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Research paper

Non-alcoholic steatohepatitis induces transient changes within the liver macrophage pool

Lindsey Devisscher[a,*,1], Charlotte L. Scott[b,c,d,*,1], Sander Lefere[a], Sarah Raevens[a], Eliene Bogaerts[a], Annelies Paridaens[a], Xavier Verhelst[a], Anja Geerts[a], Martin Guilliams[b,c,1], Hans Van Vlierberghe[a,1]

[a] Department of Gastroenterology and Hepatology, Ghent University, Belgium
[b] Laboratory of Myeloid Cell Ontogeny and Functional Specialization, VIB-UGent Center for Inflammation Research, Ghent, Belgium
[c] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
[d] Centre for Immunobiology, Institute of Infection, Immunity and Inflammation, College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, UK

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ABSTRACT

Kupffer cells (KCs) and monocyte-derived macrophages are implicated in non-alcoholic steatohepatitis (NASH) pathogenesis but their functions remain unclear due to the lack of specific markers to distinguish between the different cell types. Additionally, it is unclear if multiple subsets of KCs are present during NASH. Here, we characterized the liver macrophage subsets during methionine/choline deficient (MCD) diet-induced NASH and recovery. We observed a significant reduced contribution of Ly6C^{lo}Clec4F^{+}Tim4^{+}KCs to the hepatic macrophage pool in MCD fed mice, which normalized during recovery. Ly6C^{lo}Clec4F^{-}Tim4^{-} monocyte-derived macrophages increased during MCD feeding and returned to baseline during recovery. Ly6C^{lo}Clec4F^{+}Tim4^{-} monocyte-derived KCs developed during initial recovery but did not self-renew as their numbers were reduced after full recovery. Initial recovery from MCD diet feeding was further characterized by increased proportions of Ki-67^{+} proliferating KCs. In conclusion, the hepatic macrophage pool undergoes substantial albeit transient changes during NASH and recovery, with the KC pool being maintained by proliferation and differentiation of short-lived monocyte-derived KCs.

1. Introduction

Kupffer cells (KCs), the resident macrophages (mϕs) of the liver, represent the largest population of mϕs in the body and the predominant mϕ population in the liver under homeostatic conditions. Recent studies have shown that KCs derive predominantly from embryonic progenitors including yolk sac mϕs and fetal liver monocytes during embryogenesis [1–3], while circulating Ly6C^{hi} bone marrow (BM) derived monocytes contribute minimally to the KC pool during normal homeostasis [1–5]. Thus KCs must self-maintain via *in situ* proliferation [5,6]. However under non-homeostatic conditions, in addition to the KC pool, which is often diminished in size (termed mϕ disappearance reaction), the liver is also infiltrated by monocyte-derived mϕs (MoMϕs) [7–9]. Furthermore, it has recently been shown that circulating Ly6C^{hi} monocytes can enter the liver and differentiate into bona fide KCs (MoKCs) when the KC niche is available, for example in the neonatal window as the liver grows [5], when KCs are completely or indeed partially depleted [5] or during aging [10]. However, whether this occurs during liver pathology remains unknown. While much is now understood regarding the origins of liver resident mϕs in the steady state, the functions of these cells remain somewhat elusive, due to the lack of tools with which they can be specifically studied. Moreover, the roles of the distinct mϕ populations present in the liver under non-homeostatic conditions are largely unknown. Understanding the function of these distinct cell types is further complicated by our lack of understanding of the composition of the mϕ pool during non-homeostatic conditions, such as inflammation, infection and injury. This lack of understanding stems from a lack of specific markers with which we can distinguish between the different mϕ populations, and only once we can discriminate between populations can we begin to understand their

Abbreviations: BM, bone marrow; CD, control diet; KCs, Kupffer cells; mϕs, macrophages; MCD, methionine and choline deficient diet; MoKCs, monocyte-derived Kupffer cells; MoMϕs, monocyte-derived mϕs; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NC, normal chow

* Corresponding authors at: Department of Gastroenterology and Hepatology, Ghent University, Belgium (Lindsey Devisscher) and Laboratory of Myeloid Cell Ontogeny and Functional Specialization, VIB-UGent Center for Inflammation Research, Ghent, Belgium (Charlotte L. Scott).
E-mail addresses: Lindsey.devisscher@ugent.be (L. Devisscher), charlotte.scott@irc.vib-ugent.be (C.L. Scott).
[1] These authors contributed equally.

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specific functions. CCR2 and CX3CR1 are two markers which have been used in the past to distinguish between MoMϕs and KCs in the liver [11–14], as MoMϕs but not KCs express these two markers. However, these markers cannot discriminate between infiltrating inflammatory MoMϕs and MoKCs which also express these markers. More recently, two markers were proposed to distinguish between MoMϕs and KCs, Clec4F and Tim4, both of which are expressed by KCs but absent from infiltrating MoMϕs [5]. Additionally, these markers can be used to discriminate between KCs and recently differentiated MoKCs as the latter do not express Tim4 in the first weeks post-differentiation. However, with time (at least under near steady state conditions) these will gain expression of Tim4 and hence a BM chimera approach is required to distinguish between original KCs and those which have recently developed from monocytes [5]. Thus, we are now in a position to examine the composition of the mϕ pool and assess the roles played by the distinct mϕ populations during liver disease.

Non-alcoholic steatohepatitis (NASH) is a severe form of non-alcoholic fatty liver disease (NAFLD) and is characterized by steatosis, liver inflammation and ballooning degeneration of hepatocytes. NASH patients often develop liver fibrosis/cirrhosis and up to 25% eventually present with hepatocellular carcinoma [15]. Multiple studies have reported on NASH pathogenesis and the multi-hit theory with parallel hits driving fat accumulation and inflammation being commonly accepted [16,17]. Recently, it has been hypothesised that liver mϕs are the initial drivers of hepatic fat accumulation and are thought to play a key role in the progression of NAFLD to NASH [18–22]. To date, reports suggest that KCs are the first responders against gut-derived bacterial compounds, toxic lipids and adipose tissue-derived pro-inflammatory cytokines in the pathogenesis of NAFLD/NASH. This is based on the observed upregulation of Toll-like receptors and pro-inflammatory cytokines expression by mϕs [18–20,23–25]. While these hepatic mϕs are often assumed to be KCs based on their expression of F4/80, these findings could also be attributed to infiltrating monocytes and MoMϕs during NASH. In addition, to date, the proposed role(s) played by KCs in NAFLD/NASH pathogenesis have been determined following experiments using clodronate liposome depletion strategies [20–22,25–28], administration of which also results in the depletion of other phagocytic cells, including monocytes and MoMϕs. The contribution of infiltrating monocytes and MoMϕs to NASH pathogenesis has been demonstrated in animal models for NASH and associated fibrosis using genetic or pharmacological inhibition of the CCL2/CCR2 axis [28–31] and this has recently been confirmed in a phase 2b clinical trial for NASH patients with fibrosis [32]. At present, it is unknown if monocytes contribute to the KC pool during NAFLD/NASH pathogenesis and/or recovery. Thus, it is evident that a better understanding of the mϕ pool during NASH is required. Here, using the recently defined markers, Clec4F and Tim4, alongside BM chimeras, we have accurately characterized the dynamic changes and origins of the distinct liver macrophage subsets in methionine and choline deficient (MCD) diet-induced NASH and recovery.

2. Materials and methods

2.1. Animals

Female wild type C57Bl/6, CD45.1 and CD45.2 mice were purchased from Harlan (Boxmeer, The Netherlands) at 6 weeks of age and reared and housed in the laboratory animal facility at University Hospital Ghent according to the institutional animal healthcare guidelines. Mice were given free access to food and water and housed in a 12 hour light/dark cycle. NASH was induced at the age of 8 weeks (wild type mice) or 10–14 weeks (chimeras) by feeding the MCD diet (Harlan Laboratories). Control mice were fed the same diet supplemented with 8.2 g/kg methionine and 1.4 g/kg choline (Harlan Laboratories, control diet, CD). Mice were followed up for weight loss and sacrificed after 2 days, 2, 4 and 8 weeks of MCD/CD feeding and after 8 weeks of diet feeding followed by 2, 4 and 6 days and 2 and 4 weeks of normal chow (NC). This study was approved by the Institutional Review Board of the Faculty of Medicine and Health Science of Ghent University (ECD 15/57 and 16/21).

2.2. Tissue sampling

Mice were anesthetized by ketamine (100mg/kg) and xylazine (10mg/kg) and a piece of the liver was isolated and fixed in 4% phosphate buffered formaldehyde solution (Klinipath, Olen, Belgium) for H & E and Sirius Red staining. The remaining liver was flushed with PBS, isolated, weighted, chopped into small pieces and incubated for 20′ in 1 mg/ml Collagenase A (Sigma) and 10 U/ml Dnase (Roche) in a heated bath (37°C). Suspensions were filtered and cells were stained with appropriate antibodies.

2.3. Histology

NAFLD/NASH was evaluated on H & E stained liver sections by two independent observers. The NAFLD activity score (NAS) was used as reference and scores represent the sum of the grade of steatosis (no steatosis = 0, < 33% microvesicular steatosis = 1, 33–66% microvesicular steatosis = 2, > 66% microvesicular + macrovesicular steatosis, 100% macrovesicular steatosis = 4), hepatocyte ballooning (non, few, many ballooning cells = 0, 1, 2 respectively) and inflammatory foci (foci/field: no foci = 0, < 2 foci = 1, 2–4 foci = 2, > 4 foci = 3). Fibrosis was quantitatively scored on Sirius Red stained liver sections by two independent observers using the CellˆD software (Olympus, Berchem, Belgium).

2.4. Pro-inflammatory cytokines/chemokines

The concentrations of monocyte chemoattractant protein 1 (MCP-1), keratinocyte chemoattractant (KC), tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) were determined by multiplex bead-based assay (Bio-Rad Laboratories) in whole liver tissue.

2.5. Flow cytometry

Cells were stained with CD45-AF700, CD45.1-PE, CD45.2-BUV737, F4/80-Biotin, Ki-67-BV786, Ly6G-PE, SiglecF-PE from BD Bioscience, CD11b-PECy7 from Biolegend, Ly6C-eFluor450 from eBioscience, Clec4F from R & D systems and anti-goat-AF647 from Invitrogen for 20′ at 4°C in the dark as previously described [5]. The expression of Ki-67 was analysed as previously described [5]. Cells were analysed with a Fortessa (BD Bioscience) and FlowJo software (TreeStar) and gated first as live $CD45^+$ single cells and subsequently as $CD11b^+Ly6G^-SiglecF^-Ly6C^+$ monocytes, $CD11b^{int}Ly6C^-Ly6G^-SiglecF^-F4/80^+Clec4F^+Tim4^+$ KCs, $CD11b^{int}Ly6C^-Ly6G^-SiglecF^-F4/80^+Clec4F^-Tim4^-$ MoMϕ and $CD11b^{int}Ly6C^-Ly6G^-SiglecF^-F4/80^+Clec4F^+Tim4^-$ KCs.

2.6. Chimeras

At the age of 6–8 weeks, CD45.2 mice were anesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg) and subjected to lethal irradiation (8 Gy) while protecting the abdomen with a lead plate. Mice were reconstituted with 10×10^6 bone-marrow from congenic CD45.1 mice 12 hours after recovery. Mice were fed the MCD or CD diet 5–6 weeks following irradiation and reconstitution for 4 and 8 weeks and for 8 weeks diet following 2 and 4 weeks of recovery (NC).

2.7. Statistics

Multiple-group comparisons were performed using one or two way analysis of variance (ANOVA) followed by a Bonferroni post-test with Prism Software (GraphPad Software). Samples were assumed to be normally distributed with similar variance between groups.