A TLR2/S100A9/CXCL-2 signaling network is necessary for neutrophil recruitment in acute and chronic liver injury in the mouse. 

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A TLR2/S100A9/CXCL-2 signaling network is necessary for neutrophil recruitment in acute and chronic liver injury in the mouse

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Introduction

Hepatic infiltration by neutrophils and their subsequent activation are a rapid response to sterile and non-sterile tissue injury [1]. Following extravasation into the parenchyma, neutrophils directly interact with hepatocytes via their surface LFA-1 (CD11b/CD18) and Mac-1 receptors. Engagement of the latter stimulates production of reactive oxygen species (ROS), which is important for host-defense but can be cytotoxic; neutrophil-derived ROS can diffuse into adjacent hepatocytes and either directly induce cellular damage or more likely cause mitochondrial dysfunction and necrosis [2]. Additionally, degranulation of neutrophils results in the release of proteases that can contribute to hepatocellular damage and death [3]. Clinical conditions, with evidence for neutrophil activation and hepatocellular death, include alcoholic hepatitis [4], acetaminophen (APAP)-induced acute liver injury [2], and ischemia-reperfusion injury [5]. The extent of neutrophil infiltration in alcoholic hepatitis is correlated with disease severity [6]. There is experimental evidence for neutrophil-mediated cell death in liver injury models including acute alcoholic hepatitis [7]. By contrast, a role for neutrophils in chronic liver disease is less clear. Cirrhosis is associated with elevated levels of circulating and hepatic neutrophil chemokines such as IL-8, while hepatic neutrophils are also described to be...
a feature of progressive fibrotic disease [8]. However, experimental models of biliary liver disease suggest a minor if any functional contribution of neutrophils to fibrogenesis [9]. But it remains likely that persistence of activated neutrophils in chronic liver disease is contributory to disease progression and outcome, including hepatocellular carcinoma [10]. As such, a better understanding of the regulation of neutrophil recruitment is important, as is the identification of molecular targets that might be exploited for therapeutic manipulation of neutrophils in diseased tissues.

McDonnell et al. reported that a dynamic multistep network of directional cues serves to recruit neutrophils to sites of cellular damage under sterile conditions. At least three key mechanisms were identified: (i) Activation of the Nlrp3 inflammasome by ATP released from necrotic cells, which promoted adherence of circulating neutrophils to sinusoids, (ii) generation of a chemokine gradient directing neutrophils to the site of cellular damage, and (iii) formyl-peptide signals from necrotic cells that help guide neutrophils through the sinusoids to the site of injury [11]. Activation of Toll-like receptors (TLRs) also facilitates neutrophil recruitment and recent studies have highlighted the increased expression and functional importance of TLR2, 4, and 9 in acute and chronic liver disease [12].

Here we have used a mouse gene knockout approach to investigate the role of TLR2 in the response to toxic (carbon tetrachloride, CCl₄) liver injury. We demonstrate a critical role for TLR2 (but not TLR4) for recruitment of neutrophils to the injured liver. TLR2 was required for expression of the CXCL-1 (KC) CXCL-2 (MIP-2) neutrophil chemokines by hepatic macrophages. In addition, the myeloid-related proteins Mrp-8 (S100A8) and Mrp-14 (S100A9) were also induced in response to liver injury and were responsive to TLR2 activation. Under normal physiological conditions S100A8 and S100A9 are expressed in neutrophils, monocytes, and eosinophils [13], but following tissue damage they are induced in epithelial and endothelial cells and upon secretion act as powerful leukocyte chemoattractants [14]. S100A8 and S100A9 form functional homodimers and heterodimers (the latter known as Calprotectin). Mice lacking S100A9 also lack S100A8 due to instability of the S100A8 protein in the absence of S100A9 protein; hence s100a9-/− mice fail to express Calprotectin under resting or injury-induced states. We show here that s100a9-/− mice display a similar phenotype to tlr2−/− animals with defective induction of hepatic CXCL-2 induction and reduced neutrophil recruitment. However, neither deficiency in TLR2 or S100A9 or antibody-mediated neutrophil depletion had any impact on activation of hepatic stellate cells (HSCs) or wound-repair. Hence, a hepatic TLR2-S100A9-CXCL-2 pathway may be an interesting target for the selective manipulation of neutrophils in acute and chronic liver disease.

Materials and methods
In vivo models of rodent chronic liver injury and fibrogenesis

Wt, tlr2−/− [15], tlr4−/− [16], and s100a9−/− [17] mice were provided by Prof M. Karin, Prof E. Seki and Prof N. Hogg. Single intraperitoneal injection of CCl₄ at a dose of 2 μl (CCl₄, olive oil, 1:3, [v:v])/g body weight was administered for 8, 24, 48, and 72 h to 8–10 week old male littermates. 8–10 week old male C57Bl/6 mice were injected with pure LTA at 250 μg/mouse 30 min prior to acute CCl₄ challenge. Mice were pre-treated with Ly-6G or Ly6G control antibody for 12 h before LTA injection. Animals were culled at 48 h post-CCl₄ injection. At least 5 animals were used per treatment group.

8–10 week old male s100a9−/− and wt littermate mice were injected with CCl₄ intraperitoneally (IP) twice a week at a dose of 2 μl (CCl₄, olive oil, 1:3, [v:v])/g body weight during 8 weeks or bile duct ligation was performed as previously described for 14 days [18]. For the chronic CCl₄ model, animals were culled either at 24 h (peak) or 7 days (recovery) after the last CCl₄ injection. At least 7 animals were used per group of treatment.

Statistical analysis

Data is expressed as mean ± S.E.M. (standard error). A minimum of 5 animals per group were used in the experimental animal models. All p values were calculated using a two tailed paired Student’s t test or a one way ANOVA and “p < 0.05 or “p < 0.01 was considered statistically significant.

Results

Neutrophils are a common feature of human liver disease

Prior to investigating the role of TLRs in neutrophil recruitment we confirmed that these cells are present in diseased human liver. As shown in representative Neutrophil elastase (NE+) stained liver sections (Fig. 1A), neutrophils are present in acute alcoholic hepatitis (AAH), acetaminophen acute liver failure (APAP ALF), primary biliary cirrhosis (PBC), primary sclerosing cholangiatis (PSC), alcoholic liver disease (ALD), and non-alcoholic steatohepatitis (NASH) suggestive of neutrophils being a common feature of the diseased liver irrespective of the cause of the underlying injury. As anticipated APAP was associated with high numbers of hepatic neutrophils relative to chronic liver diseases, of the latter neutrophils were highest in PBC livers (Fig. 1B).

TLR2 is essential for optimal neutrophil recruitment to the damaged liver

To determine if TLR2 is a regulator of neutrophil recruitment we administered CCl₄ to tlr2−/− mice and compared their acute response with wt and tlr4−/− mice at 24, 48, and 72 h. Serum ALT measurements indicated no significant differences in the degree of hepatic damage between the three phenotypes with the exception of tlr4−/− at 72 h where there was a trend towards higher levels of damage (Supplementary Fig. 1A). IHC staining (NIMP-1) and counting of neutrophils demonstrated the anticipated appearance of high numbers of hepatic neutrophils at 24 h in wt and tlr4−/− injured mice (Fig. 1C). By contrast there was a 3-fold lower number of neutrophils in tlr2−/− livers at this time point. In all genotypes, hepatic neutrophils declined to near base-line levels by 48 h. To assess effects of TLR deletions on the acute fibrogenic and regenerative responses following liver damage we performed morphometric analysis of α-SMA+ myofibroblasts (Fig. 1D) and counted PCNA+ hepatocytes (Fig. 1E) respectively. As expected these IHC markers were elevated at 48 and 72 h but no differences were observed between the three genotypes suggesting normal wound-healing in tlr2−/− liver. A normal fibrogenic response in tlr2−/− was confirmed by similar induction of transcripts for α-SMA and Collagen I between the genotypes (Supplementary Fig. 1B–C). We conclude that TLR2 is required for optimal recruitment of neutrophils to the hepatic parenchyma, but is dispensable for subsequent wound-repair/ fibrogenesis and regenerative responses. However, as previously
Published [19,20] neutrophil depletion dramatically reduced APAP-induced liver damage (Supplementary Fig. 2A–C).

Since the role of neutrophils has not been formally addressed in the CCl4 injury model, we determined the effects of Ly-6G antibody-mediated depletion of circulating neutrophils on fibrogenesis at the 48 h time point in wt animals. Ly-6G treatment led to a dramatic reduction in numbers of circulating CCL2-induced hepatic neutrophils (Fig. 1F and Supplementary Fig. 3A–C). Specificity was confirmed by lack of effect of Ly-6G on hepatic macrophages (Supplementary Fig. 3D). IHC and mRNA analysis of α-SMA revealed no requirement for neutrophils in the fibrogenic response (Fig. 1G and H). Moreover, treatment of CCL2-injured mice with the TLR2 agonist LTA was also without effect on fibrogenesis (Fig. 1G and H).

TLR2 is required for hepatic induction of neutrophil attractants CXCL-2 and TNF-α

Expression CXCL-1 and CXCL-2 can be found across a broad range of human liver injuries implicating these chemokines in the recruitment of hepatic neutrophils (Supplementary Fig. 4). The expression of CXCL-1 and CXCL-2 and the murine neutrophil chemokine attractants TNF-α and S100A9 was examined in CCl4 damaged mouse livers. Hepatic CXCL-2, TNF-α, and S100A9 transcripts were all robustly induced in wt at 24 h post-CCl4 with subsequent decline in their expression (Fig. 2A and B). By contrast, CXCL-1 was only modestly induced and peaked at 48 h (Fig. 2B). Absence of TLR2 was associated with blunted CXCL-2 and TNF-α responses (Fig. 2A), but at this time point had no impact on the induction of S100A9 (Fig. 2B). ELISA confirmed TLR2 is required for induction of CXCL-2 protein expression (Supplementary Fig. 1D). We next determined the combined effects of LTA and CCl4 in hepatic neutrophil chemokine receptors comparing wt with 

This result contrasted with our earlier observation (Fig. 2B) where CCl4 alone at 24 h post-injury was associated with TLR2-independent induction of S100A9 (Fig. 2B). We therefore confirmed the potential for TLR2 activation to stimulate S100A9 by treating cultured ex vivo neutrophils with LTA. This treatment increased S100A9 expression at the protein level and could be blocked by incubation of neutrophils with anti-TLR2 antibody (Supplementary Fig. 5A). Phosphorylation of P38 was monitored as a positive assay control. As expected, P38 phosphorylation was successfully blocked by an anti-TLR2 antibody after LTA treatment (Supplementary Fig. 5B). IHC analysis confirmed that liver injury was associated with de novo induced expression of S100A9 in hepatocytes in addition to the anticipated expression in neutrophils (Fig. 2H).

Induction of neutrophil recruitment and hepatic CXCL-2 requires Calprotectin

As Calprotectin is induced by CCl4, we were interested to determine a role in the hepatic wound healing response. However, absence of S100A9 made no impact on CCL2-induced liver damage (Fig. 3A) or the fibrogenic response as determined by hepatic α-SMA protein expression (Fig. 3B), morphometry of α-SMA+, and Collagen I gene expression (Fig. 3C–D). Normal induction of PCNA expression in S100A9−/− livers suggested no major role for S100A9 in the regenerative response (Fig. 3B). However, CCL2-induced neutrophil recruitment was defective in S100A9−/− livers (Fig. 3E). This phenotype was associated with a trend towards reduced expression of CXCL-2 at 24 h (Fig. 3F), which by IHC staining was mainly expressed in macrophages (Fig. 3G). CXCL-1 was also reduced at the later time point of 48 h (Fig. 3H) and was expressed in hepatocytes and macrophages (Supplementary Fig. 6A). These data suggest that both TLR2 and Calprotectin function in the recruitment of hepatic neutrophils and are required for optimal induction of CXCL-2. Given this signalling cross-talk, we determined if hepatic TLR2 expression is influenced by S100A8/S100A9. As shown in Supplementary Fig. 6B, we observed no impact of S100A9 knockout on hepatic TLR2 transcript expression in control uninjured (olive oil) or 24 h injured mice, this suggests that Calprotectin does not operate upstream of TLR2 in controlling neutrophil recruitment. However, we did observe a substantial 10-fold induction of TLR2 transcript at 48 h in wt liver, which was completely absent in s100a9−/− mice. We conclude that at the whole tissue level there is likely to be complex time-dependent, two-way signalling cross-talk between TLR2 and Calprotectin.

Defective neutrophil recruitment and CXCL-2 expression do not impact on wound-healing in chronic liver disease

To determine the impact of suppressed neutrophil recruitment in a more complex model of chronic liver disease we investigated the effects of S100A9 deletion on wound-repair and regeneration in an 8-week model of iterative CCl4-induced liver disease. Two end-points were selected; 1-day (peak disease) and 7-day
Fig. 2. TLR2 is required for hepatic induction of the neutrophil attractants CXCL-2 and TNF-α. (A) CXCL-2, TNF-α, (B) S100A9, CXCL-1 mRNA expression in whole liver of wt, tlr2<sup>−/−</sup>, tlr4<sup>−/−</sup> mice after acute CCl<sub>4</sub> treatment for 24, 48, 72 h. (C) Average NIMP-1+ cells/field. (D) CXCL-1, (E) TNF-α, (F) S100A8, (G) CXCL-2, and (H) S100A9 mRNA expression in whole liver and representative 400× pictures of (G) CXCL-2 and (H) S100A9 IHC of wt, and tlr2<sup>−/−</sup> after acute CCl<sub>4</sub> treatment for 8 h ± LTA. *p ≤ 0.05; **p ≤ 0.01.
**A**

Blot quantification indicating changes in protein expression over time.

**B**

α-SMA, PCNA, and β-actin protein expression levels.

**C**

Graph showing the average percentage of α-SMA+ area per field.

**D**

Graph showing the relative liver damage (RLTD) fold of liver oil.

**E**

Graph showing the average number of NIMP-1+ cells per field.

**F**

Graph showing the relative liver damage (RLTD) fold of CXCL-2.

**G**

Immunohistochemistry images showing changes in protein expression.

**H**

Graph showing the relative liver damage (RLTD) fold of CXCL-1.
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(recovery) post-final CCl4 administration, this to allow us to determine effects of S100A9 deletion on wound healing and subsequent spontaneous repair and regeneration following cessation of injury. As anticipated we observed high numbers of hepatic neutrophils in wt mice at peak injury that dramatically declined with recovery (Fig. 4A). Numbers of neutrophils at peak injury in s100a9+/− livers were at roughly 50% of the levels found in wt, again these declined to base-line with recovery. Despite this reduced level of hepatic neutrophils we observed no differences in collagen deposition by Sirius Red staining (Fig. 4B and D), numbers of hepatic macrophages by F4/80 IHC (Fig. 4C and D), numbers of αSMA+ myofibroblasts (Fig. 4D and E) or expression of collagen I mRNA and αSMA protein (Fig. 4F and F). In addition, we detected similar levels of PCNA between wt and s100a9+/− livers (Fig. 4F), qRT-PCR profiling (Fig. 4G) revealed high levels of cytokines (IL6, TNF-α) and chemokines (CCL-2, CCL-5, CCL-1, and CXCL-2) at peak disease, all of which declined to low baseline levels with recovery. In s100a9+/− we observed significantly reduced levels of CXCL-2 compared with wt, this in agreement with our earlier observation of reduced chemokine expression in acute injury (Fig. 3F). Additionally a trend towards lower expression of the neutrophil attractants TNF-α and CXCL-1 was also noted in the absence of S100A9. ELISA measurements confirmed the induction of CXCL-2 and the requirement of s100a9 for this response (Fig. 4H). BDL-induced fibrosis was also relatively unaffected in s100a9+/− mice (Supplementary Fig. 7A-D) although we did observe a non-significant trend towards reduced levels of collagen deposition. These data suggest that in chronic liver disease targeting neutrophil recruitment via the TLR2/Calprotectin/CXCL-2 pathway would have minimal impact on efficiency of wound-repair and regeneration.

Discussion

Neutrophils are recruited to the hepatic sinusoids in acute liver injury and then migrate into the hepatic parenchyma in response to macrophage-derived CXC chemokines and other immune mediators released from dying/dead hepatocytes [21]. In alcoholic liver disease, APAP-induced acute liver injury and during ischemia-reperfusion, activated neutrophils within the parenchyma are potentially harmful as they can promote hepatocellular stress and necrosis contributing to liver failure [2]. Hence, illuminating the molecular regulators of neutrophil recruitment and extravasation is of interest for a better understanding of hepatic immunity and for developing strategies aimed at limiting collateral tissue damage caused by activated neutrophils. Here we report that TLR2 and S100A9 are required in a non-redundant manner for optimal induction of hepatic CXCL-2 and recruitment of neutrophils in response to hepatocellular damage. The expression of TLR2 has been detected on a number of different resident liver cell types including Kupffer cells, hepatic stellate cells, hepatocytes, cholangiocytes and sinusoidal endothelial cells [22]. In previous studies by the Seki lab employing TLR2 bone marrow chimeric mice, Kupffer cells were found to be the dominant cell type through which hepatic inflammation is mediated by TLR2 [23]. Most likely from our IHC studies CXCL-2 is chiefly induced within Kupffer cells via intracellular TLR2 signalling with more modest, possibly secondary expression appearing in damaged hepatocytes, which would be in agreement with other reports in the literature [24,25]. However the cellular source of CXCL-2 may be dependent on the nature of the liver injury. Xu et al. showed that α-Naphthilothioisocyanate (ANIT), which causes severe cholestatic injury in the mouse was associated with rapid induction of CXCL-2 primarily from periportal hepatocytes [26]. Although, of note, the authors observed only a modest induction of CXCL-2 in cultured hepatocytes directly exposed to ANIT [26]. S100A9 is mainly associated with granulocytes, but was induced along with S100A8 in hepatocytes following liver damage. This observation is in keeping with others who have documented inducible expression of S100A8 and S100A9 in epithelial cells of injured tissues [14]. Furthermore, S100A8 and S100A9 are expressed by HCC tumour cells as well as by other tumours of epithelial origin including lung, breast, gastric, and prostate [27]. Hence, we propose that dual signalling via TLR2 on Kupffer cells and S100A8/S100A9 from hepatocytes combine to generate CXCL-2 chemokine gradients for guidance of neutrophils into the hepatic sinusoids and parenchyma respectively.

Marques et al. recently showed that CXCR2 antagonism in mice injured with APAP suppressed hepatic neutrophil recruitment by 50% [28]. In this latter study combined antagonism of CXCR2 and PPR1 resulted in more substantive suppression of neutrophil migration; this is in agreement with McDonald et al., who reported a cooperation between CXCR2 chemokines and mitochondrial formyl peptides for guidance of neutrophils to sites of necrosis [29]. In the ANIT cholestasis model a 50% reduction in the influx of neutrophils was observed in cxcr2+/− mice [26], which closely agrees with data from Marques et al. when employing a pharmacological approach for blockade of CXCR2 in the APAP model. Hence, CXCR2 chemokines are not absolutely required for neutrophil influx to injured liver, but instead cooperate with other neutrophil attractants such as ATP, formyl peptides and TNF-α to ensure optimal neutrophil guidance [11]. Our data build on these findings by revealing that TLR2 and S100A8/S100A9 operate in neutrophil guidance most likely upstream of CXCL-2 (and to a lesser extent CXCL-1 and TNF-α) by regulating its expression in response to tissue damage. Signalling pathways downstream of TLR2 and S100A8/S100A9 both converge on activation of NF-κB, for which CXCL-1 and CXCL-2 are known target genes [30], this providing one plausible explanation for the similar regulatory functions of TLR2 and S100A8/S100A9 in neutrophil recruitment.

A key finding of our work was that acute repair and regenerative responses were normal in TLR2 and S100A9 knockout mice, and furthermore fibrosis caused by chronic injury with CCl4 was unaffected in S100A9 knockout mice. Previous studies have shown little or no influence of neutrophilic inflammation on fibrosis [9], in addition ANIT-induced fibrosis is unaffected in cxcr2+/− animals [26]. Hence, our data are supportive of neutrophils and CXCR2 chemokines being redundant for fibrogenesis. The role of TLR2 in fibrosis is unclear since there are apparently contradictory data in the recent literature. Seki et al. observed that while itrlr4−/− mice are attenuated for liver fibrosis induced by CCl4 and BDL,

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Fig. 3. S100A9 is required for an effective neutrophil recruitment and hepatic expression of CXCL-2. (A) Serum analysis of ALT levels, (B) α-SMA, PCNA and β-actin western blot of whole liver lysates, (C) morphometric analysis of α-SMA+ area/field, (D) Coll1A1 mRNA expression in whole liver, (E) average NIMP-1+ cells/field with representative pictures at 400×, (F) CXCL-2 mRNA expression, (G) CXCL-2 representative IHC pictures at 400×, cytosolic macrophage staining (black arrows), and (H) CXCL-1 mRNA expression in whole liver of wt and s100a9+/− mice after acute CCl4 treatment for 24, 48, 72 h. *p < 0.05; **p < 0.01.
by contrast \(\text{tlr}2^{-/-}\) developed BDL-induced fibrosis in a similar manner to wt mice [31]. However, in subsequent studies Hartmann and colleagues found that TLR2 deficient mice re-activated and housed in specific pathogen-free (sp-f) protocols were resistant to BDL- and CCL4-induced fibrosis [32]. In the latter study, it was suggested that TNF-\(\alpha\) produced by TLR2+ monocytes in the intestinal lamina propria mediates intestinal barrier disruption, resulting in translocation of bacteria and their products across the mucosal barrier and on to the liver via the portal circulation, where they enhance fibrogenesis. The Seki lab also recently reported that \(\text{tlr}2^{-/-}\) mice are protected from progression of CDAA-induced NASH to fibrosis and they suggested this was due to an impaired inflammatory reaction associated with reduced expression of NLRP3 inflammasome components [23]. Hence, neither of these latter studies suggest a direct role for TLR2 in hepatic stellate cell activation, but instead indicate that absence of TLR2 results in failure of inflammatory pathways that are upstream of our observation. Normal induction of \(\alpha\)-SMA and \(\text{collagen I}\) expression in \(\text{tlr}2^{-/-}\) following acute injury with CCL4 argues against a direct role for TLR2 in hepatic stellate cell activation.

In summary we have advanced current knowledge regarding the mechanisms by which neutrophils are guided to the injured liver and identified TLR2 and S100A8/S100A9 as key regulators of hepatic CXCL-2 expression and neutrophil recruitment. This new information may be of use in developing strategies for limiting neutrophil-mediated tissue damage in acute liver injuries.

**Conflict of interest**

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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**Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013.12.005.

**Fig. 4.** \(s100a9^{-/-}\) present a normal wound-healing response despite defective neutrophil recruitment and CXCL-2 expression. (A) Average NIMP-1+ cells/field with representative pictures of peak time point at 400×. Morphometric analysis of (B) Sirius Red, (C) F4/80 positive area/field, (D) Representative pictures at 100× of \(\alpha\)-SMA, F4/80 and Sirius Red staining from peak group of wt and \(s100a9^{-/-}\) mice. (E) Morphometric analysis of \(\alpha\)-SMA+ area/field and Col1A1 mRNA expression. (F) \(\alpha\)-SMA, PCNA and \(\beta\)-actin western blot of whole liver from peak group of wt and \(s100a9^{-/-}\) mice. (G) IL6, CCL-2, CCL-5, TNF-\(\alpha\), CXCL-1 and CXCL-2 mRNA expression. (H) CXCL-2 ELISA in whole liver of 8 week CCl4 treated wt and \(s100a9^{-/-}\) mice. \(p<0.05; \quad **p<0.01.\)


