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Role of Hepatic Resident and Infiltrating Macrophages in Liver Repair after Acute Injury

Qiang You[1], **Michael Holt**[3], **Hao Yin**[4], **Guiying Li**[5], **Cheng-Jun Hu**[6], and **Cynthia Ju**[1,2,*]

[1]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus

[2]Integrated Department of Immunology, University of Colorado Anschutz Medical Campus

[3]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health

[4]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology

[5]Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education, College of Life Science, Jilin University

[6]Department of Craniofacial Biology, University of Colorado Anschutz Medical Campus

Abstract

Treatment of liver disease, caused by hepatotoxins, viral infections, alcohol ingestion, or autoimmune conditions, remains challenging and costly. The liver has a powerful capacity to repair and regenerate, thus a thorough understanding of this tightly orchestrated process will undoubtedly improve clinical means of restoring liver function after injury. Using a murine model of acute liver injury caused by overdose of acetaminophen (APAP), our studies demonstrated that the combined absence of liver resident macrophages (Kupffer cells, KCs), and infiltrating macrophages (IMs) resulted in a marked delay in liver repair, even though the initiation and extent of peak liver injury was not impacted. This delay was not due to impaired hepatocyte proliferation but rather prolonged vascular leakage, which is caused by APAP-induced liver sinusoidal endothelial cell (LSEC) injury. We also found that KCs and IMs express an array of angiogenic factors and induce LSEC proliferation and migration. Our mechanistic studies suggest that hypoxia-inducible factor (HIF) may be involved in regulating the angiogenic effect of hepatic macrophages (Macs), as we found that APAP challenge resulted in hypoxia and stabilization of HIF in the liver and hepatic Macs. Together, these data indicate an important role for hepatic Macs in liver blood vessel repair, thereby contributing to tissue recovery from acute injury.

Keywords

Acetaminophen; Kupffer cells; microvasculature; angiogenesis

*To whom correspondence should be addressed. Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, V20-2121, 12850 East Montview Blvd, Aurora, CO 80045. Phone: (303) 724-4019. Fax: (303) 724-7266. CYNTHIA.JU@UCDENVER.EDU.

1. Introduction

Liver disease is a significant health problem, affecting millions of people in the world. Currently, transplantation is the only effective treatment for acute liver failure and end-stage chronic liver injury. However, transplantation is costly, challenging, and well-matched donor tissues are not readily available. Therefore, there is a pressing need to develop pharmacological treatments. One area of research that holds promise of breakthrough discoveries is better understanding of the liver's own repair and regeneration processes. The majority of studies of liver regeneration have used animal models of partial hepatectomy. However, hepatectomy often lacks the cell death and tissue inflammation that are commonly observed in acute and chronic liver injury. Therefore, the present study aimed to investigate liver repair processes after overdose of acetaminophen (APAP)-induced liver injury (AILI), which itself is a significant clinical problem [1]. Macrophages /monocytes play important roles in liver injury and liver repair. They produce pro- and anti-inflammatory mediators which can trigger hepatocytic cell death pathways as well as activate protective signaling pathways [2] [3]. Damage-associated molecular pattern molecules (DAMPS), such as high-mobility group box 1 protein and heat shock proteins [4] [5], are released during liver injury and activate macrophages to produce cytokines and chemokines. Macrophage-derived soluble mediators can exacerbate cellular injury or inhibit cell death and promote hepatocyte proliferation [2]. Another important function of macrophages is to phagocytose dead cells and cellular debris [6] [7] [8]. As result, these cells produce angiogenic factors and growth factors, thereby promoting tissue repair.

There are at least two types of macrophages in the liver after acute injury, resident macrophages (Kupffer cells, KCs) and infiltrating macrophages. We have reported that APAP challenge causes hepatic recruitment of circulating monocytes in C-C chemokine receptor type 2 (CCR2)-dependent manner [9]. Our studies also demonstrated that the lack of infiltrating macrophages (IMs) in $CCR2^{-/-}$ mice resulted in a slight delay in tissue recovery from APAP-induced liver injury (AILI). However, the observation that liver repair was completed by 72 h in $CCR2^{-/-}$ mice (versus 48 h in WT mice) suggested that resident Kupffer cells are also involved in liver repair and can compensate for the lack of IMs. Hence, the present study aims to investigate the combined role of resident and infiltrating hepatic Macs during tissue repair.

We developed a mouse model in which either or both populations of hepatic Macs (resident KCs and IMs) could be depleted. We found that the liver repaired and histology returned to normal by day 3 after APAP in mice with intact hepatic Macs or lack of either population of Macs; however, necrotic areas remained prominent in mice with the combined absence of both populations of Macs. Moreover, our data revealed an important angiogenic function of hepatic Macs that plays an integral role in liver repair from AILI.

2. Materials and Methods

2.1. Animal treatment and assessment of hepatotoxicity

Seven – 10 week old male BALB/cJ wild-type (WT, Jackson Laboratories, Bar Harbor, ME) and $CCR2^{-/-}$ mice [on Balb/cJ background, provided by Cara L. Mack, M.D. (Department of Pediatrics, School of Medicine, University of Colorado Denver)] were used. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus. Animals were fasted overnight for approximately 16 h to deplete glutathione levels, prior to intraperitoneal (i.p.) injection of PBS or APAP (Sigma, St. Louis, MO; 300 mg/kg, dissolved in PBS).

Mice were divided into 4 groups containing none, either, or both populations of the hepatic Macs (Table 1). To deplete hepatic KCs, animals were intravenously (i.v.) injected with liposome/clodronate (lipo/cld, Sigma) 2 days prior to APAP challenge. Lipo/cld was prepared as previously described [10]. Control animals were i.v. injected with liposome/PBS.

Blood was collected by retro-orbital puncture. Sera samples were collected for colorimetric measurement of alanine aminotransferase (ALT) using a Colorimetric ALT Assay Kit (Teco Diagnostics, Anaheim, CA). Liver samples were obtained at various time points after APAP challenge and paraffin-embedded tissue sections were prepared and stained with hematoxylin and eosin (H&E).

2.2. Evaluation of hepatocyte proliferation and liver sinusoid permeability

To measure hepatocyte proliferation, mice were injected i.p. with BrdU (Sigma, 100 mg/kg) 1 h prior to sacrifice. Paraffin-embedded liver tissue sections were prepared and stained for BrdU immunohistochemically (IHC) using an anti-BrdU antibody (Sigma). As an additional approach, IHC staining for the proliferating cell nuclear antigen (PCNA) was also performed using an anti-PCNA antibody (Sigma). BrdU- or PCNA-positive cells were counted under a light microscope, and the average number of positive cells in 3 high power fields (field = 1008 μm) was plotted.

To measure liver sinusoid integrity, mice were i.p. injected with Evans blue dye (Sigma) at a dose of 20 mg/kg (dissolved in saline). Four h after injection, mice were anesthetized and liver tissues were perfused *in situ* with Hank's Balanced Salt Solution (HBSS) for 5 min to remove excess Evans blue dye remaining in the circulation. Livers were excised and placed in formamide (4 mL/g tissue) and incubated at 37 °C for 24 h to allow dye extraction. The supernatant was analyzed using spectrophotometry at a wavelength of 630 nm. The amount of extravasated Evans blue dye in the tissue was calculated from a standard curve of known Evans blue concentrations.

2.3. Isolation of mouse KCs and IMs

Mouse liver nonparenchymal cells (NPCs) were isolated following a previously established method [9]. In brief, a 20G catheter was put through mouse superior vena cava, the inferior vena cava was clamped and the portal vein was cut. The liver was perfused *in situ* with HBSS followed *in vitro* digestion with digestion buffer [1× HBSS supplemented with 0.05% collagenase (Type IV, Sigma), 1.25 mM $CaCl_2$, 4 mM $MgSO_4$ and 10 mM HEPES]. Single cell suspensions were filtered through a 100 μm cell strainer, and the cells were fractionated using 30 % (w/v) Nycodenz (Axis-Shield PoC AS, Oslo, Norway) at 1.155 g/mL to yield liver NPCs and further purified using 30 % Percoll (Sigma) at 1.04 g/mL.

To further purify hepatic Macs, the cells were incubated with the following antibodies from eBioscience (San Diego, CA): fluorescein isothiocyanate (FITC) conjugated anti-mouse CD45, phycoerythrin (PE) conjugated anti-mouse CD11b, and allophycocyanin (APC) conjugated anti-mouse F4/80. Subsequently, KCs ($CD45^+F4/80^{high}CD11b^{low}$) and IMs ($CD45^+F4/80^{low}CD11b^{high}$) were isolated by using a MoFlo High Performance Cell Sorter (Cytomation, Fort Collins, CO).

2.4. Immunoblot and qPCR analyses

To measure protein expression levels of hypoxia-inducible factor (HIF)-1α and HIF-2α, liver tissues were homogenized within 5 min after sacrificing the animals. Fifty μg of tissue homogenate samples were diluted in Laemmli sample buffer (Bio-Rad, Hercules, CA) under reducing conditions, boiled for 5 min, and resolved on 12% polyacrylamide gels. After

being transferred onto nitrocellulose membranes, nonspecific binding was blocked with 5% nonfat milk. The blots were probed with either anti-mouse HIF-1α or anti-mouse HIF-2α antibody (Novus Biologicals, Littleton, CO).

Total RNA was isolated from liver tissues or hepatic Macs using RNeasy Kits (Qiagen, Valencia, CA) as described by the manufacturer. 0.5 μg of liver tissue RNA or 0.2 μg of hepatic Macs RNA was reverse-transcribed to cDNA in 20 μL of volume and amplified using GoTaq® qPCR Master Mix (Promega, Madison, WI) and gene-specific primers (Table 2) for GAPDH, Glut-1 (glucose transporter-1), VEGF (vascular endothelial growth factor), EPO (erythropoietin), MCP-1, PAI-1 (plasminogen activator inhibitor-1), CA-9 (carbonic anhydrase-9), interleukin (IL)-1α, IL-1β, IL-8, STAT4 (signal transducer and activator of transcription 4), ADM (adrenomedullin), CXCR4 (C-X-C chemokine receptor type 4), ADORA2A (adenosine receptor A2a), angiopoietin-like (ANGPTL)2 and 4, FLT1 (VEGF receptor-1), and matrix metalloproteinase (MMP)-2, 7, 8, 9, and 13. All PCR products were measured using a Real-Time PCR 7500 SDS system and software (Life Technologies, Grand Island, NY).

2.5. Evaluation of the angiogenic effects of hepatic Macs

Transformed sinusoidal endothelial cells (TSECs, a generous gift from Dr. Vijay Shah, Mayo Clinic, Minnesota) were seeded on the bottom of 12-well plates at a density of 50,000 cells/well. Hepatic Macs (including KCs and IMs) were isolated from male BALB/cJ mice treated with APAP for 24 h, and cultured in 0.4 μm inserts (50,000 cells/well). After 1, 2, and 3 days, the inserts were removed and the numbers of TSECs were counted to measure TSEC proliferation.

To examine the effect of hepatic Macs on TSEC migration, TSECs (3×10^5 cells/well) were seeded in 12-well plates and cultured in DMEM medium containing 0.5% fetal calf serum. When cells were confluent, a "+" sign was made by scratching each well with a pipette tip. Subsequently, hepatic Macs (5×10^4 cells) were added in 0.4 μm inserts and co-cultured with the TSECs. Images of each well were captured by an inverted microscope imaging system after 0, 6, 12, 18 and 24 h. The cells that migrated into the scratched region were counted.

2.6. Statistical analysis

Data are presented as mean ± SEM. Two-tailed Student's t-test was used to compare two groups. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) with Bonferroni post-hoc test of significance between individual groups. The statistical analysis was performed by GraphPad Prism 5.0 (La Jolla, CA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. The combined absence of KCs and IMs markedly delays liver repair after AILI

In the present study we developed an *in vivo* approach to assess the roles of KCs and IMs in the progression and regression of AILI by using 4 groups of mice having none, either, or both populations of the hepatic Macs (see method section).

At 24 h after APAP treatment, the ALT levels (Fig. 1B) were similar and liver histopathology (Fig. 1A, 24 h) was comparable across the 4 groups. These data suggested that the extent of initial hepatic injury was not impacted by the absence of either or both populations of hepatic Macs. However, the most striking observation was that although the liver repaired and histology returned to normal by day 3 after APAP in Groups I, II and III,

massive necrosis remained prominent in Group IV even by day 6 and day 9 after APAP (Fig. 1A). This significant delay in liver repair was also evident as judged by gross liver appearance (Fig. 1C), further indicating the prominent role of both KCs and IMs together in this process.

3.2. Lack of hepatic Macs does not affect hepatocyte proliferation but prolongs sinusoid leakage during AILI

It has been reported that KCs contribute to the production of growth factors, including TNF-α, IL-6, HGF, and TGF-β [11] that promote hepatocyte proliferation after hepatectomy. We injected mice with BrdU 2 days after APAP challenge to examine hepatocyte proliferation. Interestingly, the data revealed that hepatocyte proliferation was not impaired in the absence of KCs, IMs, or the combined absence of KCs and IMs (Fig. 2A). Furthermore, PCNA staining of liver tissue from mice with intact hepatic Macs (Group I) and depletion of both populations of Macs (Group IV) showed no difference (Fig. 2B).

In addition to the well characterized mechanism of APAP-induced direct hepatotoxicity, it has been reported that APAP causes liver sinusoidal endothelial cell (LSEC) damage and disrupts microcirculation, which play a critical role in the pathogenesis of AILI [12]. Because it has become increasingly clear that angiogenesis is an integral part of tissue repair, we examined hepatic sinusoid integrity by injecting mice with Evan's blue at various time points. Evan's blue dye binds to intravascular albumin and is retained within the circulation under normal endothelial integrity and homeostasis. During vascular disruption or injury, Evan's blue penetrates into the tissue. Thus, the concentration of Evans blue dye within the liver tissue can be a reliable indicator of sinusoid permeability. In naïve mice, the level of Evan's blue in the liver is approximately 10 μg/g tissue (data not shown). APAP challenge caused a significant increase in tissue accumulation of Evan's blue and the levels were similar between Groups I and IV mice. Interestingly, the APAP-induced vascular leakage was prolonged when both KCs and IMs were absent (Fig. 3), suggesting an impaired re-vascularization in Group IV mice.

3.3. Hepatic Macs exhibit angiogenic functions

Our data are consistent with the accumulating evidence suggesting that Macs play a critical role in angiogenesis during wound-healing and tumorigenesis [13;14]. To investigate whether hepatic Macs directly promote angiogenesis, we isolated KCs and IMs from the mouse liver at 24 h after APAP by FACS, as previously described [9]. Isolated hepatic Macs were co-cultured with TSEC for various time points. The data demonstrated that in the presence of hepatic Macs, TSEC proliferation (Fig. 4A) and migration (Fig. 4B) were significantly increased. Because the Macs were seeded in culture inserts, the findings suggest that the angiogenic effect of hepatic Macs does not require cell-cell contact, but rather mediated by soluble factors.

We next examined the expression of various angiogenic factors from isolated hepatic Macs by qPCR analysis. Compared with KCs isolated from naïve mice, KCs and IMs isolated from APAP-challenged mice expressed dramatically higher levels of a number of angiogenic genes, including MMP 2, 7, 8, 9, and 13, VEGF, FLT1, ANGPTL2 and ANGTL4 (Fig. 5). With the exception of VEGF, MMP8, and MMP9, all other genes were expressed at higher levels in KCs compared to IMs.

3.4. APAP challenge induces HIF stabilization in the liver and hepatic Macs

We have previously reported that APAP treatment results in a profound hypoxia in the liver [15]. Macs are known to accumulate in large numbers within hypoxic areas of injured tissues, and it is hypoxia that dictates the phenotype of the cells [16;17]. Our data

demonstrated that hepatic Macs exhibit a distinct hypoxia-induced transcription profile characterized by the up-regulation of interleukin (IL)-1α, IL-1β, KC (IL-8), STAT4, ADM, CXCR4, and ADORA2A (Fig. 6).

Hypoxia triggers stabilization of HIFα protein, which promotes the transcription of numerous genes that convey an adaptive response by the cells under hypoxia. HIF is a key transcription factor that induces angiogenic genes, many of which were up-regulated in hepatic Macs as shown in Fig. 5. These data suggest that HIF stabilization during AILI may be important in regulating the angiogenic function of hepatic Macs. We measured HIF-1α and HIF-2α protein expression in the mouse liver after APAP treatment. Due to the instability of HIF-1α and HIF-2α, we prepared liver homogenates immediately (within 5 min) after sacrificing the mice. Our data revealed an induction of HIF-2α in APAP-treated mice, compared with naïve controls. (Fig. 7) However, we were unable to detect HIF-1α, most likely due to its extremely low stability *ex vivo*.

As an alternative approach to evaluate HIF stabilization during AILI, we measured mRNA expression levels of HIF-1α and HIF-2α target genes. Our data clearly demonstrated that both HIF-1α target genes (Glut-1, CA-9, and MCP-1), HIF-2α target genes (EPO, and PAI-1) and the HIF1α/HIF2α common target VEGF were induced in liver tissue (Fig. 8A), as well as in hepatic Macs (Fig. 8B).

4. Discussion

Liver disease caused by various etiologies, such as viral infection, alcohol or drug ingestion, metabolic disorders, and autoimmunity affect approximately 150,000 new patients in the U.S. each year [18]. Although liver transplantation is an effective treatment for chronic and acute liver failure, the limited options for therapeutic interventions remain a pressing issue of treating liver disease. A better understanding the liver's own repair and regeneration processes may lead to discoveries of therapeutic targets and strategies. The majority of studies in this area have focused on the role of hepatocyte viability and proliferation in tissue recovery. In addition to hepatocytes, the tightly orchestrated process of liver repair involves multiple cell types, including LSECs, stellate cells, and hepatic Macs. Moreover, hepatic microcirculation disorder is a complication in many cases of severe liver injury, and blood vessel repair represents a critical and integral step of the tissue recovery process. The present report describes an important role of hepatic Macs in promoting blood vessel repair and regeneration, thereby contributing to liver recovery from acute injury.

We chose a mouse model of overdose acetaminophen (APAP)-induced liver injury (AILI) to study the role of hepatic Macs in liver repair after an acute injury. AILI is a significant clinical problem, causing more than 56,000 emergency room visits, 2,600 hospitalizations, and an estimated 458 deaths each year in the U.S [19]. Ample evidence supports that APAP bioactivation, protein adduct formation, mitochondrial dysfunction, oxidant stress, peroxynitrite formation and nuclear DNA fragmentation are critical intracellular events that contribute to hepatocytes damage [2]. Following tissue injury, neutrophils and monocytes are recruited into the liver. Although pro-inflammatory mediators are released by these cells, their overall function is to promote tissue repair, rather than exacerbating tissue damage[2].

We have demonstrated that IMs are recruited into the liver of WT, but not CCR2−/− mice, after APAP challenge [9]. Lipo/cld is a widely used agent to deplete Macs. We showed that i.v. injection of mice with lipo/cld 48 h prior to APAP challenge effectively depletes resident KCs without affecting the hepatic recruitment of IMs [9]. Thus, using CCR2−/− mice and the lipo/cld approach, we are able to deplete either or both populations of hepatic Macs. In the present study our data demonstrate that the combined absence of hepatic Macs,

but not either population individually, dramatically delays liver recovery from AILI. As shown in Fig. 1, both gross liver appearance and liver histology returned to normal by day 3 after APAP in Groups I, II and III, while massive necrosis remained prominent by day 6 and day 9 after APAP in Group IV. Increasing evidence suggests that Macs are involved in wound healing and tissue repair and regeneration [14;20]. It has been demonstrated that Macs play critical roles in: i) clearance of necrotic cells from injured areas to initiate tissue repair; ii) promotion of cellular proliferation through the production and release of various cytokines and growth factors; and iii) production of pro-angiogenic factors thereby facilitating neovascularization. Our observation of comparable numbers of PCNA- or BrdU-positive hepatocytes at 48 h after APAP challenge across all 4 groups (Fig. 2) suggest that hepatocyte proliferation was not impaired in the absence of KCs, IMs, or the combined absence of both populations of hepatic Macs. It is known that failure of necrotic cell clearance results in mineralization thereby leading to dystrophic calcification. We observed marked dystrophic calcification in the liver of lipo/cld-treated CCR2$^{-/-}$ mice (Group IV) at day 8 post APAP (Fig. 1A), a phenomenon that was absent in Groups I, II and III. This is a strong indication that in Group IV mice, necrotic cells are not removed in a timely manner, due to the absence of both KCs and IMs. The presence of dystrophic calcification in the liver has also been observed in two studies using CCl_4 treatment of plasminogen$^{-/-}$ and plasminogen activator$^{-/-}$ mice [21;22]. In agreement with our data, these studies demonstrated that the delayed liver regeneration observed in both types of plasmin-deficient mice did not correlate with impaired hepatocyte proliferation. Plasmin plays a critical role in degradation of extracellular matrix during clearance of necrotic cells. Therefore, in the plasmin-deficient mice, it is likely that although Macs are present, their ability to effectively clear cellular debris is abrogated.

It has become increasingly clear that angiognesis is an integral part of tissue repair and regeneration, as functional microvessels facilitate the delivery of nutrients, oxygen, and infiltrating immune cells to the damaged tissue. It has been demonstrated that Macs, through phagocytosis of dead cells, are activated to produce pro-angiogenic factors, thereby contributing to blood vessel repair and regeneration [23]. Tumor-associated Macs (TAMs) have been implicated in the angiogenic switch involving the dramatic increase in vessel density and cell migration during the benign-to-malignant tumor transition [24]. The relationship between TAMs and tumor angiogenesis, and the correlation with an overall poor prognosis has been reported in a number of human cancers and experimental animal models [24;25]. The critical role of Macs in wound healing is supported by the observation of delayed angiogenesis and impaired muscle regeneration in CCR2$^{-/-}$ mice, in which Macs recruitment was impaired [14].

We demonstrated that hepatic Macs isolated from APAP-challenged mice promote TSEC proliferation and migration. These data directly support a strong angiogenic function of these cells. Furthermore, both KCs and IMs express a panel of angiogenic factors, such as VEGF, MMPs, and ANGPTL2 and 4. VEGF is a well-documented angiogenic mediator that can promote endothelial cell growth, maturation, and survival [26]. The VEGF family consists of a number of factors, of which VEGF-A has been identified as a potent regulator of neovascularization during both embryonic development as well as wound healing. Studies have shown that VEGF contributes to the proliferation of LSEC via the up-regulation of VEGF receptors during liver regeneration [27]. Recently, it was found that VEGFR-1 signaling promotes liver repair through restoration of liver microvasculature after acetaminophen hepatotoxicity [28]. MMPs represent a family of 24 zinc-dependent, matrix degrading enzymes. Overwhelming evidence supports a critical role for MMPs in angiogenesis during wound healing, tissue remodeling, and tumorigenesis. Through mediating extracellular matrix (ECM) remodeling, MMPs promote endothelial cell and pericyte migration, and release pro-angiogenic factors [29;30]. ANGPTL2 and 4 belong to a

family of 7 proteins structurally similar to angiopoietins [31]. The transcription of ANGPTL2 and 4 is induced by hypoxia, suggesting their pro-angiogenic effect [32;33]. Both knockdown and overexpression studies have demonstrated an association of ANGPTL2 and 4 with pro-angiogenic functions [33;34].

Hypoxia is known to trigger the stabilization of HIF, which is a key transcription factor critical in inducing the expression of many angiogenic factors. We have previously noted that APAP challenge causes profound hypoxia in the liver [15], and here, we further demonstrated the induction HIF. Both HIF-1α and HIF-2α target genes were expressed in the liver tissue as well as in isolated hepatic Macs of APAP-challenged mice (Fig. 8), suggesting an important role of HIF in regulating the angiogenic and tissue-repair function of hepatic Macs. We detected hepatic protein expression of HIF-2α, but not HIF-1α, probably due to the increased stability of HIF-2α compared to HIF-1α [35]. It has been reported that APAP treatment of mice causes HIF-1α stabilization in the liver [36]. Although we observed a band at approximately 129 kDa, consistent with the molecular weight of HIF-1α, this band was not detected in cells cultured under hypoxia, which served as a positive control for HIF-1α expression. It is known that HIF-1α is extremely unstable, which may be the reason we were unable to detect HIF-1α even though we prepared liver homogenates within 5 min after sacrificing the mice.

In APAP-induced liver injury (AILI), APAP-induced direct hepatotoxicity is the predominant mechanism of tissue injury. As result, leukocytes (neutrophils and macrophages) are recruited into the liver and they are activated by cytokines and damage-associated molecular pattern molecules released from injured/stressed hepatocytes. The activated neutrophils and macrophages produce pro- and anti-inflammatory cytokines, chemokines, and other soluble mediators. Another important function of these cells is to phagocytose dead cells and cellular debris. As result, these cells produce angiogenic factors and growth factors, thereby promoting tissue repair. During acute tissue injury, such as the case of AILI, the overall effect of hepatic macrophages is facilitating tissue repair and regeneration. However, if the injury persists, some of the macrophage-derived mediators, such as TNF-α and TGF-β, may contribute to detrimental chronic inflammation and fibrogenesis.

In summary, the present study demonstrates that the combination of resident and infiltrating Macs in the liver plays a critical role in liver vasculature repair and overall tissue recovery from injury. The data showed that hepatic Macs express angiogenic factors and promote LSEC proliferation and migration. The angiogenic effect of hepatic Macs appears to be mediated by up-regulation of HIF under APAP-induced hypoxia environment of the liver. Although hepatic microcirculation disruption occurs in various models of severe liver injury, studies of liver repair and regeneration have focused on mechanisms that govern hepatocyte proliferation. Our data demonstrate that hepatocyte proliferation and LSEC repair are independent events, and that impaired angiognesis leads to significant delays in overall liver regeneration. Although the angiogenic effect of Macs is well-recognized in tumorigenesis, our study revealed the critical contribution of these cells in re- and neo-vascularization within the liver. Findings of this study bridge a significant knowledge gap in understanding the underlying mechanism of liver repair. Further studies of the role of HIF, and perhaps other factors, in regulating the angiogenic function of hepatic Macs will lead to the identification of new therapeutic strategies that aim to restrain injury progression and accelerate tissue repair.

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Abbreviations

APAP	acetaminophen
KCs	Kupffer cells
IMs	infiltrating macrophages
LSECs	liver sinusoidal endothelial cells
HIF	hypoxia-inducible factor
Macs	macrophages
AILI	acetaminophen (APAP)-induced liver injury
MCP-1	macrophage chemotactic protein-1
i.p.	intraperitoneal
i.v.	intravenous
lipo/cld	liposome/clodronate
NPCs	nonparenchymal cells
Glut-1	glucose transporter-1
VEGF	vascular endothelial growth factor
EPO	erythropoietin
PAI-1	plasminogen activator inhibitor-1
CA-9	carbonic anhydrase-9
STAT4	signal transducer and activator of transcription 4
ADM	adrenomedullin
CXCR4	C-X-C chemokine receptor type 4
ADORA2A	adenosine receptor A2a
ANGPTL	angiopoietin-like
MMP	matrix metalloproteinase
TAMs	Tumor-associated Macs

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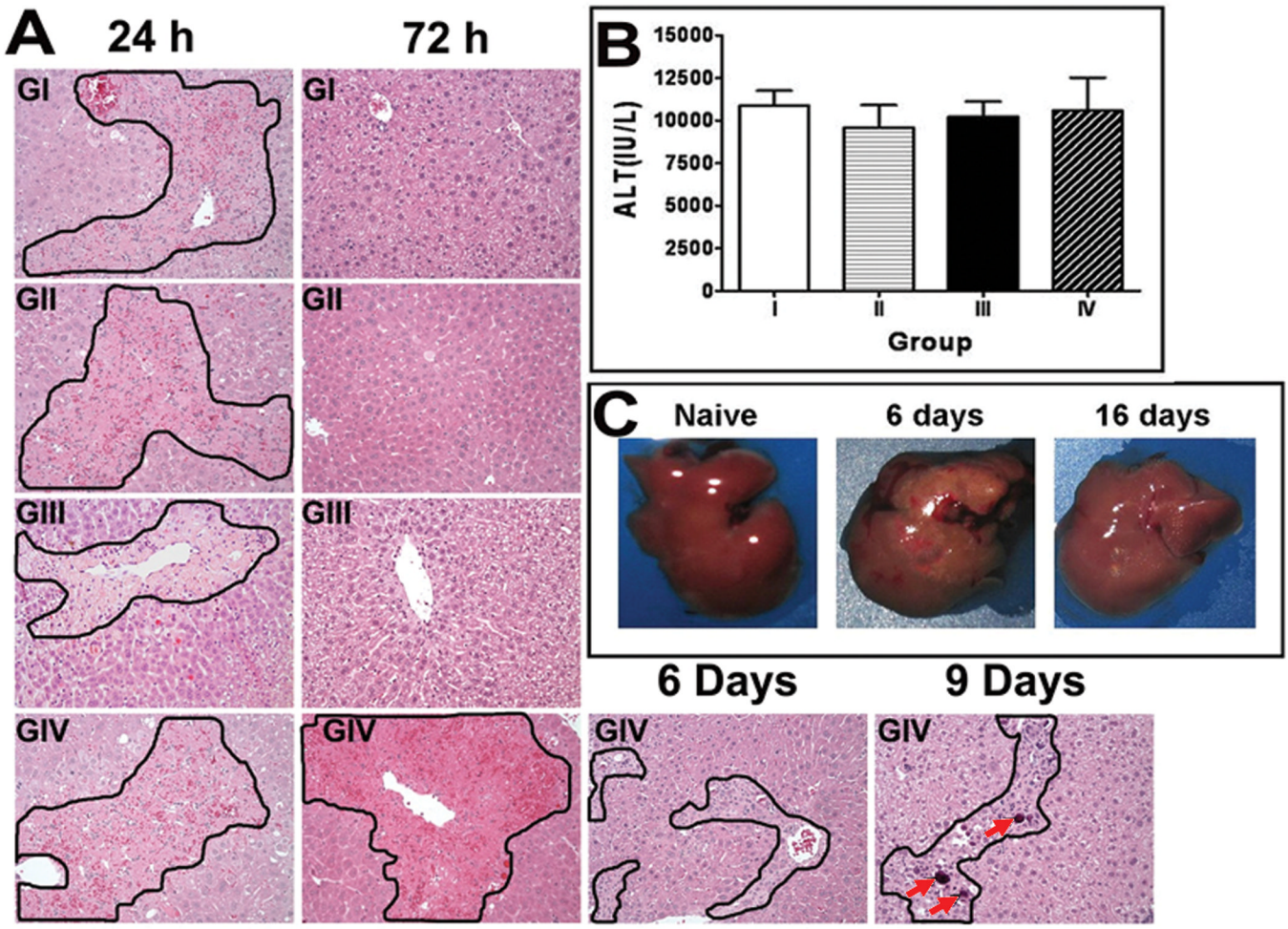

Figure 1. Prolonged tissue damage during AILI in the combined absence of KCs and IMs

(A) Formalin-fixed, paraffin-embedded sections were prepared from the livers of mice from Groups I, II, III, and IV at 24 h and 72 h after APAP and from Group IV at 6 days and 9 days after APAP. Representative liver sections from 3 mice per group were stained with H&E (original magnification, 200×). Injured areas are outlined. Arrows indicate areas of dystrophic calcification. **(B)** Analysis of serum ALT levels of mice from Groups I, II, III, and IV at 24 h after APAP. Levels represent means ± SEM of at least 5 mice per group. **(C)** Digital images of representative livers of mice (3 per time points) from Group IV were taken prior to (naïve) and at 6 days and 16 days after APAP.

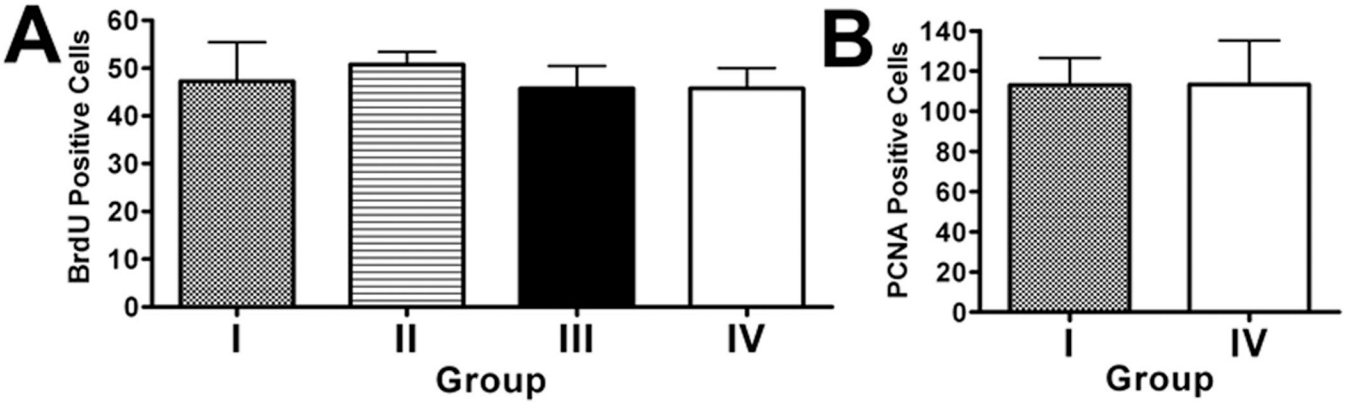

Figure 2. Absence of KCs and/or IMs does not affect hepatocyte proliferation during AILI

Mice were treated with APAP and livers were harvested at 48 h after APAP. Paraffin-embedded sections were stained for BrdU (**A**) or PCNA (**B**). The numbers of BrdU- or PCNA-positive cells were counted and expressed as the average number in 4 high power fields of each sample. A total of 5 liver tissue samples obtained from 5 mice in each group were counted.

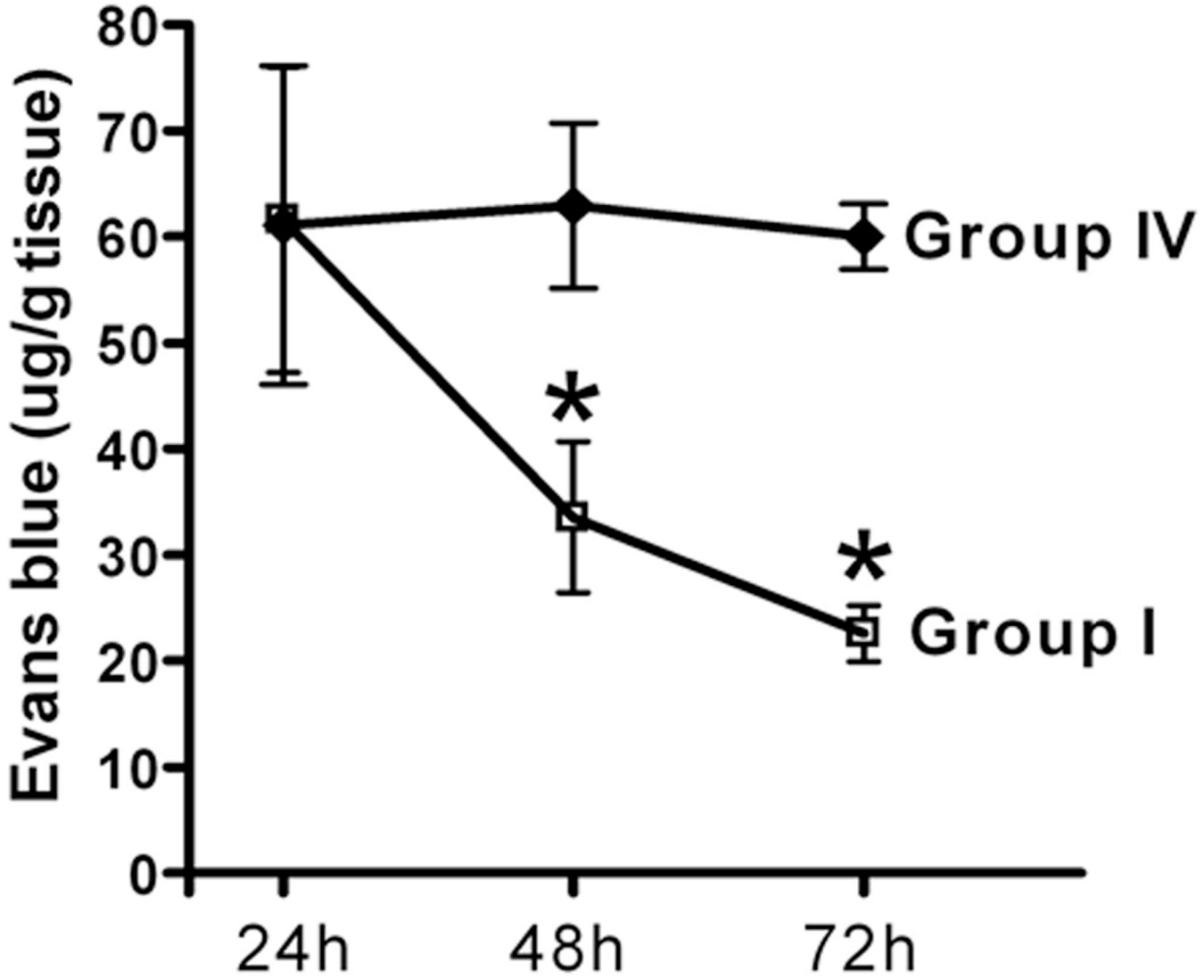

Figure 3. Combined absence of KCs and IMs causes prolonged vascular leakage upon APAP challenge

The extravasation of Evans blue dye into the liver was measured in mice from Group I and Group IV at 24, 48, and 72 h after APAP. *, $p < 0.05$, compared with Group IV. Data shown represent mean ± SEM of 4 mice per group. The baseline level of Evans blue measured in the liver of saline-treated mice is 10 ± 2 μg/g tissue.

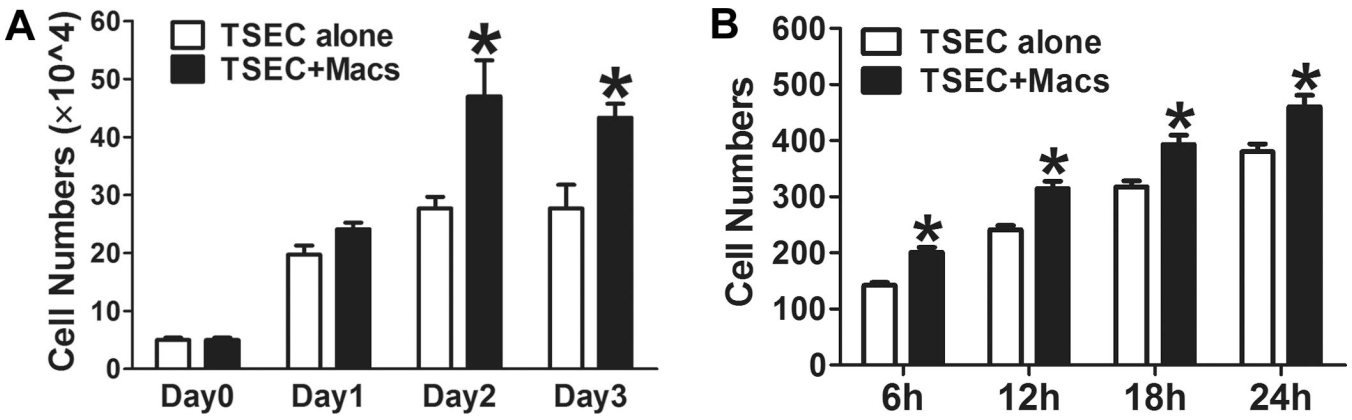

Figure 4. Hepatic Macs promote TSEC proliferation and migration

Hepatic Macs (including KCs and IMs) were isolated from male BALB/cJ mice treated with APAP for 24 h. **(A)** TSECs (50,000 cells/well) were seeded on the bottom of 12-well plates. In the TSEC/Mac co-cultures, hepatic Macs (50,000 cells/well) were cultured in 0.4 μm inserts. After 1, 2, and 3 days, the inserts were removed and the number of TSECs were counted. *, $p < 0.05$ compared with TSECs cultured alone. **(B)** TSECs (300,000 cells/well) were seeded in 12-well plates. A "+" sign was made by scratching each well containing confluent cells with a pipette tip. Subsequently, hepatic Macs (50,000 cells/well) were added in 0.4 μm inserts and co-cultured with TSECs. Images of each well were captured by an inverted microscope imaging system after 0, 6, 12, 18 and 24 h. The cells that migrated into the scratched region were counted.

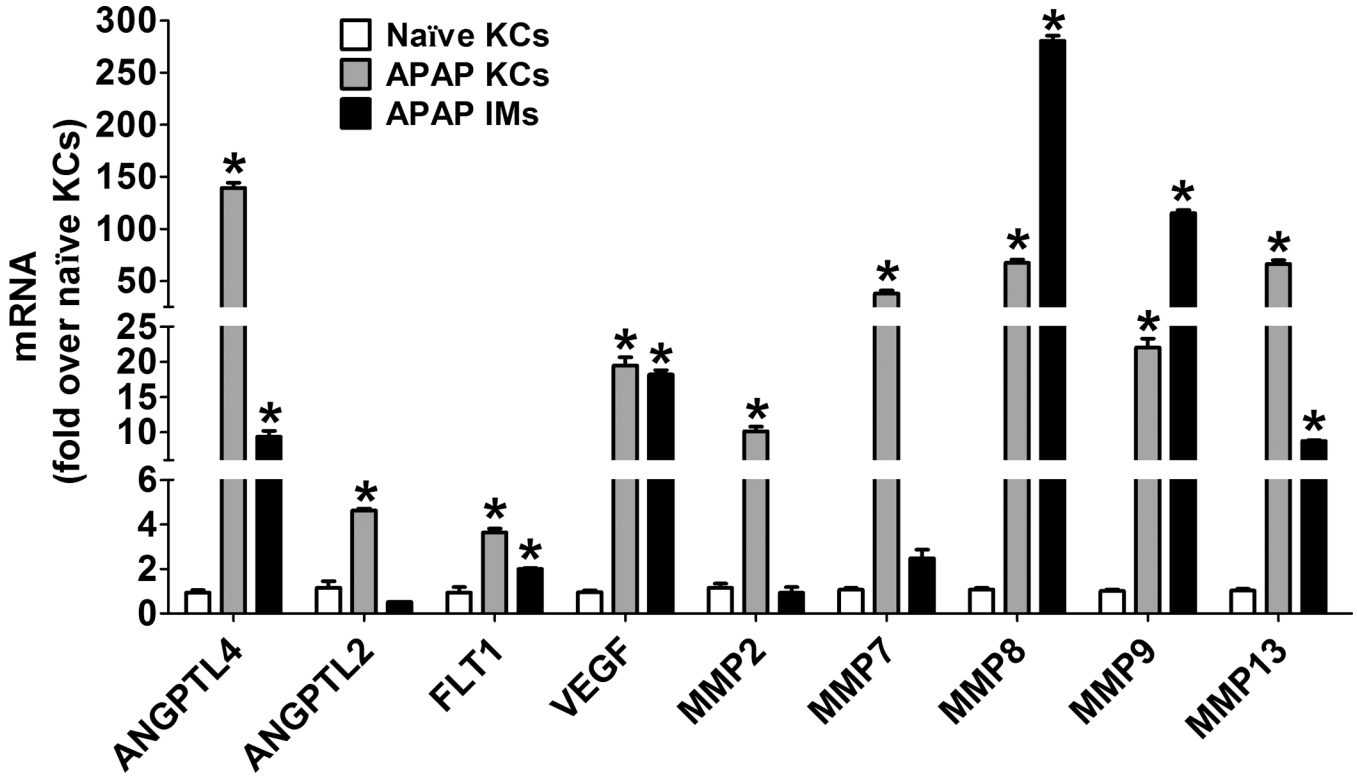

Figure 5. Expression of angiogenic genes by hepatic Macs isolated from APAP-treated mice

At 24 h after APAP challenge, liver KCs and IMs were isolated and separated. Naïve KCs were also isolated from non-treated mice. mRNA expression levels of the following angiogenic genes were measured by qPCR: ANGPTL2 and 4, FLT1, VEGF, and MMP-2, 7, 8, 9, and 13. *, $p < 0.05$ compared with naïve KCs.

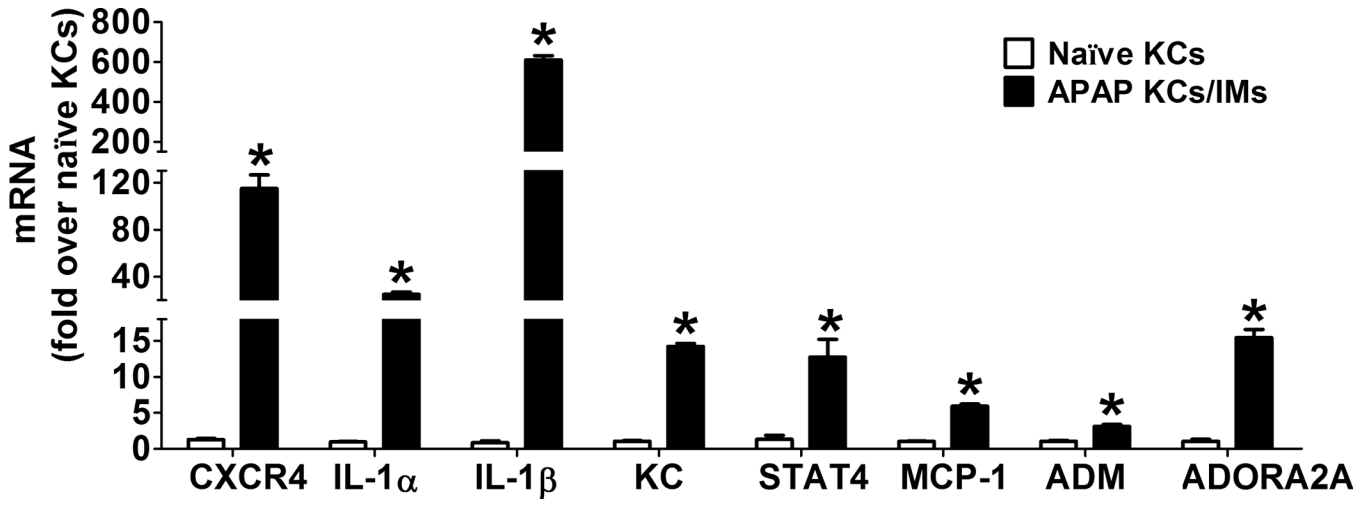

Figure 6. Hepatic Macs exhibit a distinct hypoxia-induced transcription profile

At 24 h after APAP challenge, liver KCs and IMs were isolated and analyzed as a combined population. Naïve KCs were also isolated from non-treated mice. mRNA expression levels for the following panel of genes characteristic of Macs under hypoxic conditions were measured by qPCR: CXCR4, IL-1α, IL-1β, KC (IL-8), STAT4, MCP-1, ADM, ADORA2A. *, $p < 0.05$ compared with naïve KCs.

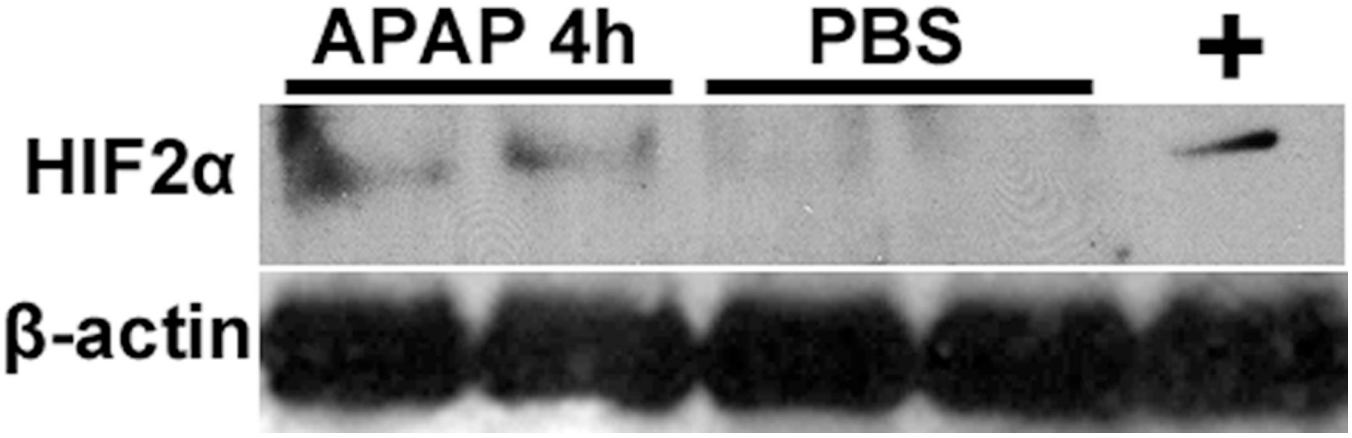

Figure 7. Detection of HIF-2α in the liver of APAP-treated mice

Mice were treated with APAP or PBS (as control) and liver tissues were harvested after 4 h. HIF-2α protein expression was detected by immunoblot analysis using an anti-HIF-2α antibody (1:500 dilution). Protein extracted from hypoxic Hep3B cells was used as a positive control (+) for HIF-2α expression. β-actin expression served as a loading control.

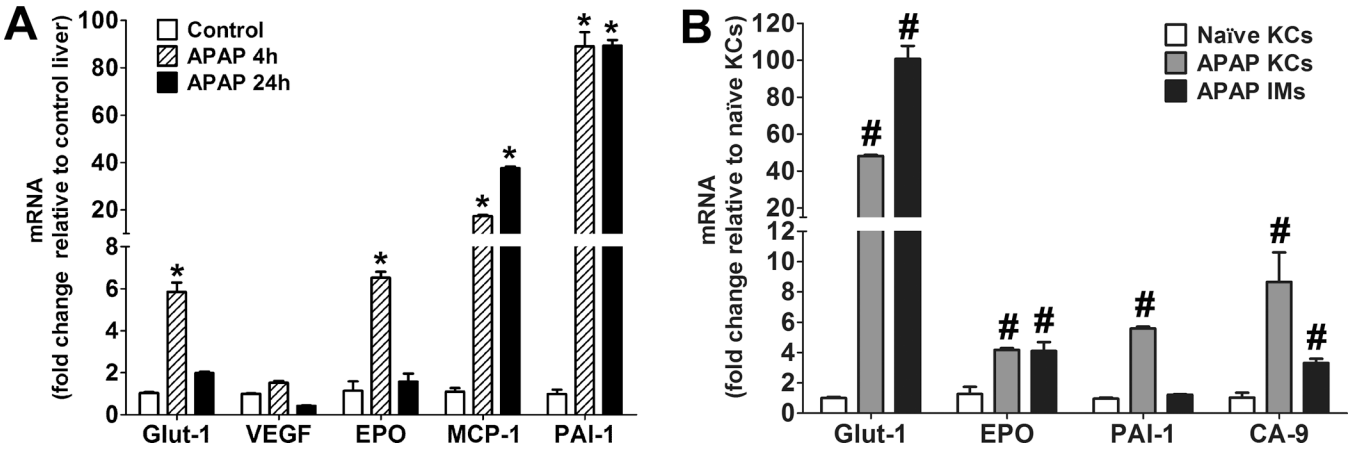

Figure 8. HIF is stabilized in the liver and hepatic Macs of mice treated with APAP

(A) Liver tissues were harvested at 0 (control), 4, and 24 h after APAP challenge. mRNA expression levels of the following HIF-target genes were measured by qPCR: Glut-1, VEGF, EPO, MCP-1, and PAI-1. *, $p < 0.05$ compared with control mice. **(B)** At 24 h after APAP challenge, liver KCs and IMs were isolated and separated. Naïve KCs were also isolated from naïve mice. mRNA expression levels of the following HIF-target genes were measured by qPCR: Glut-1, EPO, PAI-1 and CA-9. #, $p < 0.05$ compared with naïve KCs.

Table 1

Comparison of liver repair after APAP treatment between four groups of mice

Group	Genotype	Treatment	Resultant Mac Populations in the liver
I	WT	empty liposome	KCs, IMs
II	WT	lipo/cld	IMs, -- (absence of KCs)
III	$CCR2^{-/-}$	empty liposome	KCs, -- (absence of IMs)
IV	$CCR2^{-/-}$	lipo/cld	(absence of both KCs and IMs)

Table 2

Target genes	sequences		Amplicon size (bp)
	Forward	Reverse	
GAPDH	AGGTCGGTGTGAACGATTTG	TGTAGACCATGTAGTTGAGGTCA	123
Glut-1	CAGTTCGGCTATAACACTGGTG	GCCCCCGACAGAGAAGATG	156
VEGF	GCACATAGAGAGAATGAGCTTCC	CTCCGCTCTGAACAAGGCT	105
EPO	ACTCTCCTTGCTACTGATTCCT	ATCGTGACATTTTCTGCCTCC	123
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT	121
PAI-1	TTCAGCCCTTGCTTGCCTC	ACATTTTACTCCGAAGTCGGT	116
CA-9	TGCTCCAAGTGTCTGCTCAG	CAGGTGCATCCTCTTCACTGG	126
IL-1α	CGAAGACTACAGTTCTGCCATT	GACGTTTCAGAGGTTCTCAGAG	126
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT	89
IL-8	CCACACTCAAGAATGGTCGC	TCTCCGTTACTTGGGGACAC	101
STAT4	TGGCAACAATTCTGCTTCAAAAC	GAGGTCCCTGGATAGGCATGT	225
ADM	CACCCTGATGTTATTGGGTTCA	TTAGCGCCCACTTATTCCACT	101
CXCR4	GAAGTGGGGTCTGGAGACTAT	TTGCCGACTATGCCAGTCAAG	125
ADORA2A	GCCATCCCATTCGCCATCA	GCAATAGCCAAGAGGCTGAAGA	122
ANGPTL-2	AGCCTGAGAATACCAACCGC	CCCTTTGCTTATAGGTCTCCCAG	134
ANGPTL-4	CATCCTGGGACGACGAGATGAACT	TGACAAGCGTTACCACAGGC	136
FLT1	TAAGCCTGGAACTCATTCT	CCAAAGATGCGACTGTAATGCTG	109
MMP2	CAAGTTCCCCGGCGATGATGTC	TTCTGGTCAAGGTCACCTGTC	171
MMP7	CTGCCACTGTCCCAGGAAG	GGGAGAGTTTTTCCAGTCATGG	175
MMP8	TCTTCCTCCACACACAGCTTG	CTGCAACCATCGTGGCATTC	150
MMP9	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG	145
MMP13	CTTCTTCTTGTTGAGCTGGACTC	CTGTGGAGGTCACTGTAGACT	173