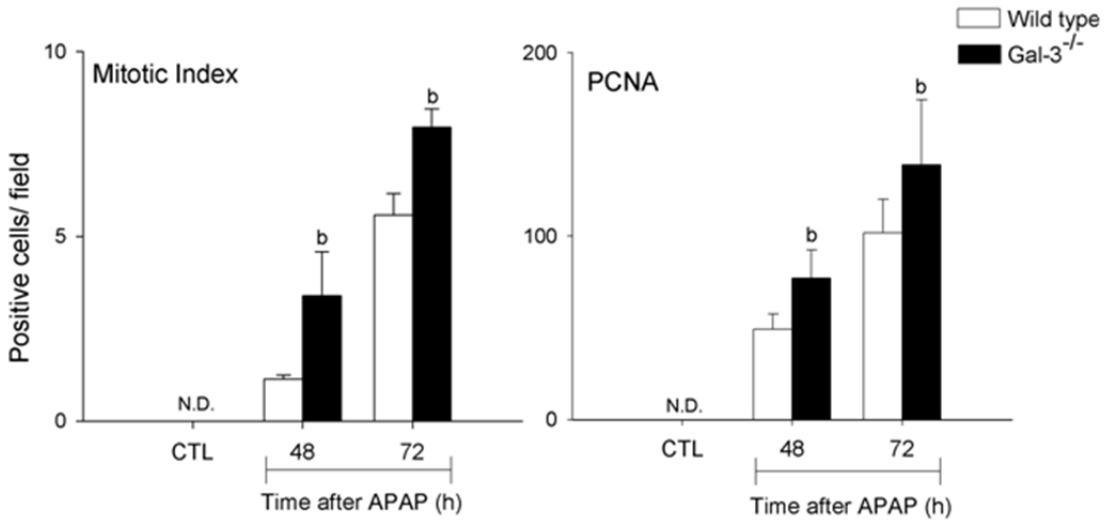
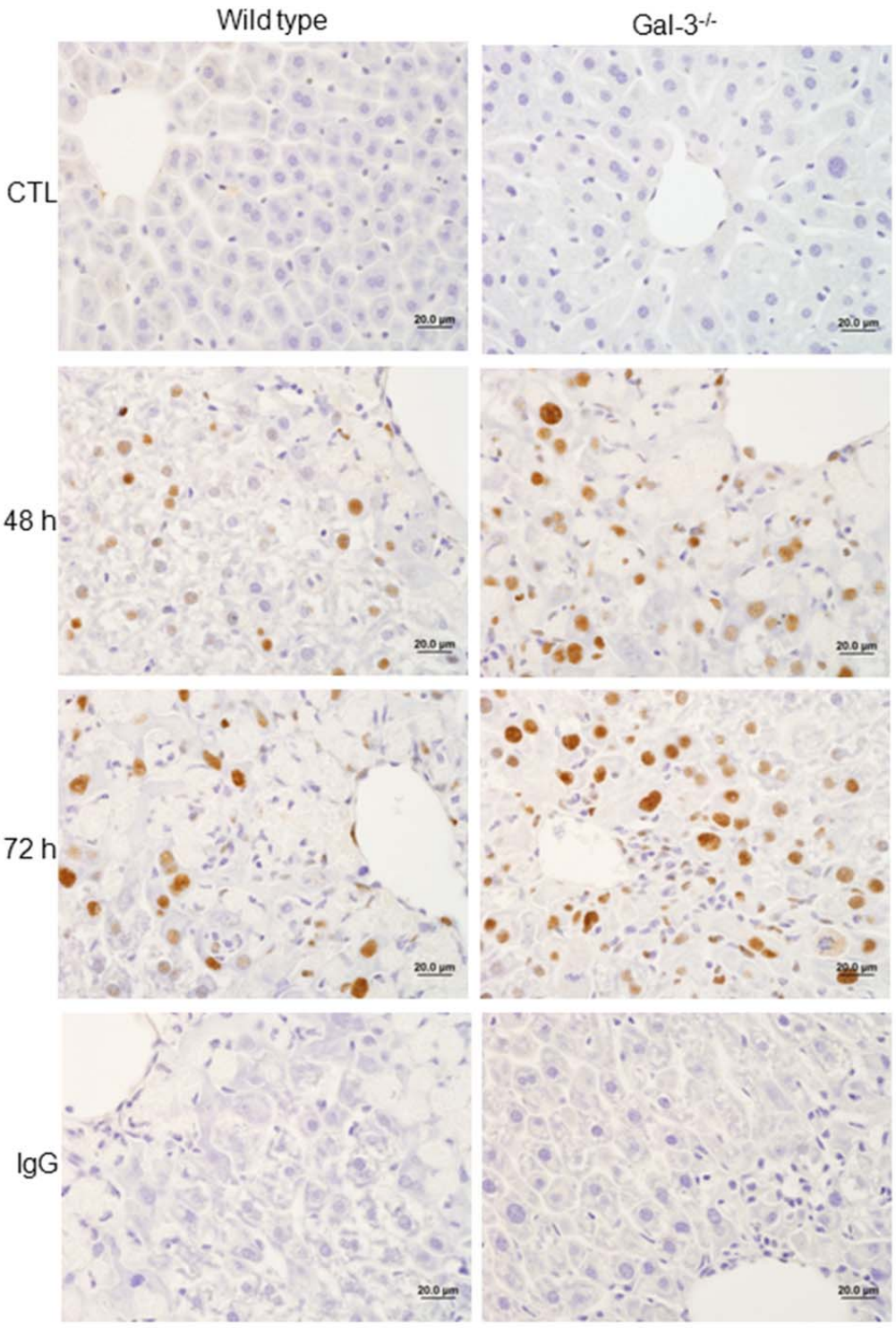

Wild type
Gal-3-/-
Mitotic Index
Positive cells/ field
10
5
0
N.D.
b
b
CTL
48
72
Time after APAP (h)
PCNA
200
100
0
N.D.
b
b
CTL
48
72
Time after APAP (h)

FIGURE 23. Effects of APAP administration on PCNA expression. Liver sections, prepared 48 to 72 h after treatment of wild-type and Gal-3$^{-/-}$ mice with APAP or PBS control, were stained with anti-PCNA antibody or IgG control. One representative section from three independent experiments is shown. Original magnification, 63x.


Wild type
Gal-3-/-
CTL
48 h
72 h
IgG
20.0 µm

SUMMARY AND CONCLUSIONS

The overall objective of these studies was to characterize mechanisms regulating macrophage activation and inflammatory mediator production in the liver during APAP-induced hepatotoxicity. We hypothesized that factors such as HMGB1, which are released from necrotic hepatocytes, and Gal-3, released from activated macrophages play important roles in promoting proinflammatory macrophage activation and hepatotoxicity. Our results demonstrate that primary mouse hepatocytes treated with cytotoxic doses of APAP release HMGB1, and that this contributes to proinflammatory macrophage activation. Thus, macrophages treated with conditioned medium from APAP-injured hepatocytes (CM-AA) produce increased quantities of ROS, and express HO-1, and catalase, as well as cyclooxygenase COX-2, 12/15-LOX, MIP-1α (CCL3) and MIP-2 (CXCL2).

Another aspect of our studies was to analyze the role of Gal-3 in inflammatory macrophage activation during APAP-induced hepatotoxicity. Following APAP intoxication, Gal-3 expression increases in the liver, predominantly in macrophages infiltrating into necrotic areas. Findings that APAP-induced hepatotoxicity is reduced in Gal-3-deficient mice, and that this correlates with decreased expression of proinflammatory proteins, including iNOS, IL-12, TNF-α, MIP-2, MMP-9, and MIP-3α, as well as the Gal-3 receptor (CD98), demonstrate that Gal-3 is important in promoting inflammation and injury in the liver following APAP intoxication. We speculated that Gal-3 plays a role in classical macrophage activation during APAP-induced hepatotoxicity. To test this, we characterized activated macrophage subpopulations present in the APAP-injured liver using techniques in immunofluorescence, flow cytometry and cell sorting. These studies revealed that both proinflammatory ($CD11b^{+}/Ly6C^{hi}$) and anti-inflammatory ($Ly6C^{lo}$) macrophages accumulated in the liver following APAP administration to wild type mice. These cells were distinct from resident $F4/80^{+}$

macrophages. Ly6C^{hi} macrophages were also found to express Gal-3. Loss of Gal-3 resulted in a decrease in the numbers of CD11b^{+}/Ly6C^{hi} macrophages accumulating in the liver after APAP. In contrast, numbers of CD11b^{+}/Ly6C^{lo} macrophages increased. This was associated with increases in liver regeneration. Taken together, these data indicate that classically and alternatively activated macrophages play distinct roles in tissue injury and repair following APAP intoxication; moreover, Gal-3 plays an important role in regulating macrophage proinflammatory phenotype.

In conclusion, these studies indicate that APAP-injured hepatocytes release of mediators such as HMGB1, which contribute to the inflammatory environment in the liver through macrophage activation. Moreover, macrophages themselves are a source of activating factors, such as Gal-3, which acts in an autocrine and paracrine manner to promote classical macrophage activation and inflammatory mediator production.

FUTURE STUDIES

The present studies identify HMGB1 as a factor released from APAP-injured hepatocytes that activates macrophages. In future studies, it would be interesting to characterize the signaling pathways that mediate the effects of HMGB1 on macrophages. Our results indicate that HMGB1-containing hepatocyte conditioned medium upregulates macrophage expression of RAGE, one of the macrophage HMGB1 receptors. Studies using macrophages isolated from $RAGE^{-/-}$ mice would allow the identification of RAGE-dependent effects of HMGB1 in this system.

Studies on the role of Gal-3 in APAP-induced hepatotoxicity and inflammation can be further pursued to assess the signaling pathways mediating the effects of Gal-3. Recent reports suggest that the proinflammatory effects of Gal-3 are mediated by IFN-γ-receptor and JAK-STAT-dependent pathways. Specifically, Gal-3 activated JAK2, STAT1, STAT3, and STAT5 (Jeon *et al*., 2010). Studies using mice deficient in these proteins would provide insight into the signaling pathways activated by Gal-3. Macrophage-specific information could be obtained by using transgenic mice where these proteins have been specifically deleted in myeloid cells. The role of CD98 in APAP-induced hepatotoxicity and inflammation could also be analyzed in future studies. While CD98 gene deletion is embryonically lethal (Tsumura *et al.*, 2003), it would be interesting to assess the effects of macrophage-specific deletion of this protein using a transgenic mouse. Another model to circumvent the lethal effects of CD98 deletion would be to use a mouse strain that expresses green fluorescent protein (GFP) coupled to the *cd98* promoter; this would result in all CD98-expressing cells becoming fluorescent

Another interesting research direction suggested by our studies is the exploration of the role of Gal-1 in APAP-induced hepatotoxicity. This can be accomplished using Gal-1-deficient mice, which are commercially available. In contrast to Gal-3, Gal-1 is considered an anti-inflammatory galectin and a potential inducer of alternatively

activated macrophages. Therefore Gal-1 is expected to play a protective role following APAP intoxication, and Gal-$1^{-/-}$ mice would exhibit increased hepatotoxicity in response to APAP.

In these studies, we characterized multiple subpopulations of activated macrophages present in APAP-injured livers. Thus, Ly6C^{hi} macrophages exhibit a proinflammatory phenotype and contribute to the persistence of injury. A convincing demonstration of the cytotoxic effect of these cells would come from experiments where Ly6C^{hi} macrophages isolated from APAP-treated livers are administered i.v. into wild type mice at early times after APAP administration (less than 6-8 h). Findings that APAP-induced hepatotoxicity is exacerbated by infusion of these cells would confirm that they contribute to tissue injury. Conversely, if Ly6C^{lo} macrophages are anti-inflammatory, infusion of these cells either before (6-8 h) or at the peak of toxicity (18-24 h), would be expected to accelerate tissue repair and restoration of homeostasis.

The identification of Ly6C-expressing cells raises several questions regarding the origin of these cells, the mechanisms responsible for their hepatic accumulation, and the relationship between Ly6C^{hi} and Ly6C^{lo} macrophages. Evidence suggests that Ly6C^{hi} cells are bone-marrow derived; however, a rigorous demonstration of their origin would require bone marrow reconstitution experiments. In these studies, irradiation of wild type mice would deplete the bone marrow compartment, which could be reconstituted with cells derived from a mouse strain that expressed GFP under the control of a ubiquitous promoter, such as actin. This would allow identification of infiltrating, GFP-positive versus resident, GFP-negative macrophages by flow cytometry or immunofluorescence. Findings that GFP-positive macrophages isolated from the livers of APAP-treated mice express high levels of Ly6C would confirm that they are derived from the bone marrow. Bone marrow reconstitution experiments would also provide information regarding the origin of Ly6C^{lo} cells. Thus, lack of GFP fluorescence in the Ly6C^{lo} subpopulation would

indicate that these cells arise from resident liver macrophages, whereas the presence of GFP in Ly6C^{lo} macrophages would suggest they are recruited from the bone marrow. In the case of concomitant recruitment of Ly6C^{hi} and Ly6C^{lo} macrophages from the bone marrow, further studies could be performed to determine whether these cells accumulate in the liver as separate subpopulations, or whether Ly6C^{hi} cells selectively recruited from the bone marrow, are converted into Ly6C^{lo} macrophages after emigration into the liver. In vivo fate tracing experiments could be used to answer these questions. For example, selective labeling of Ly6C^{lo} monocytes can be achieved by i.v. administration of fluorescent latex beads, whereas Ly6C^{hi} labeling is obtained by administration of clodronate liposomes 18 h before bead injection (Tacke *et al.*, 2006).

Similar bone marrow reconstitution experiments would allow identification of the mechanisms responsible for Ly6C^{hi} and/or Ly6C^{lo} macrophage recruitment into the liver after APAP intoxication. Reconstitution of bone marrow compartment with cells from mice deficient in chemokine receptors, including CCR2, CX3CR1, and CCR1, would provide information on the mechanisms for macrophage influx into the APAP-injured liver. Furthermore, the use of cells from mice deficient in pro- or anti-inflammatory cytokines would allow a more detailed investigation on the role of specific mediators in the pathogenesis of APAP-induced toxicity. For example, findings that Ly6C^{hi} macrophages from TNF-α$^{-/-}$ mice infused into APAP-treated wild type mice do not increase injury would indicate that Ly6C^{hi} macrophage-derived TNF-α is an important contributor to the pathogenesis of toxicity.

Previous reports suggest that APAP administration results in the hepatic accumulation of multiple subpopulations of alternatively activated macrophages. It is therefore likely that Ly6C^{lo} cells represent a heterogeneous group. It would also be interesting to further characterize Ly6C^{lo} macrophages in the liver following APAP intoxication with respect to their expression of different markers of alternative activation.

Previous studies have used macrophage inhibitors, such as gadolinium chloride and clodronate liposomes, to analyze the role of macrophages in APAP-induced hepatotoxicity, with conflicting results. It is thought that these discrepancies are due to differential effects on classical and alternatively activated macrophages. It would be interesting to determine the effects of these inhibitors on different subpopulations of activated macrophages accumulating in the liver after APAP.

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