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Infiltrating Monocyte-Derived Macrophages and Resident Kupffer Cells Display Different Ontogeny and Functions in Acute Liver Injury

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The liver has a remarkable capacity to regenerate after injury; yet, the role of macrophages (MF) in this process remains controversial mainly due to difficulties in distinguishing between different MF subsets. In this study, we used a murine model of acute liver injury induced by overdose of N-acetyl-p-aminophenol (APAP) and defined three distinct MF subsets that populate the liver following injury. Accordingly, resident Kupffer cells (KC) were significantly reduced upon APAP challenge and started recovering by self-renewal at resolution phase without contribution of circulating Ly6C\textsuperscript{hi} monocytes. The latter were recruited in a CCR2- and M-CSF–mediated pathway at the necroinflammatory phase and differentiated into ephemeral Ly6C\textsuperscript{lo} MF subset at resolution phase. Moreover, their inducible ablation resulted in impaired recovery. Microarray-based molecular profiling uncovered high similarity between steady-state KC and those recovered at the resolution phase. In contrast, KC and monocyte-derived MF displayed distinct prorestorative genetic signature at the resolution phase. Finally, we show that infiltrating monocytes acquire a prorestorative polarization manifested by unique expression of proangiogenesis mediators and genes involved with inhibition of neutrophil activity and recruitment and promotion of their clearance. Collectively, our results present a novel phenotypic, ontogenic, and molecular definition of liver-MF compartment following acute injury. The Journal of Immunology, 2014, 193: 000–000.
manner and controlled by M-CSF, massively infiltrated the injured liver, becoming the dominant liver-MF subset at both necroinflammatory and early resolution phases and had no contribution to the recovery of resident KC. Selective ablation of monocyte-derived MF (MoMF) resulted in impaired recovery manifested by greater hepatotoxic damage. Finally, microarray-based molecular profiling of MF subsets uncovered that resident KC and infiltrating MoMF acquire a distinct gene-expression signature, suggesting different restorative functions of these cells.

Materials and Methods

Mice
The following 8–12-wk-old male mouse strains were used: C57BL/6 mice were purchased from Harlan Laboratories and Zbtb40+/mice from The Jackson Laboratory (B6; 129P2-Zbtb40tm2LittJX). CD45.1 and CD45.2 Cx3cr1R+/+ and Ccr2+/– Cx3cr1R+/+ mice were bred at the Sourasky Medical Center animal facility. All murine experiments were approved by the Sourasky Medical Center Animal Care Committee.

AILI
Mice were fasted overnight for 12 h prior to administration of vehicle or 300 mg/kg APAP (Tiptipot Novimol; CST, Kiryat Malakhi, Israel; 100 mg paracetamol/ml) by oral gavage. Water was returned with APAP administration and the food 2 h later.

Isolation of hepatic nonparenchymal cells
Perfused livers were cut into small fragments and incubated (37°C, 250 rpm for 40 min) with 5 ml digestion buffer (5% FBS, 0.5 mg/ml collagenase VIII [Sigma-Aldrich, Rehovot, Israel], and 0.1 mg/ml DNase I [Roche] in PBS) at 400 rpm from which the supernatant was taken, omitting the parenchymal cell population. Flow cytometry analysis was performed using FACSAria machine (BD Biosciences). Flow cytometry analysis, sorting of liver-MF subsets, and ELISA

Abs used for liver-MF characterization included: CD45 (30-F11), CD45.2 (104), CD45.1 (A20), Ly6C (HK.14), CD64 (S45-57.1), CD11c (N418), CD11b (M1/70), IAb (Af6-120.1), CD19 (HIB19), CR3/4 (H57-597), B220 (RA3-6B2), and MPDC1A (927) (all from BioLegend, San Diego, CA); NK.1.1 (PK136) (BD Biosciences, San Jose, CA); F4/80 (Cl: A3-1) (AbD Serotech); and Ki67 (SolA15; eBioscience, San Diego, CA). Abs used for the sorting of bone marrow (BM) monocytes used in the adoptive transfer experiments included: CD45.1 (A20), CD11b (M1/70), CD115 (AF598), Ly6C (HK.14), and c-Kiit (2BB). Cells were analyzed by LSR Fortessa or FACSCTII flow cytometer (BD Biosciences) and sorted with an FACSAria machine (BD Biosciences). Flow cytometry analysis was performed using FlowJo software (TreeStar, Ashland, OR). PGE2 levels were examined in supernatants isolated from overnight incubated sorted Ly6C+ monocytes from APAP 24 h-challenged livers using the PGE2 ELISA Kit-Monoclonal (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

BrdU pulsing
Mice received three i.p. injections of 2 mg BrdU (BD Biosciences) 30 min apart. To assess BrdU incorporation, isolated nonparenchymal cells were stained, and scanned with the Affymetrix GeneChip Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA). Hybridized chips were stained, washed, and scanned with the Affymetrix GeneChip 3000 7G plus scanner (Affymetrix). Microarray analysis was performed using Partek Genomics Suite version 6.6 (Partek, St. Louis, MO). Data were normalized and summarized with the robust multivariate regression method (31). Heat maps and Venn diagrams were performed using Partek Genomics Suite software with Pearson’s similarity correlation and average linkage methods. Functional enrichment analysis was performed using DAVID and GOEAST tools.

Microarray performance
Total RNA was extracted from freshly frozen liver-sorted liver-MF subsets with the microRNAeasy Kit (Qiagen, Venlo, Limburg, Netherlands). Infiltrating Ly6C+ monocytes were sorted from a pool of five mice at the inflammatory peak of the necrotic phase, 24 h post-AILI (3× biological repeats). Their Ly6Cin MoMF descendants were sorted at the recovery phase, 72 h post-AILI from pool of seven mice (3× biological repeats). Finally, resident KC were sorted from both pool of seven steady-state mice (2× biological repeats) and a pool of seven mice at 72 h post-AILI (3× biological repeats), for a total of 11 arrays. RNA purity was assessed with BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA). The cDNA was hybridized to GeneChip Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA). Hybridized chips were stained, washed, and scanned with the Affymetrix GeneChip 3000 7G plus scanner (Affymetrix). Microarray analysis was performed using Partek Genomics Suite version 6.6 (Partek, St. Louis, MO). Data were normalized and summarized with the robust multivariate regression method (31). Heat maps and Venn diagrams were performed using Partek Genomics Suite software with Pearson’s similarity correlation and average linkage methods. Functional enrichment analysis was performed using DAVID and GOEAST tools. All microarray data have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus public database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE55606.

Histopathological quantification of hepatic damage
Liver samples were obtained at steady state and 24 and 48 h after AILI, fixed (4% paraformaldehyde), paraffin embedded, sectioned, and stained with H&E, and pathologic evaluation was performed by a pathologist (E.B.). Necrosis was scored as 0 (no necrosis), 1 (spotty necrosis), 2 (confluent, zone 3 necrosis), 3 (confluent, zone 2 plus 3 necrosis), or 4 (panlobular necrosis). Bridging necrosis was scored as 0 (absent) or 1 (present) and ballooning of hepatocytes as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe).

Statistical analysis
Data were analyzed either by ANOVA followed by Bonferroni’s multiple comparison test or by unpaired, two-tailed t test with GraphPad Prism 4 (GraphPad, San Diego, CA). Data are presented as mean ± SEM; p values < 0.05 were considered statistically significant.

Results
Phenotypic characterization of liver-MF compartment in steady state and following AILI

Given the broad expression of the fractalkine receptor Cx3cr1 gene within the mononuclear phagocyte system, we characterized the composition of the liver-MF compartment using the Cx3cr1GFP reporter mice (32) and multiparameter flow cytometry analysis. Liver-MF were defined as cells mutually positive for CD45, CD11b, MHC class II (MHC II), F4/80, and the FcyR1 (CD64) markers. Using this gating strategy, we show that Cx3cr1-CR1-GFP expression could discriminate under homeostatic conditions between a predominant population of Cx3cr1GFP+/+ KC and a sig-
significantly smaller population of CX3CR1hi MF (Fig. 1A, 1B). At 24 h following AILI, there was a massive infiltration of circulating monocytes characterized as Ly6C\(^hi\)CD11b\(^hi\)MHC II\(^neg\)CX3CR1-GFP\(^+\) cells concomitantly with marked reduction in the frequency of resident KC. Notably, at 72 h following AILI, the liver-MF compartment was dominated by CX3CR1-GFP\(^+\)Ly6C\(^hi\)–infiltrating MF, whereas KC did not yet fully recover (Fig. 1A, 1B). At 120 h following AILI, the KC returned to be the predominant liver-MF subset, similar to pre-APAP administration, whereas the CX3CR1\(^+\) Ly6C\(^lo\)–infiltrating MF were hardly detectable (Fig. 1A, 1B).

Importantly, flow cytometry analysis of APAP 72-h livers of Zbtb46\(^{gfp/+}\) transgenic mice, in which cells positive for the dendritic cell (DC)–associated transcription factor Zbtb46 are labeled with GFP (33), confirmed that GFP\(^+\) liver-DC are excluded from our gating strategy for liver-MF (Supplemental Fig. 1A). Moreover, staining for TCR\(\beta\), NK1.1, plasmacytoid DC Ag-1, SiglecF, and Ly6G confirmed that our gating strategy excludes contamination with T cells, NK cells, plasmacytoid DC, eosinophils, and neutrophils, respectively (Supplemental Fig. 1B).

Ly6C\(^lo\) monocytes are recruited in a CCR2- and M-CSF–dependent manner and give rise to the CX3CR1\(^+\)Ly6C\(^lo\) liver-MF subset

To investigate the contribution of circulating Ly6C\(^lo\) monocytes to the ontogeny of liver-MF subsets following AILI, we performed adoptive transfer experiments of purified BM-derived CD45.1 Cx3cr1\(^{gfp/+}\)Ly6C\(^hi\) monocytes into APAP-challenged wild-type recipients. The monocyte graft was administered by i.v. injection at 12 h following AILI, and recipient mice were sacrificed at following consecutive time points (Fig. 2A). The engrafted Ly6C\(^hi\) monocytes infiltrated the injured liver already by 24 h following APAP challenge and, subsequently, started differentiating toward the Ly6C\(^hi\)F4/80\(^+\)CX3CR1-GFP\(^+\) MF population we named MoMF. At 96 h post–APAP administration, the MoMF could hardly be detected, suggesting that these cells are ephemeral (Fig. 2B). Importantly, at all examined time points, the engrafted Ly6C\(^hi\) monocytes did not differentiate to CX3CR1-GFP\(^{neg}\) cells.

The chemokine receptor CCR2 is required for emigration of Ly6C\(^hi\) monocytes from the BM (34). Flow cytometry analysis of Ccr2\(^{-/-}\)Cx3cr1\(^{gfp/+}\) mice subjected to APAP overdose revealed a failure of these animals to accumulate Ly6C\(^hi\) monocytes (24 h) and Ly6C\(^lo\)CX3CR1-GFP\(^+\) MoMF (72 h) in their livers (Fig. 2C, 2D). Importantly, maintenance and recovery of KC were not affected by CCR2 deficiency (Fig. 2C, 2D). M-CSF controls the differentiation, proliferation, and survival of certain MF subsets (35). Quantitative PCR (qPCR) analysis of M-CSF expression in APAP-challenged livers revealed a 4-fold increase at 24 h that remained above baseline level during the recovery phase (72–120 h) (Fig. 3A). Interestingly, we could not detect any significant change in the expression of other factors associated with MF differentiation including IL-4, IL-34, and GM-CSF during the course of AILI (Fig. 3B). Injection of recombinant mouse M-CSF resulted in a significant increase in the cell number of liver

**FIGURE 1.** Phenotypic characterization of mouse liver-MF compartment in health and following AILI. (A) Flow cytometry analysis of nonparenchymal Cx3cr1\(^{gfp/+}\) liver cells isolated from steady state or at 24, 72, and 120 h following AILI. (B) Dynamics of liver-MF subsets in steady state and at different time points following AILI: resident CX3CR1-GFP\(^{neg/lo}\) KC (circle), infiltrating Ly6C\(^hi\)CX3CR1-GFP\(^+\) monocytes (square), and Ly6C\(^lo\)CX3CR1-GFP\(^{hi}\) MF (triangle), presented as percentage out of CD45\(^+\) living cells (mean [SEM]; n \(\geq\) 5 for any time point).
Ly6Chi monocytes and MoMF, but had no effect on KC levels (Fig. 3C, 3D). Collectively, these results show that the recovery of KC does not rely on CCR2+Ly6Chi monocytes or M-CSF.

Liver-KC recover by self-renewal following AILI

To investigate whether KC can self-renew by local proliferation, we resorted to BrdU pulsing regimen as it is empirically incorporated into dividing cell. BrdU labeling was performed at 48 h following AILI with the initiation of KC recovery (Fig. 1). Flow cytometry analysis 24 h later revealed incorporation of BrdU in 5% of KC and 2% of MoMF (Fig. 4A). To delineate whether BrDU+ KC were recently derived from proliferating precursor or, alternatively, are capable of self-renewal, we stained for the Ki67 proliferation marker present only during active phases of cell cycle. Flow cytometry analysis revealed a 7-fold increase in the fraction of Ki67+ KC at 72 h following AILI (Fig. 4B, 4C). In contrast, we could not observe any increase in the fraction of proliferating cells within the MoMF compartment. Furthermore, introduction of rM-CSF had no effect on the frequency of Ki67+-proliferating KC or MoMF (Fig. 4B, 4C).

Ablation of Ly6Chi monocytes and their MoMF descendants impairs the recovery from AILI

To investigate the contribution of Ly6Chi monocytes and MoMF in AILI, we took advantage of the anti-CCR2 MC21-depleting Ab (11, 24, 36). MC21 treatment specifically eliminated the Ly6Chi monocytes and their MoMF progenies, but had no effect on resident KC (Fig. 5A, 5B). Histopathological analysis revealed more extensive damage, with bridging necrosis and ballooning degeneration in livers of MC21-treated mice at both 24 and 48 h following APAP administration (Fig. 5C). Pathological scoring confirmed a significant prolonged hepatic damage (Fig. 5D). Thus, MoMF contribute to the recovery from liver injury.

Molecular characterization of resident KC, infiltrating liver monocytes and their MoMF descendants

We next sought to molecularly define the distinct liver-MF subsets during the inflammatory and resolution phases of liver injury. To do that, we performed a transcriptome microarray analysis of highly purified cell populations freshly sorted from the livers of APAP-challenged Cx3cr1gfp/+ mice (Supplemental Fig. 2). Specifically, Ly6Chi-infiltrating liver monocytes were sorted from APAP 24-h livers, whereas KC and MoMF were sorted from APAP 72-h livers. These were compared with resident liver KC from unchallenged mice. Microarray analysis performed with a stringent statistical significance cutoff of \( p < 0.05 \) (with false discovery rate correction) revealed very high resemblance between steady-state KC and recovered KC, with a Pearson correlation coefficient of 0.975 in support of their self-renewal (Fig. 6A, 6B). Nevertheless, there were 2196 genes that were differentially expressed between the distinct liver-MF subsets with at least 2-fold change. In particular, there was high variability between steady-state KC and both Ly6Chi monocytes and their MoMF progenies, with a Pearson correlation coefficient of 0.38 and 0.73, respectively (Fig. 6B). Indeed, we identified 2059 genes that exhibited at least a 2-fold change in expression between Ly6Chi monocytes and steady-state...
KC and 802 genes between MoMF and steady-state KC (Fig. 6C). In addition, the Ly6Chi monocytes induced a major gene expression shift upon their differentiation into MoMF manifested by a Pearson correlation coefficient of 0.76 and 1267 genes with at least 2-fold change in their expression (Fig. 6B). Importantly, our microarray results show that the expression of CD169 (Siglec1), F4/80 (Emr1), CD64 (Fcgr1), MER receptor tyrosine kinase (Mertk), macrosialin (CD68), the AXL receptor tyrosine kinase, and MHC II, all extensively used in the literature as definitive markers of resident tissue MF, were similarly expressed in KC and infiltrating MoMF. Instead, the molecular profiling revealed 603 genes that were differentially expressed by at least a 2-fold change between these subsets. Among them, there were various cell-surface markers that can be used for discrimination (Fig. 6D).

Infiltrating liver Ly6C<sup>hi</sup> monocytes regulate neutrophil recruitment, activity, and removal

Corroborating previous results (13), we show in this study a significant increase in the frequency of neutrophils in the livers of Ccr2<sup>−/−</sup> mice at 24 h following APAP administration (Fig. 7A). Supportively, the inducible ablation of Ly6C<sup>hi</sup> monocytes and their MoMF descendants resulted in a profound increase in neutrophil levels (Fig. 7B), suggesting that the former negatively regulate the latter. PGE2 has been recently established as a potent inhibitor of neutrophil activation (10). In this relation, the Ly6C<sup>hi</sup> monocytes expressed uniquely high levels of the Ptgs2 gene, which encodes for cyclooxygenase-2 (COX2), and of the microsomal PGE synthase-1 (Ptges), both of which constitute key enzymes in PGE2 synthesis (37, 38), as well as lower levels of the Hpgd gene encoding for the PG-degrading enzyme hydroxyl-PG dehydrogenase 15-(NAD), overall resulting in higher PGE2 levels. Moreover, both Ly6C<sup>hi</sup> monocytes and MoMF produced Anxa1 (Fig. 7C), a positive regulator of PGE2 synthesis, a potent anti-inflammatory mediator in MF and an inhibitor of neutrophil migration (39). Conversely, KC expressed higher levels of Ptgs1 gene.

FIGURE 3. M-CSF specifically promotes the differentiation of MoMF. (A and B) Graphical summary of qPCR analysis showing mRNA expression of M-CSF, GM-CSF, IL-34, and IL-4 in livers isolated from steady-state mice and at 24, 48, 72, and 120 h following AILI (mean [SEM]; n = 5). (C) Flow cytometry analysis of Cx3cr1<sup>10<sup>6</sup>+</sup> mouse livers at 72 h following AILI gated on CD45<sup>+</sup>CD11b<sup>+</sup>MHC II<sup>+</sup>CD64<sup>+</sup>F4/80<sup>+</sup> liver-MF subsequent to treatment with rM-CSF or saline. (D) Graphical summary showing the cell number of indicated liver-MF subsets normalized for liver tissue weight (mean [SEM]; n = 5). *p < 0.05.

FIGURE 4. KC recovery by local proliferation following AILI. (A) Representative flow cytometry analysis images of nonparenchymal cells isolated from perfused livers of Cx3cr1<sup>10<sup>6</sup>+</sup> mice at 72 h post-AILI and 24 h post–3× consecutive pulses with BrdU. Bottom panel: graphical summary indicating the percentage of BrdU<sup>+</sup> cells out of indicated cell subsets (mean [SEM]; n = 3). (B) Graphical summary showing percentage of Ki67<sup>+</sup> cells out of KC or MoMF at steady state or AILI 72 h, with or without concomitant treatment with M-CSF (mean [SEM]; n = 5). (C) Flow cytometry analysis of Ki67 proliferation marker in KC and MoMF in steady-state Cx3cr1<sup>10<sup>6</sup>+</sup> mouse livers and at 24, 48, and 72 h following AILI. *p < 0.05.
encoding for COX1 and of the Hpgds gene encoding for hematopoietic PGD synthase that catalyzes the conversion of PGH2 to PGD2 (Fig. 7C). qPCR and ELISA analyses further confirmed the unique activation of the PGE2 metabolic pathway in Ly6ChI monocytes (Fig. 7D, 7E).

The Ly6ChI monocytes also expressed the product of TNF-stimulated gene-6 (Tnfaip6), a potent inhibitor of neutrophil migration (40) and a positive regulator of COX2 expression in MF (41). Moreover, they expressed Lcn2 that induces cell death in neutrophils (42) and plays a significant hepatoprotective role in liver injury (43) (Fig. 7C). Validating qPCR analysis confirmed the expression of Lcn2 and Tnfaip6 specifically in Ly6ChI monocytes (Fig. 7D). The Ly6ChI monocytes and MoMF also expressed genes involved in the removal of apoptotic neutrophils such as thrombospondin-1 (Thbs1) and its receptor CD36 (44) and the αL (Itgal) and β2 (Itgb2) integrin components of the lymphocyte function-associated Ag 1 that is involved with engulfment of ICAM-3+ apoptotic neutrophils (45). In this relation, they also expressed the guanine-nucleotide exchange factor Vav3, which is essential for the β2 integrin–dependent MF phagocytosis of apoptotic neutrophils in wound healing (46) (Fig. 7C). Collectively, these results suggest a hepatoprotective role for infiltrating liver monocytes in mediating neutrophil recruitment, activity, and clearance.

Hepatoprotective polarization of Ly6ChI monocytes and MoMF

Shaping of MF function is an essential component of tissue damage and repair (47, 48). The Ly6ChI monocytes expressed exclusively proinflammatory genes associated with classical activation M1 phenotype including triggering receptor expressed on myeloid cells-1 (TREM1), NO synthase 2 (Nos2), COX2 (Ptgs2), and the chemokines Ccl2 and Ccl7. Nevertheless, they expressed a wider panel of genes associated with both alternatively activated regulatory M2 and prorestorative wound-healing MF phenotypes (48), including arginase 1 (Arg1), Lcn2, chitinase-3-like protein 3 (Chil3l3, also known as Ym1), IL-4R subunit α (Il4ra), TNF ligand superfamily member 14 (Tnfsf14), TNF, TNFAIP3-interacting protein 3 (Tnip3, also named Abin3), and the α-induced protein 3 (Tnfaip3, also named A20). They also uniquely expressed oncostatin M (Osm) reported to be indispensable for liver regeneration following injury (49, 50) (Supplemental Fig. 3A, 3B). Upon their differentiation into MoMF, the Ly6ChI monocytes downregulated their expression of the proinflammatory mediators mentioned above while upregulating the expression of other prorestorative wound healing genes including Trem2, Gpmb, insulin-like growth factor 1 (Igf1), mannose receptor 1 (Mrc1), the resistin-like molecule α (Retna, also known as Fizz-1), Scarf1, and Cd163 (Supplemental Fig. 3).

Interestingly, Ly6ChI monocytes expressed high levels of key proangiogenesis mediators including vascular endothelial growth factor-A (VEGF-A; Vegfa), semaphorin 4A and D (Sem4a and Sem4d), hypoxia-inducible factor-1α (Hif1a), the plasminogen activator urokinase receptor gene (Plaur), Tbias1, Vas1, Anxa2, ephrin type A receptor 2 (Epha2), fibroentin 1 (Fn1), the cytokines TGF-β1 (Tgfb1), IL-6 (Il6), and IL-1β (Il1b) as well as the extracellular matrix (ECM) remodeling enzymes matrix metalloproteinase (MMP) 18, a disintegrin and metalloprotease domain (ADAM) 8, and heparanase (Hps3) (Supplemental Fig. 3A). Collectively, these results establish that following AILI, monocytes undergo genetic reprogramming, acquiring alternatively activated and proresolution phenotypes important for wound healing.
**KC and MoMF display distinct prorestorative molecular signature**

The microarray results revealed major gene expression differences that strongly imply task division between KC and MoMF in the resolution from liver injury. In this relation, functional enrichment analysis based on the DAVID and GOEAST bioinformatics tools revealed variable expression of wound healing associated genes between the recruited MoMF and liver-resident KC (Supplemental Table I). Deeper overview revealed that both liver-MF subsets displayed variable expression of pattern recognition receptors including scavenger receptors and C-type lectins important for the clearance of apoptotic cells and cellular debris (Supplemental Fig. 3C). Specifically, KC displayed exclusive expression of the T cell Ig mucin 4 (gene Timd4) and stabilin 2 (Stab2) receptors, both of which mediate the engulfment of phosphatidylserine-expressing apoptotic cells (51, 52). They also expressed higher levels of the scavenger receptors Marco, CD163, Colec12, and mannose receptor (Mrc1) as well as of the C-type lectin genes Clec1b, Clec4f, and Clec9a and DC-specific ICAM3-grabbing nonintegrin isoform Cd209f. In contrast, Ly6C\textsuperscript{hi} monocytes and MoMF expressed uniquely high levels of T cell Ig mucin 3 (Havcr2), TLR2 gene (Tir2), the C-type lectin genes Clec4d, Clec4e, and Clec5a, and DC-specific ICAM3-grabbing nonintegrin isoform Cd209a. They also expressed uniquely the C1q receptor CD93, shown to be important for the removal of apoptotic cells (53).

Extracellular matrix (ECM) remodeling is of major importance in resolution from liver injury. The microarray results revealed variable expression of ECM-remodeling enzymes. Accordingly, KC expressed higher levels of genes encoding for matrix metalloproteinases-12 and -13 (Mmp12 and -13) and ADAM-23 as well as Adamdec1, Tgm1, and Timp2 and -3. In contrast, Ly6C\textsuperscript{hi} monocytes and MoMF expressed higher levels of MMP-8, -14, and -19, ADAM-8, -15, and -19, and heparanase (Hps). With respect to ECM structural components, they also expressed higher levels of the thrombospondin-1 (Tbhs1), fibronectin-1 (Fn1), versican (Vcn), emilin-2, and embigin (Emb) genes, whereas KC expressed higher levels of nidogen-1, 2 (Nid1 and -2), biglycan (Bgn), and osteonectin (Sparc) (Supplemental Fig. 3D). Finally, the activation of coagulation and complement cascades are also important for resolution. The microarray results revealed variable expression of.

**FIGURE 6.** Molecular characterization of liver-MF subsets. (A) Affymetrix microarray hierarchical clustering performed on mRNA of sorted liver-MF subsets. A colored bar indicating the standardized log2 intensities accompanies the expression profile. (B) Correlation matrix with Pearson correlation coefficient performed for all genes above background and above 2-fold change. (C) Venn diagram of liver-MF subsets showing the distribution of the 2196 genes with above 2-fold expression difference in comparison with steady-state KC. (D) Heat map analysis showing the differential raw expression level of MF-associated genes.
coagulation mediators and complement system factors (Supplemental Fig. 3E).

Discussion
Recent studies have highlighted marked heterogeneity in the origins of tissue MF. Fate-mapping approaches revealed that most tissue-resident MF have a prenatal origin and do not rely on blood monocytes for their renewal under steady-state conditions (3–9). We have shown that intestinal lamina propria MF are exceptional and maintained by Ly6C<sup>hi</sup> monocytes (11, 54). In respect to KC, their ontogeny and maintenance is disputed. Thymidine incorporation experiments revealed that KC are in mitosis at any given time in the healthy liver (21, 55). Yet, other studies suggested that KC are generated by BM-derived cells or by local proliferation, depending on the nature of stimulation (56–59). Nevertheless, emerging studies have shown that liver KC are established prenatally by yolk sac–derived precursors and can persist in adult mice independently of hematopoietic stem cells (3, 4). Yet the question whether circulating monocytes can contribute to the renewal of KC following inflammatory insults remained elusive. In this study, using several complementary approaches, including adoptive transfer, selective ablation, and molecular profiling, we report that following liver injury, Ly6C<sup>hi</sup> monocytes infiltrate the liver in a CCR2- and M-CSF-dependent manner and give rise to a phenotypically and transcriptionally distinct MF population and do not contribute to the replenishment of resident KC. Instead, the latter are replenished transitorically by yolk sac–derived precursors and can persist in adult mice independently of hematopoietic stem cells (3, 4).

Ly6C<sup>hi</sup> monocytes display high plasticity and are directly involved in both the establishment and resolution of inflammatory reactions. Previous studies based on the use of Ccr2<sup>-/-</sup> and Cx2<sup>2-/-</sup> transgenic mice revealed an impaired recovery from AILI, strongly suggesting that infiltrating MF play a pivotal hepatoprotective role in the resolution phase (13, 25, 60). In this study, we further substantiated these findings, demonstrating that inducible and selective ablation of Ly6C<sup>hi</sup> monocytes and MoMF results in impaired recovery manifested by greater hepatotoxic damage. Indeed, our microarray profiling provides compelling evidence that Ly6C<sup>hi</sup> monocytes and MoMF express a genetic signature associated with alternatively activated and prorrestorative phenotypes. Worth mentioning is the unique expression of key angiogenesis mediators by the Ly6C<sup>hi</sup> monocytes. These include VEGF-A, which is critically important to the process of hepatocyte regeneration and restoration of liver microvasculature following AILI (61). Moreover, VEGF-A induces the paracrine release of hepatocyte growth factor, IL-6, and other hepatotrophic molecules by liver sinusoidal endothelial cells and the protection from hepatotoxic damage (62). Moreover, they expressed the urokinase plasminogen activator and its receptor, which are central players in wound healing and angiogenesis and in liver repair (63, 64).

Neutrophils recruited to inflammatory sites release a diverse set of deleterious substances and take substantial part in the collateral damage to the tissue. Thus, opportune termination of neutrophil activity is essential for resolution from liver injury. Holt et al. (13) were the first to make the association between absence of infiltrating MF and increased frequency of neutrophils following AILI. Substantiating these results, we show in this study increased accumulation of neutrophils at 24 h following APAP administration in Ccr2<sup>-/-</sup> mice and in mice subjected to inducible ablation of the Ly6C<sup>hi</sup> monocytes. Our results revealed several hepatoprotective mechanisms used by these cells to regulate neutrophil activity, apoptosis, and removal. In particular, we show their unique activation of the PGE<sub>2</sub> synthesis pathway recently shown in a murine model of acute mucosal infection with Toxoplasma gondii to inhibit neutrophil activation (10).
Impaired recovery from AILI has been demonstrated in mice lacking KC (65) or MoMF (13) and was greater in mice deficient for both MF subsets (23), suggesting that both play critical role in this process. Nevertheless, the question of whether MoMF compensate functionally for the reduced presences of KC at the initial phase of recovery, or, oppositely, these MF subsets are functionally distinct has not been addressed. In an effort to unravel new insights on the task division between KC and MoMF in the recovery from liver injury, we have performed in-depth gene-expression microarray analysis at 72 h postinduction of AILI, when both MF subsets coexist. Our molecular profiling revealed 603 genes that were differentially expressed by at least 2-fold change between these subsets. Among them, using bioinformatics tools, we were able to provide a list of genes associated with wound healing that were differently expressed between the resident KC and the infiltrating monocyte-derived MF and may hence include known and novel subset-specific resolution markers. Specifically, we chose to demonstrate their variable expression of scavenger receptors, as timely removal of apoptotic cells and cellular debris is critical to the initiation of tissue repair. Another important phase of wound resolution is remodeling of the ECM, which is capable of modulating cellular behavior and phenotype, sequestering and activating specific cytokines and growth factors, as well as organizing the geometric framework that controls cell migration and interactions. We show in this study variable expression of ECM-remodeling enzymes as well as glycoproteins and proteoglycans by the distinct liver-MF subsets. Thus, these results imply strongly that the infiltrating MoMF and resident KC contribute differently to the resolution from liver injury, when according to the kinetics, the MoMF dominate the early phase and KC the later phase once they complete their recovery.

Infiltrating monocytes and resident MF play crucial and distinct roles in liver homeostasis and immunity, but also contribute to a broad spectrum of liver pathologies and are thus attractive therapeutic targets. Potential intervention strategies aiming at manipulation of these cells require in-depth insights of their origins and division of labor. In this study, we distinguish phenotypically and molecularly among three distinct MF subsets that populate the liver through the inflammatory and resolution phases of acute liver injury. We show that during the necrotic phase of AILI, the liver is massively infiltrated by Ly6Chi monocytes in a CCR2- and M-CSF–mediated manner. Subsequently, these cells differentiate into Ly6CLo MoMF to become the dominant liver-MF population at the early resolution phase. In contrast, resident KC are significantly reduced upon APAP challenge and recover at the resolution phase by self-renewal. Moreover, we show that the monocyte-derived MF critically contribute to the resolution from AILI. Finally, microarray based molecular profiling uncover substantial gene-expression differences among these three liver-MF subsets. Specifically, we show that infiltrating Ly6Chi monocytes acquire a proinflammatory phenotype mediated by expression of proangiogenesis mediators and negative regulation of neutrophil activity. Moreover, KC and MoMF isolated at the resolution phase display distinct pattern of proresorptive genes. Importantly, our up-to-date phenotypic, ontogenic, and molecular definition of liver-MF compartment under inflammatory settings refers to the context of AILI and differences related to the representation and function of liver-MF subsets are possible in other liver disorders and should be elucidated in the future.

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Disclosures

The authors have no financial interests of conflict.

References

MACROPHAGE HETEROGENEITY IN ACUTE LIVER INJURY


