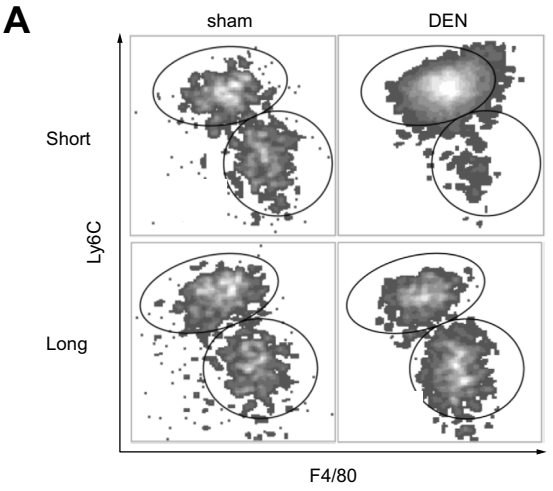
JOURNAL OF HEPATOLOGY

Lack of Kupffer cell depletion in diethylnitrosamine-induced hepatic inflammation

To the Editor:

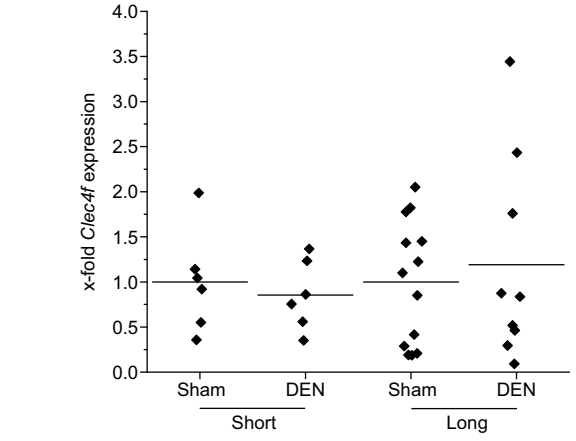
Recent evidence published in the *Journal of Hepatology* shed light on the fate of Kupffer cells (KCs) during hepatic inflammation. The study by Borst *et al.* elegantly demonstrated that KCs are rapidly depleted and then replaced by monocyte-derived macrophages in the course of viral hepatitis.[1] This report is quite in contrast to the dogma that KCs, as resident tissue macrophages, are self-maintaining cells. Similar observations were reported for hepatic inflammation induced by paracetamol, CCl_4, or bacterial infection.[2–4] All of these studies show a temporary decline of KC numbers. An editorial in the *Journal of Hepatology* raised the question "of whether all liver injuries lead to KC loss".[5] We would, therefore, like to report data on the hepatic immune cell composition in one of the most frequently used animal models in liver cancer studies: diethylnitrosamine (DEN)-induced hepatocellular carcinoma in mice. Although it has been shown before that short-term DEN treatment drives severe liver injury and acute hepatic inflammation,[6] the effects of DEN administration on the composition of myeloid cells are largely unknown. Therefore, we performed a comprehensive flow cytometric analysis upon short-term high-dose DEN treatment to model acute severe liver damage and long-term low-dose DEN treatment to induce hepatocarcinogenesis.

The proportion of leukocytes ($CD45^+$ cells) in liver cell suspensions was significantly higher in the DEN-treated animals in the short-term (2.5 ± 0.4-fold in DEN-treated compared to sham-treated livers; $p = 0.004$), as well as the long-term model (2.10 ± 0.26-fold in DEN-treated compared to sham-treated livers; $p = 0.003$). The proportion of hepatic macrophages ($CD11b^+$ $CD11c^-$ $NK1.1^-$ $Ly6G^-$ $Ly6C^{lo}$ $F4/80^{hi}$) within the leukocyte fraction was significantly decreased in the short-term DEN-treated mice (Fig. 1A). Accordingly, the monocyte/macrophage ratio was highly elevated (19.1 ± 2.0 in DEN-treated mice compared to 2.2 ± 0.4 sham-treated mice; $p = 2.39E{-}7$). This finding might be interpreted as a KC depletion. However, normalisation of monocyte and macrophage counts to total cell number showed that, while there was indeed an increase in the number of monocytes, no macrophage depletion in the short-term DEN model can be concluded (Fig. 1A). Concordantly, the expression analysis of the C-type lectin domain family 4, member F (*Clec4f*), which is a specific marker for KCs, revealed no differences between the DEN- and sham-treated animals, neither in the short-term nor in the long-term model ($p = 0.62$ and $p = 0.66$, respectively; Fig. 1B). In long-term DEN-treated mice, an increase in both hepatic monocyte and macrophage counts was observed (Fig. 1A). This might be a result of monocytes giving rise to tissue-resident macrophages. Since *Clec4f* was not elevated in the long-term DEN model, the source and characteristics of the increased proportion of hepatic macrophages, *i.e.*, whether they are derived from infiltrated monocytes and whether they represent a transient state of differentiation towards Clec4f expressing KCs,[7] will be a challenge for future studies. Infiltrated monocytes may also become short-lived



Short		% of $CD45^+$	% of total hepatic cells
Monocytes ($Ly6C^{high}$ $F4/80^{low}$)	Sham	2.79 ± 0.34	0.02 ± 0.002
	DEN	17.55 ± 1.74***	0.23 ± 0.002***
Macrophages ($Ly6C^{high}$ $F4/80^{low}$)	Sham	1.91 ± 0.36*	0.01 ± 0.002
	DEN	0.97 ± 0.09	0.01 ± 0.001

Long		% of $CD45^+$	% of total hepatic cells
Monocytes ($Ly6C^{high}$ $F4/80^{low}$)	Sham	2.32 ± 0.23	0.02 ±0.002
	DEN	2.82 ± 0.26	0.05 ± 0.005**
Macrophages ($Ly6C^{high}$ $F4/80^{low}$)	Sham	1.03 ± 0.16	0.01 ± 0.001
	DEN	1.30 ± 0.24	0.02 ± 0.005*



Keywords: Liver; Hepatitis; Hepatocellular carcinoma; Ly6C; Hepatic leukocyte composition; Flow cytometry.

Fig. 1. Effects of DEN on hepatic macrophages. For short-term treatment, 9-week-old male C57BL/6 mice were intraperitoneally injected with either 100 mg/kg body weight DEN in 0.9% NaCl (Sigma Aldrich, Taufkirchen, Germany) or a 0.9% NaCl solution as a sham-control. The mice were sacrificed 48 h after injection. For long-term treatment, 2-week-old male C57BL/6 mice were intraperitoneally injected with either 5 mg/kg body weight DEN in 0.9% NaCl (Sigma Aldrich, Taufkirchen, Germany) or a 0.9% NaCl solution as a sham-control. All animal procedures were performed in accordance with the local animal welfare committee (37/2014). Statistics were performed using the Origin software (OriginPro 8.1G; OriginLabs, Northampton, MA, USA). Statistical differences were estimated by independent 2-sample *t* test or Mann-Whitney *U* test depending on normal distribution. All tests are 2-sided, and differences were considered statistically significant when *p* values were less than 0.05. (A) Flow cytometric analysis of livers from mice treated with either DEN or 0.9% NaCl (sham) for 48 h (short) or 22 weeks (long). Macrophages ($Ly6C^{lo}$ $F4/80^{hi}$) and monocytes ($Ly6C^{hi}$ $F4/80^{lo}$) are shown. The table indicates the relative amounts of monocytes and macrophages in NaCl- and DEN-treated mice normalised to $CD45^+$ and total liver cells. n = 10 per treatment group. Statistical difference between DEN-treated and sham-treated mice: $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Preparation of hepatic cell suspensions was performed according to Wang *et al.*[9]. For determination of leukocyte composition, the liver was cut into small pieces and digested with 0.04% collagenase type IV. The suspension was used to assess the proportion of $CD45^+$ cells. The remaining cells were separated by Percoll centrifugation. After adding FCS-EDTA solution, the cells were resuspended in MACS buffer (0.5% BSA and 2 mM EDTA in PBS), and $CD45^+$-cells were isolated using microbeads (10 μl per 10^7 cells, Miltenyi, Bergisch Gladbach, Germany). The cells were counted, diluted (500,000/vial), centrifuged, and resuspended in flow cytometry buffer (FCB; PBS containing 2.5% (v/v) bovine calf serum and 0.05% (w/v) NaN_3) and incubated with mouse BD Fc Block (BD Biosciences, Heidelberg, Germany). APC rat anti-mouse Ly6G Clone 1A8 (#560599), APC-R700 rat anti-mouse CD11b Clone M1/70 (#564985), BV421 rat anti-mouse Ly6C Clone AL-21 (#562727), BV510 mouse anti-mouse NK 1.1 Clone PK136 (#563096), PE hamster anti-mouse CD11c Clone HL3 (#55740, all from BD Biosciences), and FITC human anti-mouse F4/80 clone REA126 (#130-102-327 from Miltenyi) were used for staining. The stained cells were examined on a BD LSRFortessa™ cell analyser (BD Biosciences), and results were analysed using FACS Diva and FACSuite software (BD Biosciences). To determine the composition of the leukocytes, the following gating strategy was applied: FSC^{low} debris and erythrocytes, and multiplets with a non-linear SSC-A/SSC-H ratio were excluded. Viability was determined by 7-AAD staining. Viable cells ($7\text{-}AAD^-$) were analysed for CD11b and Cd11c expression. Neutrophils were identified as $Ly6G^+$ cells within the $CD11b^+$ $CD11c^-$ population. $CD11b^+$ $CD11c^-$ $Ly6G^-$ $NK1.1^-$ cells were further divided into subpopulations according to their Ly6C and F4/80 expression, *i.e.* macrophages ($Ly6C^{lo}$ $F4/80^{hi}$) and monocytes ($Ly6C^{hi}$ $F4/80^{lo}$). All gates were defined by using fluorescence minus one controls. (B) *Clec4f* mRNA expression in livers from mice, which underwent the short-term (left) and long-term (right) DEN-treatment model, by qPCR normalised to *Csnk2a2* mRNA. *Csnk2a2* was identified as the most stable housekeeping gene out of 3 housekeeping genes. Data are displayed as individual values with mean indicated by a horizontal line (short-term: n = 6 per treatment group; long-term: n = 13 sham, n = 9 DEN). Primer sequences can be found in the Supplementary CTAT Table. The study was in compliance with the local animal welfare committee (approval no. 37/2014). Animals were kept in IVC system cages (Blue Line Sealsafe, Tecniplast) under controlled conditions regarding temperature (22 ± 2 °C), humidity (55 ± 10%), and 12 h day/night cycle with unrestricted access to food and water. Cages contained an enriched environment using igloos and paper towels.

pro-inflammatory macrophages distinct from KCs as reported for non-alcoholic fatty liver disease.[8]

Based on the assessment of cytokine expression in isolated KCs, resident liver macrophages were concluded to be the primary source of DEN-induced inflammatory cytokine expression leading to DEN-induced liver injury.[6] However, no experiments investigating *in vivo* cytokine expression in the presence and absence of KCs had been performed in this study.[6] Our data on the massive influx of monocytes upon short-term DEN treatment suggests that monocytes and monocyte-derived macrophages considerably contribute to the inflammatory response upon DEN treatment.

In conclusion, the replenishment of KCs found by Borst *et al.* does not necessarily happen in all types of liver damage, but a specifically altered composition of hepatic myeloid cells is a common event in liver injury. However, an understanding of changes in immune cell composition for each specific liver pathology will be essential to gain insight into pathomechanisms and to develop novel therapeutic strategies.

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest concerning this manuscript.

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

Authors' contribution

SMK and JH (Hoppstädter) performed experiments, analysed data and wrote the manuscript. KH performed experiments. SL analysed data. JH (Haybaeck) analysed liver tissues. AKK supervised and concepted the study. All authors reviewed the final version of the manuscript.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2018.11.018.

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Sonja M. Kessler[1,†]
Jessica Hoppstädter[1,†]
Kevan Hosseini[1]
Stephan Laggai[1]
Johannes Haybaeck[2,3,4]
Alexandra K. Kiemer[1,*]
[1]*Department of Pharmacy, Pharmaceutical Biology, Saarland University, Saarbrücken, Germany*
[2]*Department of Pathology, Medical Faculty, Otto-von-Guericke University Magdeburg, Magdeburg, Germany*
[3]*Department of Pathology, Neuropathology and Molecular Pathology, Medical University of Innsbruck, Innsbruck, Austria*
[4]*Institute of Pathology, Medical University of Graz, Graz, Austria*
*Corresponding author. Address: Saarland University, Department of Pharmacy, Pharmaceutical Biology, Campus C2 3, 66123 Saarbrücken, Germany.
E-mail address: pharm.bio.kiemer@mx.uni-saarland.de

† Both authors contributed equally to this work.

Reply to: "Lack of Kupffer cell depletion in diethylnitrosamine-induced hepatic inflammation"

To the Editor:

"Stay here or disappear": Kupffer cell behaviour during liver injury

Kupffer cells (KC) are liver-resident macrophages that during homeostasis display an overall anti-inflammatory phenotype.[1] Upon infection, KCs critically shape the local inflammatory immune milieu, either by supporting inflammation and controlling the infection, or by maintaining tolerance.[2] Liver injury mostly results from infection or intoxication and is typically accompanied by local inflammatory reactions of varying quality and quantity. Recent studies demonstrated fulminant KC loss during viral or bacterial infection, as well as after toxic hepatic injury.[3–6] Furthermore, upon diphtheria toxin-induced KC depletion infiltrating monocytes can differentiate to monocyte-derived KCs (MoKCs).[7] Similarly, KCs that were depleted during virus-induced hepatitis and then replenished by F4/80$^+$-CLEC4F$^+$ MoKCs were indistinguishable from embryo-derived KCs, when analysed by cell surface receptor expression.[3] Alazawi and Knolle speculated that transient KC loss could be generally associated with liver injury.[8] This hypothesis was followed up by Kessler *et al.* who demonstrated stable KC presence in the model of diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC), even during the acute phase of hepatic inflammation. The authors conclude that different causes of liver inflammation may lead to distinct immune reactions, which do not always result in KC loss. Furthermore, different liver injuries might induce specific alterations in the composition of hepatic myeloid cell subsets as demonstrated in the letter by Kessler *et al.*

Indeed, the DEN model studied by Kessler *et al.* is very different when compared with infection models. This is highlighted by the fact that pathogens not only actively induce, but also modulate immune responses. Therefore, characteristics of the pathogens that induce hepatitis presumably influence the onset and course of liver inflammation and subsequent KC immunity. In particular, direct infection of KCs, as well as inflammation-induced apoptosis, bystander necrosis, or necroptosis may induce KC loss.[4] For several infection-induced cytokines even opposing functions have been identified within the liver, *e.g.* type I interferon might act either in a pro- or anti-inflammatory manner by regulating the composition of other cytokines and chemokines. This way myeloid cell function, including KC disappearance, might be directly and indirectly influenced by different cytokines.[3,9–13]

The liver is a complex and highly structured organ. Therefore, the anatomical site of inflammation might influence KC immunity. KCs are mainly localised in periportal regions. Acute viral hepatitis is often associated with disseminated inflammation throughout the liver. In contrast, toxic inflammation mostly occurs around the portal veins, whereas carcinoma-associated inflammation should primarily occur locally at sites of tumour development. Interestingly, Kessler *et al.* did not detect KC depletion in the DEN model at tumour proximal sites.

Future research should focus on the detailed characterisation of KC development. As outlined above, infiltrating monocytes can replenish KCs by differentiating to CLEC4F$^+$ MoKCs. However, they can also differentiate to short-lived inflammatory macrophages, depending on the kind and extent of liver damage. The functional differences of myeloid cell subsets, their impact on the induction and resolution of liver inflammation as well as their role in liver repair will be of key relevance in order to be able to predict disease course.

Additionally, it is not clear why upon similar liver injuries individuals may show very divergent disease outcomes ranging from entire liver recovery to liver cirrhosis and HCC. Recent studies indicated that individual host characteristics might account for variations in cytokine responses against certain stimuli and infections.[11,12] Thus, in individuals it is possible that selected pathogens induce variable inflammatory responses that are associated with hepatitis and KC depletion, or not. Therefore, it remains necessary to better understand the molec-