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Unveiling the depletion of Kupffer cells in experimental hepatocarcinogenesis through liver macrophage subtype-specific markers

To the Editor:

We read with great interest the recent study by Borst *et al.* characterizing the rapid loss of Kupffer cells (KCs) and their subsequent replacement by monocyte-derived macrophages in the setting of acute viral hepatitis.[1] This phenomenon of KC depletion has previously been reported following other inflammatory stimuli such as acetaminophen intoxication, bacterial infection and non-alcoholic steatohepatitis (NASH).[2–4] In response to these findings, and to an Editorial in the *Journal of Hepatology*[5] questioning whether this pattern is observable in all liver diseases, Kessler *et al.* published their experience in the diethylnitrosamine (DEN) model of hepatocarcinogenesis.[6] They showed that, despite a decrease in the ratio of macrophages over monocytes, the number of hepatic macrophages (defined as $CD11b^{+}$ $CD11c^{-}$ $NK1.1^{-}$ $Ly6G^{-}$ $Ly6C^{lo}$ $F4/80^{hi}$ cells) remained stable after short-term exposure to DEN, while both monocytes and macrophages were increased in the liver 22 weeks after DEN injection. The authors interpreted this as a lack of KC depletion in both the acute and chronic liver injury model. Furthermore, they pointed to the characterization of macrophages and infiltrating monocytes in experimental hepatocellular carcinoma (HCC) as an outstanding research question.[6]

The hepatic macrophage population consists of different subsets, however, which could not be distinguished using the set of markers employed by Kessler *et al.*[6] Depletion of KCs following an acute insult is indeed well-established,[3] while the composition of liver monocytes/macrophages during DEN-induced HCC, and hepatic carcinogenesis in general, is less clear.

To complement the data of Kessler *et al.*, we performed flow-cytometric analysis on the livers of acetaminophen-overdosed mice, using the KC-specific marker Tim4 to distinguish between resident KCs and infiltrating monocyte-derived macrophages.[4,7] We detected a marked infiltration of $Ly6C^{hi}$ monocytes while the monocyte-derived $Ly6C^{lo}$ $F4/80^{+}$ $Tim4^{-}$ macrophage population remained unchanged. However, we did observe a marked depletion of $Ly6C^{lo}$ $F4/80^{+}$ $Tim4^{+}$ KCs, confirming previous reports on KC depletion during acute liver injury[3] (Fig. 1A).

In order to shed light on the liver monocyte/macrophage population in experimental HCC, we performed flow-cytometric analysis in the DEN-induced HCC model, using a different protocol with weekly DEN injections resulting in advanced HCC (Fig. 1B), employing the same subset-specific markers. In accordance with Kessler *et al.*, we observed a stark increase in $Ly6C^{hi}$ monocytes and $Ly6C^{lo}$ $Tim4^{-}$ macrophages following repeated DEN administration and tumor formation. Nevertheless, whereas the total macrophage proportion remained more or less stable, KCs were in fact depleted. This was compensated by the 2-fold rise in $Tim4^{-}$ monocyte-derived macrophages (Fig. 1B).

To ascertain whether this is a general phenomenon in HCC development, we validated these findings in a model for NASH-associated HCC, obtained by injecting streptozotocin followed by Western diet feeding.[8] The results were similar to DEN-induced carcinogenesis, with an even more apparent depletion of KCs and hepatic infiltration with monocytes and monocyte-derived macrophages (Fig. 1C).

The hypothesis of Alazawi and Knolle that all types of inflammatory hepatic injury may be associated with KC loss[5] is therefore still a possibility. Importantly, this concept depends on the use of subset-specific markers and could only have been formulated after these markers became available.

That said, the homeostatic reaction to KC loss is not necessarily always the same. As in the study of Borst *et al.*, monocytes can differentiate into monocyte-derived KCs, which can be hard to distinguish from original KCs.[7,9] Local proliferation of KCs that fill the available niche is another way of restoring the KC population. Interestingly, both processes were observed during recovery from diet-induced NASH.[4]

In summary, the depletion of KCs and the simultaneous infiltration of the liver with bone marrow-derived pro-inflammatory $Ly6C^{+}$ monocytes, and their differentiation into monocyte-derived macrophages, constitute major pathophysiological events in various hepatic disorders. Our data suggest that these mechanisms are also at play in models of NASH-associated and chemically induced HCC.

Future work in this field could focus on the sequence of events during active hepatocarcinogenesis, as well as elucidate how KCs are replenished and what phenotype the resulting cells acquire. These inquiries could broaden our understanding of specific roles played by various macrophage populations during liver injury and recovery, thus paving the way for therapeutic applications.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Keywords: Kupffer cells; Inflammatory macrophages; Hepatocellular carcinoma; NASH; Flow cytometry.

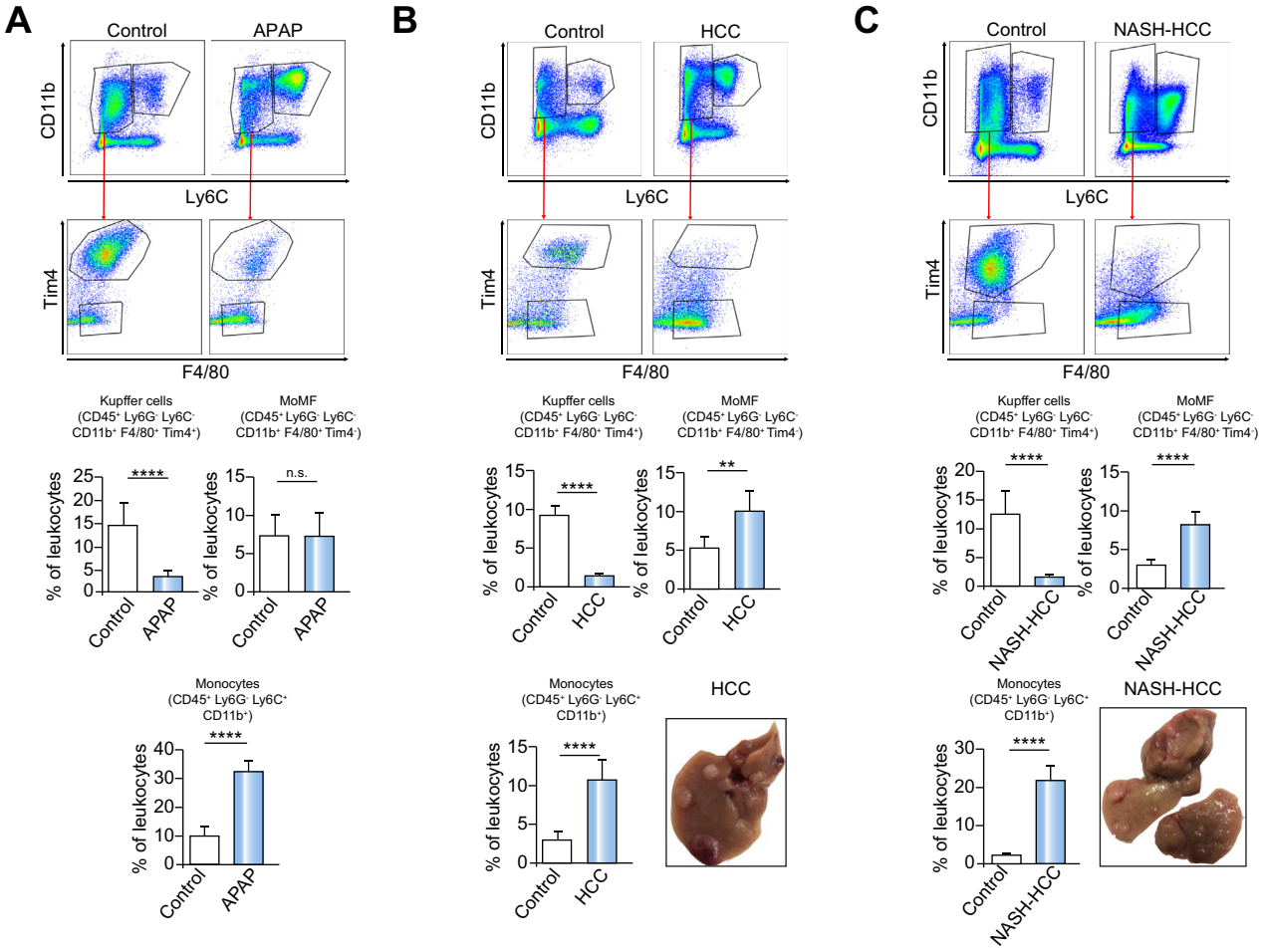

Fig. 1. Hepatic macrophage populations during acute liver injury and hepatocarcinogenesis. (A) Wild-type male C57BL/6J mice were fasted for 20 h prior to an intraperitoneal injection of 300 mg/kg APAP (Sigma, Diegem, Belgium) or PBS. The mice were sacrificed after 24 hours. Representative flow cytometric analysis with the quantification of hepatic KCs, MoMFs and monocytes are shown. (B) Wild-type male 129/Sv mice were injected intraperitoneally weekly with 35 mg/kg DEN (Sigma) or 0.9% NaCL for 30 weeks, starting at 5 weeks of age. Representative flow cytometric analysis with the quantification of hepatic KCs, MoMFs and monocytes, and a picture of a liver with DEN-induced HCC lesions are shown. (C) Wild-type C57BL/6J mice were injected subcutaneously with 200 μg streptozotocin (Sigma) 2 days after birth to destroy the pancreatic β cells, and the males were fed a high-fat, high-sucrose, high-cholesterol diet (Western diet, Ssniff, Uden, The Netherlands, TD.08811 + 1% added cholesterol) from 4 weeks up to 16 weeks of age. Control mice did not receive STZ injections and were fed a normal chow diet. Representative flow cytometric analysis with the quantification of hepatic KCs, MoMF and monocytes, and a picture of a NASH liver with HCC lesions are shown. For flow cytometry, the liver was flushed with PBS, homogenized and incubated for 20 min in 1 mg/ml Collagenase A (Sigma) and 10 U/ml DNAse (Sigma) at 37 °C. After antigen blockade with Fc block (BD Biosciences, Erembodegem, Belgium), the cells were stained with CD45/APC-Cy7, Ly6C/V450, CD11b/PE-Cy7, Ly6G/PerCP-Cy5.5, Tim4/PE (BD Biosciences) and F4/80/FITC (Thermo Scientific, Merelbeke, Belgium) for 20 min at 4 °C in the dark. $^{**}p < 0.01$; $^{****}p < 0.0001$. Statistical analysis was performed in Graphpad Prism 6 (GraphPad Software Inc., La Jolla, CA, US), using unpaired *t* tests. A 2-sided *p* value < 0.05 was considered statistically significant. APAP, acetaminophen; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; KCs, Kupffer cells; MoMFs, monocyte-derived macrophages; NASH, non-alcoholic steatohepatitis. (This figure appears in colour on the web.)

Authors' contributions

SL analyzed data and wrote the manuscript. HD performed experiments. HVV supervised the study and secured funding. LD conceived and supervised the study and wrote the manuscript. All authors reviewed the final version of the manuscript.

Ethics statement

This study was approved by the Experimental Animal Ethical Committee of the Faculty of Medicine and Health Science at Ghent University. Animals were kept under controlled conditions with a stable temperature (20–24 °C), humidity (45–65%) and a 12 h day/night cycle with unrestricted access to food and water.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.03.016.

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Author names in bold designate shared co-first authorship

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Reply to: "Unveiling the depletion of Kupffer cells in experimental hepatocarcinogenesis through liver macrophage subtype-specific markers"

"Who are you?", cell surface marker expression profiles of tissue-resident Kupffer cells and liver infiltrating myeloid cells

To the Editor:

During the last years, great efforts have been undertaken to uncover the origin and function of tissue-resident macrophages. Kupffer cells (KCs) are embryo-derived liver-resident macrophages, which are essential to combat liver infection, and which are critically involved in liver regeneration.[1–3] Recent data indicate that KCs have a distinct origin and function when compared with monocyte-derived macrophages. During homeostasis, KCs are self-maintained by local proliferation, with no additional input from bone marrow-derived cells.[1–4] However, upon activation of the immune system either by infection, carcinogenesis, or intoxication, the composition of the hepatic macrophage pool can change dramatically. A lively discussion about the fate of KCs after liver insult is currently ongoing, as highlighted by the comment of Lefere *et al.* in the current issue.[5] While we and others discovered a rapid KC loss upon infection,[6,7] this phenomenon was initially not detected in diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC).[8] In contrast, KC loss was apparent in 2 other HCC models.[5] These observations led to the question of whether KC loss after liver injury is a general phenomenon, or whether the KC fate is dependent on the nature of the liver insult.[9,10]

Recently new surface markers suitable for classification of tissue-resident macrophages, including KC, were identified, which allow discrimination of local KCs and inflammatory macrophages.[4,11] One of these markers, Clec4F, was established as a specific KC marker, whereas Tim4 is expressed by a variety of long-lived tissue-resident macrophage subsets.[12–14] While, KC classification under homeostatic conditions is well established, we and others discovered that under inflammatory conditions KC classification is more challenging (Table 1).[4,6,8] Using the recently identified tissue-resident macrophage marker Tim4, Lefere *et al.* followed up on the question of whether the local KC pool is depleted upon acute acetaminophen (APAP) - induced liver injury and chronic inflammation. In accordance with previous data, they detected a profound KC loss during both, acute and chronic liver inflammation.[5,15] Additionally, they observed infiltration of monocyte-derived cells. Thus, in contrast to Kessler *et al.*, Lefere *et al.* concluded that KC loss is also present in the APAP model. Therefore, it is tempting to speculate that, as proposed earlier by Alazawi and Knolle,[9] KC loss is a general phenomenon during liver injury. The differences detected in the studies by Kessler *et al.* and Lefere *et al.* could have several explanations. Since it is rather likely that KC loss in the APAP model is transient, it is crucial to study KC disappearance at several time points after liver injury. While KC loss can be monitored by immunolabeling with the aforementioned cell surface markers Clec4F and Tim4, it is not possible to dissect the origin of reappearing KC with these markers, since upon liver infiltration their expression can also be upregulated on bone marrow-derived macrophages. Therefore, to address the origin of myeloid cells found in tissues, fate-mapping approaches are needed. Under acute inflammatory conditions, we and others used CX3CR1-GFP mice to dissect the origin of myeloid cell populations.[6,15] However, these mice are not suitable for studying chronic conditions, such as HCC. For the analysis of chronic inflammation, the use of inducible fate-mapping models such as CX3CR1-CreERT2 mice or a Cre-recombinase-driven barcoding system that generates tens of thousands of individual barcodes in a tissue- and time-controlled manner[16] could be suitable approaches. Scott *et al.* generated Clec4F-Cre mice which support KC-specific gene targeting.[17] This mouse will be instrumental in future studies specifically addressing KC functions.