COMMD10 is critical for Kupffer cell survival and controls Ly6C<sup>hi</sup> monocyte differentiation and inflammation in the injured liver

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In brief
Cohen et al. demonstrate a pivotal role for COMMD10 in homeostatic maintenance of Kupffer cells and other resident macrophages. COMMD10 is also involved in dictating Ly6C<sup>hi</sup> monocyte differentiation fates and restraining their inflammatory activity in the injured liver.

Highlights
- COMMD10 deficiency impedes liver Kupffer cell homeostatic survival
- COMMD10 is crucial for the survival of diverse tissue-resident macrophages
- COMMD10 restrains the hepatotoxic activity of Ly6C<sup>hi</sup> monocytes in the injured liver
- COMMD10 deficiency skews monocyte differentiation toward NeuMos and LAMs
COMMD10 is critical for Kupffer cell survival and controls Ly6C^hi monocyte differentiation and inflammation in the injured liver

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SUMMARY

Liver-resident macrophages Kupffer cells (KCs) and infiltrating Ly6C^hi monocytes both contribute to liver tissue regeneration in various pathologies but also to disease progression upon disruption of orderly consecutive regeneration cascades. Little is known about molecular pathways that regulate their differentiation, maintenance, or inflammatory behavior during injury. Here, we show that copper metabolism MURR1 domain (COMMD)10-deficient KCs adopt liver-specific identity. Strikingly, COMMD10 deficiency in KCs and in other tissue-resident macrophages impedes their homeostatic survival, leading to their continuous replacement by Ly6C^hi monocytes. While COMMD10 deficiency in KCs mildly worsens acetaminophen-induced liver injury (AILI), its deficiency in Ly6C^hi monocytes results in exacerbated and sustained hepatic damage. Monocytes display unleashed inflammasome activation and a reduced type I interferon response and acquire "neutrophil-like" and lipid-associated macrophage differentiation fates. Collectively, COMMD10 appears indispensable for KC and other tissue-resident macrophage survival and is an important regulator of Ly6C^hi monocyte fate decisions and reparative behavior in the diseased liver.

INTRODUCTION

Most tissue-resident macrophages are established prenatally and self-maintain independently of bone marrow (BM) monocytes. Instructed by local cues, these cells adopt unique transcriptional modules that impart tissue-specific functional identity (Varol et al., 2015). In the liver, Kupffer cells (KCs) dominate the homeostatic macrophage pool. They reside in the sinusoidal vessels and in the space of Disse, interacting with hepatic stellate cells (HSCs) and hepatocytes (Bonnardel et al., 2019), where they act as sentinels and perform specialized accessory functions involving iron and lipid metabolism (Scott and Guilliams, 2018). Upon depletion, resident KCs (ResKCs) are replaced by BM Ly6C^hi monocyte-derived KCs (MokKCs) that adopt ResKC functions during homeostasis and disease (Bonnardel et al., 2019; Remmerie et al., 2020; Sakai et al., 2019; Scott et al., 2016; Seidman et al., 2020; Tran et al., 2020).

Hepatic macrophages play a central role in the initiation, progression, and resolution of various liver diseases (Krenkel and Tacke, 2017). KCs play a major role in initiating inflammation but have limited plasticity. In contrast, Ly6C^hi monocytes and their derived macrophage descendants (MoMFs) prevail in acute or chronic liver injury, display marked transcriptional variance, and provide crucial functional plasticity (Krenkel et al., 2018; Ramachandran et al., 2019; Zigmond et al., 2014). A specific example of MoMFs is lipid-associated macrophages (LAMs) involved in progression of metabolic liver disease (Remmerie et al., 2020). Recent studies have uncovered binary developmental trajectories of monocytes in the BM. Neutrophil-like monocytes (NeuMos) and dendritic cell (DC)-like monocytes (DCMos) differentiate downstream of granulocyte-macrophage progenitors (GMPs) and macrophage-DC progenitors (MDPs), respectively (Weinreb et al., 2020; Yáñez et al., 2017). While the impact of Ly6C^hi monocyte bifurcation on liver diseases...
Figure 1. COMMD10-deficient ResKC are replaced by MoKCs in the healthy liver

(A–I) KCs from Commd10fl/fl and Clec4fCommd10 healthy livers were compared. (A) qRT-PCR gene expression of Commd10 (n > 3).
(B) Mean fluorescence intensity (MFI) of CLEC4F (n > 6).
(C) Confocal microscopy of livers showing expression of CLEC4F (red), Desmin (yellow), CD31 (cyan), and nuclei (blue). Original magnification, x 40. Scale bars, 50 μm.
(D) KC numbers normalized to liver mass (n > 9).
(E) Frequency of TIM4+ out of total KCs.
(F) qRT-PCR gene expression of Timd4 in KCs (n > 3).
(G) Frequency of CD163+ and VSIG4+ KCs (n > 12).
(H) Normalized cell counts of VSIG4+ and CD163+ KCs (n > 6).

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remains enigmatic, these findings challenge our current comprehension of macrophage heterogeneity in the diseased liver and stress the importance of identifying factors that govern macrophage and monocyte fate decisions.

The COMMD (copper metabolism MURR1 domain) family includes 10 evolutionarily conserved proteins. Functions of COMMD proteins are still being defined, but they seem to play non-redundant roles in regulating transcription and protein trafficking (Bartuzi et al., 2013; Burstein et al., 2005). COMMD1, the prototype of this family, negatively regulates nuclear factor κB (NF-κB) (Li et al., 2014; Maine et al., 2007) and hypoxia inducible factor-1 (HIF-1α) (Vonk et al., 2011) (van de Sluis et al., 2010). COMMD proteins are essential components of the COMMD/CDC22/CDC93 complex, which modulates endolysosome architecture and trafficking of transmembrane proteins (Bartuzi et al., 2016; Phillips-Krawczak et al., 2015). Embryonic lethality of COMMD10 deficiency has hampered the study of its function. Yet, utilizing conditional COMMD10 knockout mice we uncovered a role for COMMD10 in limiting inflammasome activation in Ly6C hi monocytes during experimental sepsis and colitis (Mouhadeb et al., 2018), and in supporting phagolysosomal biogenesis and maturation in KCs and BM-derived macrophages infected with Staphylococcus aureus (Ben Shlomo et al., 2019). These studies mark COMMD10 as a candidate mediator of monocyte and macrophage fate and immune responses.

Here, we uncover an important role for COMMD10 in homeostatic maintenance of KCs and other fetal tissue-resident macrophages. We also show that COMMD10 is involved in determining Ly6C hi monocyte differentiation fates and in restraining their inflammatory response in the injured liver.

**RESULTS**

**COMMD10 is required for homeostatic maintenance of KCs**

To target COMMD10 deficiency to KCs we crossed Clec4f Δcre mice (Scott et al., 2018) with Commdd10 Δf/f mice (Mouhadeb et al., 2018) (Clec4f Δf/f/Commdd10 Δf/f). KCs were defined as CD45+CD11b+Ly6G+Ly6C− cells, also expressing the KC markers TIM4, VSIG4, CLEC2, and CLEC4F (Figure S1A). Commdd10 gene expression decreased by about 50% in Clec4f−/Commdd10− KCs (Figure 1A). COMMD10 deficiency had no effect on KC expression of CLEC4F (Figures 1B and S1B) or on their localization in the niche, where they intimately interacted with Desmin+ HSCs, hepatocytes, and CD31+ liver sinusoidal endothelial cells (SECs) (Figures 1C, S1C, and S1D). Yet, KC counts were significantly lower in Clec4f Δf/f/Commdd10 Δf/f versus Commdd10 Δf/f/Δf/f litter (Figure 1D). Expression of TIM4, an apoptotic cell clearance receptor selectively expressed by ResKCs in the liver, was diminished in Clec4f Δf/f/Commdd10− KCs at both protein (Figure 1E) and gene (Figure 1F) levels. Expression of CD163 and VSG4, additional ResKC-associated cell surface receptors, was also altered in Clec4f Δf/f/Commdd10− KCs with an increase in the fractions of CD163+ and VSG4+ cells (Figures 1G and S1E) and VSG4+ counts, and a reduction in CD163+ counts (Figure 1H). Similar results were obtained when Cx3cr1 Δf/Δf mice were used to delete COMMD10 in KCs (Cx3cr1 Δf/Δf/Commdd10 Δf/f). Cx3cr1 Δf/Δf/Commdd10 Δf/f KCs expressed normal levels of CLEC4F (Figure S1F) but had diminished expression of TIM4 already at the perilatinal stage (Figure S1G), and later during maturation (Figure S1H) and intracellularly (Figure S1I). Cx3cr1 Δf/Δf/Commdd10 Δf/f KCs from older livers (24–30 weeks) displayed increasing numbers of TIM4+ cells, although most remained TIM4− (Figure S1J). In contrast, TIM4+ KCs remained barely detectable even in older Clec4f Δf/f/Commdd10 Δf/f (24–30 weeks) (Figure 1J).

TIM4 is a discriminating marker between TIM4+ ResKCs and TIM4− MoKCs (Remmerie et al., 2020; Tran et al., 2020). We next studied whether the persistently low expression of TIM4 by Clec4f Δf/f/Commdd10− KCs is due to their replacement by MoKCs. Fetal-derived KCs are relatively radioreistant while MoKCs are radiosensitive (Soysa et al., 2019). Hence, if TIM4− Clec4f Δf/f/Commdd10− KCs are MoKCs, they would be expected to lose radiosensitivity. Clec4f Δf/f/Commdd10 Δf/f mice were lethally irradiated and reconstituted with congenic CD45.1+ wild-type (WT) BM graft. CD45.2+ radiosensitive KCs constituted about 40% of KCs in reconstituted Commdd10 Δf/f/Δf/f livers but were barely detectable in Clec4f Δf/f/Commdd10 livers (Figure 1J), confirming replacement of COMMD10-deficient KCs with radiosensitive MoKCs. Given the radiosensitivity of KCs in Clec4f Δf/f/Commdd10 mice, we next subjected these mice to a competitive mixed BM chimerism experiment aiming to compare the fate of Clec4f Δf/f/Commdd10 versus WT KCs in the same environment. Nine weeks following lethal irradiation and reconstitution with mixed CD45.1+ WT and CD45.2+ Clec4f Δf/f/Commdd10 BM, circulating and liver Ly6C hi monocytes displayed mixed chimerism, with ~60% being of CD45.2+ Clec4f Δf/f/Commdd10 origin. In contrast, only 20% of reconstituted KCs were of CD45.2+ Clec4f Δf/f/Commdd10 origin (Figure 1K). This could be the result of COMMD10 deficiency hampering the ability of monocytes to reconstitute the KC niche and/or shortening MoKC survival. To examine this question, lethally irradiated CD45.2+ WT mice were reconstituted with mixed CD45.1+ WT and CD45.2+ Clec4f Δf/f/Commdd10 BM, and 8 weeks later clodronate liposomes were applied to effectively deplete the entire KC compartment (Figure S1K) (Soya et al., 2019). Four weeks later, Ly6C hi monocytes and KCs displayed similar mixed chimerism, suggesting that COMMD10 deficiency had no effect on the ability of MoKCs to replenish the niche. However, 8 weeks following KC depletion, although chimism proportions of the Ly6C hi monocytes

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(i) Frequency of TIM4−/total KCs in 24- to 30-week-old livers (n ≥ 4).

(j–l) Quantification of % chimerism (j) in KCs at 8 weeks after irradiation (n = 4), (k) in blood and liver monocytes and KCs at 8 weeks after irradiation (n = 4), and (l) in blood and liver monocytes and KCs as assessed 4/8 weeks after clodronate (n ≥ 4).

(M) Quantification of BrdU+ cell frequency among KCs (n > 7).

(N) Quantification of Ki-67+ cell frequency among KCs (n > 4).

Data were analyzed by an unpaired, two-tailed t test and are presented as mean ± SEM with significance: *p < 0.05, **p < 0.01, ***p < 0.001. Experiments were repeated twice (A, B, H–J, and M), once (C, E, F, K, L, and N), or at least three times (D and G).
remained essentially the same, there was a significant reduction in the fraction of MoKCs of CD45.2+ Clec4f\textsuperscript{Commd10\textsuperscript{+}} origin, strongly indicating their shorter survival (Figure 1L). We next evaluated the proliferation rate of Clec4f\textsuperscript{Commd10\textsuperscript{+}} versus Commd10\textsuperscript{−/−} KCs by measuring 5-bromo-2'-deoxyxyridine (BrdU) incorporation. Clec4f\textsuperscript{Commd10\textsuperscript{+}} MoKCs showed a higher proliferation rate (Figure 1M). This was also evident by increased Ki-67 staining (Figure 1N). Altogether, these results show that the absence of COMMD10 impedes KC survival.

### COMMD10-deficient KCs acquire liver-specific identity but display increased stress and mitochondrial dysfunction

To examine the ability of COMMD10-deficient MoKCs to acquire KC-associated transcriptional programs, we performed a MARS-seq transcriptome analysis of Clec4f\textsuperscript{Commd10\textsuperscript{+}} versus Commd10\textsuperscript{−/−} KCs. There were 259 differentially expressed genes (DEGs) (≥1.5 fold, adjusted p [p.adj] < 0.05), among which 144 were upregulated and 115 were downregulated. Clec4f\textsuperscript{−/−} Commd10\textsuperscript{−/−} KCs acquired a similar expression pattern of KC core genes and associated transcription factors (Figures 2A and 2B), with only 12% of ~70 KC-associated genes (Scott et al., 2018) being significantly downregulated (Table S1). Further supporting their recent ontogeny from Ly6C\textsuperscript{hi} monocytes, they displayed higher expression of MoKCs-associated genes (Figure 2C). KCs specialize in lipid metabolism and iron recycling (Scott and Guilmant, 2018). With respect to lipid metabolism, Clec4f\textsuperscript{Commd10\textsuperscript{+}} KCs exhibited altered expression of several genes involved with lipid uptake (Cd36, Lp), and cholesterol processing and export (Abca1, Apobec1, Apoc1) (Figure S2A). Yet, BODIPY staining for neutral lipids revealed no significant differences under homeostatic conditions (Figure S2B). Triglyceride levels were also identical in healthy Clec4f\textsuperscript{Commd10\textsuperscript{+}} versus Commd10\textsuperscript{−/−} livers (Figure S2C). Some iron storage and transport genes were differentially expressed, with increased expression of Ile and ferroportin 1 (Slc40a1), and reduced expression of ferritin light and heavy chains (Fth1 and Fth1, respectively) and Marco (Figure S2D). Nevertheless, iron deposits were hardly detectable in healthy Clec4f\textsuperscript{Commd10\textsuperscript{+}} and Commd10\textsuperscript{−/−} livers, suggesting normal physiological iron recycling (Figure S2E).

In contrast, Ingenuity Pathway Analysis (IPA) and MouseMine pathway and Gene Ontology analyses (p < 0.05, 1.5 fold change [FC]) revealed among the top canonical pathways significant gene expression alterations involved with regulation of translation and EIF2/EIF4 signaling, as well as protein ubiquitination and degradation (Figure 2D). Accordingly, Clec4f\textsuperscript{Commd10\textsuperscript{+}} KCs displayed profound reduction in genes involved with translation initiation and ribosomal biogenesis (Figure 2E), and in genes belonging to the ubiquitin system and proteasome machinery (Figure 2F). Among the most significant pathways were also mitochondrial dysfunction and oxidative phosphorylation (Figure 2D), with reduction in many genes encoding for key enzymes and protein complexes mediating the mitochondrial respiratory chain and protecting cells from cytotoxicity elicited by oxidative stress (Figure 2G). Interestingly, Clec4f\textsuperscript{Commd10\textsuperscript{+}} KCs exhibited also increased expression of genes associated with interleukin (IL)-1 production and inflammasomes (Figure S2F), and with antigen processing and presentation (Figure 2H), a feature that was also associated with MoKCs (Bonnardel et al., 2019; Seidman et al., 2020). Collectively, these results show that although COMMD10 is dispensable for the ability of MoKCs to acquire KC identity, its deficiency is associated with transcriptional alterations that may facilitate their shortened survival.

### COMMD10 is vital for the survival of diverse tissue-resident macrophages

We next examined whether COMMD10 is important for the survival of other tissue-resident macrophages. We utilized Lyz2\textsuperscript{cre} mice to delete COMMD10 (LysM\textsuperscript{Commd10\textsuperscript{+}} mice) in large peritoneal macrophages (LPMs) and alveolar macrophages (AMS) (Abram et al., 2014). In the peritoneum, Cd11b+major histocompatibility complex class II (MHC class II)\textsuperscript{+}/F4/80\textsuperscript{+}ICAM2\textsuperscript{+} LPMs (Figure 3A) from LysM\textsuperscript{−/−} mice versus LysM\textsuperscript{Commd10\textsuperscript{+}} versus LysM\textsuperscript{Commd10\textsuperscript{−/−}} mice were compared. Expression of Commd10 was diminished in LysM\textsuperscript{Commd10\textsuperscript{−/−}} LPMs, but expression of the LPM core transcription factor GATA-binding protein 6 (GATA6) was unaltered (Figure 3B), suggesting that COMMD10 deficiency does not alter their tissue-specific identity. There was reduced representation of LPMs in the LysM\textsuperscript{Commd10\textsuperscript{−/−}} peritoneum (Figures 3C and 3D). TIM4 expression in LPMs revealed three subsets: TIM4+, TIM4\textsuperscript{−/−}, and TIM4\textsuperscript{hi}. In LysM\textsuperscript{Commd10\textsuperscript{−/−}} peritoneum, TIM4\textsuperscript{−/−} LPMs were replaced with TIM4\textsuperscript{hi} LPMs (Figure 3E). Cd11c+ Siglec F\textsuperscript{+}/F4/80\textsuperscript{+} AMs were isolated by bronchoalveolar lavage of Commd10\textsuperscript{−/−} or LysM\textsuperscript{Commd10\textsuperscript{+}} mice. AM counts were similar, although LysM\textsuperscript{−/−} AMs exhibited reduction in the expression of Cd11c (Figures 3F and 3G). Revisiting the mixed BM chimera approach, WT mice were lethally irradiated and reconstituted with mixed Cd45.1\textsuperscript{+} WT and Cd45.2\textsuperscript{+} LysM\textsuperscript{Commd10\textsuperscript{−/−}} BM. Nine weeks later, circulating Ly6C\textsuperscript{hi} monocytes displayed mixed chimerism with nearly 60% being of Cd45.2\textsuperscript{+} LysM\textsuperscript{Commd10\textsuperscript{−/−}} origin. In contrast, Cd45.2\textsuperscript{−} LPMs and AMs dropped to 15% (Figure 3H). Similarly to COMMD10-deficient KCs, LysM\textsuperscript{Commd10\textsuperscript{−/−}} LPMs exhibited increased proliferation as depicted by BrdU incorporation (Figure 3I) and Ki-67 staining (Figure 3J). Similar results were obtained using Cx3cr1\textsuperscript{Commd10\textsuperscript{−/−}} mice, with reduction in LPM counts (Figure S3A), exchange of TIM4\textsuperscript{hi} in TIM4\textsuperscript{−/−} LPMs in both young (8 weeks) and older (24–30 weeks) mice (Figure S3B), and a significant reduction in the fraction of Cd45.2\textsuperscript{+} Cx3cr1\textsuperscript{Commd10\textsuperscript{−/−}} versus Cd45.1\textsuperscript{+} WT LPMs in a mixed BM chimera experiment (Figure S3C).

TIM4 is also a marker of resident macrophages in the splenic marginal zone and red pulp (Fujiiyama et al., 2019). Splenic macrophages were defined as Cd11b\textsuperscript{+}/F4/80\textsuperscript{+}CD64\textsuperscript{+} cells. Indeed, there was significant depletion of TIM4\textsuperscript{hi} splenic macrophages in both young and older Cx3cr1\textsuperscript{Commd10\textsuperscript{−/−}} mice (Figure S3D). The gut lamina propria macrophage (lpMF) compartment also contains fetal TIM4\textsuperscript{hi} macrophages that decline with age (Shaw et al., 2018). LpMFs, defined as Cd45\textsuperscript{−}/Cd11b\textsuperscript{+}/Cd64\textsuperscript{+}MHc class II\textsuperscript{+}Cx3cr1\textsuperscript{+} cells, exhibited a significant reduction in TIM4\textsuperscript{hi} fetal lpMFs in the colons of Cx3cr1\textsuperscript{Commd10\textsuperscript{−/−}} mice (Figure S3E). In contrast to LPMs, colonic lpMFs from the mixed BM chimera exhibited Cd45.1\textsuperscript{+} WT and Cd45.2\textsuperscript{+} Cx3cr1\textsuperscript{Commd10\textsuperscript{−/−}} ratios similar to circulating Ly6C\textsuperscript{hi} monocytes (Figure 3K), as expected given...
COMMD10 deficiency delays clearance of dying cells by KCs and LPMs

Being in direct contact with hepatocytes, KCs are the primary cells that sense and react to hepatic damage by initiating a...
Figure 3. Impeded survival of other COMMID10-deficient tissue-resident macrophages

(A) Gating strategy of large peritoneal macrophages (LPMs) defined as CD45^+F4/80^+CD11b^+ICAM2^+.
(B–G) LPMs or alveolar macrophages (AMs) from Commid10^fl/fl^ and LysM^D^Commd10^mice were compared.
(B) qRT-PCR gene expression of Commid10 and Gata6 (n > 5).
(C) Fraction of LPMs among CD45^+ cells.
(D) LPM cell counts normalized to extracted volume (n > 6).
(E) Frequency of TIM4^+, TIM4^lo, and TIM4^−/C0 cells among LPMs (n > 4).
(F) Frequency of AMs (defined as CD45^+F4/80^+CD11C^+Siglec-F^+) among CD45^+ cells (n > 3).
(G) MFI of CD11c expression by AMs (n = 3).
(H) Quantification of % chimerism in blood Ly6C^hi monocytes, LPMs, and AMs assessed in mixed CD45.1 WT/CD45.2 LysM^D^Commd10^BM chimeras at 8 weeks after irradiation (n > 9).

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restorative response (Krenkel and Tacke, 2017). Hence, we next sought to examine the effect of COMMD10 deficiency on KC survival and ability to recruit Ly6Ch monocytes and neutrophils to the injured liver, using acetaminophen-induced liver injury (AILI) as a model. For AILI 24 h, plasma levels of the liver enzymes alanine and aspartate transaminases (ALT, AST) were similar in Clec4fl/fl versus Commd10fl/fl mice (Figure 4A). In the Clec4fl/fl livers the histopathological score was mildly increased (Figure 4B), KC counts were still reduced (Figure 4C), and KCs were TIM4+ (Figure 4D). Ly6Ch monocytes and neutrophil cell numbers were lower (Figure 4E), yet expression levels of their recruitment chemokines as well as inflammatory cytokines were similar (Figure 4F). Despite the increased expression of genes involved with IL-1 production in Clec4fl/fl KCs at steady state (Figure S2F), there was no evidence for increased inflammasome and caspase-1 activation in Clec4fl/fl livers (Figure 4G). KCs are involved in clearing dying cells during AILI. In addition to these fewer KCs, the reduction in TIM4 may compromise their ability to clear phosphatidylserine (PtSer)-expressing apoptotic cells. To directly assess the effect of COMMD10 deficiency on the ability of KCs to clear dying cells, apoptotic CFSE (carboxyfluorescein succinimidyl ester)-labeled thymocytes were injected into the portal vein. After 15 min, Commd10fl/fl KCs were already able to engulf apoptotic thymocytes, while these could not be detected in the Cx3cr1fl/fl KCs (Figure 4H). TIM4 also mediates the recognition, engulfment, and clearance of PtSer-expressing apoptotic cells by LPMs (Wong et al., 2010). Hence, the depletion of TIM4 in COMMD10-deficient LPMs (Figures 3D and S3B) may compromise this function. Indeed, Cx3cr1fl/fl LPMs displayed delayed uptake and clearance of engrafted CFSE-labeled dying thymocytes (Figure 4). This was further directly confirmed in ex vivo-isolated Cx3cr1fl/fl LPMs (Figure 4J). In agreement with the delayed clearance of dying cells by COMMD10-deficient KCs there was increased accumulation of TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling)-dying hepatocytes in Clec4fl/fl livers (Figure 4K). Overall, COMMD10 deficiency in KCs interferes with their clearance of dying cells.

Increased hepatic damage and inflammation are induced by COMMD10 deficiency in Ly6Ch monocytes

COMMD10 deficiency in Ly6Ch monocytes profoundly aggraves colonic damage and inflammation (Mouhadeb et al., 2018). Ly6Ch monocytes and their MoMF progenies play important roles in resolution of AILI (Graubardt et al., 2017; Zigmond et al., 2014), yet their inflammatory behavior must be strictly controlled to avoid collateral damage. Hence, we next endeavored to study the effect of COMMD10 deficiency in Ly6Ch monocytes on hepatic damage and inflammation during AILI. In LysMfl/fl mice, about 50% of liver-infiltrating Ly6Ch monocytes become COMMD10 deficient (Mouhadeb et al., 2018), and indeed there was a 50% reduction in Commd10 gene expression in liver-infiltrating LysMfl/fl Ly6Ch monocytes sorted at AILI 24 h (Figure 5A). LysMfl/fl mice displayed profoundly increased hepatic damage as manifested by increased plasma levels of ALT/AST at AILI 24 and 48 h (Figure 5B). Histopathological scoring revealed increased necrotic damage in LysMfl/fl livers with extended areas of panlobular and bridging necrosis (Figure 5C). There was profoundly higher accumulation of infiltrating Ly6Ch monocytes and neutrophils in LysMfl/fl livers (Figure 5D), and this was linked to elevated expression of various inflammatory genes, including tumor necrosis factor (TNF)-α, IL-1β, and the Ly6Ch monocyte and neutrophil recruitment chemokines CCL2 and CXCL1/CXCL2, respectively (Figure 5E).

COMMD10 negatively regulates both canonical and non-canonical inflammasome pathways in Ly6Ch monocytes, but not tissue-resident macrophages, during endotoxic shock and experimental colitis (Mouhadeb et al., 2018). We therefore speculated that locally released death-associated molecular patterns (DAMPs) might incite hyper-inflammasome activation in liver-infiltrating LysMfl/fl Ly6Ch monocytes. Indeed, there was increased caspase-1 and caspase-11 activation in the LysMfl/fl livers at AILI 24 h (Figure 5F).

To pinpoint the contribution of Ly6Ch monocytes (out of total myeloid cells) to the increased hepatotoxicity in LysMfl/fl livers, some of the LysMfl/fl mice were treated prior to AILI with the anti-CCR2 antibody MC-21 (Mack et al., 2001), which efficiently blocks the arrival of Ly6Ch monocytes to the injured liver (Graubardt et al., 2017; Zigmond et al., 2014) (Figure 5E). Their inducible ablation was sufficient to ameliorate hepatic damage and inflammation to that of Ccr2−/− mice as depicted by normalization of plasma ALT/AST levels (Figure 5B), histopathological score (Figure 5C), expression of inflammatory genes such as Ccl2 (Figure 5E), and inflammasome activation (Figure 5F). We implemented a complementary BM chimera approach in which Ccr2−/− recipients are subjected to low-dose irradiation and transplanted with BM cells from CCR2+ donors. This mild irradiation spares KCs, and given the established reliance of Ly6Ch monocytes on CCR2, enforces liver engraftment by CCR2+ Ly6Ch monocytes of donor BM origin, while other resident and infiltrating immune cells remain largely of recipient origin (Tran et al., 2020). Indeed, 2 weeks after transplantation, Ly6Ch monocytes were 90% of donor origin, while only 15%–20% of neutrophils and CD11b− lymphocytes and a minor fraction of KCs (<2%) were of donor origin in blood and liver at AILI 24 h (Figures S4A and S4B). Next, irradiated Ccr2−/− Cx3cr1gfp/+ mice were engrafted with BM from LysMfl/fl (BM LysMfl/fl) or Commd10fl/fl (BM LysMfl/fl) mice (Figure S4C), and 2 weeks later were subjected to AILI 24 h. About 90% of Ly6Ch monocytes in BM Commd10fl/fl and BM LysMfl/fl livers were of donor BM origin (Cx3cr1-gfp+) (Figure S4D). Importantly, BM
Figure 4. Clec4f<sup>DComm10</sup> mice display mildly worse hepatic damage in response to acetaminophen-induced liver injury (AILI)

Commd10<sup>fl/fl</sup> and Clec4f<sup>DComm10</sup> mice were injected with APAP (300 mg/kg) and sacrificed 24 h later.

(A) ALT and AST serum levels (n > 5).
(B) Histopathological score and representative images of H&E-stained liver sections. Original magnification, x4. Scale bars, 50 μm (n > 9).
(C) Quantification of normalized KC counts (n > 7).
(D) MFI of TIM4 in KCs (n > 12).
(E) Quantification of normalized Ly6Chi monocyte and neutrophil counts (n > 8).
(F) Gene expression of inflammatory cytokines and chemokines quantified by qRT-PCR (n > 8).
(G) Immunoblot of total liver tissue protein showing caspase-1 activation. GAPDH was used as a loading control (n > 3).
(H and I) Frequency of CFSE-labeled apoptotic thymocytes comparing Commd10<sup>fl/fl</sup> and Cx3cr1<sup>DComm10</sup> mice (H) in KCs 15 min after injection of 2 × 10<sup>7</sup> apoptotic cells into the portal vein (n > 2), and (I) in LPMs at 20 and 30 min after intraperitoneal injection of 2.5 × 10<sup>6</sup> apoptotic cells (n > 3).

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LysM^+/Commd10 mice had significantly increased plasma ALT/AST levels (Figure 5G), histopathological score (Figure 5H), and infiltration of Ly6C^hi monocytes and neutrophils (Figure 5I). Moreover, expression of TNF-α (Tnfa) and CCL2 (Ccl2) increased in BM LysM^+/Commd10 livers (Figure 5J). Collectively, these results illuminate an important role for COMMD10 in tuning the recruitment and inflammatory activity of Ly6C^hi monocytes in AILI.

**COMMD10-deficient Ly6C^hi monocytes exhibit a reduction in type I interferon response genes**

To gain molecular insight into the immunoregulatory role played by COMMD10, LysM^+/Commd10 and Commd10^+/m Ly6C^hi monocytes were sorted from livers at AILI 24 h and subjected to bulk RNA sequencing (RNA-seq) analysis. Among 651 DEGs (≥1.5 fold, padj < 0.05), 374 genes were significantly downregulated. The most pronounced GO functional enrichments (MouseMine) among downregulated genes were associated with type I interferon response and production (Figures 6A and 6B; Table S2). These included genes related to sensing (Tlr3, Tlr9, Ddx58), transcriptional activators of type I interferon response (Irf7, Stat1, Stat2), and many interferon-stimulated genes. Many of these genes were significantly upregulated during the transition of Ly6C^hi monocytes (AILI 24 h) into pro-restorative MoMFs at the resolution phase (AILI 72 h) (Figure 6C; Table S3). An additional profoundly reduced GO enrichment pathway with antigen processing and presentation of exogenous peptide antigen (Figure 6A). Indeed, LysM^+/Commd10 Ly6C^hi monocytes exhibited reduction in genes encoding for HLA class II β and β chain paralogs, and molecules involved with transport of antigens (Figure 6D; Table S2). Functional enrichment analyses of 277 upregulated genes revealed pathways associated with an abnormal response to injury, abnormal innate immunity, abnormal macrophage physiology, abnormal liver regeneration, extracellular matrix organization, and glucose metabolic process (Figure 6A; Table S4). In this respect, LysM^+/Commd10 Ly6C^hi monocytes exhibited higher expression of inflammatory genes, including Ccl2 and Cxcl2, and genes encoding for extracellular matrix (ECM) core proteins and remodeling enzymes (Figure 6E).

**COMMD10 deficiency promotes NeuMo fate of Ly6C^hi monocytes**

Among upregulated genes there was also enrichment for neutrophil-associated pathways, such as neutrophil degranulation, abnormal neutrophil morphology and neutrophil migration (Figure 6A; Table S4). Given the recent discovery of NeuMos and DCMos (Weinreb et al., 2020; Yáñez et al., 2017), we speculated that COMMD10 deficiency in Ly6C^hi monocytes might favor enrichment for NeuMos. Indeed, comparison with Yáñez et al. (2017) NeuMo and DCMo gene signatures revealed in LysM^+/Commd10 Ly6C^hi monocytes a significant upregulation of about 40 NeuMo genes (e.g., S100a8, S100a9, Elane, Lcn2, and Prtn3), while about 80 DCMo genes were significantly reduced (Figure 6F; Table S5). In an additional comparison with Weinreb et al. (2020), the top 50 most significant NeuMo and DCMo signature genes revealed similar enrichment for the NeuMo phenotype (Figure S5; Table S3). Further RNA-seq analysis revealed that the differentiation bias of COMMD10-deficient Ly6C^hi monocytes toward NeuMo fate initiates already in the steady-state BM (Figure 6G). These results outline a role for COMMD10 in regulating Ly6C^hi monocyte differentiation pathways.

**Single-cell RNA-seq reveals distinct differentiation traits of COMMD10-deficient Ly6C^hi monocytes**

To further dissect the transcriptional changes evoked by COMMD10 deficiency, we next performed single-cell (SC) RNA-seq analysis of purified Ly6C^hi monocytes sorted from LysM^+/Commd10 and Commd10^+/m livers at AILI 24 h. Seurat (PC 1:18, resolution 0.5) was used to generate a uniform manifold approximation and projection (UMAP) revealing nine (0–8) significant (>100 cells) clusters (Figure 7A). Commd10^+/m Ly6C^hi monocytes mainly distributed in cluster 0, while LysM^+/Commd10 Ly6C^hi monocytes mainly distributed in cluster 1 (Figures 7B and 7C). Top marker genes of cluster 0 (Aif1, Itpb5, Cx3crl1, and Cd174) (Figures 7D and S6A; Table S6) were reduced in LysM^+/Commd10 Ly6C^hi monocytes in the bulk RNA-seq (Figure 7E), while top markers of cluster 1 (Arg1, Srgn, Adam8, Tgm2, F10, Ccl2, and Cxcl2) (Figures 7D and S6B; Table S6) were significantly upregulated (Figure 7E). These results confirm a strong bias of COMMD10-deficient monocytes toward the cluster 1 phenotype.

There was also a higher fraction of COMMD10-deficient Ly6-C^hi monocytes in cluster 6 (Figure 7C), annotated by CellMarker as stage I neutrophils and characterized by their distinctive expression of NeuMo markers (S100a8, Lcn2, Prtn3, and Vcan) (Figures 7D and S6C; Table S6). In fact, S100a8 and S100a9 were among the 10 most highly variable genes (Figure S6D). About 93 NeuMo signature genes were detected in this SC count, and hypergeometric testing revealed the most significant enrichment of these genes in clusters 6 (p = 0.00038) and 1 (p = 0.015), further highlighting the differentiation bias of LysM^+/Commd10 monocytes toward NeuMos. Cluster 8 comprised a minor fraction of total monocytes and was unique in its expression of type I interferon genes (Figure 7D; Table S6). In agreement with the reduced expression of type I interferon response genes in LysM^+/Commd10 Ly6C^hi monocytes (Figure 6B), there was a reduction in the fraction of cluster 8 among these cells (Figure 7C).

Osteopontin gene (Spp1) was among the 10 most highly variable genes (Figure S6D). Cluster 1-associated genes Spp1 and Cd9 were recently defined as markers of Ly6C^hi monocyte-derived LAMs appearing in the metabolically diseased liver
Figure 5. COMM10 deficiency in Ly6C\textsuperscript{hi} monocytes aggravates hepatic damage during AILI

(A–F) Commd10\textsuperscript{fl/fl} and LysM\textsuperscript{D\textsuperscript{Commd10}} were injected with APAP (300 mg/kg) and sacrificed 24 or 48 h later. Several LysM\textsuperscript{D\textsuperscript{Commd10}} mice were injected with MC-21 anti-mouse CCR2 antibody.

(A) qRT-PCR of Commd10 expression in liver Ly6C\textsuperscript{hi} monocytes at 24 h of AILI (n = 5).

(B) ALT (24 and 48 h) and AST (24 h) serum levels (n > 5).

(C) Histopathological score of H&E-stained liver sections at 24 h. Original magnification, $\times 4$. Scale bars, 50 $\mu$m (n > 14).

(D) Normalized Ly6C\textsuperscript{hi} monocyte and neutrophil counts at 24 h (n > 17).

(E) Gene expression of inflammatory cytokines and chemokines quantified by qRT-PCR (n > 8).

(F) Immunoblots of total liver tissue showing caspase-1 and caspase-11 activation at 24 h. GAPDH was used as a loading control.

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(Remmerie et al., 2020). Examination of top hepatic LAM markers (Remmerie et al., 2020) revealed that many of them appear in clusters 1, 2, and 6 (Figure 7F; Figure S6E) and were also significantly upregulated by LysM<sup>Cre/+Commd10</sup> Ly6Ch<sup>+</sup> monocytes in the bulk RNA-seq analysis (Figure 7G; Table S7). All 35 top LAM signature genes were present in this SC count, and hypergeometric testing revealed the most significant enrichment of these genes in clusters 1 (p = 2.9E−15), 2 (p = 5.7E−13), and 6 (p = 4.7E−13). Further qRT-PCR analysis confirmed the significant upregulation of Spp1, Trem2, and Arg1 in MoMFs sorted from LysM<sup>Cre/+Commd10</sup> versus Commd10<sup>+/+</sup> livers at AILI 48 h (Figure 7H). Altogether, these results outline an important role for COMMD10 in dictating Ly6C<sup>+</sup> monocyte fate decisions in the injured liver.

**DISCUSSION**

The KC niche is composed of HSCs, hepatocytes, and LSECs. This ménage à trois conveys signals that imprint KC identity (Bonnardel et al., 2019; Sakai et al., 2019), yet the molecular pathways involved with homeostatic maintenance of KC survival remain largely unclear. Our findings highlight COMMD10 as a pivotal intracellular intersection molecule indispensable for KC survival. COMMD10 deficiency does not interfere with KC tissue specialization. Furthermore, Clec4F<sup>Cre/+Commd10</sup> KCs normally express CLEC4F, and high-resolution imaging demonstrates their intact ability to closely interact with the aforementioned niche cells. KCs perform accessory lipid metabolic functions and iron recycling (Scott and Guilliams, 2018). Clec4F<sup>Cre/+Commd10</sup> mice exhibit normal KC lipid content, liver triglyceride levels, and trace iron deposits. This suggests that KC-targeted COMMD10 deficiency has limited impact on homeostatic functions, despite altered expression of linked genes. However, COMMD10 deficiency leads to a significant reduction in KC counts and their entire replacement of fetal radioresistant KCs with radiosensitive KCs. Clec4F<sup>Cre/+Commd10</sup> KCs remain TIM4<sup>+</sup> also in 6-month-old livers. As reconstituting MoKCs renew TIM4 expression already 30 days following KC depletion (Scott et al., 2016), these results suggest that Clec4F<sup>Cre/+Commd10</sup> KCs do not survive long enough to upregulate TIM4 expression, obliging continuous renewal by Ly6C<sup>+</sup> monocytes. Indeed, Clec4F<sup>Cre/+Commd10</sup> KCs express MoKC-associated genes and contain a larger fraction of Vsig4<sup>+</sup> and CD163<sup>+</sup> cells, probably pre-MoKCs at an earlier differentiation phase. Of note, MARS-RNA-seq analysis did not reveal reduced expression of Timd4, Vsig4, and Cd163 genes in Clec4F<sup>Cre/+Commd10</sup> KCs. This may be due to the inferior accuracy of MARS-seq in comparison with qRT-PCR and flow cytometry methods used to obtain the results above. BM chimerism experiments further reveal complete replacement of fetal radiosensitive KCs with radiosensitive MoKCs in Clec4F<sup>Cre/+Commd10</sup> livers. Moreover, in mixed BM chimerism experiments, Clec4F<sup>Cre/+Commd10</sup> KCs evenly compete with their WT counterparts on the ability to replenish the KC niche following ablation, but later exhibit impaired survival. In agreement, Clec4F<sup>Cre/+Commd10</sup> KCs also exhibit higher homeostatic proliferation, originating from the constant need for replenishment. In agreement with these results, there is profound reduction in normalized counts of KCs in Clec4F<sup>Cre/+Commd10</sup> livers. This may be also a result of their increased fragility to the isolation protocol.

Clec4F<sup>Cre/+LysM<sup>Cre/+</sup></sup> mice used in our studies are a well-established tool to target KCs by virtue of their selective expression of the c-type lectin CLEC4F (Bonnardel et al., 2019; Scott et al., 2018). We show that Clec4F<sup>Cre/+Commd10</sup> KCs express the KC-associated CLEC4F protein similarly to control KCs, therefore allowing their efficient targeting in Clec4F<sup>Cre/+Commd10</sup> mice. Cx3cr1<sup>+/+</sup> mice are also used to target ResKCs. However, adult KCs are Cx3cr1<sup>+</sup> and Cx3cr1<sup>Cre/+LysM<sup>Cre/+</sup></sup> mice target 25% of Ly6C<sup>+</sup> monocytes (Yona et al., 2013). As it remains unknown to what extent MoKCs express Cx3cr1 during their differentiation pass, we speculated that the Cx3cr1<sup>Cre/+</sup> mice would be less efficient in targeting Commd10 gene deletion in MoKCs. Indeed, in contrast to the uniform loss of TIM4 expression in 6-month-old Clec4F<sup>Cre/+Commd10</sup> KCs, there are about 30% of TIM4<sup>+</sup> cells among Cx3cr1<sup>Cre/+Commd10</sup> KCs. In addition, other liver-resident macrophages, such as CLEC4F<sup>+</sup> Cx3Cr1<sup>+</sup> liver capsular macrophages (LCMs) (Sierra et al., 2017), will be targeted in Cx3cr1<sup>Cre/+LysM<sup>Cre/+</sup></sup> mice. Therefore, Clec4F<sup>Cre/+</sup> mice were preferentially used in our studies to specifically target KCs.

Transcriptional analysis of Clec4F<sup>Cre/+Commd10</sup> KCs revealed a notable reduction in genes encoding for translation initiation and ribosomal complex proteins, for ubiquitin and the proteosome protein complex, and for key proteins of the mitochondrial respiration chain. These transcriptional alterations allude to protein stress and mitochondrial dysfunction, and they hint at the reason of Clec4F<sup>Cre/+Commd10</sup> KC premature death. Further defining the role of COMMD10 in macrophage homeostatic maintenance, we demonstrate that its deficiency impedes survival of other tissue-resident macrophages. COMMD10-deficient LPMs display significantly reduced counts, replacement of TIM4<sup>+</sup> with TIM4<sup>+</sup> LPMs, and compensatory increased proliferation. Mixed BM chimerism further confirmed impaired survival of LysM<sup>Cre/+Commd10</sup> versus WT LPMs and AMs.

KC are the main sentinel phagocytes located in juxtaposition to hepatocytes, and therefore they initiate a reparative immune response while facing hepatocyte damage (Krenkel and Tacke, 2017). Despite a notable delay in clearance of dying cells, COMMD10 deficiency in KCs had moderate effects on hepatic damage. In contrast, COMMD10 deficiency in Ly6C<sup>+</sup> monocytes profoundly aggravated hepatic damage and inflammation. In
Figure 6. COMM10-deficient Ly6C<sup>hi</sup> monocytes exhibit an altered transcriptional profile associated with neutrophil-like monocytes (NeuMos)

(A, B, and D–F) Ly6C<sup>hi</sup> monocytes were sorted from Comm10<sup>fl/fl</sup> and LysM<sup>D</sup> Comm10 livers at 24 h following AILI and subjected to RNA-seq analysis. (A) Top pathways with significant gene expression alterations.

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agreement with our previous studies during endotoxic shock and colitis (Mouhadeb et al., 2018), COMMD10 deficiency unleashes canonical and non-canonical inflammasome activation in liver-infiltrating Ly6C\textsuperscript{hi} monocytes in AILI. Moreover, LysM\textsuperscript{Commd10}\textsuperscript{-}Ly6C\textsuperscript{hi} monocytes display upregulation of various inflammatory mediators and of ECM components and remodeling enzymes, which may contribute to the collateral damage. Specifically, their increased production of CCL2 may be linked to their increased accumulation in LysM\textsuperscript{Commd10}\textsuperscript{-}livers. In contrast, they dramatically reduce expression of genes associated with type I IFN production and response. During AILI, the release of DAMPs from damaged hepatocytes triggers activation of type I interferon pathways in liver immune cells (Araujo et al., 2018). Interestingly, similar type I IFN monocytes have been recently described in the metabolically diseased liver (Remmerie et al., 2020), during acute liver failure (Kolodziejczyk et al., 2020), and in injured peripheral nerves (Ydens et al., 2020), and hence may be functionally involved in regulating tissue inflammation and resolution.

Recent studies have indicated binary differentiation pathways of BM Ly6C\textsuperscript{hi} monocytes yielding distinct NeuMo- and DCMo-like monocyte progenies (Weinreb et al., 2020; Yáñez et al., 2017). Based on the NeuMo and DCMo marker genes described in these two studies we show here that COMMD10 deficiency in Ly6C\textsuperscript{hi} monocyte generates a clear bias toward NeuMos. This differentiation bias initiates already in the steady-state BM. We also noticed a bias of LysM\textsuperscript{Commd10}\textsuperscript{-}Ly6C\textsuperscript{hi} monocytes toward a cluster of cells characterized by the expression of markers associated with LAMs, a MoMF population accumulating in high damage areas in the metabolically diseased liver (Remmerie et al., 2020). We intend to pursue further the immune-metabolic functions of COMMD10 in these cells during chronic models of metabolic liver diseases.

**Limitations of the study**

Our findings highlight the impaired homeostatic survival of COMMD10-deficient KCs and suggest their continuous renewal by Ly6C\textsuperscript{hi} monocytes. Yet, the BM chimerism approach used in this study does not directly examine the lineage of COMMD10-deficient KCs. Classical fate mapping studies, such as shielded liver irradiation, parabiosis, or transgenic approaches allowing discrimination between fetal or BM-derived macrophages, are required to directly prove the renewal of COMMD10-deficient KCs by Ly6C\textsuperscript{hi} monocytes.

**Future directions**

COMMD proteins, and specifically COMMD10, are poorly defined mechanistically. To define the mechanistic link between COMMD10 deficiency and altered resident macrophage cell survival, it would be important to develop tools that allow investigation of the COMMD10 interactome. Our comprehension of Ly6C\textsuperscript{hi} monocyte heterogeneity, of how COMMD10 mediates Ly6C\textsuperscript{hi} monocyte differentiation, and of whether the biased differentiation fates of COMMD10-deficient monocytes are responsible for the aggravated necro-inflammatory response during liver injury remains limited. Establishing methods that enable phenotypic and functional discrimination between distinct Ly6C\textsuperscript{hi} monocyte-derived effector cells will help clarify these questions.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.110026.
Figure 7. Higher representation of NeuMos and lipid associated macrophages (LAMs) among COMMD10-deficient Ly6C\textsuperscript{hi} monocytes at AILI 24 h

Ly6C\textsuperscript{hi} monocytes sorted from LysM\textsuperscript{DCommd10} and Commd10\textsuperscript{fl/fl} livers (AILI 24 h) were compared by single-cell RNA-seq analysis.

(A) Uniform manifold approximation and projection (UMAP) showing the distribution of Ly6C\textsuperscript{hi} monocytes among 12 distinct clusters.

(B) UMAPs displaying distinct patterns of cluster distribution.

(C) Table displaying % of Ly6C\textsuperscript{hi} monocytes in each of the main 0–8 clusters (>100 cells).

(D) Expression level and % of specific marker genes across the main clusters.

(E) Volcano plot displaying the expression of cluster 0 (blue) and cluster 1 (red) top marker genes in LysM\textsuperscript{DCommd10} versus Commd10\textsuperscript{fl/fl} Ly6C\textsuperscript{hi} monocytes (AILI 24 h), as depicted from bulk RNA-seq analysis.

(F) Expression levels and percentages of LAM genes across the main clusters.

(G) Volcano plot displaying the expression of LAM genes among LysM\textsuperscript{DCommd10} versus Commd10\textsuperscript{fl/fl} Ly6C\textsuperscript{hi} monocytes (AILI 24 h), as depicted from bulk RNA-seq analysis.

(H) qRT-PCR gene expression analysis of LAM genes, comparing MoMFs sorted from Commd10\textsuperscript{fl/fl} and LysM\textsuperscript{DCommd10} livers (AILI 48 h) (n > 6). Analysis was performed on differentially expressed genes (≥1.5-fold, p < 0.05) (n > 3).
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AUTHOR CONTRIBUTIONS

K.C., N.G., and C.V. conceived the study, performed and analyzed experiments, and wrote the manuscript. O.M., S.B.S., M.L., A.N., I.M., and R.P. assisted K.C. with in vivo experiments and analyses. N.E. assisted with confocal microscopy. D.J.K. performed the portal vein injections. E.B. scored histopathological analyses. M.G. provided the Clec4f<sup>fl<sup>−</sup></sup> mice. H.S.G. provided gene expression signatures of NeuMos and DCMos. N.G. and C.V. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Abram, C.L., Roberge, G.L., Hu, Y., and Lowell, C.A. (2014). Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. J. Immunol. Methods. 389, 89–100.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq—A Python framework to work with high-throughput sequencing data. Bioinformatics. 31, 166–169.

Araujo, A.M., Antunes, M.M., Mattos, M.S., Diniz, A.B., Alvarenga, D.M., Nakagaki, B.N., Carvalho, É., Lacerda, V.A.S., Carvalho-Gontijo, R., Goulart, J., et al. (2018). Liver immune cells release type 1 interferon due to DNA sensing and amplify liver injury from acetonphen overdose. Cells. 7, 88.

Bartuzi, P., Hofker, M.H., and van de Sluis, B. (2013). Tuning NF-κB function and clearance in acetaminophen-induced liver injury. Front. Immunol. 7, 88.

Ben Shlomo, S., Mouhadeb, O., Cohen, K., Varol, C., and Gluck, N. (2019). COMMD10-guided phagolysosomal maturation promotes clearance of Staphylococcus aureus in macrophages. iScience. 14, 147–163.

Bonnardel, J., T’Jonck, W., Govaere, O., Abdallah, A.T., Mossanen, J.C., Kohlhepp, M., Liepelt, A., Lefevbre, E., Luedde, T., Hellerbrand, C., et al. (2018). Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. Hepatology. 67, 1270–1283.

Li, H., Chan, L., Bartuzi, P., Melton, S.D., Weber, A., Ben-Shlomo, S., Varol, C., Raetz, M., Mao, X., Starokadomsky, P., et al. (2014). Copper metabolism domain-containing 1 represses genes that promote inflammation and protects mice from colitis and colitis-associated cancer. Gastroenterology. 147, 184–195.e3.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.

Mack, M., Cihak, J., Simonis, C., Luckow, B., Proudfoot, A.E., Plachy, J., Bruhl, H., Frink, M., Anders, H.J., Vielhauer, V., et al. (2001). Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. J. Immunol. 166, 4987–4970.

Maine, G.N., Mao, X., Komarck, C.M., and Burstein, E. (2007). COMMD1 promotes the ubiquitination of NF-κB subunits through a cullin-containing ubiquitin ligase. EMBO J. 26, 436–447.

Motenko, H., Neuhauser, S.B., O’Keefe, M., and Richardson, J.E. (2015). MouseMine: A new data warehouse for MGI. Mamm. Genome. 26, 325–330.

Mouhadeb, O., Ben Shlomo, S., Cohen, K., Farkash, I., Gruber, S., Mahshar, N., Halpern, Z., Burstein, E., Gluck, N., and Varol, C. (2018). Impaired COMMD10-mediated regulation of Ly6C<sup>+</sup> monocyte-driven inflammation disrupts gut barrier function. Front. Immunol. 9, 2623.

Phillips-Krawczak, C.A., Singla, A., Starokadomsky, P., Deng, Z., Osborne, D.G., Li, H., Dick, C.J., Gomez, T.S., Koenecke, M., Zhang, J.S., et al. (2015). COMMD1 is linked to the WASH complex and regulates endosomal trafficking of the copper transporter ATP7A. Mol. Biol. Cell. 26, 91–103.

Ramachandran, P., Dobie, R., Wilson-Kanamori, J.R., Dora, E.F., Henderson, B.E.P., Luu, N.T., Portman, J.R., Matchett, K.P., Brice, M., Marwick, J.A., et al. (2019). Resolving the fibrotic niche of human liver cirrhosis at single-cell level. Nature. 575, 512–518.

Remmerie, A., Martens, L., Thoné, T., Castoldi, A., Seurinck, R., Pavie, B., Roels, J., Vanneste, B., De Prijck, S., Vanhockerhout, M., et al. (2020). Osteo-pontin expression identifies a subset of recruited macrophages distinct from Kupffer cells in the fatty liver. Immunity. 53, 641–657.e14.

Sakai, M., Troutman, T.D., Seidman, J.S., Ouyang, Z., Spann, N.J., Abe, Y., Ego, K.M., Bruni, C.M., Deng, Z., Schlachetzki, J.C.M., et al. (2019). Liver-derived signals sequentially reprogram myeloid enhancers to initiate and maintain Kupffer cell identity. Immunity. 51, 655–670.e8.

Scott, C.L., and Guilliams, M. (2018). The role of Kupffer cells in hepatic iron and lipid metabolism. J. Hepatol. 69, 1197–1199.

Scott, C.L., Zheng, F., De Baetselier, P., Martens, L., Saesy, Y., De Prijck, S., Lippens, S., Abeel, C., Schoonooge, S., Raes, G., et al. (2016). Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. Nat. Commun. 7, 10321.
Scott, C.L., T’Jonck, W., Martens, L., Todorov, H., Sichien, D., Soen, B., Bonnardel, J., De Prijck, S., Vandamme, N., Cannoodt, R., et al. (2018). The transcription factor ZEB2 is required to maintain the tissue-specific identities of macrophages. Immunity 49, 312–325.e5.

Seidman, J.S., Troutman, T.D., Sakai, M., Gola, A., Spann, N.J., Bennett, H., Bruni, C.M., Ouyang, Z., Li, R.Z., Sun, X., et al. (2020). Niche-specific reprogramming of epigenetic landscapes drives myeloid cell diversity in nonalcoholic steatohepatitis. Immunity 52, 1057–1074.e7.

Shaw, T.N., Houston, S.A., Wemyss, K., Bridgeman, H.M., Barbera, T.A., Zangerle-Murray, T., Strangward, P., Ridley, A.J.L., Wang, P., Tamoutounour, S., et al. (2018). Tissue-resident macrophages in the intestine are long lived and defined by Tim-4 and CD4 expression. J. Exp. Med. 215, 1507–1518.

Sierro, F., Evrard, M., Rizzetto, S., Melino, M., Mitchell, A.J., Florido, M., Beattie, L., Walters, S.B., Tay, S.S., Lu, B., et al. (2017). A liver capsular network of monocyte-derived macrophages restricts hepatic dissemination of intraperitoneal bacteria by neutrophil recruitment. Immunity 47, 374–388.e6.

Soysa, R., Lampert, S., Yuen, S., Douglass, A.N., Li, W., Pfeffer, K., and Crispe, I.N. (2019). Fetal origin confers radioresistance on liver macrophages via p21cip1/WAF1. J. Hepatol. 71, 553–562.

Tran, S., Baba, I., Poupel, L., Dussaud, S., Moreau, M., Gélinaud, A., Marcelin, G., Magrèau-Davy, E., Ouhachi, M., Lesnik, P., et al. (2020). Impaired Kupffer cell self-renewal alters the liver response to lipid overload during non-alcoholic steatohepatitis. Immunity 53, 627–640.e5.

van de Sluis, B., Mao, X., Zhai, Y., Groot, A.J., Vermeulen, J.F., van der Wall, E., van Diest, P.J., Hofker, M.H., Wijmenga, C., Klomp, L.W., et al. (2010). COMMD1 disrupts HIF-1α/β dimerization and inhibits human tumor cell invasion. J. Clin. Investig. 120, 2119–2130.

Varol, C., Mildner, A., and Jung, S. (2015). Macrophages: Development and tissue specialization. Annu. Rev. Immunol. 33, 643–675.
## STAR METHODS

### KEY RESOURCE TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| APC/Cy7 anti mouse CD45 (30-F11) | BioLegend | Cat #103116; RRID: AB_312981 |
| PE/Cy7 anti mouse CD11b (M1/70) | BioLegend | Cat #101216; RRID: AB_312799 |
| PerCP/Cy5.5 anti mouse Ly6C (HK1.4) | BioLegend | Cat #128012; RRID: AB_1659241 |
| PB anti mouse Ly6G (1A8) | BioLegend | Cat #127612; RRID: AB_2251161 |
| PE anti mouse CD115 (AF598) | BioLegend | Cat #135505; RRID: AB_1937254 |
| APC anti mouse TIM4 (RMT4-54) | BioLegend | Cat #130008; RRID: AB_2271648 |
| APC anti mouse CD163 (TNKUPJ) | ThermoFisher Scientific | Cat #12-1631-80; RRID: AB_2716923 |
| PE anti mouse F4/80 (REA126) | Miltenyi | Cat #130-116-499; RRID: AB_2727574 |
| APC anti mouse VSIG4 (NLA14) | ThermoFisher Scientific | Cat #25-5752-82; RRID: AB_2637431 |
| Unconjugated anti mouse CLEC4F | R&D Systems | Cat #AF2784; RRID: AB_2081339 |
| PE/Cy7 anti mouse CD64 (X54-5/7.1) | BioLegend | Cat #139314; RRID: AB_2563904 |
| PE anti mouse CLEC2 (17D9) | BioLegend | Cat #146104; RRID: AB_2562383 |
| APC anti mouse CD45.1 (A20) | BioLegend | Cat #117013; RRID: AB_313502 |
| CFL 647 donkey anti goat IgG | Santa Cruz Biotechnology | Cat # sc-362285 |
| Alexa flour 488 anti mouse CX3CR1 (SA011F11) | BioLegend | Cat #149022; RRID: AB_2565705 |
| Alexa flour 647 anti mouse SiglecF (E50-2440 (RUO)) | BD | Cat # BD62680; RRID: AB_2687570 |
| PE anti mouse F4/80 (BM8) | BioLegend | Cat #123110; RRID: AB_893486 |
| PE/Cy7 anti mouse TIM4 (RMT4-54) | BioLegend | Cat #130009; RRID: AB_2565718 |
| Alexa flour 647 anti mouse CD102 (3C4 (MIC2/4)) | BioLegend | Cat #105611; RRID: AB_2121883 |
| Alexa flour 488 donkey anti rat IgG (H+L) | ThermoFisher Scientific | Cat # A21208; RRID: AB_2535794 |
| Ki-67 eFluor 660 (SolA15) | Invitrogen | Cat #50-5698-82; RRID: AB_2574235 |
| Anti-Desmin | Abcam | Cat #ab15200; RRID: AB_301744 |
| Alex flour 555 goat anti rabbit IgG (H+L) | Abcam | Cat #ab150078; RRID: AB_2722519 |
| Anti CD31 (MEC 13.3) | BD | Cat #BD550274; RRID: AB_393571 |
| PB anti mouse CD11c (N418) | BioLegend | Cat #117321; RRID: AB_755987 |
| MC-21 anti mouse CCR2 | Jung Lab (Weizmann Institute of Science) (Mack et al., 2001) | Clone: MC-21 |

| **Chemicals, peptides, and recombinant proteins** |        |            |
|-------------------------------------------------|--------|------------|
| APC BRDU FL1W KIT | BD | Cat # BD552598 |
| APAP | Merck | Cat # A7085 |
| BODIPY 493/503 | ThermoFisher Scientific | Cat # D3922 |
| CFSE | Merck | Cat # 65-0850 |
| Collagenase VIII from Clostridium histolyticum | Merck | Cat # C5138-500MG |
| Dexamethasone | Merck | Cat # D4902-25MG |
| Dnase I (Deoxyribonuclease I from bovine pancreas) | Merck | Cat # 10104159001 DN25-100MG |
| DPBS | Biological Industries | Cat # 02-023-1A |
| DTT (Dithiothreitol) | Formidum | Cat # 3483-12-3 |
| EDTA (Ethyleneediaminetetraacetic acid disodium salt dihydrate) | Merck | Cat # E5134 |
| Fetal Bovine Serum | Biological industries | Cat # 04-001-1A |
| Fixation/Permeabilization Kit | BD | Cat # BD554714; RRID: AB_2869008 |
| HBSS /-/- | gibo | Cat # 2183113 |
| Clodronate | Liposoma | Cat # C-005 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Paraformaldehyde 32% | Electron microscopy sciences | Cat# 15714 |
| Protease inhibitor | Merck | Cat# P8340 |
| RIPA buffer | Cell Signaling | Cat# C-9806S |
| Tissue-Tek O.C.T | Scigen | Cat# 23-730-625 |
| TRIzol® reagent | Merck | Cat# T9424-100ML |

**Critical commercial assays**

- cDNA reverse transcription kit: Applied Biosystems, Cat# 4368814
- miRNeasy Mini Kit: Qiagen, Cat# 217084
- SYBR Green PCR Master Mix kit: Applied Biosystems, Cat# 4309155
- TUNEL staining: Promega, Cat# G3250

**Deposited data**

- KCs steady state: Bulk MARS-Seq: This paper, GEO: GSE183493
- Ly6C<sup>hi</sup> monocytes AILI 24 h: Bulk RNAseq: This paper, GEO: GSE183494
- BM Ly6C<sup>hi</sup> monocytes: Bulk RNAseq: This paper, GEO: GSE183495
- Ly6C<sup>hi</sup> monocytes AILI 24 h: Single cell RNAseq: This paper, GEO: GSE183367
- mRNA SuperSeries bundle of all four datasets: This paper, GEO: GSE183756
- RAW western blot images (refer to Figures 4G and 5F): This paper, Mendeley Data: https://doi.org/10.17632/kfst2hy224.1

**Experimental models: Organisms/strains**

- Mouse: **Commd10**<sup>fl/fl</sup>: Generated by our lab, (Mouhadeb et al., 2018) N/A
- Mouse: **Cx3cr1**<sup>cre/+</sup>: Jung Lab (Weizmann Institute of Science) (Yona et al., 2013) Stock No: 025524
- Mouse: **Lyz2**<sup>cre/+</sup>: The Jackson Laboratories Stock No: 004781
- Mouse: **Clec4f**<sup>cre/+</sup>: Guilliams Lab (Ghent University) (Scott et al., 2018) N/A
- Mouse: CD45.1 (B6.SJL-Ptprca Pepcb/Boy): The Jackson Laboratories Stock No: 002014
- Mouse: CD45.2 C57BL/6JOlalHsd male mice: Envigo (Israel) Stock No: 000664
- Mouse: **Ccr2**<sup>−/−</sup>: **Cx3cr1**<sup>fl/fl</sup>: Jung Lab (Weizmann Institute of Science) (Zigmond et al., 2012) N/A

**Oligonucleotides**

- Commd10 FWD: GAGAGCCCCAGCATGAAGAA: Merck N/A
- Commd10 REV: AATCCCGGCTGAGAAGTGTG: Merck N/A
- Ripl0 FWD: TCCAGCAGGTGTGGACAA: Merck N/A
- Ripl0 REV: CCATCTGCAGCACACACT: Merck N/A
- Arg1 FWD: CAGAAGAATGGAAGATGTCG: Merck N/A
- Arg1 REV: CAGATATGCAAGGAGTCCAC: Merck N/A
- Trem2 FWD: GGCCCATGCCCCCGGTGGTGTG: Merck N/A
- Trem2 REV: CCAGAGATCTCCAGACATC: Merck N/A
- Spp1 FWD: AGGAGAAGAATCTTCCAAGCAA: Merck N/A
- Spp1 REV: TGAGATCTGGCAGATCTCCAG: Merck N/A
- More Oligonucleotides appear in Figure S7 N/A

**Software and algorithms**

- FlowJo 10.7.1: Flow jo https://www.flowjo.com/solutions/flowjo/downloads
- GraphPad Prism 5: GraphPad Software https://www.graphpad.com/
- ImageJ v1.51j: NIH https://imagej.nih.gov/ij/

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Chen Varol (chenv@tlvmc.gov.il).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- All Single-cell RNA-seq, Bulk RNA-seq and MARS-RNA-seq data have been deposited at GEO and are publicly available. Accession numbers are listed in the key resources table. Original western blot images were deposited in Mendeley, and associated link appears in the key resources table. Original microscopy data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse strains
Animal experiments were performed with male adult mice (C57BL/6 background). Animals were maintained in specific pathogen-free animal facility, and experiments were performed according to protocols approved by the Animal Care Use Committee of the Sourasky Medical Center. Mice were housed with 12-h light cycles and a constant temperature of 22°C. LysMCre/+, Cx3cr1Cre/+, and Clec4fCre/+, mice, and their individual littermate Commd10−/− controls, were generated by crossing Lyz2Cre/+, (B6.129P2-Lyz2tm1(Tgcre)jo, Stock No: 004781, The Jackson Laboratory), Cx3cr1tm1(yona et al., 2013), and Clec4f−/− mice (Scott et al., 2018) mice...
with Commd10^{fl/fl} mice (Mouhadeb et al., 2018), respectively. Ccr2^{-/-}/Cx3cr1^{grf/+} (Zigmond et al., 2012) were provided by the Jung laboratory. For chimera mice experiments, six-week-old CD45.1 (B6.SJL-Ptprca Pepcb/Boy, Stock No: 002014, The Jackson Laboratory) and CD45.2 C57BL/6OLaHsd male mice (Envigo, Israel) were used.

**METHOD DETAILS**

**Acetaminophen-induced liver injury (AILI)**

Mice were fasted overnight for 12 h prior to intraperitoneal (i.p.) administration of 300 mg/kg acetaminophen (APAP). Water was re- turned concomitantly with APAP administration and chow 2 h later.

**Quantification of hepatic damage**

Liver samples were obtained at 24/48 h after AILI, fixed (4% paraformaldehyde), paraffin-embedded, sectioned, and stained with H&E of liver sections. Blinded pathologic evaluation was performed by a liver pathologist. 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). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a ADVIA Chemistry XPT analyzer.

**MC-21-mediated ablation of Ly6C<sup>hi</sup> monocytes**

When monocyte ablation was required, mice received an i.p. injection of 400 μL conditioned media of anti-mouse CCR2 mAb (clone MC-21) (Mack et al., 2001)(about 29 μg Ab/mL). Injections were performed at 12 h prior to APAP challenge.

**Isolation of hepatic non-parenchymal cells**

Isolation of hepatic non-parenchymal cells was performed as previously described (Ben Shlomo et al., 2019; Graubardt et al., 2017; Zigmond et al., 2014). In brief, mice were anesthetized and perfused livers were collected, cut into small fragments, and incubated with 5 mL digestion buffer composed of 5% fetal bovine serum, 0.5 mg/mL collagenase VIII from Clostridium histolyticum, 0.1 mg/mL Deoxyribonuclease I from bovine pancreas in Dulbecco’s phosphate-buffered saline with calcium and magnesium (PBS<sup>+/-</sup>), in a shaker-incubator at 250 rpm, 37°C for 45 min. Samples were then subjected to three cycles of washing with Dulbecco’s phosphate-buffered saline without calcium and magnesium (PBS<sup>-/-</sup>) at 400 rpm, 4°C for 5 min from which the supernatant was kept, omitting the parenchymal cell pellet. Subsequently, the supernatant was centrifuged at 1,400 rpm, 4°C for 5 min and the cell pellet was lysed for erythrocytes by 2 min incubation with Ammonium-Chloride-Potassium (ACK) buffer composed of 0.15 M NH<sub>4</sub>Cl and 0.01 M KHCO<sub>3</sub> and washed with PBS<sup>-/-</sup>.

**Isolation of splenic macrophages**

The spleen was perfused with 1ml of digestion buffer composed of 5% fetal bovine serum, 0.5 mg/ml, collagenase VIII from Clostridium histolyticum, 0.1 mg/mL Deoxyribonuclease I from bovine pancreas in PBS<sup>+</sup> and incubated in 37°C for 40 min. The supernatant was centrifuged at 1,400 rpm, 4°C for 5 min and the cell pellet was washed with PBS<sup>-/-</sup>.
Isolation of blood and BM Ly6C<sup>hi</sup> monocytes
Blood peripheral blood mononuclear and polymorphonuclear cells were isolated using the BD FACS Lysing Solution, according to the manufacturer’s instructions. For isolation of BM cells, femurs and tibia were carefully dissected and flushed with cold PBS/C0/C0.

Protein immunoblotting
Total protein from liver was extracted in ice cold RIPA buffer containing protease inhibitors. Proteins were detected by immunoblotting using standard techniques. Antibodies used: caspase-11 (sc-374615), GAPDH (sc-47724), from Santa Cruz; caspase-1(AG-20B-0042) from Adipogen. Blots were incubated with HRP-conjugated secondary antibodies and subjected to chemiluminescent detection using the MicroChemi imaging system (DNR Bio-Imaging Systems, Israel).

Liver triglyceride measuring
Liver tissues were harvest and homogenized in 5% NP-40 using homogenizer. The lysates were heated to 100°C for 5 minutes and then cooled down to room temperature twice. The lysates were measured using a ADVIA Chemistry XPT analyzer.

Staining for iron deposits in the liver
Liver samples were obtained at steady state, fixed (4% paraformaldehyde), paraffin-embedded, sectioned, and stained with Perl’s stain for Iron of liver sections. Blinded pathologic evaluation was performed by a liver pathologist.

Quantitative real-time PCR
Total RNA was extracted from tissues with TRIzol® reagent, and from sorted Ly6C<sup>hi</sup> monocytes or Kupffer cells using the miRNeasy Mini Kit. RNA was reverse transcribed with a high-capacity cDNA reverse transcription kit. All PCR reactions were performed with SYBR Green PCR Master Mix kit and Applied Biosystems 7300 Real-Time PCR machine. Quantification of PCR signals of each sample was performed by the ΔCt method normalized to RpLp0 housekeeping gene. Oligonucleotide sequences appear in the Key Resources Table and Figure S7.

Flow cytometry analysis
Cells (0.5–5 × 10<sup>6</sup>) isolated from liver, peritoneum, alveolar space, spleen, colon, BM, or peripheral blood were stained with appropriate antibodies (listed in the key resource table) at 4°C in the dark for 20 min and were analyzed with BD FACSCanto II (BD Bioscience). Flow cytometry analysis was performed using FlowJo™ software (Ashland, OR, Becton, Dickinson & Company USA). Cell sorting was performed using the BD FACSAria™ FUSION cell sorter (BD Bioscience).

Confocal microscopy
Perfused livers were fixed using 4% PFA for 24 h, dehydrated in 30% sucrose, and subsequently embedded in OCT freezing media. About 13 µm sections were sliced with cryostat (Thermo fisher) and blocked with a blocking buffer containing 2% BSA for 1h. Sections were stained with directly conjugated antibodies or appropriate primary and secondary antibodies 1h or overnight, respectively. For detection of in situ cell death (TUNEL staining), staining was performed using the supplier’s protocol (Promega). Sections were mounted with fluorescence medium containing DAPI. Images were taken with ZEISS Confocal Microscope LSM700 (MicroImaging GmbH, ZEISS, Germany). Image processing was performed with ZEN 2011 SP7 software. TUNEL quantification was calculated by subtraction of the background from each slide and an average was performed.

Clearance of apoptotic cells, in vivo assay
To generate apoptotic cells, thymocytes isolated from 6- to 8-weeks-old C57BL/6 mice were labeled with CFSE (Merck), and then incubated for 16 hr in 1 mM dexamethasone (Merck), washed several times with PBS, spun through a FCS cushion to eliminate the dexamethasone, and resuspended in 1% FCS in RPMI or PBS. Peritoneal macrophages were isolated from Commd10<sup>fl/fl</sup> and Cx3cr1<sup>ΔCommd10</sup> mice, 20 or 30 minutes after intraperitoneal injection of 2 × 10<sup>7</sup> apoptotic thymocytes. Resident peritoneal macrophages were obtained by peritoneal lavage with ice-cold PBS. KCs were isolated from the livers of Commd10<sup>fl/fl</sup> or Cx3cr1<sup>ΔCommd10</sup> mice, 15 minutes after injection to the portal vein of 2 × 10<sup>7</sup> CFSE-labeled apoptotic thymocytes. KCs were isolated as described above.

Clearance of apoptotic cells, in vitro assay
Resident macrophages were isolated from the peritoneum of Commd10<sup>fl/fl</sup> and Cx3cr1<sup>ΔCommd10</sup> mice by peritoneal lavage with ice-cold 4% FCS in PBS/C0/C0 and plated on coverslips in 24-well plates for immunofluorescent confocal microscopy. Macrophages were allowed to rest for 2 hours at 37°C at 5% CO<sub>2</sub> before starting experiments. Macrophages were incubated with apoptotic cells in 1% FCS in RPMI for 2 hours at a ratio of 1:5 at 37°C. Afterward, unbound cells were gently washed away with ice-cold PBS. For immunofluorescence microscopy, macrophages on coverslips were stained as indicated and mounted on glass slides. Images were taken with ZEISS Confocal Microscope (MicroImaging GmbH, ZEISS, Germany). Processing was performed with ZEN 2011 software.
Total body irradiation and BM transplantation

1. Ccr2−/− mice (C57BL/6 background) were exposed to 3Gy total body irradiation. BM cells were harvested from donor mice the day after by gently flushing their femurs, and 5×10⁶ cells were intravenously injected into each of recipient mice. A two-week recovery period was sufficient to ensure donor BM engraftment and blood monocyte reconstitution.

2. WT C57BL/6, Commd10floflo or Clec4f−/−Commd10 mice were exposed to 9 Gy total body irradiation. Mice were reconstituted the day after by intravenous administration of 5×10⁶ BM cells from congenic CD45.1 WT BM, or of 5×10⁶ mixed 1:1 CD45.1 WT/CD45.2 Clec4f−/−Commd10, CD45.1 WT / CD45.2 LysM−/−Commd10 or CD45.1 WT / CD45.2 Cx3cr1−/−Commd10 BM cells. An eight to nine weeks recovery period was sufficient to ensure donor BM engraftment and reconstitution of macrophage compartments in the liver, peritoneum, alveolar space or colonic lamina propria.

Clodronate liposome-mediated depletion of KCs

To deplete KCs, mice were injected intravenous (10ul/gr body weight) of the liposome suspension (Liposoma, the Netherlands) to the lateral tail vain. The mice were sacrificed 4/8 weeks post injection.

Extraction of microarray data

Date was extracted from our previously established Affymetrix GeneChip database deposited at the National Center for Biotechnology Information Gene Expression Omnibus public database (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE55606 (Zigmond et al., 2014). Ly6C⁰ monocytes were sorted at the inflammatory peak of the necrotic phase, 24 h post-AILI (n = 3). Their Ly6C⁰ MoMF descendants were sorted at the recovery phase, 72 h post-AILI (n = 3). Heatmaps were performed using Partek Genomics Suite software.

Bulk RNA-seq

RNAseq and bioinformatics were performed as a service at The Crown Genomics and The Mantoux Bioinformatics institutes of the Nancy and Stephen Grand Israel National Center for Personalized Medicine (GINCPM), Weizmann Institute of Science. All analyses were performed by Dr. Dayana Yahalomi. Sequencing: Libraries were prepared using the G-INCPM-mRNA-seq protocol (Weizmann Institute of Science). PolyA fraction (mRNA) was purified from 500 ng of total RNA following by fragmentation and the generation of double-stranded cDNA. Then, end repair, A base addition, adaptor ligation and PCR amplification steps were performed. Libraries were evaluated by Qubit (Thermo fisher scientific) and TapeStation (Agilent). Sequencing libraries were constructed with barcodes to allow multiplexing of samples. Around 20 million single-end 60-bp reads were sequenced per sample on Illumina SR 60 v4, High Output, one lane.

Bioinformatics

Poly-A/T stretches and Illumina adapters were trimmed from the reads using Cutadapt; and resulting reads shorter than 30bp were discarded. Reads were mapped to the M. musculus reference genome GRCm38, supplied with gene annotations downloaded from Ensembl (Version 92) (and with End-To-End option and outFilterMismatchNoverLmax was set to 0.05). Deduplication was carried out by flagging all reads that were mapped to the same gene and had the same UMI. Counts for each gene were quantified using htseq-count (Anders et al., 2015), with the betaPrior, cooksCutoff and independent Filtering parameters set to False. Raw P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg. Pipeline was run using snakemake (Koster and Rahmann, 2012).

Bulk MARS-seq

A bulk adaptation of the MARS-Seq protocol (Jaitin et al., 2014; Keren-Shaul et al., 2019) was used to generate RNA-Seq libraries for expression profiling. Briefly, mRNA was purified from ~10,000 cells of each sample using Dynabeads mRNA Direct purification kit (ThermoFisher), barcoded during reverse transcription and pooled. Following Agencourct Ampure XP beads cleanup (Beckman Coulter), the pooled samples underwent second strand synthesis and were linearly amplified by T7 in vitro transcription. The resulting RNA was fragmented and converted into a sequencing-ready library by tagging the samples with Illumina sequences during ligation, RT, and PCR. Libraries were quantified by Qubit and TapeStation as well as by qPCR for mouse ActB gene. Sequencing was done on a Nextseq 75 cycles high output kit (Illumina).

Bioinformatics

Poly-A/T stretches and Illumina adapters were trimmed from the reads using Cutadapt; and resulting reads shorter than 30bp were discarded. Remaining reads were mapped onto 3’ UTR regions (1000 bases) of M. musculus, mm10 genome according to Refseq annotations, using STAR (Dobin et al., 2013) with End-To-End option and outFilterMismatchNoverLmax was set to 0.05. Deduplication was carried out by flagging all reads that were mapped to the same gene and had the same UMI. Counts for each gene were quantified using htseq-count (Anders et al., 2015), using the gtf above and corrected for UMI saturation. Differentially expressed genes (DEGs) were identified using DESeq2 (Love et al., 2014), with the betaPrior, cooksCutoff and independent Filtering parameters set to False. Raw P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg. Pipeline was run using snapemake (Koster and Rahmann, 2012). MouseMine bioinformatics tool (Motenko et al., 2015) and Ingenuity pathway analysis (IPA) (QIAGEN) was used to determine pathway and gene ontology (GO) enrichments among the DEGs.
Single-cell RNA-seq

Cells were counted and diluted to a final concentration in PBS supplemented with 0.04% BSA. Cellular suspension was loaded onto Next GEM Chip G and then ran on a Chromium Controller instrument to generate GEM emulsion (10x Genomics). Single-cell 3’ RNA-seq libraries were generated according to the manufacturer’s protocol (10x Genomics Chromium Single Cell 3’ Reagent Kit User Guide v3/v3.1 Chemistry). Final libraries were quantified using NEBNext Library Quant Kit for Illumina (NEB) and high sensitivity D1000 TapeStation (Agilent). Libraries were pooled according to targeted cell number, aiming for ~50,000 reads per cell. Pooled libraries were sequenced on a NovaSeq 6000 instrument using an SP 100 cycles reagent kit (Illumina).

Bioinformatics

Cell Ranger v3 with default parameters was used for alignment, filtering, barcode counting, and UMI counting. The Seurat package in R was used for downstream analysis and visualization. Log normalization was used to normalized the reads. Dimension reduction was done using PCA. Clustering was done using KNN graph and visualization and non-linear reduction was done using UMAP. Marker genes were found by performing differential expression based on the non-parametric Wilcoxon rank sum test (Seurat default). CellMarker (http://biocc.hrbmu.edu.cn/CellMarker/) (Zhang et al., 2019) was used to annotate each cluster according to the top 10 marker genes. Cell type enrichment was carried out using hypergeometric test. Each cluster annotation was determined according to cell type with the best p value.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical differences between two groups were determined according to unpaired two-tailed Student’s t test. Statistical differences between three groups and above were determined using one way ANOVA with Tukey post-tests. All experiments were analyzed using Prism 7 (GraphPad Software). Graphical data was shown as mean values with error bars indicating SEM. Significance was defined if p value was less than 0.05 as following: * p < 0.05; ** p < 0.01; *** p < 0.001. Statistical details of experiments can be found in figure legends.