

Chronic alcohol ingestion modulates hepatic macrophage populations and functions in mice

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ABSTRACT

Hepatic Macs, consisting of resident KCs and infiltrating monocytes/IMs, are thought to play an important role in the pathogenesis of ALD. Previous work has focused on KCs or studied hepatic Macs as one cell population. The aim of the current study is to distinguish IMs from KCs and to compare their phenotypes and functions. We show here that a 4-week ethanol feeding of C57BL/6J mice causes recruitment of IMs into the liver. KCs and IMs can be distinguished based on their differential expression of F4/80 and CD11b. IMs can be divided further into two subsets based on their differential expression of Ly6C. KCs and two subsets of IMs were separately purified by FACS. The phagocytosis abilities and the expression profiles of genes related to various functions were compared among different populations of hepatic Macs. Ly6C^{low} IMs exhibit an anti-inflammatory and tissue-protective phenotype; in contrast, Ly6C^{hi} IMs exhibit a proinflammatory, tissue-damaging phenotype. The ratio of Ly6C^{hi}/Ly6C^{low} increases when mice chronically fed ethanol were binged, which significantly enhanced liver injury. Moreover, upon phagocytosis of apoptotic hepatocytes, Ly6C^{hi} IMs switch to Ly6C^{low} IMs. Taken together, chronic ethanol feeding induces the recruitment of two subsets of hepatic IMs, which play different or even opposite roles in

regulating liver inflammation and repair. These findings may not only increase our understanding of the complex functions of Macs in the pathogenesis of ALD but also help us to identify novel therapeutic targets for the treatment of this disease. *J. Leukoc. Biol.* **96**: 657–665; 2014.

Introduction

In the United States, ALD affects more than 10 million people and accounts for 48% of liver cirrhosis-associated deaths [1]. Given the significant economic and health impact of ALD, it is imperative to understand better the underlying pathogenesis to develop new therapeutic strategies. Alcohol metabolism, which generates toxic-reactive metabolites and oxidative stress, is important in the initiation of liver injury. Emerging evidence suggests that subsequent activation of the innate immune system is a critical aspect of the underlying mechanism. An increased number of hepatic Macs have been observed in different stages of ALD [2, 3]. However, the phenotypes and functions of hepatic Macs, as well as their roles in the pathogenesis of the disease, are not yet understood.

It has been demonstrated that alcohol-induced endotoxemia activates and sensitizes liver-resident KCs through TLRs and that activated KCs contribute to alcohol-induced liver injury in mice [4–6]. Factors that regulate KC activation, such as adiponectin, IL-10, STAT3, and sirtuin 1, have been shown to ameliorate ALD [7–10]. Although KCs represent the predominant population of Macs in naive/healthy liver, during infectious or sterile tissue stress, there is often an influx of bone marrow-derived monocytes that plays critical roles in eliminating pathogens and promoting tissue repair and regeneration. Studies of acute and chronic liver injury models have demonstrated the hepatic recruitment of IMs and their roles in the progression and resolution of tissue damage and fibrosis [11–

Abbreviations: ALD=alcohol liver disease, ALT=alanine aminotransferase, APAP=acetaminophen, Arg1=arginase 1, ClITA=class II MHC transactivator, COX=cyclooxygenase, EtOH=ethyl alcohol, Fizz1=resistin-like molecule α 1, HGF=hepatocyte growth factor, HSP=heat shock protein, IGF=insulin growth factor, IL-1R2=IL-1R, type II, IL-1RA=IL-1R antagonist, IM=infiltrating macrophage, IP-10=IFN-inducible protein 10, KC=Kupffer cell, M1=classically activated type 1, M2=alternatively activated type 2, Macs=macrophages, MDSC=myeloid-derived suppressor cell, MMP=matrix metalloproteinase, Mrc1=mannose receptor C type 1, NPC=nonparenchymal cell, Nr4a1=nuclear receptor subfamily 4 group A member 1, Nr4a1^{-/-}=nuclear receptor subfamily 4 group A member 1-deficient, PC=phosphatidylcholine, PS=phosphatidylserine, qPCR=quantitative PCR, VEGF=vascular endothelial growth factor, Ym1=chitinase 3-like 3

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14]. The goals of the current study are to (1) determine whether alcohol ingestion causes hepatic recruitment of Macs and (2) examine the phenotypes of IMs and compare with those of resident KCs.

Based on *in vitro* studies of cytokine stimulation, Macs are divided into M1 (stimulated by LPS and IFN- γ) and M2 (stimulated by IL-4) Macs. M1 cells have antimicrobial and proinflammatory functions, and they also induce matrix degradation and cause tissue destruction. In contrast, M2 cells have anti-inflammatory activities and promote angiogenesis, matrix synthesis, and tissue remodeling. Although useful, the M1/M2 phenotyping of Macs is oversimplified, owing to the substantial plasticity of Macs *in vivo*. Macs have a unique ability to alter their phenotypes and thus functions, depending on the tissue microenvironmental cues, such as cytokines, growth factors, pathogen-associated molecular pattern molecules, and damage-associated molecular pattern molecules. The heterogeneity of Macs is reflected on their differential, sometimes opposing, roles in various diseases. Proinflammatory Macs are responsible for eliminating bacteria and viruses; however, they also play a pathological role in autoimmune diseases (such as arthritis and multiple sclerosis) and metabolic diseases, including insulin resistance, diabetes, and atherosclerosis [15]. In contrast, the wound-healing and immune regulatory Macs, when dysregulated, are often associated with chronic diseases, such as atopic dermatitis, asthma, tissue fibrosis, and cancer [16, 17]. Therefore, the investigation of the phenotypes and functions of Macs is crucial in understanding the pathogenesis and uncovering therapeutic targets of ALD.

In the current study, we detected IMs in the liver of alcohol-treated mice. The IMs (CD11b^{hi}F4/80^{int}Ly6C⁺) and resident KCs (CD11b^{low}F4/80^{hi}Ly6C⁻) can be distinguished by their differential expression of cell surface markers. Moreover, we uncovered two subsets of IMs that have very distinct gene expression profiles. The data suggest that the Ly6C^{hi} subset is proinflammatory and that upon phagocytosis of apoptotic hepatocytes, they can switch to the Ly6C^{low} subset, which may be important in tissue repair.

MATERIALS AND METHODS

Animal treatment

Female and male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were maintained under pathogen-free conditions in the Center for Laboratory Animal Care at the University of Colorado Anschutz Medical Campus (Aurora, CO, USA). For all experiments, mice were divided randomly into pair-fed and EtOH-fed groups. EtOH-fed groups were allowed free access to the ethanol-containing Lieber-DeCarli liquid diet (Bio-Serv, Flemington, NJ, USA). Ethanol content was introduced gradually by increasing 1.6% (v/v) every 2 days until 5%. All mice will then be fed the liquid diet containing 5% ethanol for 4 weeks (or longer), as described previously [7, 18]. The pair-fed control group received equal amount of calories in a liquid diet, in which maltose dextrins were used to substitute isocalorically for EtOH.

Isolation of liver NPCs

Liver NPCs were isolated following a method established previously [11]. Briefly, a 20-G catheter was put through mouse superior vena cava, the inferior vena cava was clamped, and the portal vein cut. The liver was per-

fused with HBSS, followed by a digestion buffer [1 \times HBSS, supplemented with 0.04% collagenase (type IV; Sigma, St. Louis, MO, USA), 1.25 mM CaCl₂, 4 mM MgSO₄, and 10 mM HEPES]. After digestion, the liver was disrupted in ACD solution (1 \times HBSS, supplemented with 0.5% FBS, 0.6% citrate-dextrose solution, and 10 mM HEPES). Single cells were passed through a 100- μ m 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.

Flow cytometry analysis and FACS

Liver NPCs were incubated with normal rat serum (Sigma) and anti-mouse Fc γ RII/III (Becton Dickinson, Franklin Lakes, NJ, USA) to minimize non-specific antibody binding. Subsequently, the cells were stained with anti-CD45, anti-Ly6C, and anti-Ly6G (Becton Dickinson) and anti-F4/80, anti-CD11b, anti-IFN- γ , and anti-TNF- α (eBioscience, San Diego, CA, USA). Cells were analyzed on a FACScan cytometer (Cytek Development, Fremont, CA, USA) using FlowJo software (Tree Star, Ashland, OR, USA). For flow cytometric analysis, CD45⁺ cells were gated to exclude endothelial cells, stellate cells, and residue hepatocytes. From CD45⁺ cells, the expressions of CD11b and F4/80 were examined.

To purify KCs, IMs, as well as Ly6C^{hi} and Ly6C^{low} IMs, liver NPCs were stained with antibodies, as described above and sorted using a MoFlo High-Performance Cell Sorter (Cytomation, Fort Collins, CO, USA). For morphological analysis, cells were cytospun onto Shandon Cytoslides and stained with the Hema 3 manual staining system (Fisher Scientific, Pittsburgh, PA, USA).

In vivo and in vitro phagocytosis

For *in vivo* phagocytosis, FITC-labeled liposomes, containing 70% PC and 30% PS, were prepared freshly and *i.v.*-injected to mice. After 30 min, liver NPCs were isolated and the cells stained with anti-Ly6C, anti-Ly6G, anti-F4/80, and anti-CD45 antibodies.

For *in vitro* phagocytosis, Ly6C-positive IMs were purified with anti-Ly6C-PE and EasySep PE selection kit (Stemcell Technologies, Vancouver, BC, Canada). Primary mouse hepatocytes were isolated from C57BL/6J mice, and apoptosis was induced by incubating with anti-mouse CD95 (Becton Dickinson) at 37°C for 4 h. This preparation yielded >90% AnnexinV-positive apoptotic hepatocytes. Ly6C-positive cells (7×10^5) were then cocultured with 7×10^5 apoptotic hepatocytes for 16 h at 37°C. Non-ingested hepatocytes were removed by washing three times with PBS, and residual adherent Macs were used for additional mRNA expression analysis.

qPCR analyses

Total RNA was extracted from liver tissues or various types of cells using RNeasy Kits (Qiagen, Valencia, CA, USA), as described by the manufacturer. RNA (0.5 μ g) was reverse-transcribed to cDNA using Moloney murine leukemia virus RT and amplified using GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and primers for specific genes. All PCR products were measured using a Real-Time PCR 7500 SDS system and software (Life Technologies, Grand Island, NY, USA).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. Differences in values were considered significant at $P < 0.05$.

RESULTS

Chronic ethanol feeding causes hepatic recruitment of IMs that can be distinguished from resident KCs

Although in a naive state, KCs represent the predominant population of Macs in the liver, there is a dynamic change of the composition of Macs under conditions of hepatic insults. To

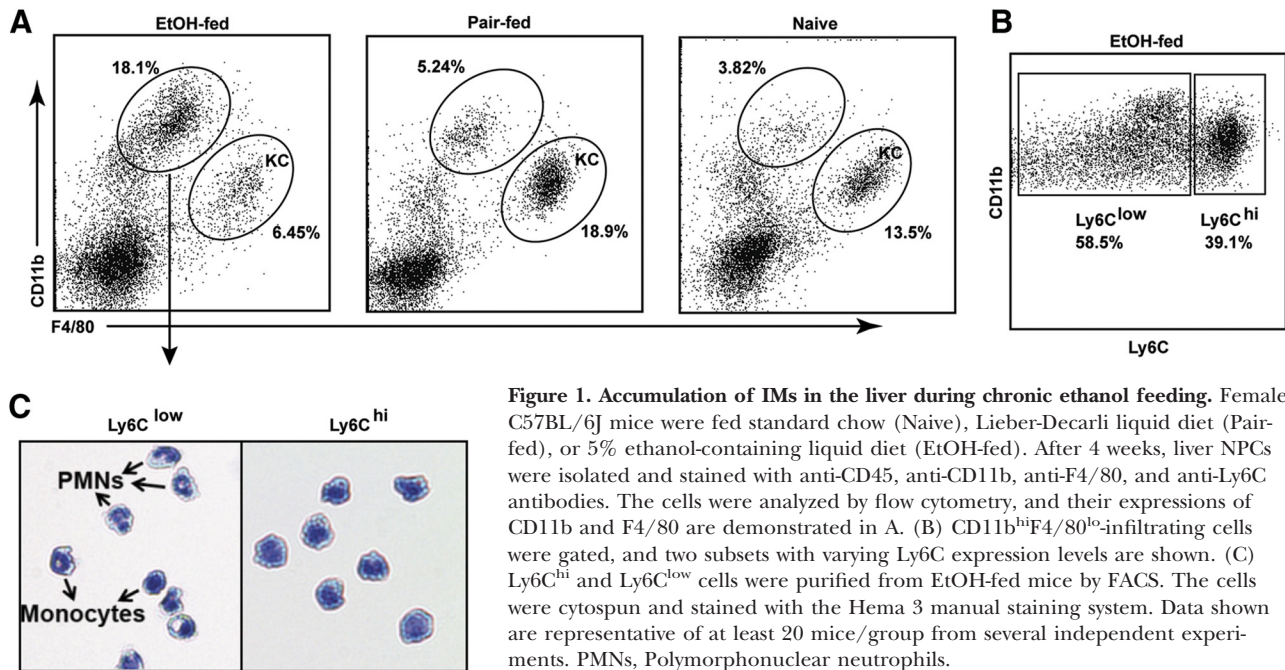


Figure 1. Accumulation of IMs in the liver during chronic ethanol feeding. Female C57BL/6J mice were fed standard chow (Naive), Lieber-Decarli liquid diet (Pair-fed), or 5% ethanol-containing liquid diet (EtOH-fed). After 4 weeks, liver NPCs were isolated and stained with anti-CD45, anti-CD11b, anti-F4/80, and anti-Ly6C antibodies. The cells were analyzed by flow cytometry, and their expressions of CD11b and F4/80 are demonstrated in A. (B) CD11b^{hi}F4/80^{lo}-infiltrating cells were gated, and two subsets with varying Ly6C expression levels are shown. (C) Ly6C^{hi} and Ly6C^{low} cells were purified from EtOH-fed mice by FACS. The cells were cytospun and stained with the Hema 3 manual staining system. Data shown are representative of at least 20 mice/group from several independent experiments. PMNs, Polymorphonuclear neutrophils.

investigate whether ethanol ingestion affects hepatic Mac populations, C57BL/6J mice were fed an ethanol-containing Lieber-Decarli diet for 4 weeks. Control mice were fed an isocaloric liquid diet for the same duration. Liver NPCs were analyzed by flow cytometry using antibodies that recognize various monocyte/Mac cell surface markers, including F4/80, CD11b, and Ly6C. We observed a marked increase of IMs in the liver of ethanol-fed compared with pair-fed and naive mice (Fig. 1A). Resident KCs express a high level of F4/80 and low level of CD11b, and they do not express Ly6C (F4/80^{hi}CD11b^{low}Ly6C⁻). In contrast, IMs express a low level of F4/80 and high level of CD11b, and they are positive for Ly6C (F4/80^{int}CD11b^{hi}Ly6C⁺). Hepatic recruitment of IMs was observed in male and female mice. Similar numbers of hepatic IMs were detected in mice fed ethanol for 3, 4, 9, or 14 weeks.

Two subsets of IMs with high and low expression levels of Ly6C were detected (Fig. 1B). The Ly6C^{hi} and Ly6C^{low} subsets were purified by FACS and the morphology examined. The Ly6C^{hi} subset consists of mainly monocytes/Macs with horse-shoe- or kidney-shaped nuclei. Aside from monocyte/Macs, the Ly6C^{low} population contains ~30% neutrophils with multilobular nuclei (Fig. 1C and Supplemental Fig. 1).

A number of mediators have been identified to be released and play a role in the recruitment and expansion of myeloid cells into inflamed, injured tissues or tumors. We found that many of these mediators, such as IL-1 β , IL-6, S100A8/A9, HSP72, and COX-2, are elevated in the liver after ethanol exposure (Fig. 2).

Characterization and comparison of the phenotypes of IMs and KCs

IMs and KCs may have similar or opposite phenotypes and functions; thus, we purified each population separately by FACS. As neutrophils were detected among Ly6C^{low} cells, anti-

body against Ly6G, which is expressed on neutrophils but not on Macs, was used to exclude neutrophils (Fig. 3A). Purified KCs (CD45⁺Ly6G^{low}F4/80^{hi}CD11b^{low}) and IMs (CD45⁺Ly6G^{low}F4/80^{int}CD11b^{hi}) were then compared for their expressions of genes known to be involved in various functions of Macs. KCs from pair-fed mice were also purified to serve as control.

The expression levels of MMPs, such as MMP9 and MMP12, were increased significantly in hepatic Macs from ethanol-fed mice compared with KCs from pair-fed mice (Fig. 3B). MMP9 expression increased more than 10-fold in IMs, and MMP12 increased nearly 60-fold in KCs. MMP9 (gelatinase B) degrades type IV collagens, which is the major structural component of basement membranes. MMP12 (macrophage elastase) de-

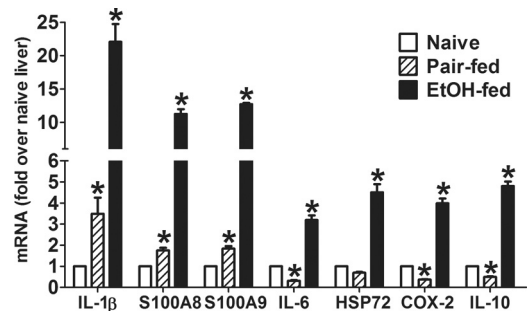


Figure 2. Ethanol treatment induces genes involved in promoting hepatic IM accumulation. Female C57BL/6J mice were fed standard chow (Naive), Lieber-Decarli liquid diet (Pair-fed), or 5% ethanol-containing liquid diet (EtOH-fed). After 4 weeks, liver tissues were harvested and RNA extracted from five mice/group. mRNA levels of various genes were determined by qPCR. * P < 0.05 compared with naive mice. Data shown are from five mice/group.

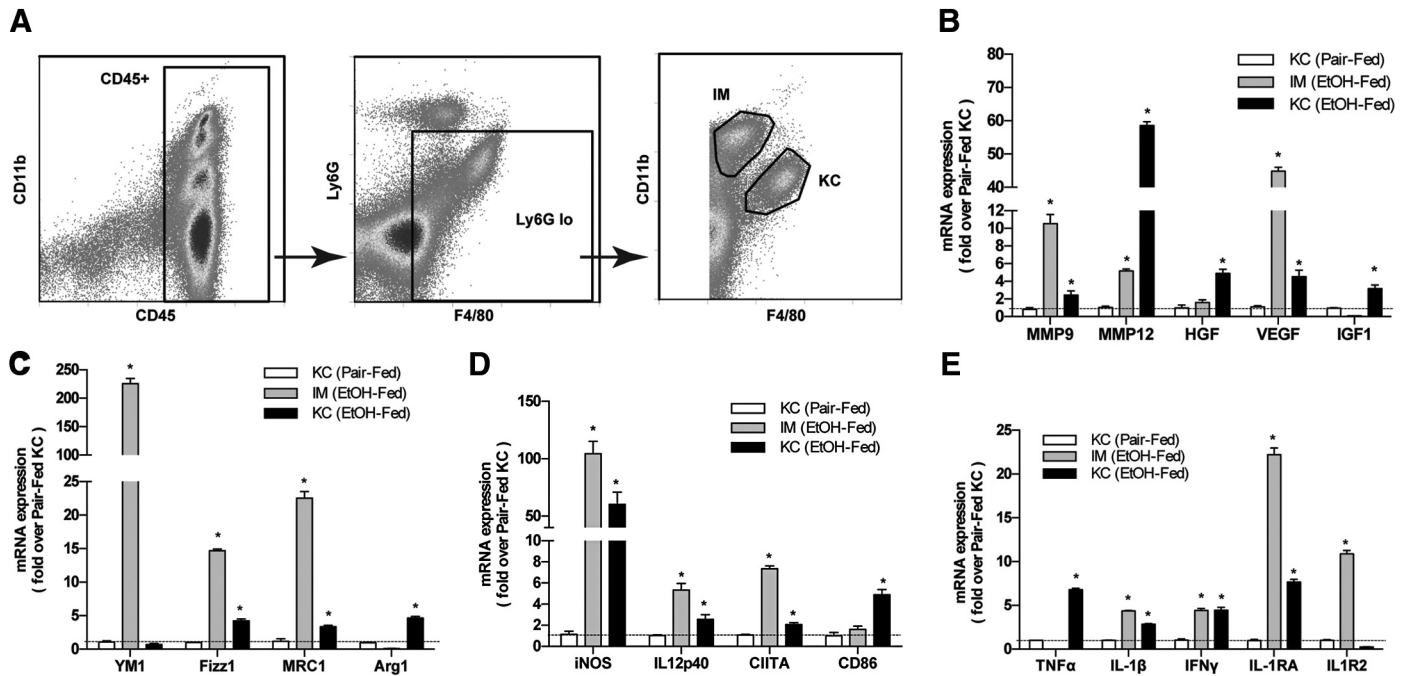


Figure 3. Phenotype comparison between IMs and KCs. Liver NPCs were isolated from pair-fed and EtOH-fed mice, and cells from eight mice/group were pooled. Subsequently, KCs from pair-fed mice and KCs and IMs from EtOH-fed mice were purified by FACS. (A) Gating strategy for purification of IMs and KCs. CD45⁺ cells were selected to eliminate nonimmune cells in the liver. Ly6G^{low} cells were selected to exclude neutrophils. (B–E) Total RNA was isolated from each type of purified cells and qPCR analyses of various genes performed. **P* < 0.05 compared with KCs from pair-fed mice.

grades soluble and insoluble elastin. The results suggest that IMs and KCs are involved in matrix remodeling during chronic ethanol exposure and that their roles are different in terms of which types of matrix they degrade.

Growth factors play a critical role during tissue regeneration. Chronic ethanol feeding led to significant increases of HGF and IGF expressions in KCs. Although VEGF expression levels were up-regulated in KCs and IMs, the increase was much more dramatic in IMs (>40-fold; Fig. 3B). The combined increase of MMP9 and VEGF expressions in IMs strongly suggests angiogenic and tissue-repair/remodeling functions of the IMs.

A number of genes that have been typically associated with M1 or M2 Macs were measured. The M2 markers Ym1, Fizz1, and Mrc1 were induced significantly in IMs. Another typical M2 gene, Arg1, is moderately up-regulated in KCs but not in IMs (Fig. 3C). Interestingly, genes that have been described as M1 markers, such as iNOS, IL-12p40, and CIITA, were also up-regulated significantly in IMs. Some of the M1 markers were also increased in KCs (Fig. 3D). Furthermore, IMs and KCs from ethanol-fed mice expressed elevated levels of proinflammatory cytokines TNF- α , IL-1 β , and IFN- γ (Fig. 3E). Although the increases of proinflammatory cytokines were modest, the expression levels of IL-1RA and IL-1R2 were up-regulated in IMs dramatically (Fig. 3E).

Ly6C^{hi} and Ly6C^{low} IMs exhibit opposite phenotypes

Our data showed that IMs express M1 and M2 markers, as well as pro- and anti-inflammatory cytokines. This mixed phenotype

may be attributed to the fact that there are two subsets of IMs. To examine this hypothesis, we purified the Ly6C^{hi} and Ly6C^{low} IMs from ethanol-fed mice and compared their phenotypes. The data show clearly distinctive gene expression profiles (Fig. 4). The Ly6C^{hi} IMs express higher levels of the majority of proinflammatory cytokines, chemokines, and their receptors, such as TNF- α , IL-1 β , CCL2, CCL24, IP-10, CCR2, and CX3CR1. In contrast, the Ly6C^{low} IMs express IL-1R2, the decoy receptor that blocks IL-1 α and IL-1 β signaling. Of all the growth factors and matrix degradation genes measured, Ly6C^{low} IMs express markedly higher levels than Ly6C^{hi} IMs. Moreover, the Ly6C^{low} IMs express significantly higher levels of the phagocytosis-related genes. These findings indicate a tissue repair/remodeling function of the Ly6C^{low} IMs (Fig. 4).

Ly6C^{low} IMs express the majority of M2 markers (Arg1, Mrc1, and Fizz1), except for Ym1. Ly6C^{hi} IMs express M1 markers, such as iNOS, CD86, CIITA, and IL-12p40. These observations are consistent with the proinflammatory phenotype of Ly6C^{hi} IMs and the anti-inflammatory phenotype of Ly6C^{low} IMs. Ym1 was first identified in peritoneal Macs in response to nematode infection [19]. Therefore, Ym1 was described as a M2 marker, although its function is not clear. Consistent with our observation, a recent study of the role of Macs in CCL₄-induced liver fibrosis also reported that Ym1 was expressed by a subset of proinflammatory Macs but not by the subset of protective or tissue-restorative Macs [13]. The mRNA expression levels of TLR1–9 were compared between Ly6C^{hi} and Ly6C^{low} IMs. The expression levels of TLR1, -2, and -5–8 were similar

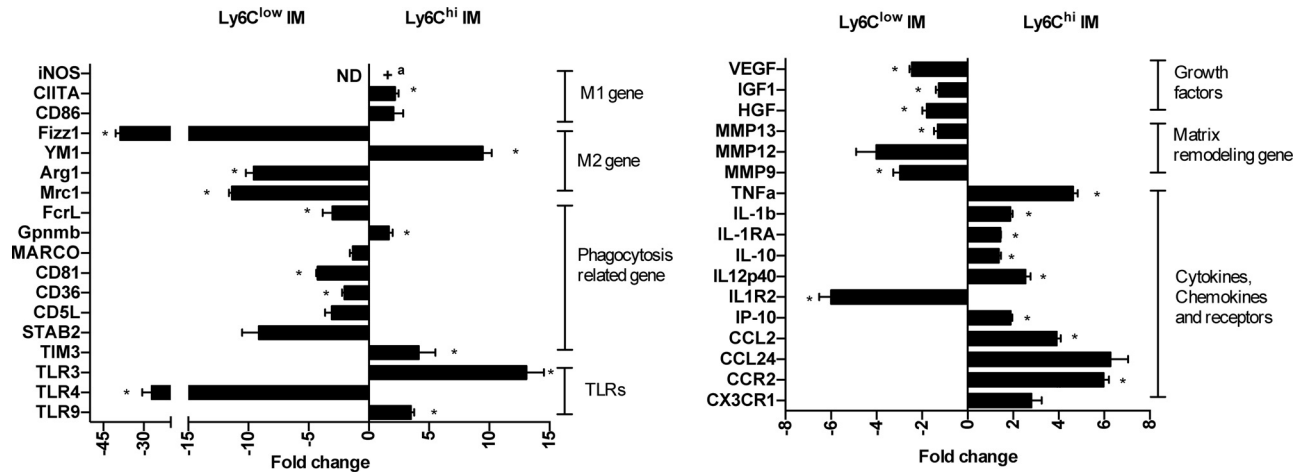


Figure 4. Ly6C^{hi} and Ly6C^{low} IMs exhibit distinct gene expression profiles. Liver NPCs were isolated from EtOH-fed mice. Ly6C^{hi} and Ly6C^{low} IMs were purified by FACS, and neutrophils were excluded using a similar gating strategy as shown in Fig. 3. Differential expressions of various genes between Ly6C^{hi} and Ly6C^{low} IMs are shown as fold changes between the two subsets. **P* < 0.05 compared with Ly6C^{lo} or Ly6C^{hi} IM; a, detectable in Ly6C^{hi} IM; ND, not detectable; Fcrl, Fc receptor-like A; Gpnmb, glycoprotein nonmetastatic melanoma protein B; MARCO, macrophage receptor with collagenous structure; CD5L, CD5 antigen-like; STAB2, stabilin 2; TIM3, T cell Ig mucin 3.

in these two subsets. Ly6C^{hi} IMs express higher levels of TLR3 and -9, but Ly6C^{low} IMs express higher levels of TLR4 (Fig. 4).

To determine protein levels of IFN- γ and TNF- α , liver NPCs were isolated from ethanol-fed mice and stimulated in vitro with 1 μ g/mL LPS overnight. Cytokine expression was measured by intracellular staining and flow cytometry. All three types of Macs produce TNF- α , although KCs express significantly lower levels. Only IMs, but not KCs, produce IFN- γ . The Ly6C^{hi} IMs express slightly higher levels of the cytokines than the Ly6C^{low} IMs (Fig. 5).

IMs and KCs are potent phagocytic cells

Phagocytosis of cellular debris is an important step during tissue repair and regeneration. Macs are known for their functions of taking up dead cells and cellular debris. Our data show that several receptors involved in recognition of dead cells were up-regulated significantly in KCs and IMs isolated from ethanol-fed mice (Fig. 6A). In fact, phagocytosis of apoptotic cells can, in turn, regulate the function of Macs [20]. To investigate this further, we synthesized liposomes that consist of 70% PC and 30% PS to mimic apoptotic cells and injected the fluorescent-labeled liposomes to pair- and ethanol-fed mice. Whereas the liposomes were mainly taken up by KCs (89%) in pair-fed mice, significant amount of liposomes were also taken up by Ly6C^{hi} and Ly6C^{low} IMs in ethanol-fed mice (Fig. 6B). It is reported that phagocytosis of apoptotic cells renders an anti-inflammatory phenotype of Macs [21, 22]. Recent studies also suggest that after recruitment to damaged tissues, monocytes switch from a proinflammatory, tissue-damaging phenotype to an anti-inflammatory, tissue-protective phenotype [23, 24]. We examined whether ingestion of hepatocytes debris could cause the switching of Ly6C^{hi} IMs to Ly6C^{low} IMs. Liver NPCs were isolated from ethanol-fed mice, and IMs were purified by MACS using anti-Ly6C antibody. The data show that compared with Ly6C⁺ IMs alone, those incu-

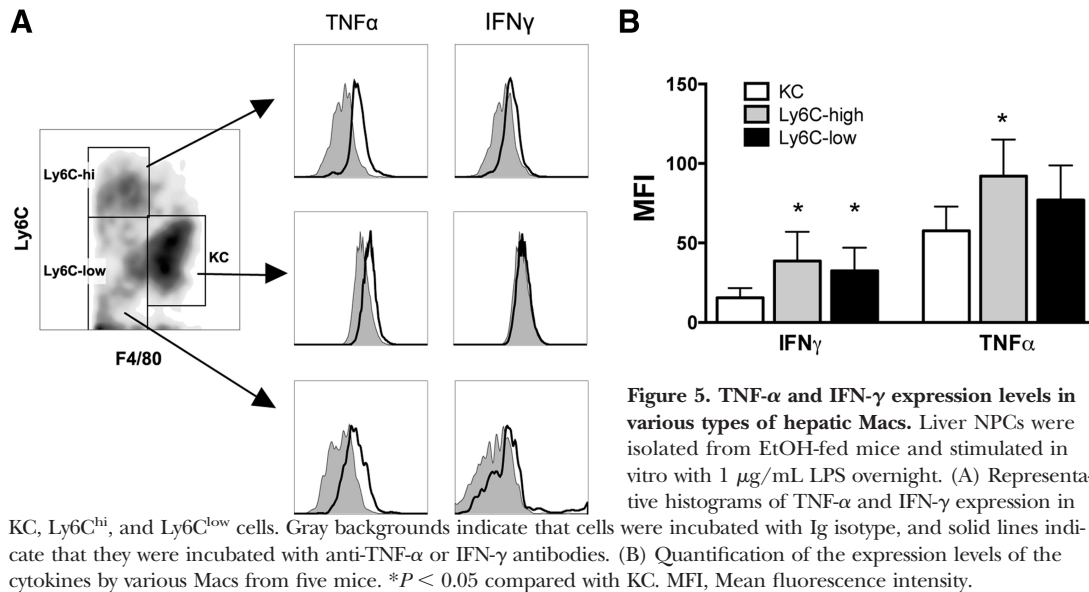
bated with apoptotic hepatocytes appear to shift toward a phenotype similar to that of Ly6C^{low} IMs, with decreased expression of proinflammatory factors (TNF- α , IL-1 β , and CCR2) and increased expression of anti-inflammatory mediators and M2 markers (Arg1, Fizz1, IL-10, and IL-1R2). Moreover, growth factors (VEGF and IGF) and tissue-remodeling MMPs (MMP9 and MMP12) were increased dramatically when Ly6C⁺ IMs were incubated with apoptotic hepatocytes (Fig. 6C).

The ratio of Ly6C^{hi}/Ly6C^{low} IMs correlates with the severity of liver injury

The phenotypes of Ly6C^{hi} and Ly6C^{low} IMs suggest that the ratio of the numbers of Ly6C^{hi}/Ly6C^{low} IMs may correlate with the severity of ALD. To examine this hypothesis, we treated mice with an acute dose of ethanol or LPS after 4 weeks of chronic ethanol feeding. The data demonstrate that LPS increased the ratio of Ly6C^{hi}/Ly6C^{low} from 1:3 to 3:1. Similarly, ethanol binge caused a significant increase of the proportion of the proinflammatory Ly6C^{hi} IMs, which correlates with the elevated ALT activity (Fig. 7). Ethanol binge and LPS challenge resemble clinical situations of acute-on-chronic alcohol consumption and an episode of bacterial infection, respectively. Our data suggest that the Ly6C^{hi} IMs may be involved in the tissue inflammation and damage under these conditions.

DISCUSSION

During tissue injury, bone marrow-derived monocytes influx the liver, resulting in heterogeneous composition of hepatic Macs. Therefore, it is necessary to investigate the respective phenotypes of various populations of hepatic Macs, as they may play different roles in liver diseases. We show that chronic ethanol feeding results in this recruitment of IMs into the liver. This observation is made possible, as we can distinguish IMs from resident KCs by



flow cytometry, based on their differential expression of several surface markers. Chronic ethanol treatment activates IMs and KCs and up-regulates their expression of genes involved in functions of phagocytosis, matrix degradation, and tissue regeneration. Within the two subsets of IMs identified, the Ly6C^{hi} IMs represent a proinflammatory phenotype, and the Ly6C^{low} IMs exhibit a tissue-protective phenotype. The data demonstrate that Ly6C^{hi} IMs can switch to Ly6C^{low} IMs upon phagocytosis of apoptotic cells. Importantly, the relative abundance of Ly6C^{hi} cells increases and correlates with increased liver injury in an acute-on-chronic ALD model.

It has been suggested that under pathological conditions, tissue-resident Macs are insufficient to mediate microbial control and subsequent tissue repair. Instead, the bone marrow or local tissue stem cells produce myeloid cells to deal with the need of microbial clearance and tissue repair. This process is called “emergency myelopoiesis,” and it is considered a common, conserved pathway found in many different types of tissue stress, such as infection, trauma, tumor, and inflammation [25]. Our previous studies have demonstrated the hepatic recruitment of IMs during the time of liver repair in APAP-treated mice [11]. In the liver of CCl₄-treated mice, an influx of bone marrow-derived monocytes, constituting up to a ten-fold increase of hepatic Macs, has also been reported [12]. A more recent study of chronic CCl₄-induced liver fibrosis described the importance of IMs in tissue restoration after injury, as well as in the progression and regression of fibrosis [13]. Similarly, the present study demonstrates the infiltration of Macs into the liver after chronic ethanol ingestion.

Furthermore, we found two subsets of IMs (Ly6C^{hi} and Ly6C^{low}) with different relative abundance in chronic versus chronic-binge alcohol administration. It is our hypothesis that the Ly6C^{hi} IMs are recruited into the liver and give rise to the Ly6C^{low} IMs in response to microenvironmental cues, such as engulfing apoptotic cells. Ample evidence supports this hypothesis. It has been demonstrated that CD62 ligand, which is required for monocytes extravasation, is only expressed by the

Ly6C^{hi} monocytes but not the Ly6C^{low} monocytes [26]. A nuclear hormone receptor, Nr4a1, is required for Ly6C^{low} monocyte production in the bone marrow; thus, circulating Ly6C^{low} monocytes are absent in Nr4a1^{-/-} mice [27]. However, two recent studies show that during muscle injury and myocardial infarction, the accumulation of Ly6C^{hi} and Ly6C^{low} IMs is similar between wild-type and Nr4a1^{-/-} mice [28, 29], suggesting that the Ly6C^{hi} IMs can give rise to Ly6C^{low} IMs. Moreover, during muscle injury, Ly6C^{hi} monocytes are recruited to the damaged muscle first and then replaced by Ly6C^{low} monocytes, which counteract inflammation and promote tissue repair [23, 30]. In a unilateral ureteral obstruction model of kidney injury, it was observed that Ly6C^{hi} monocytes are recruited to the injured kidney and differentiate into functionally distinct Ly6C^{low} cells [31]. In the liver of CCl₄-induced fibrosis, it was demonstrated that the Ly6C^{low} IMs, which were derived from the Ly6C^{hi} IMs, play an important role in resolving inflammation and fibrosis and restoring tissue homeostasis [13]. Our data suggest that during chronic alcohol administration, Ly6C^{hi} cells are recruited continuously and become Ly6C^{low} cells, and a steady state is reached with a ratio of Ly6C^{hi}/Ly6C^{low} ~1:3. Alcohol binge causes an acute damage to the tissue and results in increased recruitment of Ly6C^{hi} cells, thereby increasing the Ly6C^{hi}/Ly6C^{low} ratio to 1:1.

Consistent with hepatic recruitment of IMs, we found that ethanol feeding caused an ~20% increase of Ly6C^{hi} monocytes in the bone marrow and 60% increase of Ly6C^{hi} monocytes in the blood (Supplemental Fig. 2). Ethanol ingestion causes an increase in gut permeability and elevation of endotoxin release. It is interesting to speculate that ethanol-induced endotoxemia may trigger the bone marrow to release monocytes into the circulation. It has been shown that injection of a minute amount of LPS (2 ng/mouse) can activate bone marrow mesenchymal stem cells and induce the release of monocytes into bloodstream within 4 h [32]. Nonetheless, a direct stimulatory effect of ethanol on the bone marrow is also possible. Further studies are warranted to delineate the mecha-

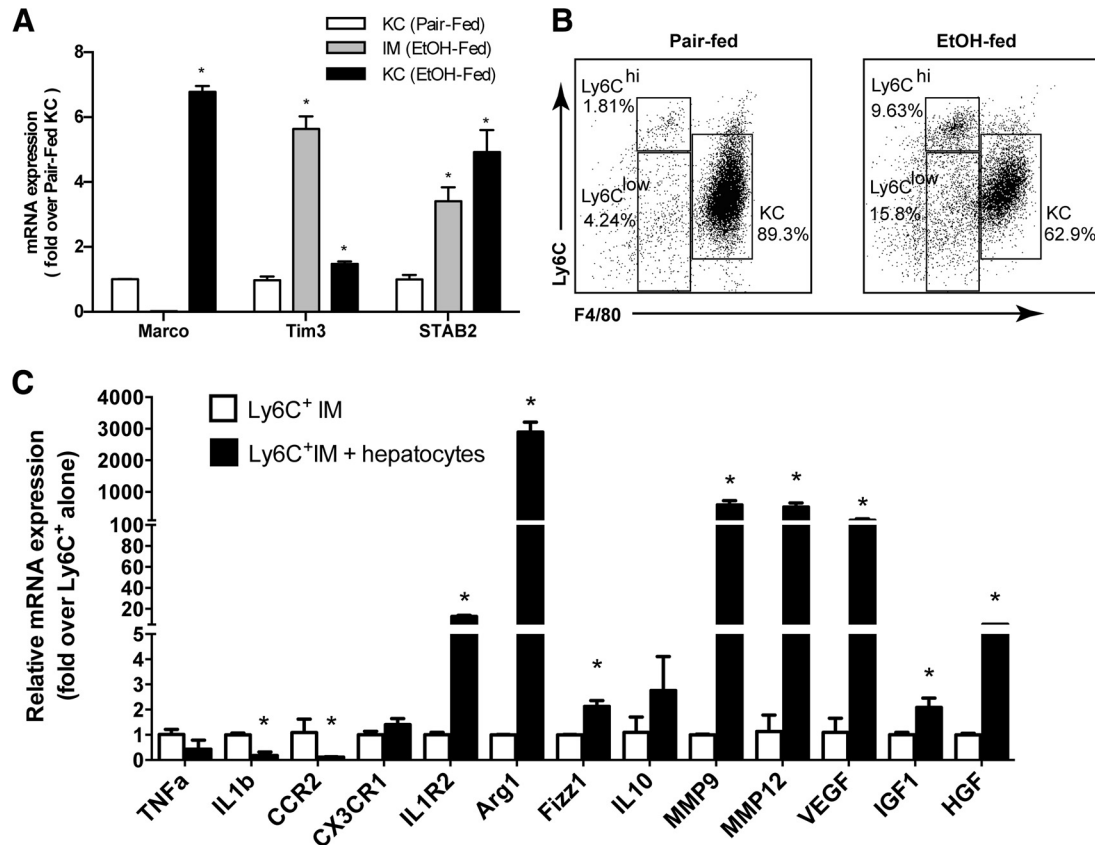


Figure 6. Phagocytosis abilities of hepatic Macs. (A) Expression of phagocytosis-related genes by IMs and KCs. (B) FITC-labeled liposomes were i.v.-injected to pair- and EtOH-fed mice. After 30 min, liver NPCs were isolated and analyzed by flow cytometry. Liposome-positive cells were gated, and the percentages of liposome-positive cells in each cell type are presented. Data shown are representative of five mice/group. (C) Liver NPCs were isolated from EtOH-fed mice. The cells are pooled from five mice, and Ly6C⁺ cells were purified by MACS and incubated with apoptotic hepatocytes for 16 h. Changes of gene expression levels compared with Ly6C⁺ cells alone are shown. * $P < 0.05$ compared with Ly6C⁺ cells alone.

nism. A number of mediators contribute to the expansion and recruitment of myeloid cells into inflamed or injured tissues [33–35]. With regard to cellular composition, morphology, and phenotype, the IMs observed in the liver of alcohol-fed

mice bear similarities to the tumor-associated MDSCs. Although the initial observation and most of the current characterization of MDSCs have come from studies of cancer, accumulating evidence demonstrates the tissue recruitment and

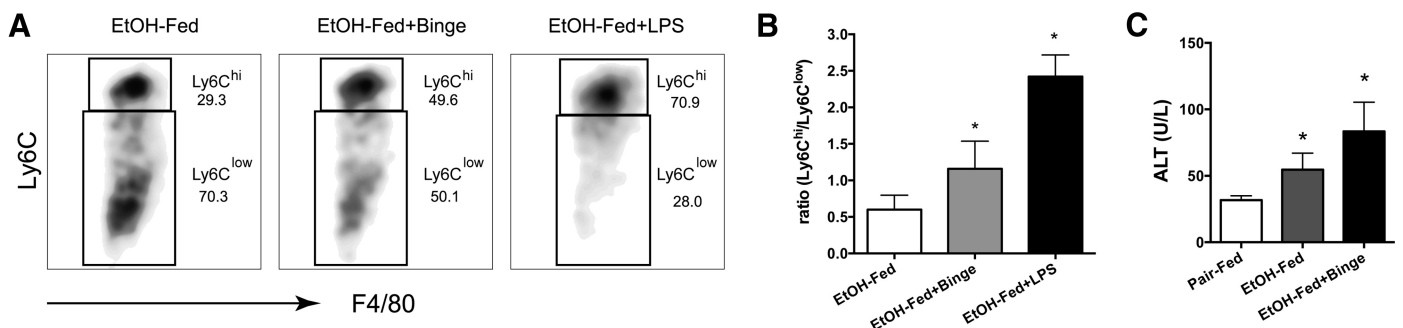


Figure 7. Ethanol binge and LPS increase the ratio of Ly6C^{hi}/Ly6C^{low} IMs in chronic ethanol-fed mice. Mice fed ethanol for 4 weeks were gavaged with one acute dose of ethanol (5 g/kg) or i.p.-injected with LPS (10 μ g/mouse). After 18 h, NPCs were isolated and analyzed by flow cytometry, as described in Fig. 1. (A) Representative density plots show the percentages of Ly6C^{hi} and Ly6C^{low} IMs within CD45⁺Ly6G⁺F4/80^{int} NPCs. Data shown is a representative of six mice/group. (B) Average ratios of Ly6C^{hi}/Ly6C^{low} IMs. * $P < 0.05$ compared with mice only fed ethanol for 4 weeks. (C) Serum ALT activities were measured at 9 h after binge with ethanol (5 g/kg) by using a colorimetric assay (Teco Diagnostics, Anaheim, CA, USA). * $P < 0.05$ compared with pair-fed mice. The results shown represent mean \pm SD of five mice/group.

expansion of MDSCs in other pathological conditions, including bacterial and parasitic infections, acute and chronic inflammation, traumatic stress, sepsis, and transplantation [33, 34, 36–41]. Therefore, we hypothesized that similar factors that govern MDSC tissue accumulation are also induced in the liver of alcohol-fed mice. We observed up-regulation of many of these mediators, such as IL-1 β , S100A8, S100A9, IL-6, IL-10, COX-2, and HSP72 (Fig. 2), in the liver of ethanol-fed mice. It is reported that IL-1 β induces the expansion of MDSCs by (1) stimulating myelopoiesis and the release of myeloid cells into the blood and (2) inducing other factors, such as COX-2 and IL-6, which directly cause MDSC accumulation [42, 43]. S100A8/A9 are cytosolic calcium-binding proteins that are highly expressed by myeloid cells, including monocytes, activated Macs, neutrophils, and MDSCs [44, 45]. S100A8/A9 have chemotactic activities in leukocyte recruitment, and they promote tumor-site migration of MDSCs by binding to their carboxylated N-glycan receptors [45]. Chronic alcohol treatment also induces hepatocytes and KCs to express MCP-1, which is an important chemokine for monocyte recruitment [46]. Furthermore, it has been demonstrated that MCP-1 contributes to alcoholic steatosis, liver injury, and inflammation [46].

We found that resident KCs from ethanol-fed mice are activated, and they exhibit pro- and anti-inflammatory properties. A recent study demonstrated that when ethanol causes minimal hepatic injury in ethanol-resistant Balb/c mice or in resveratrol-treated mice, KCs show a predominantly M2 phenotype, and the M2 KCs induce apoptosis of M1 KCs [47]. With regard to the two subsets of IMs, our data show that Ly6C^{hi} IMs express typical M1 genes (such as iNOS, CD86, CITA), as well as inflammatory cytokines, chemokines, and receptors (such as TNF- α , IL-12p40, IL-1 β , CCL2, IP-10, and CCR2). In contrast, Ly6C^{low} IMs express typical M2 genes, such as Arg1, Mrc1, Fizz1, and IL-1R2, a decoyed receptor that neutralizes the proinflammatory effects of IL-1 α and IL-1 β . Ym1 has been described as a marker for M2 cells. However, our data show that Ym1 is highly expressed in Ly6C^{hi} but not Ly6C^{low} IMs. This observation is consistent with a recent report of Ym1 expression in the proinflammatory Ly6C^{hi} monocytes but not the Ly6C^{low} tissue-restorative monocytes [13]. Ym1 is a heparin-binding protein that can bind to the extracellular matrix, thereby mediating cell-to-cell and cell-to-matrix interactions. Thus, it is possible that the Ym1 expression on Ly6C^{hi} IMs allows migration of these cells to the site of injury in the tissue. Furthermore, our data show that the Ly6C^{low} IMs express genes that are involved in tissue growth (HGF, IGF), remodeling (MMP9, MMP12), and angiogenesis (VEGF), strongly suggesting a role for these cells in tissue repair. In a murine model of APAP-induced liver injury, we have demonstrated a critical role for hepatic Macs in angiogenesis and promoting tissue repair after acute liver injury [11, 14]. Interestingly, the genes related to tissue repair and regeneration were up-regulated significantly when IMs ingested apoptotic hepatocytes (Fig. 6). In a murine model of CCl₄-induced fibrosis, it was also demonstrated that phagocytosis of cellular debris could switch proinflammatory and profibrotic Macs to an anti-inflammatory and antifibrotic phenotype [13]. These findings support the hypothesis that during tissue injury, Ly6C^{hi} IMs mi-

grate to the site of tissue damage, perhaps through Ym1, and that upon clearance of cellular debris, they switch to the Ly6C^{low} IMs that promote tissue repair.

In summary, the current study demonstrates that chronic ethanol treatment activates liver-resident KCs and causes recruitment of IMs. The two subsets of IMs exhibit distinctive gene expression profiles correlating with opposing functions. Phagocytosis of apoptotic hepatocytes by Ly6C^{hi} proinflammatory IMs causes their switch to the anti-inflammatory and tissue-protective Ly6C^{low} IMs. These findings suggest that in the liver microenvironment of alcohol-induced mild tissue stress/damage, the proinflammatory Ly6C^{hi} IMs develop progressively into the anti-inflammatory, tissue-protective Ly6C^{low}. However, an acute ethanol binge or LPS treatment increases the ratio of Ly6C^{hi}/Ly6C^{low} IMs. These results suggest that in cases of acute-on-chronic ethanol consumption and concurrent infections, the phenotype switching from Ly6C^{hi} to Ly6C^{low} IMs may be blocked, thereby resulting in persistent hepatic inflammation and impaired tissue repair. The knowledge gained is important in further studies to develop ALD therapies targeting the switch of Ly6C^{hi} to Ly6C^{low} IMs, thereby slowing down or reversing disease progression.

AUTHORSHIP

M.W. and Q.Y., contributed to the study design, performed the experimental work, analyzed data, and drafted the manuscript. K.L. and F.C. performed the experimental work and analyzed data. B.G. contributed to the study design, discussed data, and revised the manuscript. C.J. supervised the study design, analyzed data, and drafted the paper.

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DISCLOSURES

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REFERENCES

1. Yoon, Y.-H., Yi, H.-y. (2012, August) *Liver Cirrhosis Mortality in the United States, 1970–2009*. Surveillance Report #93. National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA.
2. Davis W. D., Jr., Culpepper, W. S. (1948) Cirrhosis of the liver associated with alcoholism; report of acute exacerbation with serial liver biopsies. *Ann. Int. Med.* **29**, 942–958.
3. McClain, C. J., Hill, D. B., Song, Z., Deaciuc, I., Barve, S. (2002) Monocyte activation in alcoholic liver disease. *Alcohol* **27**, 53–61.
4. Thakur, V., McMullen, M. R., Pritchard, M. T., Nagy, L. E. (2007) Regulation of macrophage activation in alcoholic liver disease. *J. Gastroenterol. Hepatol.* **22** (Suppl. 1), S53–S56.
5. Thurman, R. G. (1998) II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. *Am. J. Physiol.* **275**, G605–G611.
6. Wheeler, M. D., Kono, H., Yin, M., Nakagami, M., Uesugi, T., Arteel, G. E., Gabele, E., Rusyn, I., Yamashina, S., Froh, M., Adachi, Y., Iimuro, Y., Bradford, B. U., Smutney, O. M., Connor, H. D., Mason, R. P., Goyert, S. M., Peters, J. M., Gonzalez, F. J., Samulski, R. J., Thurman, R. G. (2001) The role of Kupffer cell oxidant production in early ethanol-induced liver disease. *Free Radic. Biol. Med.* **31**, 1544–1549.
7. Horiguchi, N., Wang, L., Mukhopadhyay, P., Park, O., Jeong, W. I., Lafdil, F., Osei-Hyiaman, D., Moh, A., Fu, X. Y., Pacher, P., Kunos, G., Gao, B. (2008) Cell type-dependent pro- and anti-inflammatory role of

- signal transducer and activator of transcription 3 in alcoholic liver injury. *Gastroenterology* **134**, 1148–1158.
8. Mandal, P., Park, P. H., McMullen, M. R., Pratt, B. T., Nagy, L. E. (2010) The anti-inflammatory effects of adiponectin are mediated via a heme oxygenase-1-dependent pathway in rat Kupffer cells. *Hepatology* **51**, 1420–1429.
 9. Mandal, P., Pritchard, M. T., Nagy, L. E. (2010) Anti-inflammatory pathways and alcoholic liver disease: role of an adiponectin/interleukin-10/heme oxygenase-1 pathway. *World J. Gastroenterol.* **16**, 1330–1336.
 10. Shen, Z., Ajmo, J. M., Rogers, C. Q., Liang, X., Le, L., Murr, M. M., Peng, Y., You, M. (2009) Role of SIRT1 in regulation of LPS- or two ethanol metabolites-induced TNF- α production in cultured macrophage cell lines. *Am. J. Physiol. Gastrointest. Liver Physiol.* **296**, G1047–G1053.
 11. Holt, M. P., Cheng, L., Ju, C. (2008) Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J. Leukoc. Biol.* **84**, 1410–1421.
 12. Karlmark, K. R., Weiskirchen, R., Zimmermann, H. W., Gassler, N., Ginhoux, F., Weber, C., Merad, M., Luedde, T., Trautwein, C., Tacke, F. (2009) Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* **50**, 261–274.
 13. Ramachandran, P., Pellicoro, A., Vernon, M. A., Boulter, L., Aucott, R. L., Ali, A., Hartland, S. N., Snowden, V. K., Cappon, A., Gordon-Walker, T. T., Williams, M. J., Dunbar, D. R., Manning, J. R., van Rooijen, N., Fallowfield, J. A., Forbes, S. J., Iredale, J. P. (2012) Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc. Natl. Acad. Sci. USA* **109**, E3186–E3195.
 14. You, Q., Holt, M., Yin, H., Li, G., Hu, C. J., Ju, C. (2013) Role of hepatic resident and infiltrating macrophages in liver repair after acute injury. *Biochem. Pharmacol.* **86**, 836–843.
 15. Lumeng, C. N., Bodzin, J. L., Saltiel, A. R. (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* **117**, 175–184.
 16. Wynn, T. A. (2003) IL-13 effector functions. *Ann. Rev. Immunol.* **21**, 425–456.
 17. Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stange, G., Van den Bossche, J., Mack, M., Pipeleers, D., In't Veld, P., De Baetselier, P., Van Ginderachter, J. A. (2010) Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res.* **70**, 5728–5739.
 18. Zhang, X., Tachibana, S., Wang, H., Hisada, M., Williams, G. M., Gao, B., Sun, Z. (2010) Interleukin-6 is an important mediator for mitochondrial DNA repair after alcoholic liver injury in mice. *Hepatology* **52**, 2137–2147.
 19. Chang, N. C., Hung, S. I., Hwa, K. Y., Kato, I., Chen, J. E., Liu, C. H., Chang, A. C. (2001) A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin. *J. Biol. Chem.* **276**, 17497–17506.
 20. Savill, J., Dransfield, I., Gregory, C., Haslett, C. (2002) A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* **2**, 965–975.
 21. Huynh, M. L., Fadok, V. A., Henson, P. M. (2002) Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF- β 1 secretion and the resolution of inflammation. *J. Clin. Invest.* **109**, 41–50.
 22. Pujalis, D., Goetsch, J., Kottas, D. J., Gerke, V., Rescher, U. (2011) Annexin A1 released from apoptotic cells acts through formyl peptide receptors to dampen inflammatory monocyte activation via JAK/STAT/SOCS signalling. *EMBO Mol. Med.* **3**, 102–114.
 23. Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J. L., Libby, P., Weissleder, R., Pittet, M. J. (2007) The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J. Exp. Med.* **204**, 3037–3047.
 24. Nahrendorf, M., Pittet, M. J., Swirski, F. K. (2010) Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation* **121**, 2437–2445.
 25. Takizawa, H., Boettcher, S., Manz, M. G. (2012) Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* **119**, 2991–3002.
 26. Jakubzick, C., Gautier, E. L., Gibbings, S. L., Sojka, D. K., Schlitzer, A., Johnson, T. E., Ivanov, S., Duan, Q., Bala, S., Condon, T., van Rooijen, N., Grainger, J. R., Belkaid, Y., Ma'ayan, A., Riches, D. W., Yokoyama, W. M., Ginhoux, F., Henson, P. M., Randolph, G. J. (2013) Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity* **39**, 599–610.
 27. Hanna, R. N., Carlin, L. M., Hubbeling, H. G., Nackiewicz, D., Green, A. M., Punt, J. A., Geissmann, F., Hedrick, C. C. (2011) The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C⁺ monocytes. *Nat. Immunol.* **12**, 778–785.
 28. Hilgendorf, I., Gerhardt, L. M., Tan, T. C., Winter, C., Holderried, T. A., Chousterman, B. G., Iwamoto, Y., Liao, R., Zirk, A., Scherer-Crosbie, M., Hedrick, C. C., Libby, P., Nahrendorf, M., Weissleder, R., Swirski, F. K. (2014) Ly-6Chigh monocytes depend on Nr4a1 to balance both inflammatory and reparative phases in the infarcted myocardium. *Circ. Res.* **114**, 1611–1622.
 29. Varga, T., Mounier, R., Gogolak, P., Poliska, S., Chazaud, B., Nagy, L. (2013) Tissue LyC6⁺ macrophages are generated in the absence of circulating LyC6⁺ monocytes and Nur77 in a model of muscle regeneration. *J. Immunol.* **191**, 5695–5701.
 30. Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R. K., Chazaud, B. (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* **204**, 1057–1069.
 31. Lin, S. L., Castano, A. P., Nowlin, B. T., Lupher M. L., Jr., Duffield, J. S. (2009) Bone marrow Ly6Chigh monocytes are selectively recruited to injured kidney and differentiate into functionally distinct populations. *J. Immunol.* **183**, 6733–6743.
 32. Shi, C., Jia, T., Mendez-Ferrer, S., Hohl, T. M., Serbina, N. V., Lipuma, L., Leiner, L., Li, M. O., Frenette, P. S., Pamer, E. G. (2011) Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating Toll-like receptor ligands. *Immunity* **34**, 590–601.
 33. Delano, M. J., Scumpia, P. O., Weinstein, J. S., Coco, D., Nagaraj, S., Kelly-Scumpia, K. M., O'Malley, K. A., Wynn, J. L., Antonenko, S., Al-Quran, S. Z., Swan, R., Chung, C. S., Atkinson, M. A., Ramphal, R., Gabrilovich, D. L., Reeves, W. H., Ayala, A., Phillips, J., Laface, D., Heyworth, P. G., Clare-Salzler, M., Moldawer, L. L. (2007) MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. *J. Exp. Med.* **204**, 1463–1474.
 34. Haile, L. A., von Wasielewski, R., Gamrekeshvili, J., Kruger, C., Bachmann, O., Westendorf, A. M., Buer, J., Liblau, R., Manns, M. P., Korny, F., Greten, T. F. (2008) Myeloid-derived suppressor cells in inflammatory bowel disease: a new immunoregulatory pathway. *Gastroenterology* **135**, 871–881, 881.e1–881.e5.
 35. Makarenkova, V. P., Bansal, V., Matta, B. M., Perez, L. A., Ochoa, J. B. (2006) CD11b+/Gr-1+ myeloid suppressor cells cause T cell dysfunction after traumatic stress. *J. Immunol.* **176**, 2085–2094.
 36. Zhu, B., Bando, Y., Xiao, S., Yang, K., Anderson, A. C., Kuchroo, V. K., Khoury, S. J. (2007) CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. *J. Immunol.* **179**, 5228–5237.
 37. Goni, O., Alcaide, P., Fresno, M. (2002) Immunosuppression during acute *Trypanosoma cruzi* infection: involvement of Ly6G(Gr1+)(CD11b+)(immature myeloid suppressor cells. *Int. Immunol.* **14**, 1125–1134.
 38. Sunderkotter, C., Nikolic, T., Dillon, M. J., Van Rooijen, N., Stehling, M., Drevets, D. A., Leenen, P. J. (2004) Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J. Immunol.* **172**, 4410–4417.
 39. Gomez-Garcia, L., Lopez-Marin, L. M., Saavedra, R., Reyes, J. L., Rodriguez-Sosa, M., Terrazas, L. I. (2005) Intact glycans from cestode antigens are involved in innate activation of myeloid suppressor cells. *Parasite Immunol.* **27**, 395–405.
 40. Ezernitchi, A. V., Vaknin, I., Cohen-Daniel, L., Levy, O., Manaster, E., Halabi, A., Pikarsky, E., Shapira, L., Baniyash, M. (2006) TCR ζ down-regulation under chronic inflammation is mediated by myeloid suppressor cells differentially distributed between various lymphatic organs. *J. Immunol.* **177**, 4763–4772.
 41. Kerr, E. C., Raveney, B. J., Copland, D. A., Dick, A. D., Nicholson, L. B. (2008) Analysis of retinal cellular infiltrate in experimental autoimmune uveoretinitis reveals multiple regulatory cell populations. *J. Autoimmun.* **31**, 354–361.
 42. Bunt, S. K., Sinha, P., Clements, V. K., Leips, J., Ostrand-Rosenberg, S. (2006) Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J. Immunol.* **176**, 284–290.
 43. Song, X., Krelm, Y., Dvorkin, T., Bjorkdahl, O., Segal, S., Dinarello, C. A., Voronov, E., Apte, R. N. (2005) CD11b+/Gr-1+ immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1 β -secreting cells. *J. Immunol.* **175**, 8200–8208.
 44. Cheng, P., Corzo, C. A., Luetteke, N., Yu, B., Nagaraj, S., Bui, M. M., Ortiz, M., Nacken, W., Sorg, C., Vogl, T., Roth, J., Gabrilovich, D. I. (2008) Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J. Exp. Med.* **205**, 2235–2249.
 45. Sinha, P., Okoro, C., Foell, D., Freeze, H. H., Ostrand-Rosenberg, S., Srikrishna, G. (2008) Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. *J. Immunol.* **181**, 4666–4675.
 46. Mandrekar, P., Ambade, A., Lim, A., Szabo, G., Catalano, D. (2011) An essential role for monocyte chemoattractant protein-1 in alcoholic liver injury: regulation of proinflammatory cytokines and hepatic steatosis in mice. *Hepatology* **54**, 2185–2197.
 47. Wan, J., Benkdane, M., Teixeira-Clerc, F., Bonnafous, S., Louvet, A., Lafdil, F., Pecker, F., Tran, A., Gual, P., Mallat, A., Lotersztajn, S., Pavoine, C. (2014) M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. *Hepatology* **59**, 130–142.

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