Manuscript number: HEP-14-2569

Title Kupffer cell-monocyte communication is required for initiating murine liver progenitor cell-mediated liver regeneration

Authors Caryn L Elsegood,[1,2] Chun Wei Chan,[3,4] Mariapia A Degli-Esposti,[5,6] Matthew E Wikstrom,[5,6] Alice Domenichini,[2] Kyren Lazarus,[2,7] Nico van Rooijen,[8] Ruth Ganss,[7] John K Olynyk,[2,9,10]* George CT Yeoh[1,7]*

* Joint senior authors.

[1] School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia, Australia

[2] School of Biomedical Science, CHIRI Biosciences Research Precinct, Curtin University, Bentley, Western Australia, Australia

[3] School of Medicine and Pharmacology, The University of Western Australia, Fremantle, Western Australia, Australia

[4] School of Biological Sciences and Biotechnology, Murdoch University, Murdoch, Western Australia, Australia

[5] Immunology and Virology Program, Centre for Ophthalmology and Visual Science, The University of Western Australia, Nedlands, Western Australia, Australia

[6] Centre for Experimental Immunology, Lions Eye Institute, Nedlands, Western Australia, Australia

[7] Harry Perkins Institute of Medical Research, QEII Medical Centre, Nedlands and Centre for Medical Research, The University of Western Australia, Crawley, Western Australia, Australia

[8] Department of Molecular Cell Biology, VU Medical Center, Amsterdam, The Netherlands

[9] Department of Gastroenterology & Hepatology, Fiona Stanley & Fremantle Hospitals, South Metropolitan Health Service, Western Australia, Australia

[10] Institute for Immunology and Infectious Diseases, Murdoch University, Murdoch, Western Australia, Australia

Keywords: Kupffer cell, monocyte-derived macrophage, liver progenitor cell, liver injury, liver regeneration

Corresponding Author: Caryn L Elsegood, PhD

School of Chemistry and Biochemistry

The University of Western Australia

35 Stirling Highway

Crawley, Western Australia, 6009

Australia

Phone: +61 8 6488 4405

Fax: +61 8 6488 1148

Email: caryn.elsegood@uwa.edu.au

Nonstandard abbreviations:

ALT alanine aminotransferase; CDE choline-deficient, ethionine-supplemented; CK cytokeratin; CL clodronate liposomes; HGF hepatocyte growth factor; HNF4α hepatocyte nuclear factor 4 alpha; KC Kupffer cell; LPC liver progenitor cell; LT lymphotoxin; MDM monocyte-derived macrophage; PL PBS liposomes

Financial Support:

This work was supported by grants from Fremantle Hospital Medical Research Foundation, Cancer Council Western Australia, and the National Health and Medical Research Council. John K. Olynyk is an NHMRC Practitioner Fellow. Ruth Ganss is an NHMRC Senior Research Fellow.

Liver progenitor cells are necessary for repair in chronic liver disease since the remaining hepatocytes cannot replicate. However, liver progenitor cell numbers also correlate with disease severity and hepatocellular carcinoma risk. Thus, the progenitor cell response in diseased liver may be regulated to optimise liver regeneration and minimise likelihood of tumorigenesis. How this is achieved is currently unknown. Human and mouse diseased liver contain two subpopulations of macrophages with different ontogenetic origins; prenatal yolk sac-derived Kupffer cells (KCs), and peripheral blood monocyte-derived macrophages (MDMs). We examined the individual role(s) of KCs and MDMs in the induction of LPC proliferation using clodronate liposome deletion of KCs and adoptive transfer of monocytes, respectively, in the choline-deficient, ethionine-supplemented diet model of liver injury and regeneration. Clodronate liposome treatment reduced initial liver monocyte numbers together with the induction of injury and LPC proliferation. Adoptive transfer of monocytes increased induction of liver injury, LPC proliferation, and TNFα production. *Conclusion:* Kupffer cells control the initial accumulation of monocyte-derived macrophages. These infiltrating monocytes are in turn responsible for the induction of liver injury, the increase in TNFα, and the subsequent proliferation of liver progenitor cells.

The liver has two avenues to regeneration. Replication of pre-existing hepatocytes is the main pathway to replace cells lost through acute damage such as that induced by acetaminophen toxicity. In contrast, an alternate pathway of hepatocyte replacement involving liver progenitor cells (LPCs) is invoked in some chronic liver injury models (1). Various murine lineage tracing models report no conversion, but others report up to 2.45% conversion of LPCs to hepatocytes (2). Different tracing strategies in diverse models may explain the variation; but importantly LPCs converted to hepatocytes in the CDE model used in this study. A recent study in humans confirmed the existence of a bipotent liver stem cell (3). The LPC pathway is induced in chronic liver diseases including alcoholic and non-alcoholic fatty liver diseases, viral hepatitis (B and C), and Hereditary Hemochromatosis (4-6). Importantly, all these liver diseases are associated with increased hepatocellular carcinoma (HCC) incidence. The propensity to develop HCC is reduced in murine models such as the TNF receptor Type 1 knockout mouse and pharmacological inhibition by c-kit inhibitor, imatinib mesylate, where LPC numbers are reduced in response to an hepatocarcinogenic diet (7, 8). Thus, regulation of LPC numbers may be a process whereby regeneration and tumorigenesis risks may be balanced.

Liver progenitor cells are bipotential cells located in the portal zone, with a high nuclear to cytoplasmic ratio and an ovoid nucleus, which are able to give rise to cholangiocytes or hepatocytes (9). LPCs express cytokeratins 7 and 19, OV-6, and EpCam (CD326). Mitogenic LPC cytokines include tumor necrosis factor (TNF) α, interleukin (IL)-6, lymphotoxin (LT) β, TNF-like weak inducer of apoptosis (TWEAK), interferon (IFN) γ, and hepatocyte growth factor (HGF) (1). Whilst there are many cellular sources of these cytokines within the liver, macrophages are likely to be key producers and/or regulators of their production. Indeed, MDM-derived TWEAK increases LPC numbers *in vivo* (10).

Liver macrophages comprise two subpopulations; the tissue-resident Kupffer cells (KCs) and infiltrating peripheral blood monocyte-derived macrophages (MDMs). Recent lineage tracing studies have shown KCs are derived prenatally from embryonic yolk-sac progenitors, rather than from hematopoietic stem cells, and under homeostatic conditions, repopulate locally rather than from peripheral blood monocytes as previously assumed (11). Importantly, the distinct ontological origins of KCs and MDMs suggest they may play functionally distinct roles in liver injury and subsequent regeneration. We have previously shown the number of KCs and MDMs both increase in the liver prior to induction of LPC proliferation in murine chronic liver injury models. KCs and MDMs also spatially co-localize with LPCs in a niche, suggesting they may regulate LPC numbers (12). Further, we and others have shown increased MDM numbers in human chronic liver diseases including alcoholic liver disease, non-alcoholic fatty liver disease, and viral hepatitis (B and C), which also have increased LPC numbers (13, 14). However, the specific roles of each liver macrophage subpopulation in the inductive phase of LPC-mediated regeneration have not been examined to date.

There is currently much interest in delivering therapy for liver disease via macrophages (15, 16). To assess the therapeutic potential of each liver macrophage population requires fundamental insights into their respective role(s). Hence, this study's aim was to determine if, and how, tissue-resident KCs and infiltrating MDMs each regulate the inductive phase of LPC proliferation in hepatocytic liver regeneration. To accomplish this, we have utilized a novel clodronate liposome injection regime, which depletes only KCs. We have then adoptively transferred monocytes to specifically assess how MDMs influence induction of LPC proliferation. Significantly, we found KCs and MDMs play interconnecting roles in induction of LPC-mediated liver regeneration.

Materials and Methods

Animals. Male C57Bl/6 and CD45.1 (B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ) mice were from the Animal Resources Centre (Murdoch, WA, Australia). All animal experiments were conducted in accordance with National Health and Medical Research Council of Australia and The University of Western Australia Animal Ethics Committee guidelines.

Kupffer Cell Depletion. Clodronate liposomes were prepared as previously described (17). To deplete KCs, five week old C57Bl/6 mice were injected intravenously with clodronate liposomes (CL; 100µl) 24 h prior to being placed on a CDE diet (18). The mice were re-injected with clodronate liposomes (100µl) 24 h after being placed on a CDE diet. Control groups were injected with PBS liposomes (PL; 100µl). Mice on normal chow diet were sham-injected with 0.9% (w/v) saline (100µl) at the same time. Mice remained on CDE or normal chow diets for times indicated. Clodronate was a gift of Roche Diagnostics.

Monocyte Adoptive Transfer. Monocytes were isolated from the bone marrow of tibia and femur of CD45.1^{+} C57Bl/6 mice by magnetic-assisted cell separation using the CD115 microbead kit (Miltenyi Biotec Australia, North Ryde, NSW, Australia). Isolated monocyte purity was confirmed by flow cytometry. Monocytes (2 X 10^{6} cells in 10 µl of PBS) or PBS (100µl) were injected intravenously into CD45.2^{+} C57Bl/6 recipient mice 2 days after mice were placed on the CDE diet.

More information on materials and methods can be found in the Supporting Information.

Results

Kupffer cell depletion delays the increase in MDM numbers. To investigate the role of KCs in LPC proliferation, we administered two intravenous clodronate liposome (CL) injections of 100µl each, rather than the commonly-used single dose of 200µl (21), to deplete KCs. The first was administered 24h prior to initiating liver injury with the choline-deficient, ethionine-supplemented (CDE) diet, and the second 24h after initiating liver injury (Fig. 1A). The drug control was PBS liposomes (PL). Mice on normal chow diet were saline sham-injected. KC depletion was confirmed by Complement Receptor for Immunoglobulin Superfamily (CRIg) and F4/80 immunostaining (Fig. 1B-D).

We then examined the effect of CL treatment on MDM numbers by CD11b immunostaining of liver sections (Fig. 1E). MDMs were reduced in CL-treated livers (CDE + CL), compared to PL-treated livers, at 2 days after diet commencement by approximately 70% (Fig. 1F). Liver MDM numbers in CL-treated livers then increased to levels similar to PL-treated livers by 4 days after diet commencement. To further investigate if KCs recruit monocytes, we examined whether CL-treatment reduced blood and/or liver MDM numbers after the second injection. Liver MDM numbers were not significantly increased at 1 day after diet commencement nor depleted by CL-treatment (Fig. S2A). Additionally, CL-treatment delayed the CDE diet-induced increase in Ly6C^{hi} MDM compared to PL-treatment (Fig. S2B). Importantly, this CL injection regime did not reduce blood monocyte numbers, but increased their numbers 10-fold compared to PL 24h after the second injection. Blood monocyte numbers remained elevated during the study (Fig. 1G). We then examined whether there were changes in the monocyte phenotype. For this analysis, we defined the Ly6C expression levels as Ly6C^{lo}, Ly6C^{int}, and Ly6C^{hi} as shown in Fig. S3A). Clodronate liposome-treated mice fed the CDE diet had a significantly higher proportion of more highly

differentiated monocytes expressing lower Ly6C (i.e. $Ly6C^{int}$) levels compared to PL-treated mice (Fig S3B), suggesting monocytes were not being recruited to peripheral tissue in the CL-treated mice and instead could differentiate to a more mature blood monocyte. Finally, we examined if CL-treatment reduced expression of the monocyte chemokine, CCL2 (Fig. S…). We found CCL2 expression was … . Taken together, these data indicate KCs are necessary for optimal early monocyte recruitment to the liver during initiation of CDE diet-induced injury and regeneration.

Kupffer cell depletion reduces LPC proliferation induction. We next examined the effect of KC-depletion on LPC numbers present after 6 days on the CDE diet. Liver progenitor cells were identified as small pan cytokeratin $(pCK)^+$ cells with oval-shaped nucleus and scant cytoplasm, whilst biliary cells were identified as pCK^+ cells in ductal structures (Fig. 2A). located within 1 cell from the portal vein. As expected, LPC numbers were significantly increased in the PL-treated mice fed the CDE diet (Fig. 2C), and were similar in CDE-fed saline-treated mice (Fig S1). The CDE diet-induced expansion in LPC numbers, however, was abrogated in CL-treated mice. Proliferating LPC ($Ki67^+pCK^+$) numbers were also significantly reduced in CL-treated mice (Fig. 2B, D). Biliary cell numbers, however, were unchanged in response to both CDE diet and CL treatment (Fig. 2E). We also found no evidence CL reduced *in vitro* LPC viability (data not shown). These data indicate KCs are required for induction of LPC proliferation.

Kupffer cell depletion reduces CDE diet-induced hepatic damage but not hepatic steatosis. The CDE diet induces hepatic steatosis which promotes liver injury required for induction of LPC proliferation. Previous literature suggests KCs regulate both steatosis and injury (22-27). CDE diet-induced steatosis, as quantitated by Oil Red O staining, was not reduced by CL

treatment (Fig. 3A-B). Serum ALT activity, an indicator of hepatic damage, was also increased by the CDE diet at day 2 but, in contrast to steatosis, tended to be reduced in CL-treated livers (Fig. 3C). Liver TUNEL staining confirmed a reduction in apoptotic hepatocyte numbers in CL-treated livers at day 2 (Fig. 3A, D). These data indicate KCs do not regulate steatosis but promote maximal liver damage.

Kupffer cell depletion reduces hepatic LPC mitogen levels. We characterized the LPC mitogens KCs secreted and/or influenced their secretion in the liver at 6 days after the mice were placed on the CDE diet. TNFα, IFNγ, and HGF mRNA levels were all significantly reduced, and IL-6 was ablated, in CL-treated livers compared to PL-treated livers (Fig. 4A-D). Lymphotoxin β and TWEAK levels were unchanged by any treatment (data not shown).

The LPC niche contains activated hepatic stellate cells (HSCs) which can secrete the LPC mitogens, TNFα, IL-6, and HGF (28). Accordingly, we determined if KCs could indirectly regulate production of these mitogens by activating HSCs. As expected, activated HSC numbers in the liver were significantly increased by the CDE diet (CDE + PL), as assessed by α-SMA immunostaining (Fig. 4E, F). Activated HSC numbers were reduced, although not statistically significantly, in CL-treated livers (CDE + CL).

We then explored whether KCs may indirectly influence LPC numbers by recruiting innate and/or adaptive immune cells which could secrete LPC mitogens (Fig. 4G-I). We focused on neutrophils which can secrete TNFα (29), and B and $CD4^+$ T cells which can secrete TNFα, IL-6, and IFNγ (30-32). Neutrophil (Fig. 4G) and B cell (Fig 4H) numbers were significantly increased in response to the CDE diet (CDE + PL), and reduced, although not statistically significantly, in CL-treated livers. In contrast, $CD4^+$ T cell numbers were increased with CDE

diet, although not significantly, but unchanged by KC-depletion (Fig. 4I). Taken together, these data indicate KCs may directly produce the LPC mitogens or indirectly regulate their levels via HSC activation or recruitment of neutrophils and B cells.

Inflammatory and non-classical monocyte numbers increase in CDE diet-injured liver. The delayed increase in liver MDM numbers (Fig. 1F) in KC-depleted livers could also reduce LPC numbers (Fig. 2C). We characterized the intrahepatic monocyte/macrophage population 6 days after the mice were placed on a CDE diet by flow cytometry analysis to determine if inflammatory or non-classical monocytes predominated. A distinct population of monocytic cells were identified using the myeloid marker, CD11b, and the macrophage marker, F4/80 (Fig. S4A). The $CD11b^{+}F4/80^{-/lo}$ cells were further classified as $Ly6G^{-}$ monocytes or $Ly6G^{+}$ neutrophils. Monocytes were then subdivided into $Ly6C^{+}$ inflammatory or $Ly6C^{-}$ non-classical cells. $Ly6C^{-}$ monocytes are derived from $Ly6C^{+}$ monocytes, at least under homeostatic conditions (11). Liver $Ly6C^{+}$ monocyte numbers were significantly increased by 7-fold in response to CDE diet-induced liver injury, whilst $Ly6C^{-}$ non-classical monocytes were increased 3.5-fold (Fig. S4B).

Monocyte adoptive transfer induces proliferation of Kupffer cells and LPCs. To ascertain if monocyte recruitment can regulate LPC proliferation, monocytes were purified from $CD45.1^{+}$ C57Bl/6 murine bone marrow by magnetic-assisted cell separation, and adoptively transferred into $CD45.2^{+}$ C57Bl/6 recipient mice fed the CDE diet. Importantly, monocytes were transferred into mice that had been fed the CDE diet for 2 days (Fig. 5A), representing the same time MDMs were reduced after KC-depletion (Fig. 1F). Monocyte purity, as determined by flow cytometry analysis of the monocyte marker, CD115, was 93% (Fig.5B). The adoptively transferred monocytes trafficked into the liver, as confirmed by CD45.1

immunohistochemistry (Fig. S5), and MDM numbers were significantly increased by adoptive transfer (Fig. 5C). Monocyte adoptive transfer also significantly increased KC numbers ($CRIg^+$, $F4/80^+$) in mice fed the CDE diet for 6 days (Fig. 5D, E), due to proliferation, as indicated by increased $Ki67^+CRIg^+$ KC numbers (Fig. 5F-G). These data indicate monocytes promote CDE diet-induced KC proliferation.

We then examined the effect of monocyte adoptive transfer on CDE diet-induced LPC proliferation. Liver progenitor cell, but not biliary cell, numbers were significantly increased by monocyte adoptive transfer (Fig 6A-B), with proliferating LPC ($Ki67^+pCK^+$) numbers also augmented (Fig. 6C). Interestingly, there was a small increase in biliary cells in response to CDE diet-induced injury when monocytes were not transferred (Fig. 6D). As the CDE diet induces hepatocyte-directed, as opposed to cholangiocyte-directed, LPC-mediated liver regeneration (33, 34), and liver macrophages have been shown to regulate hepatocytic specification (35), we examined if monocytes can explicitly promote this specification. Monocyte adoptive transfer enhanced hepatocyte specification of the LPCs, as evidenced by the significant increase in $HNF4\alpha^+$ LPC numbers (Fig. 6E-F). These data indicate monocytes induce LPC proliferation and their differentiation into hepatocytes *in vivo*.

Monocyte adoptive transfer induces CDE diet-induced hepatic damage. As KCs are required for initial monocyte recruitment (Fig. 1F) and maximal induction of liver damage (Fig. 3C-D), we examined if monocytes could induce hepatic damage. We found monocyte adoptive transfer increased hepatic damage as evidenced by elevated serum ALT levels (Fig. 7A), and this was accompanied by a significant increase in weight loss (Fig. 7B). Similarly to KC-depletion (Fig. 3B), there was no corresponding change in steatosis as determined by Oil Red O staining (Fig 7C).

TNFα expression is enhanced by monocyte adoptive transfer. Kupffer cell depletion attenuated LPC numbers and reduced TNFα, IL-6, IFNγ, and HGF levels (Figs. 2C, 4A-D). As monocyte adoptive transfer increased LPC proliferation (Fig. 6C), we explored which LPC mitogen(s) was up-regulated by this treatment. We also examined if TWEAK expression increased, as monocytes may directly provide TWEAK. Surprisingly, monocyte adoptive transfer only enhanced TNFα expression (Fig. 7D). This suggests TNFα is the critical cytokine for induction of LPC proliferation, and is consistent with our previous report TNF receptor Type I signalling is required for LPC proliferation and HCC development (7).

Monocyte adoptive transfer induces hepatic stellate cell activation and immune cell recruitment. As KC depletion tended to reduce hepatic stellate cell activation (Fig 4E-F), we investigated if monocyte adoptive transfer also affected activation of these cells. The number of activated hepatic stellate cells, as assessed by αSMA immunohistochemistry, significantly increased in response to monocyte adoptive transfer (Fig. 7E).

We then examined if monocytes could, similarly to KCs, increase numbers of other immune cells which may supply TNFα to the LPCs. Monocyte adoptive transfer significantly elevated liver neutrophil, B cell, and $CD4^+$ T cell numbers (Fig. 7F-H). Taken together, these data indicate monocytes activate HSCs and induce immune cell recruitment which may contribute to liver TNFα levels.

Discussion

We have previously shown KC and MDM numbers increase prior to the increase in LPCs (12). We have now used a novel clodronate liposome injection regime, in which only half the usual dose of clodronate liposomes was injected, to selectively deplete KCs, but not blood monocytes, to assess how KCs specifically regulate the induction of LPC-mediated liver repair. We then examined how monocytes regulate LPC proliferation by adoptively transferring monocytes. Importantly, we have shown KCs are required for the initial maximal CDE diet-induced liver injury and monocyte recruitment. The monocytes induce TNFα production, with concomitant initiation of LPC proliferation and hepatocyte specification (Fig. 8).

Our study indicates monocytes initiate CDE diet-induced liver injury. However, KCs increase injury by initially enhancing monocyte recruitment. Other as yet unidentified liver cells may also contribute to monocyte recruitment since MDM numbers are only transient reduced when KCs are depleted. Potential candidates include sinusoidal endothelial cells and HSCs which are both able to produce monocyte chemotactic protein-1 (or CCL2) (36, 37). Monocytes also contribute to acetaminophen- and carbon tetrachloride-induced liver injury, but KC involvement in monocyte recruitment in these liver injury models is unknown (38, 39). Similarly, KCs promote both ethanol- and Concanavalin A-induced liver injury, but these studies did not address whether injury was via KC-mediated monocyte recruitment (25-27). Importantly, monocyte numbers correlate with liver injury severity in chronic viral hepatitis (B and C) and primary biliary cirrhosis patients (13, 40). Our data suggests these monocytes may contribute to ongoing liver damage in patients with these diseases.

Furthermore, adoptive transfer in our study demonstrates monocytes promote LPC proliferation. Monocytes may influence LPC proliferation by one or a combination of the following mechanisms. Firstly, LPC proliferation requires an unspecified signal from apoptotic hepatocytes (41) and, we have shown in our study monocytes induce hepatocyte death. Secondly, monocytes may directly secrete the LPC mitogen(s). Thirdly, monocytes increase the number of activated HSCs, and immune cells such as neutrophils, B cells, and T cells in the liver which may secrete the LPC mitogen(s). Further, our study suggests TNFα is crucial to induction of LPC proliferation as it is the only cytokine that positively correlates with LPC numbers when KC or monocyte numbers are modulated. Importantly, we have previously shown monocytes express approximately five times the level of TNFα compared to KCs (12), suggesting monocytes rather than KCs are key providers of TNFα. We have also previously shown TNF receptor Type I is critical for increased LPC numbers in vivo (7), and TNFα directly modulates LPC proliferation (42).

Our data differ from that of van Hul and colleagues (2010) who reported KCs did not influence LPC numbers. Importantly, van Hul used a larger (150 µl) dose of clodronate liposomes administered at 3 day intervals throughout the study, and evaluated LPC numbers at 10 days after the mice were placed on the CDE diet, without reporting the status of MDMs. Our approach allows for a more precise documentation of the sequence of events following KC depletion.

Lipid deposition is crucial for CDE diet-induced liver injury, and studies in methionine and choline-deficient and high fat diet models suggest liver macrophages are required for lipid deposition (22-24). However, in our study, neither KCs nor monocytes altered lipid

deposition. This disparity could reflect differences in hepatic lipid deposition mechanisms as a result of the different diets used.

The CDE diet is the only liver injury model in which LPCs differentiate into hepatocytes (33, 34). A seminal study by Forbes and colleagues (35) showed that acquisition of a hepatocellular fate by LPCs, as opposed to a cholangiocytic fate, required macrophages and the Wnt signaling pathway. However, Forbes and colleagues did not distinguish the liver macrophage subpopulation involved in this process. We now show monocytes can induce LPC specification to hepatocytes as indicated by increased numbers of HNF4α^{+} LPCs (Fig. 6F). We suggest monocytes promote LPC specification to hepatocytes by inducing both hepatocyte apoptosis and LPC proliferation.

Studies of macrophage functions in liver pathology have typically not distinguished between KCs and MDMs, as the methods used likely depleted both subpopulations, or focused only on MDMs. Importantly, our study now distinguishes between and examines KC and MDM interactions in LPC-mediated liver regeneration (Fig. 9). Kupffer cells have distinctive functions in LPC-mediated liver regeneration, when compared to MDMs, including participation in blood monocyte recruitment to initiate the liver injury as well as promotion of liver IL-6, IFNγ, and HGF production. On the other hand, MDMs promote KC proliferation, which may be a homeostatic response to clear apoptotic hepatocytes (43). Kupffer cells and MDMs both promote TNFα production, LPC proliferation, and HSC activation, although KC regulation of these is likely due to monocyte recruitment. Thus, KCs and MDMs perform both different and complementary roles.

In situ therapy to prevent further liver injury and/or to regenerate liver is urgently required. Transplant of whole bone marrow improves liver function in human disease as well as mouse models of liver fibrosis (44, 45). Both hematopoietic and mesenchymal stem cells, derived from bone marrow, improve liver function, although the mechanism(s) of action is presently unknown. More recent work by Forbes and colleagues suggests injection of macrophages derived from bone marrow in the presence of colony stimulating factor-1 may be a viable treatment for liver regeneration by reducing fibrosis and increasing LPC numbers (46). Our study indicates the source of macrophages for transplant to improve liver function may be extremely important; for whilst bone marrow monocytes enhance LPC-mediated liver regeneration, they also significantly increase liver damage. The major difference between our study and that of Forbes and colleagues is they use CSF-1 to convert the bone marrow monocytes into comparatively less inflammatory macrophages.

In conclusion, this study demonstrates KCs and monocytes play interacting roles in the inductive phase of LPC-mediated liver regeneration. Importantly, monocytes play a crucial role in development of liver injury which results in LPC proliferation. As monocyte numbers are increased in much liver pathology, reducing their numbers in diseased liver warrants serious consideration as a strategy to balance the development of cirrhosis/HCC resulting from chronic liver injury and liver repair through LPC proliferation.

Acknowledgements:

This research is dedicated to the memory of Matthew Morrison and the courage of his family who gave so others might live.

The authors acknowledge the facilities, and scientific and technical assistance of the National Imaging Facility at the Centre for Microscopy, Characterisation & Analysis, University of Western Australia, and CHIRI Biosciences Research Precinct core facility, Curtin University. The authors thank Dr Alison Rose and Carla Smith at Fremantle Hospital Biological Research Facility for their technical assistance.

The authors declare they have no competing financial interests to disclose.

REFERENCES

1. Viebahn CS, Yeoh GC. What fires prometheus? The link between inflammation and regeneration following chronic liver injury. Int J Biochem Cell Biol 2008;40:855-873.
2. Lemaigre FP. Determining the fate of hepatic cells by lineage tracing: Facts and pitfalls. Hepatology 2015;61:2100-2103.
3. Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. Cell 2015;160:299-312.
4. Lowes KN, Brennan BA, Yeoh GC, Olynyk JK. Oval cell numbers in human chronic liver diseases are directly related to disease severity. Am J Pathol 1999;154:537-541.
5. Hsia CC, Evarts RP, Nakatsukasa H, Marsden ER, Thorgeirsson SS. Occurrence of oval-type cells in hepatitis B virus-associated human hepatocarcinogenesis. Hepatology 1992;16:1327-1333.
6. Eleazar JA, Memeo L, Jhang JS, Mansukhani MM, Chin S, Park SM, Lefkowitch JH, et al. Progenitor cell expansion: an important source of hepatocyte regeneration in chronic hepatitis. J Hepatol 2004;41:983-991.
7. Knight B, Yeoh GC, Husk KL, Ly T, Abraham LJ, Yu C, Rhim JA, et al. Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice. J Exp Med 2000;192:1809-1818.
8. Knight B, Tirnitz-Parker JE, Olynyk JK. C-kit inhibition by imatinib mesylate attenuates progenitor cell expansion and inhibits liver tumor formation in mice. Gastroenterology 2008;135:969-979.
9. Duncan AW, Dorrell C, Grompe M. Stem cells and liver regeneration. Gastroenterology 2009;137:466-481.
10. Bird TG, Lu WY, Boulter L, Gordon-Keylock S, Ridgway RA, Williams MJ, Taube J, et al. Bone marrow injection stimulates hepatic ductular reactions in the absence of injury via macrophage-mediated TWEAK signaling. Proc Natl Acad Sci U S A 2013;110:6542-6547.
11. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, Strauss-Ayali D, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity 2013;38:79-91.
12. Viebahn CS, Benseler V, Holz LE, Elsegood CL, Vo M, Bertolino P, Ganss R, et al. Invading macrophages play a major role in the liver progenitor cell response to chronic liver injury. J Hepatol 2010;53:500-507.
13. Leicester KL, Olynyk JK, Brunt EM, Britton RS, Bacon BR. Differential findings for CD14-positive hepatic monocytes/macrophages in primary biliary cirrhosis, chronic hepatitis C and nonalcoholic steatohepatitis. Liver Int 2006;26:559-565.
14. Zimmermann HW, Seidler S, Nattermann J, Gassler N, Hellerbrand C, Zernecke A, Tischendorf JJ, et al. Functional contribution of elevated circulating and hepatic non-classical CD14CD16 monocytes to inflammation and human liver fibrosis. PLoS One 2010;5:e11049.
15. Forbes SJ, Newsome PN. New horizons for stem cell therapy in liver disease. J Hepatol 2012;56:496-499.
16. Tacke F, Luedde T, Trautwein C. Inflammatory pathways in liver homeostasis and liver injury. Clin Rev Allergy Immunol 2009;36:4-12.
17. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994;174:83-93.
18. Akhurst B, Croager EJ, Farley-Roche CA, Ong JK, Dumble ML, Knight B, Yeoh GC. A modified choline-deficient, ethionine-supplemented diet protocol effectively induces oval cells in mouse liver. Hepatology 2001;34:519-522.
19. Cook AD, Turner AL, Braine EL, Pobjoy J, Lenzo JC, Hamilton JA. Regulation of systemic and local myeloid cell subpopulations by bone marrow cell-derived granulocyte-macrophage colony-stimulating factor in experimental inflammatory arthritis. Arthritis Rheum 2011;63:2340-2351.

20. Tirnitz-Parker JE, Viebahn CS, Jakubowski A, Klopcic BR, Olynyk JK, Yeoh GC, Knight B. Tumor necrosis factor-like weak inducer of apoptosis is a mitogen for liver progenitor cells. Hepatology 2010;52:291-302.
21. Gehring S, Dickson EM, San Martin ME, van Rooijen N, Papa EF, Harty MW, Tracy TF, Jr., et al. Kupffer cells abrogate cholestatic liver injury in mice. Gastroenterology 2006;130:810-822.
22. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, Scott DK, et al. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes 2010;59:347-357.
23. Jarvelainen HA, Fang C, Ingelman-Sundberg M, Lukkari TA, Sippel H, Lindros KO. Kupffer cell inactivation alleviates ethanol-induced steatosis and CYP2E1 induction but not inflammatory responses in rat liver. J Hepatol 2000;32:900-910.
24. Tosello-Trampont AC, Landes SG, Nguyen V, Novobrantseva TI, Hahn YS. Kuppfer cells trigger nonalcoholic steatohepatitis development in diet-induced mouse model through tumor necrosis factor-alpha production. J Biol Chem 2012;287:40161-40172.
25. Goldin RD, Ratnayaka ID, Brown IN, Wickramasinghe SN. The effect of Kupffer cell elimination on ethanol-induced liver damage in mice. Int J Exp Pathol 1995;76:353-359.
26. Nakashima H, Kinoshita M, Nakashima M, Habu Y, Shono S, Uchida T, Shinomiya N, et al. Superoxide produced by Kupffer cells is an essential effector in concanavalin A-induced hepatitis in mice. Hepatology 2008;48:1979-1988.
27. Schumann J, Wolf D, Pahl A, Brune K, Papadopoulos T, van Rooijen N, Tiegs G. Importance of Kupffer cells for T-cell-dependent liver injury in mice. Am J Path 2000;157:1671-1683.
28. Hu J, Srivastava K, Wieland M, Runge A, Mogler C, Besemfelder E, Terhardt D, et al. Endothelial cell-derived angiopoietin-2 controls liver regeneration as a spatiotemporal rheostat. Science 2014;343:416-419.
29. Bennouna S, Denkers EY. Microbial antigen triggers rapid mobilization of TNF-alpha to the surface of mouse neutrophils transforming them into inducers of high-level dendritic cell TNF-alpha production. J Immunol 2005;174:4845-4851.
30. Boussiotis VA, Nadler LM, Strominger JL, Goldfeld AE. Tumor necrosis factor alpha is an autocrine growth factor for normal human B cells. Proc Natl Acad Sci U S A 1994;91:7007-7011.
31. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, Johnson LL, et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol 2000;1:475-482.
32. Feldmann M, Londei M, Haworth C. T cells and lymphokines. Br Med Bull 1989;45:361-370.
33. Espanol-Suner R, Carpentier R, Van Hul N, Legry V, Achouri Y, Cordi S, Jacquemin P, et al. Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 2012;143:1564-1575 e1567.
34. Rodrigo-Torres D, Affo S, Coll M, Morales-Ibanez O, Millan C, Blaya D, Alvarez-Guaita A, et al. The biliary epithelium gives rise to liver progenitor cells. Hepatology 2014;60:1367-1377.
35. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, Ridgway RA, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med 2012;18:572-579.
36. Sprenger H, Kaufmann A, Garn H, Lahme B, Gemsa D, Gressner AM. Differential expression of monocyte chemotactic protein-1 (MCP-1) in transforming rat hepatic stellate cells. J Hepatol 1999;30:88-94.
37. Lalor PF, Herbert J, Bicknell R, Adams DH. Hepatic sinusoidal endothelium avidly binds platelets in an integrin-dependent manner, leading to platelet and endothelial activation and leukocyte recruitment. Am J Physiol - Gastrointest Liver Physiol 2013;304:G469-478.
38. Holt MP, Cheng L, Ju C. Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. J Leuk Biol 2008;84:1410-1421.
39. Zamara E, Galastri S, Aleffi S, Petrai I, Aragno M, Mastrocola R, Novo E, et al. Prevention of severe toxic liver injury and oxidative stress in MCP-1-deficient mice. J Hepatol 2007;46:230-238.

40. Zhang JY, Zou ZS, Huang A, Zhang Z, Fu JL, Xu XS, Chen LM, et al. Hyper-activated pro-inflammatory CD16 monocytes correlate with the severity of liver injury and fibrosis in patients with chronic hepatitis B. PLoS One 2011;6:e17484.
41. Jung Y, Witek RP, Syn WK, Choi SS, Omenetti A, Premont R, Guy CD, et al. Signals from dying hepatocytes trigger growth of liver progenitors. Gut 2010;59:655-665.
42. Viebahn CS, Tirnitz-Parker JE, Olynyk JK, Yeoh GC. Evaluation of the "Cellscreen" system for proliferation studies on liver progenitor cells. Eur J Cell Biol 2006;85:1265-1274.
43. Henson PM, Hume DA. Apoptotic cell removal in development and tissue homeostasis. Trends Immunol 2006;27:244-250.
44. Couto BG, Goldenberg RC, da Fonseca LM, Thomas J, Gutfilen B, Resende CM, Azevedo F, et al. Bone marrow mononuclear cell therapy for patients with cirrhosis: a Phase 1 study. Liver Int 2011;31:391-400.
45. Sakaida I, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, Okita K. Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. Hepatology 2004;40:1304-1311.
46. Thomas JA, Pope C, Wojtacha D, Robson AJ, Gordon-Walker TT, Hartland S, Ramachandran P, et al. Macrophage therapy for murine liver fibrosis recruits host effector cells improving fibrosis, regeneration, and function. Hepatology 2011;53:2003-2015.

Figure Legends

Figure 1. Clodronate liposomes ablate Kupffer cells. (A) Experimental design. Mice were injected with saline, PBS liposomes (PL), or clodronate liposomes (CL). They were then placed on normal chow (NC) or CDE diet 24h after the injections. The mice were again injected with saline, PL or CL 24h after being placed on diet, and sacrificed at times indicated. (B) Representative CRIg immunohistochemistry (bar, 50μm). (C) $CRIg^+$ and (D) $F4/80^+$ cell numbers. Data represent means ± SEM, n = 3-4. *p <0.05. ND, not detected. (E) Representative CD11b immunohistochemistry (bar, 50μm). (F) $CD11b^+$ cell numbers. (G) Flow cytometry quantitation of blood monocytes. Data represent means ± SEM, n = 3-4. *p <0.05.

Figure 2. Kupffer cell depletion reduces induction of LPC proliferation. Mice were injected with saline, PBS liposomes (PL), or clodronate liposomes (CL). They were then placed on normal chow (NC) or CDE diet 24h after the injections. The mice were again injected with saline, PL, or CL 24h after being placed on diet, and sacrificed after 6 days on diet. (A) Representative immunohistochemistry for pan cytokeratin (pCK)-positive cells which were classified as LPCs (arrow heads) or biliary cells (arrows) based on morphology (arrows) (bar, 50μm). (B) Representative immunofluorescence for $Ki67^+$ pCK^+ LPCs (arrows) (bar, 20μm). (C) pCK^+ LPC, (D) $Ki67^+pCK^+$ LPC, and (E) pCK^+ biliary cell numbers. Data represent pooled means ± SEM of two independent experiments, n = 6-8. *p <0.05.

Figure 3. Kupffer cell depletion reduces hepatic damage. Mice were injected with saline, PBS liposomes (PL), or clodronate liposomes (CL). They were then placed on normal chow (NC) or CDE diet 24h after the injections. The mice were again injected with saline, PL, or CL 24h after being placed on diet, and sacrificed at times indicated. (A) Representative Oil Red O

histochemistry and TUNEL immunohistochemistry after 2 days on the diet (bars, 50μm). Quantitation of (B) steatosis, as indicated by Oil Red O staining, (C) serum ALT activity, (D) $TUNEL^+$ hepatocytes. Data represent means ± SEM, n = 3-4. *p <0.05.

Figure 4. Kupffer cell depletion reduces hepatic LPC mitogen levels. Mice were injected with saline, PBS liposomes (PL), or clodronate liposomes (CL). They were then placed on normal chow (NC) or CDE diet 24h after the injections. The mice were again injected with saline, PL, or CL 24h after being placed on diet, and sacrificed after 6 days on diet. (A) TNFα, (B) IFNγ, (C) HGF, and (D) IL-6 mRNA expression levels. (E) Representative αSMA liver immunohistochemistry after 6 days on diet (bar, 100μm). (F) αSMA quantitation. (G) $Ly6G^+$ neutrophil, (H) $B220^+$ B cell, and (I) $CD4^+$ T cell numbers. Data represent pooled means ± SEM of two independent experiments, n = 6-8. *p <0.05.

Figure 5. Monocyte adoptive transfer induces KC proliferation. (A) Experimental design. Mice were placed on normal chow or CDE diet. Monocytes were isolated from CD45.1 mouse bone marrow and injected intravenously into $CD45.2^+$ C57Bl/6 recipients 2 days after mice were placed on CDE diet. Another group on the CDE diet and mice on normal chow were injected with PBS. Mice were sacrificed after 6 days on diet. (B) Enrichment of $CD115^+$ monocytes after MACS separation. (C) $CD11b^+$ monocyte, (D) $CRIg^+$, and (E) $F4/80^+$ Kupffer cell numbers. (F) Representative immunofluorescence showing $Ki67^+CRIg^+$ Kupffer cells, as indicated by white arrows (bar, 20μm). (G) $Ki67^+CRIg^+$ Kupffer cell numbers. Data represent means ± SEM, n = 4-5. *p <0.05.

Figure 6. Monocyte adoptive transfer induces LPC proliferation. Mice were placed on normal chow or CDE diet. Monocytes were isolated from CD45.1 mouse bone marrow and injected

intravenously into CD45.2$^+$ C57Bl/6 recipients 2 days after mice were placed on CDE diet. Another group on the CDE diet and mice on normal chow were injected with PBS. Mice were sacrificed after 6 days on diet. (A) Representative pan cytokeratin (pCK) immunohistochemistry (bar, 50μm). (B) pCK$^+$ LPCs, (C) Ki67$^+$ pCK$^+$ LPCs, and (D) pCK$^+$ biliary cell numbers. (E) Representative pCK and HNF4α immunohistochemistry on serial sections. Arrows indicate HNF4α$^+$ pCK$^+$ LPCs. (F) HNF4α$^+$ pCK$^+$ LPC numbers. Data represent means ± SEM, n = 4-5. *p <0.05.

Figure 7. Monocyte adoptive transfer enhances hepatic damage, and increases TNFα expression. Monocytes were isolated from CD45.1 mouse bone marrow and injected into CD45.2$^+$ C57Bl/6 recipients 2 days after mice were placed on CDE diet. Another group on the CDE diet and mice on normal chow were injected with PBS. Mice were sacrificed after 6 days on diet. (A) Serum ALT activity, (B) weight loss ($^{\#}$p<0.05 vs ND + CDE, $^{\wedge}$p<0.05 vs CDE + PBS), (C) steatosis, as indicated by Oil Red O staining. (D) TNFα mRNA levels. (E) αSMA quantitation. (F) Ly6G$^+$ neutrophil, (G) B220$^+$ B cell, (H) CD4$^+$ T cell numbers. Data represent means ± SEM, n = 4-5. *p <0.05.

Figure 8. Kupffer cell and monocyte-derived macrophage interaction during induction of LPC-mediated liver regeneration. Kupffer cells (KC) are identified immunohistochemically by CRIg and F4/80 expression, and monocyte-derived macrophages (MDM) by CD11b expression. Kupffer cells initiate MDM accumulation, which in turn induces hepatocyte apoptosis. Monocyte-derived macrophages also induce KC proliferation, and neutrophil, B cell, and T cells recruitment. Monocyte-derived macrophages and KCs activate hepatic stellate cells (HSC). Monocyte-derived macrophages, KCs, HSC, neutrophils, and B cells provide TNFα for LPC proliferation.