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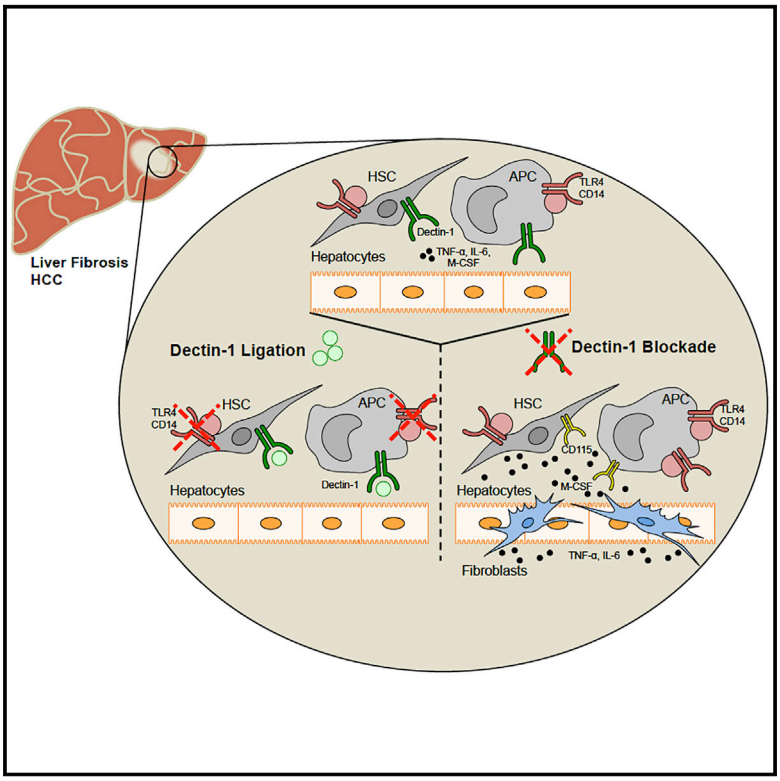
Dectin-1 Regulates Hepatic Fibrosis and Hepatocarcinogenesis by Suppressing TLR4 Signaling Pathways

Graphical Abstract



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In Brief

Seifert et al. show Dectin-1 protects against chronic liver disease by suppressing TLR4 signaling via CD14 and M-CSF. This suggests that Dectin-1 is an attractive target for experimental therapeutics in hepatic fibrosis and transformation with implications for a role for Dectin-1 in suppression of sterile inflammation, inflammation-induced oncogenesis, and endotoxemia.

Highlights

- Dectin-1 expression is upregulated in hepatic fibrosis and liver cancer
- Deletion of Dectin-1 exacerbates liver fibrosis and accelerates hepatocarcinogenesis
- Dectin-1 protects against liver disease by suppressing TLR4 signaling
- Dectin-1 mitigates TLR4 and CD14 expression, which is regulated by M-CSF expression

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Cell Reports
Article

Dectin-1 Regulates Hepatic Fibrosis and Hepatocarcinogenesis by Suppressing TLR4 Signaling Pathways

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SUMMARY

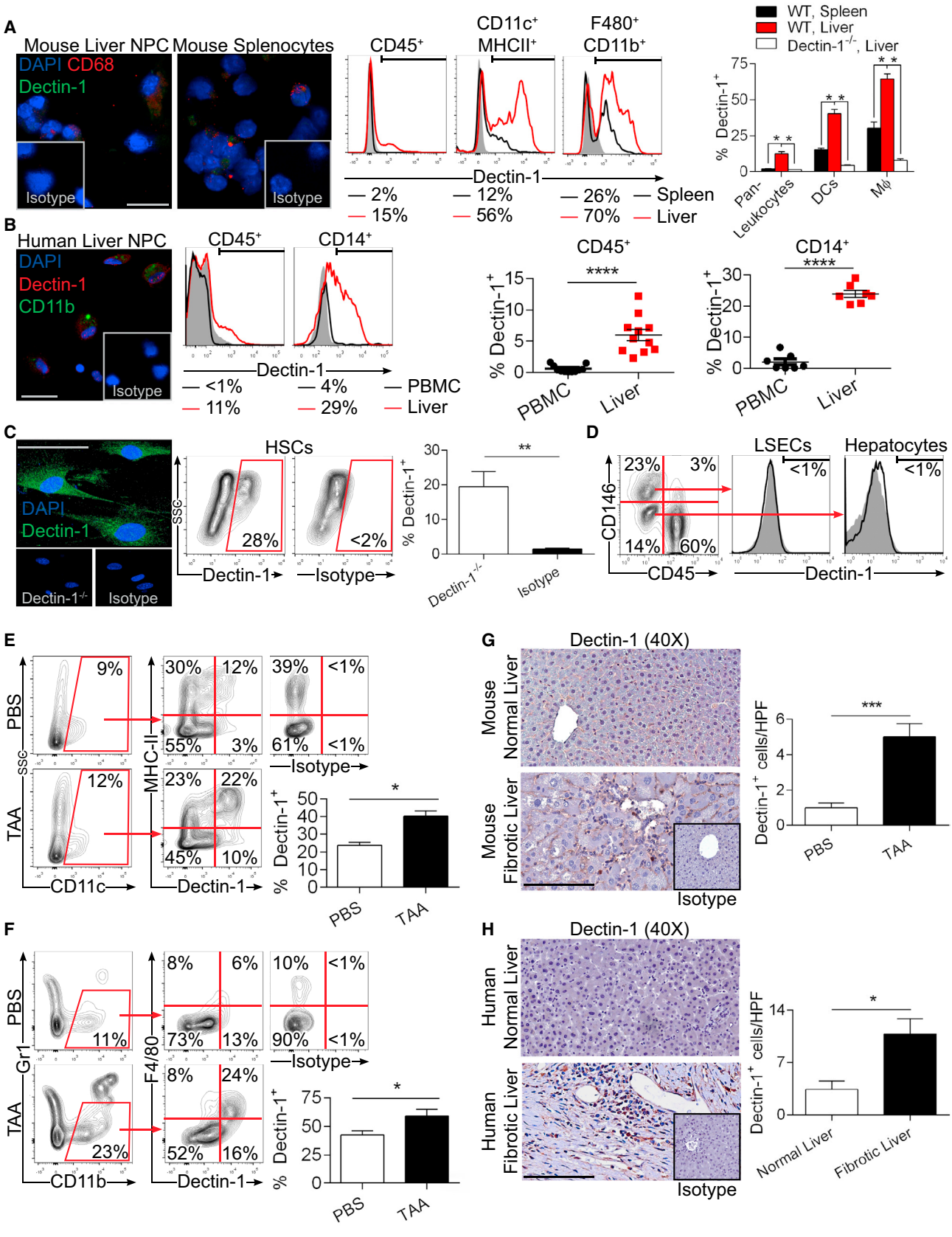
Dectin-1 is a C-type lectin receptor critical in antifungal immunity, but Dectin-1 has not been linked to regulation of sterile inflammation or oncogenesis. We found that Dectin-1 expression is upregulated in hepatic fibrosis and liver cancer. However, Dectin-1 deletion exacerbates liver fibro-inflammatory disease and accelerates hepatocarcinogenesis. Mechanistically, we found that Dectin-1 protects against chronic liver disease by suppressing TLR4 signaling in hepatic inflammatory and stellate cells. Accordingly, *Dectin-1*$^{-/-}$ mice exhibited augmented cytokine production and reduced survival in lipopolysaccharide (LPS)-mediated sepsis, whereas Dectin-1 activation was protective. We showed that Dectin-1 inhibits TLR4 signaling by mitigating TLR4 and CD14 expression, which are regulated by Dectin-1-dependent macrophage colony stimulating factor (M-CSF) expression. Our study suggests that Dectin-1 is an attractive target for experimental therapeutics in hepatic fibrosis and neoplastic transformation. More broadly, our work deciphers critical cross-talk between pattern recognition receptors and implicates a role for Dectin-1 in suppression of sterile inflammation, inflammation-induced oncogenesis, and LPS-mediated sepsis.

INTRODUCTION

Hepatic fibrosis—the end result of repeated liver injury—is one of the most significant public health concerns worldwide (Lim and Kim, 2008). Liver injury resulting from a variety of etiologies, including viral hepatitis, toxins, or metabolic disorders, primes hepatocytes to regenerate to replace necrotic or apoptotic hepatic parenchymal cells and simultaneously triggers a robust inflammatory response, which induces hepatic stellate cells (HSCs) to transdifferentiate and express extracellular matrix (ECM) proteins. If injury persists, regeneration eventually fails, and the hepatocytes are replaced by abundant ECM leading to fibrosis and eventually cirrhosis (Bataller and Brenner, 2005). Liver fibrosis is clinically associated with the development of hepatocellular carcinoma (HCC), the third leading cause of cancer-related death worldwide (Franceschi and Raza, 2009).

Toll-like receptor (TLR) ligation is a primary mechanism by which intra-hepatic innate inflammatory cells and HSCs are activated after hepatic injury (Paik et al., 2003; Seki et al., 2007). TLRs belong to a broader category of evolutionarily conserved pattern recognition receptors (PRRs), which link inflammatory responses to pathogenic or sterile inflammatory stimuli (Kawai and Akira, 2010). A primary role of TLR4 expressed on innate hepatic leukocytes is to respond to lipopolysaccharide (LPS) (De Creus et al., 2005). In addition, in the context of chronic liver injury, TLRs can ligate intra-hepatic "danger molecules," which include by-products of inflammatory injury and cellular necrosis, collectively denoted damage-associated molecular patterns (DAMPs) (Paik et al., 2003; Seki et al., 2007). As such, TLR ligation has a critical role in perpetuating sterile inflammation and tissue damage in chronic liver disease. For example, ligation of either TLR4 or TLR9 markedly promotes liver fibrosis, whereas deletion of either receptor is protective (Aoyama et al., 2010). Similarly, TLR4 ligation by LPS derived from selected intestinal microbiota promotes hepatocellular carcinogenesis (Dapito et al., 2012).

Dectin-1 is a trans-membrane receptor and member of the C-type Lectin family of PRRs. Dectin-1 is required for the innate immune response to fungal pathogens (Vautier et al., 2012). Ligation of fungal wall β-glucans by Dectin-1 recruits the CARD9 adaptor protein, which phosphorylates Syk, thereby initiating an anti-fungal immune response (Gross et al., 2006; Strasser et al., 2012). However, Dectin-1 does not have an established role in modulating sterile inflammation in liver fibrosis



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or in any physiologic context. Our preliminary findings revealed that Dectin-1 is highly upregulated in liver fibrosis and in HCC in both mice and humans. Based on these data, we postulated that Dectin-1 signaling modulates hepatic fibro-inflammatory disease and oncogenesis. We found that Dectin-1 deletion accelerates hepatic fibrosis and hepatocellular tumorigenesis, whereas Dectin-1 ligation is protective. Our mechanistic work suggests that Dectin-1 protects against liver fibrosis via suppression of TLR4 activation by directly mitigating expression of TLR4 and its co-receptor CD14. We show that Dectin-1 modulation of CD14 expression on hepatic innate inflammatory cells is contingent on macrophage colony stimulating factor (M-CSF). This is direct evidence of a role for Dectin-1 in non-pathogen-driven sterile inflammation and suggests that targeting Dectin-1 may be an attractive strategy for experimental therapeutics in hepatic fibrosis and HCC. Moreover, our work has pleiotropic implications for understanding reciprocal regulation between families of PRRs, which is critical for maintaining physiologic homeostasis in health and disease.

RESULTS

Dectin-1 Expression Is Increased in Liver Fibrosis

To assess the potential impact of Dectin-1 signaling in liver disease, we examined Dectin-1 expression in both hepatic inflammatory and parenchymal cells. Liver leukocytes, specifically dendritic cells (DCs) and macrophages, expressed high Dectin-1 compared with their counterparts in the murine spleen (Figure 1A). Similarly, human liver leukocytes, and in particular $CD14^+$ monocytic cells, expressed elevated Dectin-1 compared with peripheral blood mononuclear cells (PBMCs) (Figure 1B). Hepatic stellate cells also expressed high Dectin-1 on immune-fluorescence microscopy and flow cytometry (Figure 1C). Conversely, liver parenchymal cells, including hepatocytes and liver sinusoidal endothelial cells, expressed minimal Dectin-1 (Figure 1D). Moreover, in hepatic fibrosis, Dectin-1 expression was upregulated in hepatic DC (Figure 1E) and macrophages (Figure 1F) compared with their expression in normal liver. Accordingly, both murine (Figure 1G) and human (Figure 1H) liver fibrosis were associated with a robust intra-hepatic influx of Dectin-1^+ cells.

Dectin-1 Regulates Hepatic Fibrosis

To test the importance of Dectin-1 signaling in modulating chronic liver disease, we induced hepatic fibrosis in wild-type (WT) or *Dectin-1*$^{-/-}$ mice using Thioacetamide (TAA) or Carbon tetrachloride (CCl_4). Non-fibrotic WT and *Dectin-1*$^{-/-}$ livers exhibited indistinguishable hepatic phenotypes (Figure S1). However, Dectin-1-deficient livers developed markedly exaggerated hepatic fibrosis on histologic analysis in both the TAA (Figures 2A and 2B) and CCl4 (Figures S2A–S2D) disease models. Serum transaminase levels were not differentially elevated in *Dectin-1*$^{-/-}$ animals (Figures 2C, 2D, and S2C). Western blotting confirmed higher hepatic expression of extracellular matrix (ECM) proteins TIMP2, TIMP4, MMP7, and MMP2 in TAA-treated *Dectin-1*$^{-/-}$ liver compared with WT (Figure 2E). Genes that regulate ECM remodeling were also expressed at higher levels in fibrotic *Dectin-1*$^{-/-}$ liver on RT-PCR analysis (Figure 2F). Accordingly, α-SMA expression was elevated in fibrotic *Dectin-1*$^{-/-}$ liver, indicative of enhanced HSC activation (Figure 2G). Furthermore, consistent with more advanced disease (Kuramitsu et al., 2013), there was diminished hepatocyte proliferation in the regenerative nodules of fibrotic *Dectin-1*$^{-/-}$ liver compared with WT (Figure 2H). Notably, mice deficient in Mincle—a C-type lectin receptor akin to Dectin-1—did not exhibit exacerbated hepatic fibro-inflammatory disease after hepatotoxin treatment suggesting specificity of the effect to Dectin-1 (Figures S2E–S2G).

Dectin-1 Regulates Intra-hepatic Inflammation

To test whether Dectin-1 suppresses hepatic inflammation in liver fibrosis, we analyzed the comparative innate immune infiltrates in TAA- or CCl_4-treated WT and *Dectin-1*$^{-/-}$ liver. Fibrotic *Dectin-1*$^{-/-}$ liver exhibited a higher pan-leukocyte infiltrate (Figures 3A, S2A, and S2D), greater neutrophilia (Figure 3B), and a higher influx of $CD68^+$ macrophages (Figure 3C) compared with WT. Consistent with these findings, serum levels of MCP-1 were elevated in fibrotic *Dectin-1*$^{-/-}$ liver (Figure 3D). We previously reported that, whereas DC do not directly influence hepatic fibrosis, they are critical orchestrators of hepatic inflammation in the fibrotic liver via tumor necrosis factor α (TNF-α) production (Connolly et al., 2009). Accordingly, we found that the fraction of DC was increased in the liver of TAA-treated *Dectin-1*$^{-/-}$ mice compared with WT (Figure 3E). Further, intracellular cytokine

Figure 1. High Dectin-1 Expression in Hepatic Leukocytes and in Liver Fibrosis in Mice and Humans

(A) Liver non-parenchymal cell (NPC) suspensions from WT mice were tested for co-expression of Dectin-1 and CD68 by confocal microscopy (scale bar, 10 μm). Similarly, Dectin-1 expression was tested in murine WT and *Dectin-1*$^{-/-}$ fibrotic liver $CD45^+$ pan-leukocytes, DC ($CD11c^+MHCII^+$), and macrophages ($CD11c^-Gr1^-F480^+CD11b^+$) compared with their counterparts in spleen by flow cytometry. Gray histograms represent isotype controls. Representative data and summary data from five separate experiments are shown.

(B) Human liver NPC were tested for co-expression of Dectin-1 and CD11b by confocal microscopy (scale bar, 10 μm). The fraction of Dectin-1^+ cells among $CD45^+$ pan-leukocytes and $CD14^+$ monocytes was compared in human liver versus PBMC by flow cytometry. Representative histograms and summary data are shown.

(C) Dectin-1 expression was tested in cultured murine hepatic stellate cells by both confocal microscopy and flow cytometry compared with *Dectin-1*$^{-/-}$ HSC and isotype control (scale bar, 10 μm).

(D) Murine $CD45^-CD146^+$ liver sinusoidal endothelial cells (LSECs) and $CD45^-CD146^-$ hepatocytes derived from fibrotic liver were tested for expression of Dectin-1 compared with isotype control (gray histogram).

(E and F) Expression of Dectin-1 was tested in $CD11c^+MHCII^+$ DC (E) and $CD11c^-Gr1^-F480^+CD11b^+$ (F) macrophages from control liver versus liver from mice with TAA-induced liver fibrosis compared with isotype control. Representative data and summary data from three experiments are shown.

(G and H) Normal and fibrotic murine (G) and human liver (H) were tested for infiltration of Dectin-1^+ cells by immunohistochemistry compared with isotype control (scale bar, 100 μm).

Each experiment was repeated at least three times with similar results (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,****$p < 0.0001$).

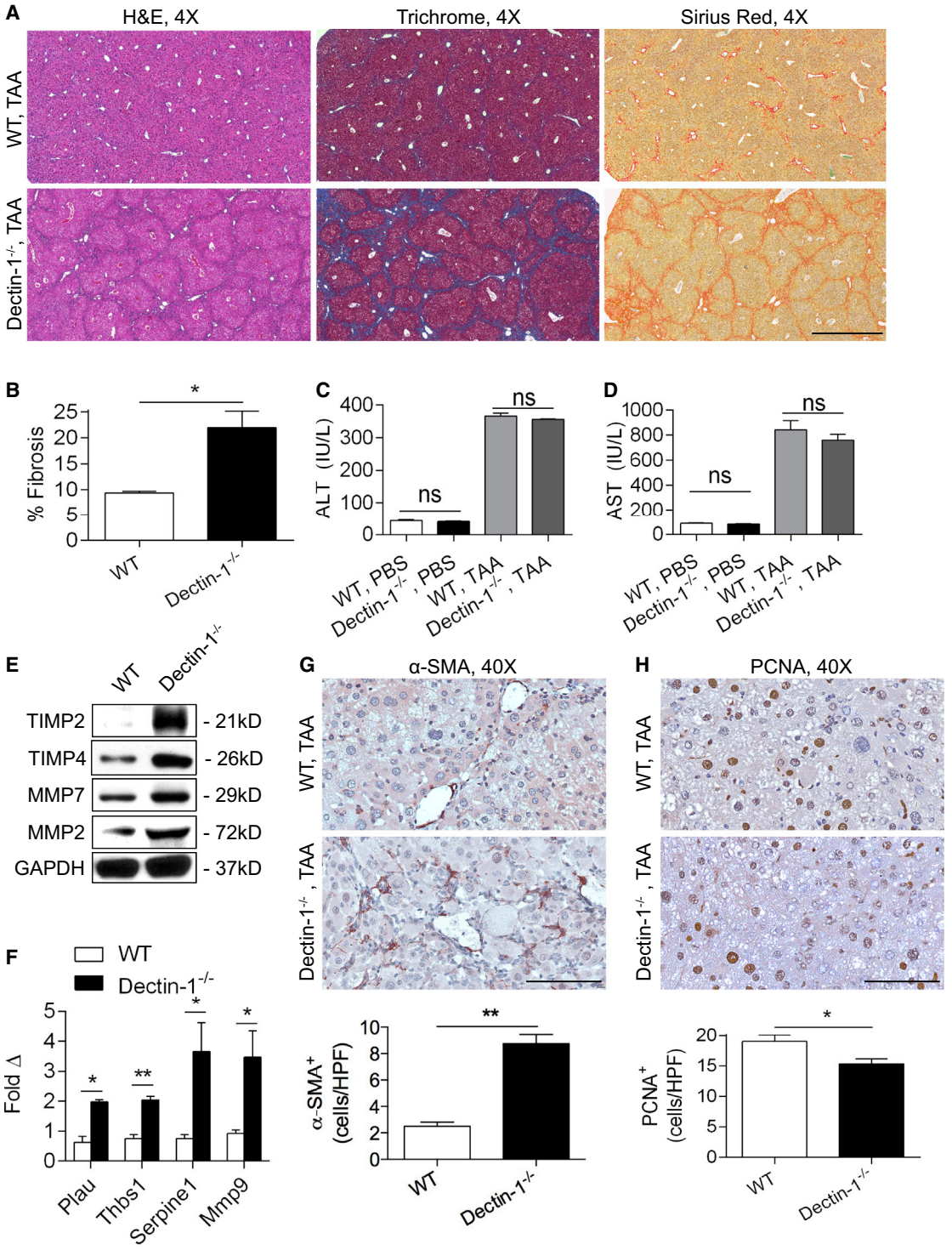

Figure 2. Dectin-1 Deletion Exacerbates Hepatic Fibrosis

WT and *Dectin-1*$^{-/-}$ mice were treated with TAA for 12 weeks.

(A) Livers were harvested and analyzed by H&E, Trichrome, and Sirius Red staining (scale bar, 1 mm).

(B) Fibrosis was quantified based on Trichrome staining using a computerized grid.

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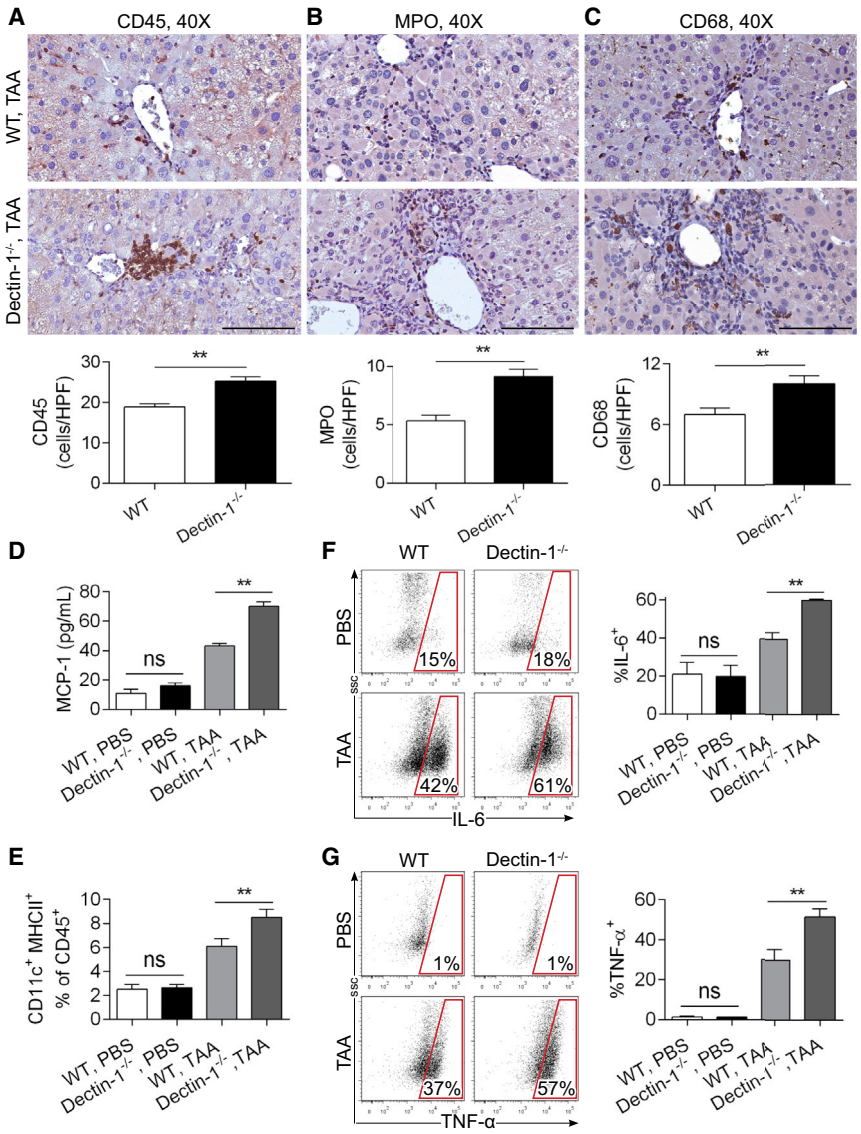

Figure 3. Dectin-1 Regulates Intra-Hepatic Inflammation in Fibrotic Liver

(A–C) WT and *Dectin-1*$^{-/-}$ mice were treated with TAA for 12 weeks. Livers were harvested and analyzed for infiltration with (A) CD45^{+} pan-leukocytes, (B) MPO^{+} neutrophils, and (C) CD68^{+} macrophages (scale bar, 100 μm) by immunohistochemistry. Representative images and summary data are shown.

(D) Serum MCP-1 levels were measured in control and fibrotic WT and *Dectin-1*$^{-/-}$ liver.

(E) The fraction of DC in hepatic NPC suspensions was compared in PBS- and TAA-treated WT and *Dectin-1*$^{-/-}$ liver.

(F and G) Liver MHC II^{+} APC from control and TAA-treated WT and *Dectin-1*$^{-/-}$ liver were tested for (F) expression of IL-6 and (G) TNF-α using intracellular cytokine analysis.

Each experiment was repeated more than three times with similar results (n = 6 mice/group; **p < 0.01).

analysis showed markedly higher interleukin 6 (IL-6) (Figure 3F) and TNF-α (Figure 3G) expression in APC in fibrotic *Dectin-1*$^{-/-}$ liver compared with WT. Collectively, these data suggest that deletion of Dectin-1 results in an expanded and more activated innate inflammatory infiltrate in hepatic fibrosis.

To further assess the effect of Dectin-1 deletion on intra-hepatic inflammation in liver fibrosis, we tested hepatic expression of inflammatory mediators by nanostring analysis. The complement system has recently been demonstrated to play a critical role in the pathogenesis of liver fibrosis in mice and humans (Vasel et al., 2014). We found higher expression of multiple components of the complement system in fibrotic *Dectin-1*$^{-/-}$ liver compared with WT (Figure S3A). Further, Dectin-1 deletion in liver fibrosis resulted in exaggerated expression of pro-inflammatory chemokines (Figure S3B), a diverse array of chemokine receptors (Figure S3C), and genes associated with IL-1, IL-6, and TGF-β signaling (Figure S3D). Notably, expression levels of soluble inflammatory mediators and chemokine receptors were not significantly different between PBS-treated WT and *Dectin-1*$^{-/-}$ liver (Figure S1D). To determine in which compartment Dectin-1 signaling modulated liver fibrosis, we irradiated WT and *Dectin-1*$^{-/-}$ mice and made these animals chimeric using bone marrow derived from WT and *Dectin-1*$^{-/-}$ donors before inducing TAA-mediated liver fibrosis 7 weeks later. Our extent of chimerism was ~95% (Figure S3E). We found that Dectin-1 deletion in both the radio-sensitive and radio-resistant compartments had additive effects on exacerbating fibrosis (Figure S3F).

Dectin-1 Protects against Hepatocellular Carcinogenesis

Hepatocellular carcinoma (HCC) develops almost exclusively in the setting of chronic liver disease (El-Serag, 2007). We found that HCC in humans is associated with a robust infiltrate of Dectin-1^{+} leukocytes (Figure 4A). Tumor cells did not express Dectin-1 (Figures 4A and 4B). However, murine DC and macrophages upregulated their expression of Dectin-1 in HCC nodules

(C–F) Serum levels of ALT and AST (D) were analyzed. Liver tissues were tested for (E) expression of TIMP2, TIMP4, MMP7, and MMP2 by western blotting and for (F) expression of *Plau, Thbs1, Serpine1*, and *Mmp9* by RT-PCR.

(G and H) HSC activation in TAA-treated WT and *Dectin-1*$^{-/-}$ liver was tested by expression of α-SMA (scale bar, 100 μm) (G), and hepatocyte proliferation was assessed using PCNA immunohistochemistry (H) (n = 20 mice/group; *p < 0.05, **p < 0.01).

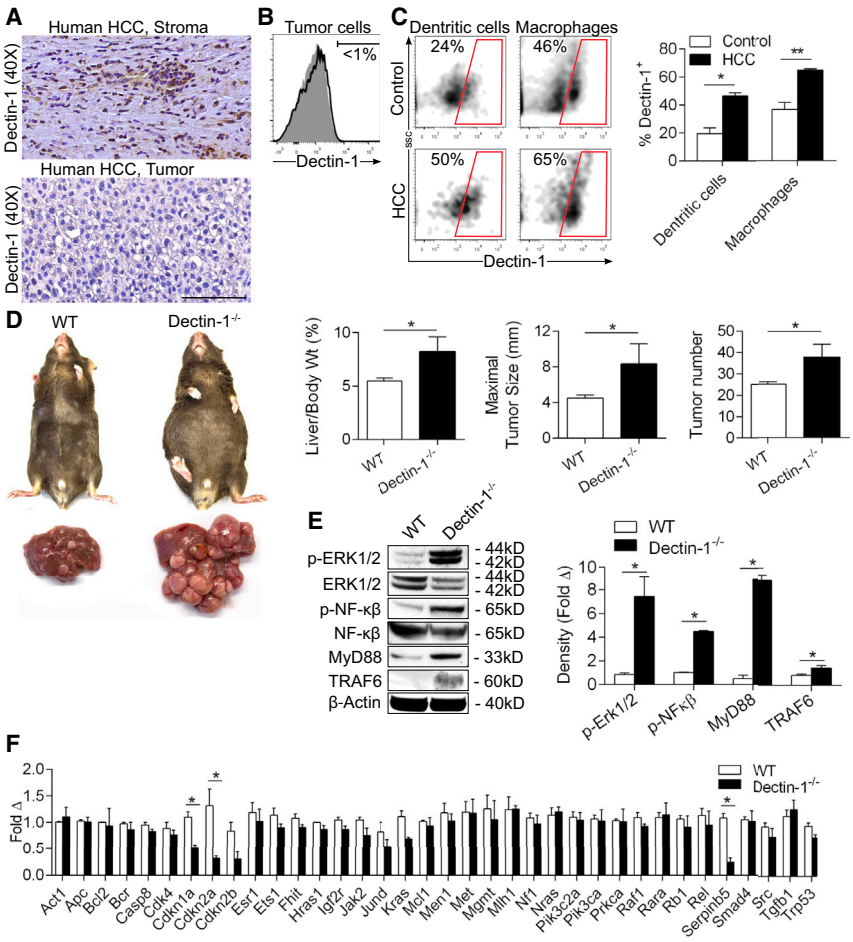

Figure 4. Dectin-1 Deletion Promotes Hepatocarcinogenesis

(A) Dectin-1 expression was tested by immunohistochemistry in human HCC. Representative interstitial and tumor cell-rich areas are shown (scale bar, 100 μm).

(B) Tumor nodules were digested with collagenase, and CD45$^-$ cells were tested for Dectin-1 expression compared with isotype control.

(C) Dectin-1 expression was compared in CD11c$^+$MHCII$^+$ DC and Gr1$^-$CD11c$^-$F4/80$^+$CD11b$^+$ macrophages from control liver versus HCC nodules in mice. Representative data and summary data from three animals are shown.

(D) WT and *Dectin-1*$^{-/-}$ mice were treated with CCl_4 + DEN. Representative images of mice and liver tumors at 24 weeks are shown. The percentage of liver weight per body weight, the maximal diameter of the largest tumor in each animal, and the number of surface liver tumors per mouse were calculated.

(E) Tumors were tested for expression of NF-κB and MAP Kinase signaling intermediates, MyD88, and TRAF6 by western blotting. Representative data and density plots based on triplicates are shown.

(F) Tumors were tested for oncogenic genes using a RT-PCR array (n = 6 mice/group; *p < 0.05, **p < 0.01).

compared with normal liver (Figure 4C). Therefore, we postulated that, besides modulating liver fibrosis, Dectin-1 may also regulate hepatocellular oncogenesis. Mice were induced to develop HCC using diethylnitrosamine (DEN) and CCl_4. WT and *Dectin-1*$^{-/-}$ animals did not exhibit differential acute responses to DEN (data not shown). However, *Dectin-1*$^{-/-}$ mice developed accelerated hepatocellular carcinogenesis based on liver weight, tumor size, and number of nodules (Figure 4D). Western blotting revealed evidence of higher MAP kinase and NF-κB signaling in *Dectin-1*$^{-/-}$ HCC tumor nodules, consistent with an aggressive malignant phenotype (Hsu et al., 1993; Nishida et al., 1994) (Figure 4E), whereas inflammatory signaling was similar in PBS-treated WT and *Dectin-1*$^{-/-}$ liver (Figure S1E). MyD88 and TRAF6 expression were also elevated in *Dectin-1*$^{-/-}$ tumor nodules (Figure 4E). Further, PCR array analysis showed that *Cdkn1*, *Cdkn2*, and *Serpinb5*—whose reduced expression is associated with a more aggressive HCC phenotype (Fujisawa et al., 2005; Hu et al., 2011; Hui et al., 2008)—were each expressed at lower levels in *Dectin-1*$^{-/-}$ tumors compared with WT (Figure 4F).

Dectin-1 Suppresses TLR4 Activation

Dectin-1 has not previously been linked to sterile inflammation or oncogenesis. However, TLR4 activation in the liver is central to the progression of hepatic fibro-inflammatory disease and carcinoma (Dapito et al., 2012; Seki et al., 2007). We found that TLR4 and Dectin-1 co-associate in liver inflammatory cells as evidenced by immunoprecipitation experiments (Figures S4A and S4B) and confocal microscopy (Figure S4C) suggesting opportunity for cross-regulation. Therefore, we postulated that suppression of TLR4 signaling may be the mechanism of Dectin-1-mediated protection in chronic liver disease. Consistent with our hypothesis, inhibition of MyD88 (Figure S5A) or TLR4 (Figure 5A) abrogated the exacerbated fibrosis associated with Dectin-1 deletion. To directly test whether Dectin-1 suppresses TLR4 signaling, we induced endotoxemia by challenging WT and *Dectin-1*$^{-/-}$ mice with TLR4 ligand LPS. Serum IL-6 levels were substantially higher and more sustained in *Dectin-1*$^{-/-}$ mice compared with WT (Figure 5B). *Dectin-1*$^{-/-}$ mice also exhibited a sharper decrease in core temperature after LPS challenge suggesting increased systemic toxicity resulting from TLR4 ligation (Figure 5C). Moreover, Kaplan-Meier analysis revealed markedly diminished survival in LPS-treated *Dectin-1*$^{-/-}$ mice compared to WT (Figure 5D). Taken together, these data suggest that Dectin-1 inhibits TLR4 activation in vivo. Similarly, in vitro LPS-treatment of splenocytes from *Dectin-1*$^{-/-}$ mice resulted in higher TNF-α production (Figure 5E) and proliferation (Figure 5F) compared with WT. Splenocyte composition was similar in WT and *Dectin-1*$^{-/-}$ animals (data not shown). LPS treatment of *Dectin-1*$^{-/-}$ HSCs also resulted in higher TNF-α (Figure 5G), IL-6 (Figure 5H), and MCP-1 (Figure 5I) production compared with LPS-treated WT HSCs. Notably, Dectin-1 deletion did not result

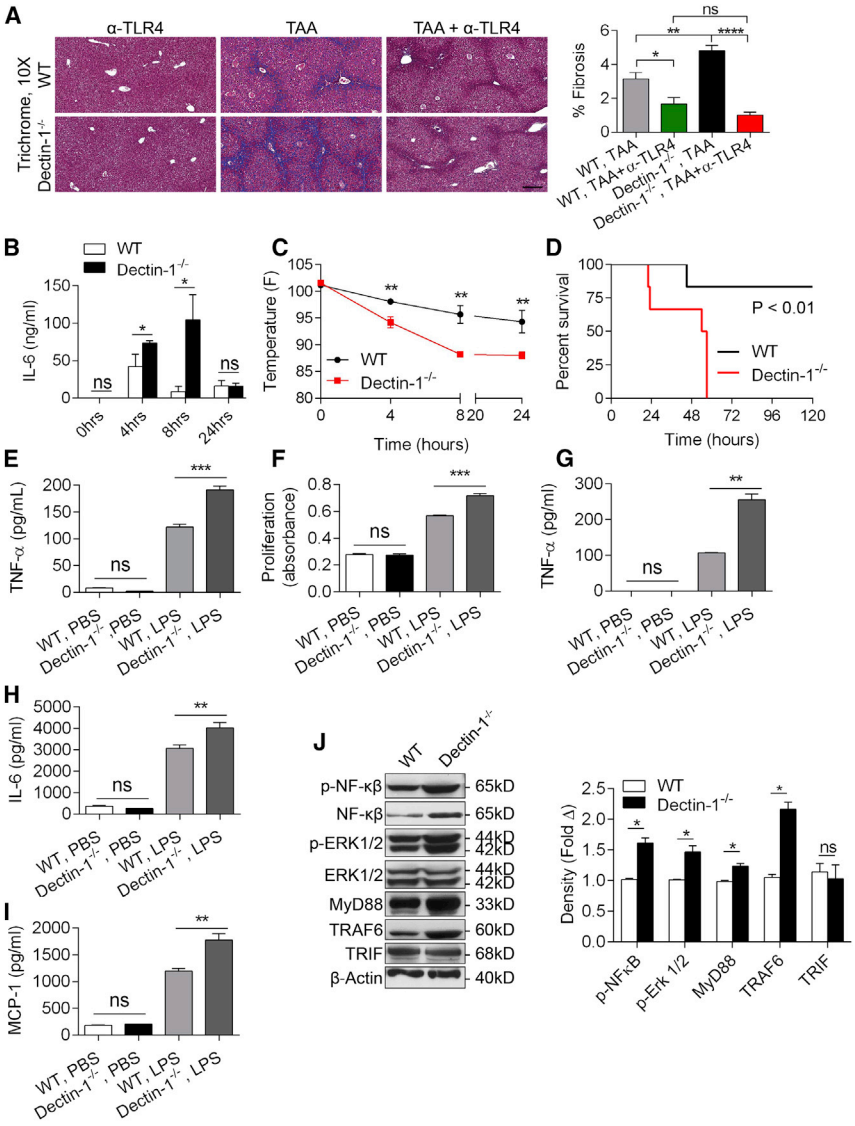

Figure 5. Dectin-1 Negatively Regulates TLR4 Activation

(A) WT and *Dectin-1*$^{-/-}$ mice were treated for 12 weeks with a TLR4 inhibitor alone, TAA alone, or both in combination (n = 5/group). Trichrome staining of representative liver sections is shown, and the fraction of fibrotic area was calculated for each cohort using a computerized grid (scale bar, 10 μm).

(B–D) WT and *Dectin-1*$^{-/-}$ mice were treated with LPS and assayed for (B) serum levels of IL-6, (C) core body temperature at serial time intervals, and (D) survival (n = 6 mice/group).

(E and F) Splenocyte suspensions derived from WT and *Dectin-1*$^{-/-}$ mice were treated in vitro with PBS or LPS and tested for both (E) TNF-α production in cell-culture supernatant and (F) cellular proliferation.

(G–I) HSCs derived from WT and *Dectin-1*$^{-/-}$ mice were treated in vitro with PBS or LPS and tested for production of (G) TNF-α, (H) IL-6, and (I) MCP-1 in cell-culture supernatant.

(J) Fibrotic *Dectin-1*$^{-/-}$ and WT liver tissue were assayed for expression of activated signaling intermediates downstream of TLR4 ligation by western blotting.

Representative data and density plots based on quadruplicates are shown. In vitro experiments were repeated at least three times using three to five mice per experimental group (*p < 0.05, **p < 0.01, ***p < 0.001).

in upregulated cytokine responses to TLR2 (HKLM) ligation (data not shown).

To test for evidence of Dectin-1 suppression of TLR4 signaling in hepatic fibrosis, tissues from fibrotic WT and *Dectin-1*$^{-/-}$ liver were probed for expression of TLR4-related signaling intermediates. We found elevated expression of TRAF6, MyD88, and activated NF-κB and MAP Kinase signaling intermediates in fibrotic *Dectin-1*$^{-/-}$ liver compared with WT (Figure 5J), consistent with higher TLR4 activation. Conversely, the TRIF adaptor protein was expressed at similar levels in both groups (Figure 5J).

Dectin-1 Critically Regulates Expression of TLR4 and CD14

We postulated that Dectin-1 may modulate TLR4 activation by suppressing TLR4 expression. Consistent with our hypothesis, whereas TLR4 was expressed at similar levels in PBS-treated WT and *Dectin-1*$^{-/-}$ hepatic APC, in liver fibrosis TLR4 was differentially upregulated in *Dectin-1*$^{-/-}$macrophages (Figure 6A) compared with WT. Further, CD14—a critical co-receptor for TLR4 (Zanoni et al., 2009)—was also expressed at elevated levels in in macrophages derived from fibrotic *Dectin-1*$^{-/-}$ liver (Figure 6B). Similar upregulations in TLR4 and CD14 expression were seen in *Dectin-1*$^{-/-}$ DC (data not shown) and on PCR of fibrotic whole-liver specimens (Figure S6A). Analysis of murine HCC tumors also revealed elevated *Cd14* expression in *Dectin-1*$^{-/-}$ tumor nodules compared with WT (Figure S6B). By contrast, Dectin-1 ligation using a variety of ligands substantially lowered macrophage expression of TLR4 (Figure 6C) and CD14 (Figure 6D). Further, Dectin-1 ligation in vivo protected animals from LPS-induced endotoxemia (Figures 6E and 6F) and liver fibro-inflammation (Figures 6G and 6H). Notably, *Dectin-1*$^{-/-}$ macrophages or DC did not exhibit elevated expression of TLR2 in hepatic fibrosis (Figures S6C and S6D) or after stimulation with TLR2 ligand HKLM compared with WT (data not shown).

To test whether Dectin-1 suppression of CD14 expression is a primary mechanism in the capacity of Dectin-1 to mitigate TLR4 responsiveness, we blocked CD14 in vivo coincident with PBS or LPS challenge in WT and *Dectin-1*$^{-/-}$ mice. CD14 blockade had no discernible effect in PBS-treated WT or *Dectin-1*$^{-/-}$ mice (Figures S6E and S6F). However, blockade of CD14 abrogated the augmented LPS-mediated inflammatory responses (Figure 6I) and systemic toxicity (Figure 6J) exclusively

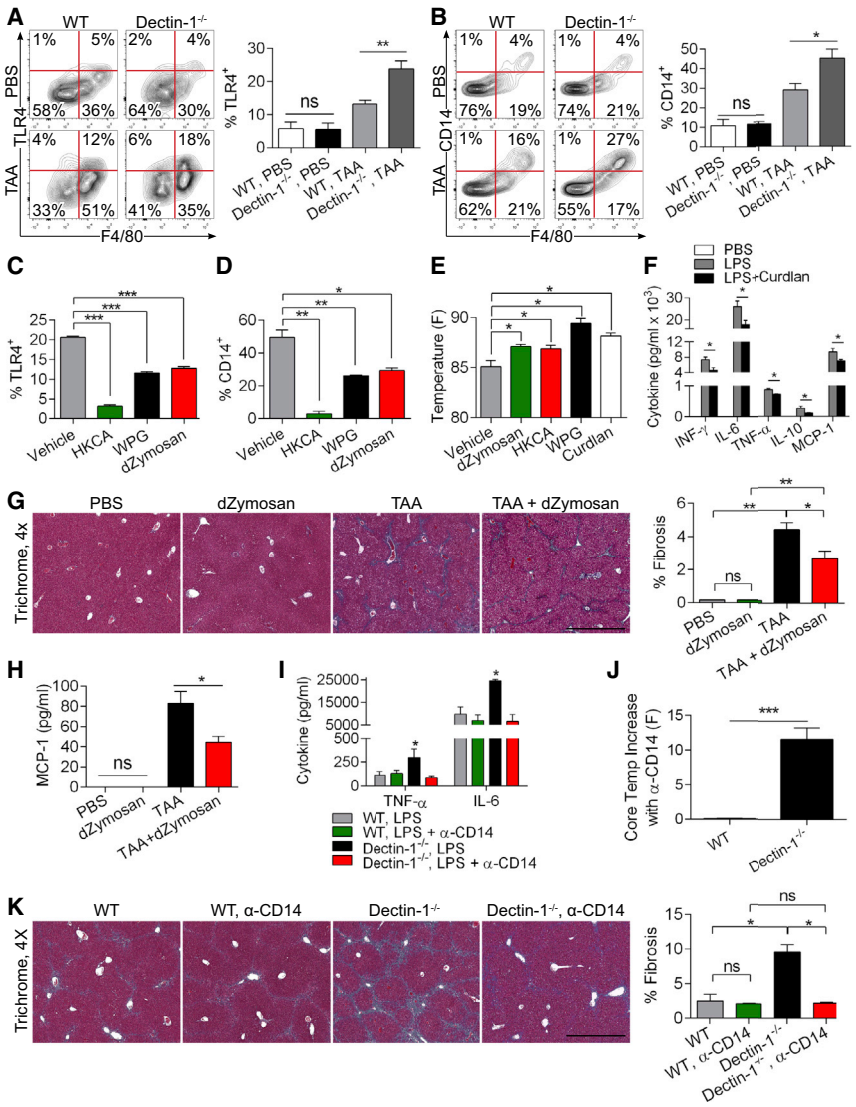

Figure 6. Dectin-1 Suppresses TLR4 and CD14 Expression, which Mediates Dectin-1 Regulation of Liver Fibrosis and Inflammation

(A and B) WT and *Dectin-1*$^{-/-}$ mice were treated for 12 weeks with PBS or TAA. $CD11c^{-}Gr1^{-}F480^{+}CD11b^{+}$ macrophages were tested for (A) TLR4 and (B) CD14 expression by flow cytometry. Representative data and summary data from five mice per group are shown.

(C and D) Macrophages were stimulated in vitro for 24 hr with an array of Dectin-1 ligands and then tested for expression of (C) TLR4 and (D) CD14.

(E) WT mice were challenged in vivo with LPS alone or in combination with an array of selective Dectin-1 ligands or vehicle. Core body temperature was measured at 12 hr.

(F) WT mice were challenged in vivo with LPS alone or LPS + Curdlan. Serum cytokines were measured at 12 hr (error bar, 1 mm).

(G and H) WT mice were treated for 12 weeks with PBS, depleted Zymosan alone, TAA alone, or depleted Zymosan + TAA. Trichrome staining of representative liver sections are shown, and the fraction of fibrotic area was calculated for each cohort using a computerized grid. (H) Serum levels of MCP-1 were calculated (n = 5/group).

(I and J) WT and *Dectin-1*$^{-/-}$ mice were challenged in vivo with LPS alone or LPS + CD14 blockade (n = 5/group). Effects of CD14 inhibition on (I) lowering serum levels of TNF-α and IL-6 and (J) increasing core body temperature compared with LPS treatment alone are shown (error bar, 1 mm).

(K) TAA-treated WT and *Dectin-1*$^{-/-}$ mice were administered isotype or a neutralizing α-CD14 mAb during the 12-week course of fibrosis induction. Trichrome staining of representative liver sections are shown, and the fraction of fibrotic area was calculated for each cohort using a computerized grid (n = 4/group; *p < 0.05, **p < 0.01, ***p < 0.001).

in *Dectin-1*$^{-/-}$ animals. Moreover, CD14 blockade was markedly protective against hepatic fibrosis in TAA-treated *Dectin-1*$^{-/-}$ liver, whereas CD14 inhibition had imperceptible effects in WT liver (Figure 6K). Similarly, CD14 inhibition reduced APC activation in fibrotic *Dectin-1*$^{-/-}$ liver but did not have anti-inflammatory effects in fibrotic WT liver (Figure S6G). In our parallel in vitro experiments, CD14 blockade was also more inhibitory in LPS-stimulated *Dectin-1*$^{-/-}$ HSCs compared with WT HSCs in terms of cellular activation (pPDGFR) and ECM protein production (MMP7, MMP9, TIMP2) (Figure S6H). Collectively, these data imply that Dectin-1 regulates TLR4 signaling in LPS sepsis as well as in liver fibrosis by modulating CD14 levels.

M-CSF Promotes CD14 Expression in Dectin-1$^{-/-}$ Liver

M-CSF can have pleiotropic effects on APC function and has recently been shown to promote fibrosis in cases of HCV infection (Kwan et al., 2007; Preisser et al., 2014). We discovered that deletion of Dectin-1 in the fibrotic liver increased expression of M-CSF in hepatic inflammatory and parenchymal cells based on immunohistochemical (Figure 7A) and mRNA (Figure S3B) analyses. Dectin-1 deletion also upregulated M-CSF receptor CD115 expression in HSC cultures (Figure S7A) and in liver APC (Figure S7B). By contrast, Dectin-1 ligation lowered M-CSF expression in vivo after LPS treatment (Figure 7B). We found that Protein Kinase C (PKC)—which can regulate M-CSF activity (Whetton et al., 1994)—was upregulated in the context of Dectin-1 deletion (Figure S7C) and PKC inhibition abrogated the higher M-CSF expression (Figure S7D). We postulated that augmented M-CSF signaling is responsible for the pathologically high CD14 expression and the exacerbated hepatic fibrosis in *Dectin-1*$^{-/-}$ liver. Consistent with our hypothesis, in vivo M-CSF blockade during fibrogenesis resulted in markedly lower CD14 expression in *Dectin-1*$^{-/-}$ hepatic APC with smaller effects in WT (Figure 7C). Similarly, in vitro M-CSF blockade mitigated the higher CD14 expression in LPS-stimulated *Dectin-1*$^{-/-}$ macrophages but had negligible effects in WT (Figure S7E).

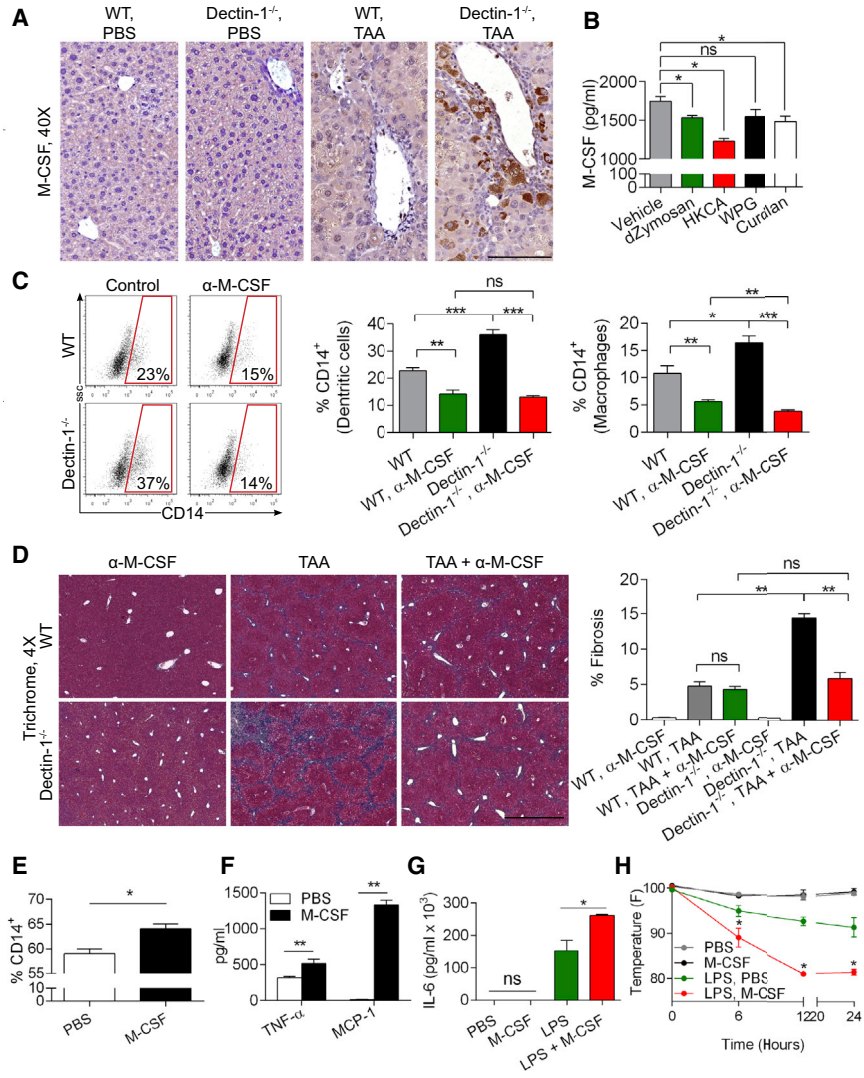

Figure 7. M-CSF Blockade Lowers CD14 Expression and Protects against Fibrosis in *Dectin-1*$^{-/-}$ Liver

(A) Paraffin-embedded liver sections from PBS- and TAA-treated WT and *Dectin-1*$^{-/-}$ mice were stained using a mAb specific for M-CSF. Representative images are shown (n = 5; scale bar, 100 μm).

(B) WT mice were challenged in vivo with LPS alone or in combination with an array of selective Dectin-1 ligands or vehicle. Serum M-CSF was measured at 12 hr (n = 3 mice/group).

(C) WT and *Dectin-1*$^{-/-}$ mice were treated with TAA for 12 weeks. Selected cohorts were additionally treated with α-M-CSF or isotype control. DC and macrophage subsets were gated by flow cytometry and tested for expression of CD14. Representative data from one liver and summary data from five mice are shown (error bar, 1 mm).

(D) WT and *Dectin-1*$^{-/-}$ mice were treated with PBS or TAA for 12 weeks. Select cohorts were additionally treated with α-M-CSF or isotype control. Liver sections were stained with Trichrome, and fibrosis was quantified using a computerized grid.

(E and F) Macrophages were treated with PBS or recombinant M-CSF and tested for (E) CD14 expression by flow cytometry and (F) TNF-α and MCP-1 expression in cell-culture supernatant.

(G and H) WT mice were co-administered LPS and M-CSF alone or in combination. (G) Serum levels of IL-6 and (H) core body temperature were measured (n = 5/group; *p < 0.05, **p < 0.01, ***p < 0.001).

Moreover, similar to CD14 blockade, M-CSF inhibition ameliorated hepatic fibrosis in the setting of Dectin-1 deletion but offered no protection in WT liver (Figure 7D). Conversely, treatment with recombinant M-CSF increased CD14 expression (Figure 7E) and upregulated MCP-1 and TNF-α expression in macrophages in vitro (Figure 7F) and exacerbated LPS-mediated sepsis in vivo (Figures 7G and 7H). TNF-α blockade prevented the M-CSF-induced differential CD14 upregulation in *Dectin-1*$^{-/-}$ macrophages (Figure S7F). Collectively, these data suggest that Dectin-1-regulated expression of M-CSF drives CD14-dependent exaggerated hepatic fibrosis and inflammatory responses in LPS-mediated sepsis.

DISCUSSION

TLR4-mediated inflammation is critical in both host defense against invading pathogens and for physiological responses to inflammatory stimuli. However, regulation of TLR4 responses is paramount in limiting end-organ injury and tissue damage in sterile inflammatory processes such as hepatic fibrosis, after septic injury, and mitigating progression toward malignancies in TLR4-dependent cancers such as HCC (Lu et al., 2008; Peri and Piazza, 2012). The understanding of negative regulation of TLR4 signaling in any of these contexts is rudimentary. In general, suppression of TLR signaling is thought to occur via three distinct mechanisms: degradation of TLR-associated adaptor proteins, dissociation of TLR-dependent signaling complexes, and regulation of transcription of soluble inflammatory mediators. Inhibitory proteins SOCS1 and SOCS3 suppress TLR4 activation by promoting the degradation of the MyD88 adaptor-like (MAL) or TRAF proteins (Fujimoto and Naka, 2010; Yoshimura et al., 2004). TANK and SHP1 each independently inhibit TRAF6 ubiquitination, and mice deficient in either of these proteins exhibit more robust inflammatory responses to TLR ligands and readily progress to organ failure in diverse TLR-dependent disease models (Kawagoe et al., 2009; Yuk et al., 2011). DAP12 and β2 integrins cross-regulate TLR responses through promotion of IL-10 expression and via activation of the E3 ubiquitin ligase Cbl-b, which degrades MyD88 and TRIF leading to reduced inflammation (Hamerman et al., 2005; Han et al., 2010; Wang et al., 2010; Yee and Hamerman, 2013). Despite these varied mechanisms of TLR regulation, cross-inhibition between individual or families of

PRRs is not well described. Our finding that Dectin-1 suppresses TLR4 activation is evidence of negative regulation between PRRs in an in vivo model of sterile inflammation or LPS-mediated endotoxemia. We show that Dectin-1 and TLR4 coassociate. This raises the question of whether the Dectin-1/TLR4 complex directly regulates TLR4 function; however, deciphering this requires more exact experimentation. Previous reports have not found augmented responses to TLR4 ligation in the context of Dectin-1 deletion; however, discrepancies with the current studies may be related to the substantially lower doses of LPS utilized in the other reports and the bone marrow-derived DC and macrophage models employed (Del Fresno et al., 2013; Saijo et al., 2007).

Dectin-1 is vital in the innate immune defense against fungal pathogens (Vautier et al., 2012). Patients with genetic deficiencies in Dectin-1 are at high risk for recurrent mucocutaneous fungal infections, such as vulvovaginal candidiasis or onychomycosis (Ferwerda et al., 2009). However, unlike their TLR cousins, a definitive role for Dectin-1 in non-pathogen mediated inflammation is lacking (Bianchi, 2007). The present study describes a protective role for Dectin-1 in liver fibrosis and hepatocarcinogenesis and, more broadly, implicates a regulatory role for Dectin-1 in modulating sterile inflammation, the inflammation-cancer paradigm, as well as LPS-mediated sepsis. We found that deletion of Mincle, an allied C-type lectin receptor, has no effect on liver fibrogenesis indicating that the observed effects are specific to Dectin-1. These data suggest that modulating Dectin-1 signaling may be an attractive target in experimental therapeutics in either inflammatory or infectious conditions mediated by TLR4 ligation or in cases of TLR4-dependant transformation such as hepatocarcinogenesis (Dapito et al., 2012). Both our in vitro data showing TLR4 hyperresponsiveness in *Dectin-1*$^{-/-}$ APC subsets and in HSCs and our in vivo data employing bone marrow chimeric mice suggest that Dectin-1 signaling in both the radio-sensitive and the radio-resistant compartments each contribute toward the exacerbated fibrotic phenotype in *Dectin-1*$^{-/-}$ liver. Importantly, HSCs may not be the only radio-resistant cellular subset to contributing to augmented hepatic fibrosis as recent work suggests that select subsets of liver macrophages are resistant to ablation by similar doses of total body irradiation (Klein et al., 2007).

We found that the exacerbated hepatic fibrosis in *Dectin-1*$^{-/-}$ liver, and augmented inflammation and toxicity in LPS-induced sepsis in *Dectin-1*$^{-/-}$ animals, is associated with elevated expression of TLR4 and its co-receptor CD14. This finding is particularly notable as expression levels of TLR4 and CD14 are similar in WT and *Dectin-1*$^{-/-}$ APC at baseline; however, in liver fibrosis, the upsurge in TLR4 and CD14 expression—which we found to be characteristic of toxin-induced hepatic injury—is muted in Dectin-1-expressing APC. Similarly, instructive are the observations that CD14 blockade mitigated the exacerbated hepatic fibrosis and LPS-induced sepsis in *Dectin-1*$^{-/-}$ mice but had negligible effects on liver fibrosis or systemic inflammation in WT animals. Given the central position of CD14 in TLR4-mediated inflammatory responses, this observation is surprising and suggests that CD14 is dispensable for both intra- and extra-hepatic TLR4-mediated inflammation in Dectin-1 competent hosts. These unanticipated findings are in line with that of Tsung et al. who found that CD14-inhibition does not lessen HMGB1-mediated hepatic ischemia-reperfusion injury in WT mice with otherwise intact TLR4 signaling (Tsung et al., 2005). It is conceivable that below a certain threshold CD14 level, CD14 expression indeed dispensable in TLR4-mediated inflammation whereas above this threshold level, CD14 blockade suppresses TLR4 responses.

We show that, via TNF-α production, M-CSF is the mechanistic influence for higher CD14 expression in activated *Dectin-1*$^{-/-}$ innate inflammatory cells. Further, M-CSF along with its receptor CD115 was robustly overexpressed in *Dectin-1*$^{-/-}$ fibrotic liver in a PKC-dependent manner. In parallel with our observations employing CD14 blockade, M-CSF inhibition protected Dectin-1-deficient mice from hepatic fibrosis but had no effect in lessening fibrotic injury in WT liver. Our current work contrasts with a very recent report suggesting that, in HCV-induced fibrosis, M-CSF plays a primary role in APC-mediated fibrogenesis in cooperation with IL-34 (Preisser et al., 2014). However, the signaling mechanisms governing HCV fibrosis and toxin-mediated chronic liver injury are likely distinct.

Besides augmenting the M-CSF-CD14-TLR4 axis, another possible mechanism for exacerbated hepatic fibrosis and accelerated carcinogenesis in the context of Dectin-1 deletion may relate to our recent finding that direct Dectin-1 ligation on $\gamma\delta$T cells drives hepatic regeneration (Rao et al., 2014). The dynamic process of liver fibrosis can be viewed as a physiological "struggle" between the drive for hepatocyte proliferation and regeneration and, conversely, the deposition of ECM proteins and collagen in place of non-viable hepatocytes. Liver regeneration—in both the context of partial hepatectomy and in the setting of chronic liver disease—requires the secretion of pro-regenerative cytokines (IL-6 and IL-22) from APC. These cytokines, in turn, induce the phosphorylation of STAT3 in hepatocytes, leading to their proliferation (Li et al., 2002). However, the triggers that promote the induction of a "regenerative phenotype" in hepatic inflammatory cells are incompletely understood. We reported that yet-uncharacterized Dectin-1 ligands released during liver regeneration bind CCL20-recruited $\gamma\delta$T cells that, in turn, release IL-22 and IL-17. IL-22 has direct mitogenic effects on hepatocytes, and IL-17 induces a pro-regenerative phenotype in hepatic APC and other leukocytes (Rao et al., 2014; Ren et al., 2010). It plausible that in liver fibrosis, akin to partial hepatectomy, Dectin-1 ligation on $\gamma\delta$T cells promotes hepatocyte proliferation in regenerative nodules. Consistent with this notion, we found evidence of markedly reduced hepatocyte proliferation in regenerative nodules in fibrotic *Dectin-1*$^{-/-}$ liver. Further, a very recent report showed that chemokine-recruited $\gamma\delta$T cells mitigate hepatic fibrosis (Hammerich et al., 2014). Hence, besides regulating TLR4 activation, Dectin-1 may additionally limit liver fibrosis via $\gamma\delta$T cell promotion of hepatic regeneration. However, as in the case in partial hepatectomy, identification of sterile Dectin-1 ligands in toxin-induced hepatic fibrosis requires additional study.

EXPERIMENTAL PROCEDURES

Animals and In Vivo Models of Liver Fibrosis, HCC, and Endotoxemia

C57BL/6 and CD45.1 mice were purchased from Jackson and bred in house. *Dectin-1*$^{-/-}$ mice were a gift from Gordon Brown (University of Aberdeen).

Mincle$^{-/-}$ mice were obtained from the MMRRC. Age-matched 6- to 8-week-old mice were used in all experiments. To induce hepatic fibrosis, female mice were treated with thrice weekly injections of TAA (250 mg/kg; Sigma) for 12 weeks as described (Connolly et al., 2009). Alternatively, mice received bi-weekly injections of CCl_4 (0.5 ml/kg; Sigma) for the same duration. To induce HCC, male mice were injected i.p. at 2 weeks of age with a single dose of DEN (15 mg/kg, Sigma) followed by bi-weekly injections of CCl_4 (0.2 ml/kg) starting at 8 weeks of age (Dapito et al., 2012). Mice were sacrificed 24 weeks later. To induce endotoxemia, male mice were injected i.p. with a single dose of LPS (15 mg/kg; Sigma). Rectal core body temperature was determined using a MicroTherma 2 temperature probe (Thermoworks). In selected experiments, MyD88 inhibitory peptide or control peptide (1 mg/kg, i.p.; Novus), α-CD14 neutralizing antibody (4 mg/kg, i.p.; 4C1, BD Bioscience), a small-molecule inhibitor of TLR4 (TAK-242, 4 mg/kg i.p.; Millipore), an α-M-CSF neutralizing antibody (2 mg/kg, i.p.; 5A1, BioXCell), recombinant murine M-CSF (250 μg/kg, i.v.; R&D Systems), Dectin-1 ligands Zymosan depleted (40 mg/kg, i.p.), HKCA (2 × 10^9cells/kg, i.p.), WGP Dispersible (8 mg/kg, i.p.), or Curdlan AL (8 mg/kg, i.p.; all Invivogen) were administered immediately preceding each TAA or LPS administration. Serum liver enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were determined using commercial kits (Sigma). M-CSF levels were measured using the mouse M-CSF Quantikine ELISA Kit (R&D). Bone marrow chimeric animals were created by irradiating mice (950 Rad) followed by i.v. bone marrow transfer (1 × 10^7 cells) from non-irradiated donors as we have described (Ochi et al., 2012b). Chimeric mice were employed in experiments 7 weeks later. All animal procedures were approved by the NYU School of Medicine IACUC.

Human and Murine Cellular Isolation and Culture

Murine hepatic non-parenchymal cells (NPCs) were collected as previously described (Connolly et al., 2009). Briefly, the portal vein was cannulated and infused with 1% Collagenase IV (Sigma). The liver was then removed, minced, and filtered to obtain single-cell suspensions. Hepatocytes were excluded with serial low speed (400 rpm) centrifugation followed by high-speed (2,000 rpm) centrifugation to isolate the NPC, which were then further enriched over a 40% Optiprep (Sigma) gradient. Human liver NPCs were isolated using a similar protocol as we have described (Ibrahim et al., 2012). Human PBMCs were isolated by overlaying whole blood diluted 1:1 in PBS over an equal amount of Ficoll. The cells were then spun at 2,100 rpm, and buffy coat was harvested to obtain the PBMCs as described (Rehman et al., 2013). Single-cell suspensions of murine splenocytes were generated by manual disruption of whole spleen and red blood cell (RBC) lysis. For HSC isolation, the liver was perfused with 1% Collagenase IV, and HSCs were enriched over a 2-layer Nicodenz (Sigma) gradient. HSCs were used for experiments on day 14 of culture. For cytokine analysis, HSCs were cultured in complete medium (DNEM F12 with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at a concentration of 1 × 10^6 cells/ml for 24 hr before supernatant harvest and analysis using a cytometric bead array according the manufactures' protocol (BD Biosciences). In vitro proliferation was measured using the XTT cell proliferation kit (Roche). PKC activity was measured using a PKC Kinase Activity Assay Kit (Abcam). In selected experiments, cellular activation was accomplished using LPS-EB Ultrapure (10 μg/ml), HKLM (10^8 cell/ml), HKCA (10^8 cells/ml), Zymosan Depleted (100 μg/ml), WGP Dispersible (100 μg/ml), Curdlan AL (100 μg/ml; all Invivogen), or recombinant murine M-CSF (100 ng/ml; R&D). In select experiments, cells were treated with mAbs directed against CD14 (10 μg/ml; 4C1), TNF-α (5 μg/ml; 2E2; Sloan-Kettering Institute), or a selective inhibitor of Protein Kinase C (GF109203X, 1 μM; Tocris Bioscience).

Flow Cytometry

Single-cell suspensions of liver or spleen cells or cultured HSCs were incubated with Fc blocking reagent (BioLegend) for 10 min followed by 30 min incubation with fluorescently conjugated mAbs directed against mouse CD11b (M1/70), CD11c (N418), CD45 (30-F11), F/480 (BM8), Gr1 (RB6-8C5), CD115 (AFS98), and MHC II (M5/114.15.2; all BioLegend). Cells were also tested for expression of Dectin-1 (2A11; Abcam), TLR4 (SA15-21; BioLegend), CD14 (Sa14-2; BioLegend), and TLR2 (6C2; eBiosciences). Human liver NPCs and PBMCs were stained with mAbs directed against CD45 (HI30), CD14 (M5E2; both BioLegend), or Dectin-1 (259931; R&D). For intracellular cytokine staining, liver NPC were incubated for 4 hr with Brefeldin A (1:1,000) before permeabilization of cells and staining using fluorescent conjugated mAbs against murine TNF-α (MP6-XT22) or IL-6 (MP5-20F3; both BioLegend). Experiments were performed using the LSRII cytometer (BD Biosciences), and analysis was done using FlowJo software (Tree Star).

Histology, Immunohistochemistry, and Immunofluorescence

Liver tissues were fixed overnight in 10% formaldehyde and were embedded in paraffin. Slides were stained with H&E, Masson's Trichrome, or Picric acid-Sirius red. For immunohistochemical analysis, paraffin-embedded or frozen sections were incubated with antibodies against mouse CD45 (30-F11; BD Bioscience), CD68 (KP1; Abcam), MPO (Rabbit polyclonal; Abcam), Dectin-1 (R1-8g7; Invivogen), PCNA (PC10; BioLegend), TLR4 (Rabbit polyclonal; Abcam), α-SMA (1A4; Abcam), Phalloidin (Cell Signaling Technology), or M-CSF (Rabbit polyclonal; Abcam). Human liver sections were stained with an antibody against Dectin-1 (Rabbit polyclonal; Abcam) or CD11b (M1/70; BioLegend). Quantification was performed by examining ten high-powered fields (HPFs) per slide. Fibrosis was quantified based on Trichrome staining using a computerized grid as described (Ochi et al., 2012a). Immunofluorescent imaging was performed using a LSM 700 confocal microscope and an Axiovert camera (Zeiss).

Western Blotting and Immunoprecipitation

For western blotting, total protein was isolated from 75-mg liver tissue by homogenization in RIPA buffer with Complete Protease Inhibitor cocktail (Roche). Proteins were separated from larger fragments by centrifugation at 14,000 × *g*. After determining total protein by the Bradford protein assay, 10% polyacrylamide gels (NuPage, Invitrogen) were equiloaded, electrophoresed at 200 V, electrotransferred to PVDF membranes, and probed with monoclonal antibodies to β-actin, MMP2, MMP7, MMP9, TIMP2, TIMP7, p-Erk1/2, Erk1/2, p-NF-κβ, NF-κβ, MyD88, TRAF6, TRIF, and pPDGFRα (all Cell Signaling Technology) using the manufacturer's recommended concentrations. Blots were developed by ECL (Thermo Scientific). For immunoprecipitation experiments, TLR4 or Dectin-1 was precipitated with protein G-agarose. Immuno-precipitates were re-suspended and heated in loading buffer under reduced condition and resolved by 10% SDS-PAGE before transfer to PVDF membranes. The presence of the co-immunoprecipitated proteins were determined by western blotting.

mRNA Analysis

For PCR analysis, total RNA was extracted using the RNEasy Mini Kit (QIAGEN), and cDNA was made using the High Capacity Reverse Transcription kit (Applied Biosystems). RT-PCR was performed for mouse CD14 and β-actin using commercially available pre-designed primers (QIAGEN). For the mouse Fibrosis PCR array, Oncogene PCR array, and Cytokine and Chemokine PCR array, mRNAs were reverse transcribed into first-strand cDNA using an RT^2miRNA First-Strand Kit (all QIAGEN). RT^2 SYBR Green/ROX Quantitative PCR Master Mix (QIAGEN) was used for amplification and the samples were run on the Stratagene Mx3005P. For Nanostring analysis, the nCounter mouse inflammation panel was employed using the nCounter Analysis System (Nanostring).

Statistical Analysis

Data are presented as mean ± SEM. Survival was measured according to the Kaplan-Meier method. Statistical significance was determined by the Student's t test and the log-rank test using GraphPad Prism 6 (GraphPad Software). p values of <0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.058.

AUTHOR CONTRIBUTIONS

L.S. (acquisition of data; analysis and interpretation; manuscript preparation; statistical analysis), M.D. (acquisition of data; analysis and interpretation),

S.A. (acquisition of data), D.A. (acquisition of data), G.W. (acquisition of data), M. Pansari (acquisition of data), M. Pergamo (acquisition of data), A.O. (technical assistance), A.T.-H. (data analysis, critical review), E.L. (acquisition of data; manuscript preparation), D.T. (technical assistance), S.H.G. (data analysis, critical review), S.T. (acquisition of data), N.N.G.L. (acquisition of data), A.E. (acquisition of data), E.v.H. (acquisition of data), A.A. (acquisition of data), R.B. (technical assistance), C.P.Z. (technical assistance), M.R. (technical assistance), D.D. (critical review), H.L.P. (critical revision and material supply), C.H. (study concept and design; study supervision; critical review), G.M. (analysis and interpretation; study concept and design; study supervision; critical review).

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REFERENCES

Aoyama, T., Paik, Y.H., and Seki, E. (2010). Toll-like receptor signaling and liver fibrosis. Gastroenterol. Res. Pract. *2010*, 2010.

Bataller, R., and Brenner, D.A. (2005). Liver fibrosis. J. Clin. Invest. *115*, 209–218.

Bianchi, M.E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. J. Leukoc. Biol. *81*, 1–5.

Connolly, M.K., Bedrosian, A.S., Mallen-St Clair, J., Mitchell, A.P., Ibrahim, J., Stroud, A., Pachter, H.L., Bar-Sagi, D., Frey, A.B., and Miller, G. (2009). In liver fibrosis, dendritic cells govern hepatic inflammation in mice via TNF-alpha. J. Clin. Invest. *119*, 3213–3225.

Dapito, D.H., Mencin, A., Gwak, G.Y., Pradere, J.P., Jang, M.K., Mederacke, I., Caviglia, J.M., Khiabanian, H., Adeyemi, A., Bataller, R., et al. (2012). Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. Cancer Cell *21*, 504–516.

De Creus, A., Abe, M., Lau, A.H., Hackstein, H., Raimondi, G., and Thomson, A.W. (2005). Low TLR4 expression by liver dendritic cells correlates with reduced capacity to activate allogeneic T cells in response to endotoxin. J. Immunol. *174*, 2037–2045.

Del Fresno, C., Soulat, D., Roth, S., Blazek, K., Udalova, I., Sancho, D., Ruland, J., and Ardavín, C. (2013). Interferon-β production via Dectin-1-Syk-IRF5 signaling in dendritic cells is crucial for immunity to C. albicans. Immunity *38*, 1176–1186.

El-Serag, H.B. (2007). Epidemiology of hepatocellular carcinoma in USA. Hepatol. Res. *37* (*Suppl 2*), S88–S94.

Ferwerda, B., Ferwerda, G., Plantinga, T.S., Willment, J.A., van Spriel, A.B., Venselaar, H., Elbers, C.C., Johnson, M.D., Cambi, A., Huysamen, C., et al. (2009). Human dectin-1 deficiency and mucocutaneous fungal infections. N. Engl. J. Med. *361*, 1760–1767.

Franceschi, S., and Raza, S.A. (2009). Epidemiology and prevention of hepatocellular carcinoma. Cancer Lett. *286*, 5–8.

Fujimoto, M., and Naka, T. (2010). SOCS1, a negative regulator of cytokine signals and TLR Responses, in human liver diseases. Gastroenterol. Res. Pract. *2010*, 2010.

Fujisawa, K., Maesawa, C., Sato, R., Wada, K., Ogasawara, S., Akiyama, Y., Takeda, M., Fujita, T., Otsuka, K., Higuchi, T., et al. (2005). Epigenetic status and aberrant expression of the maspin gene in human hepato-biliary tract carcinomas. Lab. Invest. *85*, 214–224.

Gross, O., Gewies, A., Finger, K., Schäfer, M., Sparwasser, T., Peschel, C., Förster, I., and Ruland, J. (2006). Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. Nature *442*, 651–656.

Hamerman, J.A., Tchao, N.K., Lowell, C.A., and Lanier, L.L. (2005). Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. Nat. Immunol. *6*, 579–586.

Hammerich, L., Bangen, J.M., Govaere, O., Zimmermann, H.W., Gassler, N., Huss, S., Liedtke, C., Prinz, I., Lira, S.A., Luedde, T., et al. (2014). Chemokine receptor CCR6-dependent accumulation of γδ T cells in injured liver restricts hepatic inflammation and fibrosis. Hepatology *59*, 630–642.

Han, C., Jin, J., Xu, S., Liu, H., Li, N., and Cao, X. (2010). Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. Nat. Immunol. *11*, 734–742.

Hsu, H.C., Tseng, H.J., Lai, P.L., Lee, P.H., and Peng, S.Y. (1993). Expression of p53 gene in 184 unifocal hepatocellular carcinomas: association with tumor growth and invasiveness. Cancer Res. *53*, 4691–4694.

Hu, H., Li, Z., Chen, J., Wang, D., Ma, J., Wang, W., Li, J., Wu, H., Li, L., Wu, M., et al. (2011). P16 reactivation induces anoikis and exhibits antitumour potency by downregulating Akt/survivin signalling in hepatocellular carcinoma cells. Gut *60*, 710–721.

Hui, L., Zatloukal, K., Scheuch, H., Stepniak, E., and Wagner, E.F. (2008). Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. J. Clin. Invest. *118*, 3943–3953.

Ibrahim, J., Nguyen, A.H., Rehman, A., Ochi, A., Jamal, M., Graffeo, C.S., Henning, J.R., Zambirinis, C.P., Fallon, N.C., Barilla, R., et al. (2012). Dendritic cell populations with different concentrations of lipid regulate tolerance and immunity in mouse and human liver. Gastroenterology *143*, 1061–1072.

Kawagoe, T., Takeuchi, O., Takabatake, Y., Kato, H., Isaka, Y., Tsujimura, T., and Akira, S. (2009). TANK is a negative regulator of Toll-like receptor signaling and is critical for the prevention of autoimmune nephritis. Nat. Immunol. *10*, 965–972.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. *11*, 373–384.

Klein, I., Cornejo, J.C., Polakos, N.K., John, B., Wuensch, S.A., Topham, D.J., Pierce, R.H., and Crispe, I.N. (2007). Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. Blood *110*, 4077–4085.

Kuramitsu, K., Sverdlov, D.Y., Liu, S.B., Csizmadia, E., Burkly, L., Schuppan, D., Hanto, D.W., Otterbein, L.E., and Popov, Y. (2013). Failure of fibrotic liver regeneration in mice is linked to a severe fibrogenic response driven by hepatic progenitor cell activation. Am. J. Pathol. *183*, 182–194.

Kwan, W.H., Boix, C., Gougelet, N., Fridman, W.H., and Mueller, C.G. (2007). LPS induces rapid IL-10 release by M-CSF-conditioned tolerogenic dendritic cell precursors. J. Leukoc. Biol. *82*, 133–141.

Li, W., Liang, X., Kellendonk, C., Poli, V., and Taub, R. (2002). STAT3 contributes to the mitogenic response of hepatocytes during liver regeneration. J. Biol. Chem. *277*, 28411–28417.

Lim, Y.S., and Kim, W.R. (2008). The global impact of hepatic fibrosis and end-stage liver disease. Clin. Liver Dis. *12*, 733–746, vii.

Lu, Y.C., Yeh, W.C., and Ohashi, P.S. (2008). LPS/TLR4 signal transduction pathway. Cytokine *42*, 145–151.

Nishida, N., Fukuda, Y., Komeda, T., Kita, R., Sando, T., Furukawa, M., Amenomori, M., Shibagaki, I., Nakao, K., Ikenaga, M., et al. (1994). Amplification and overexpression of the cyclin D1 gene in aggressive human hepatocellular carcinoma. Cancer Res. *54*, 3107–3110.

Ochi, A., Graffeo, C.S., Zambirinis, C.P., Rehman, A., Hackman, M., Fallon, N., Barilla, R.M., Henning, J.R., Jamal, M., Rao, R., et al. (2012a). Toll-like receptor 7 regulates pancreatic carcinogenesis in mice and humans. J. Clin. Invest. *122*, 4118–4129.

Ochi, A., Nguyen, A.H., Bedrosian, A.S., Mushlin, H.M., Zarbakhsh, S., Barilla, R., Zambirinis, C.P., Fallon, N.C., Rehman, A., Pylayeva-Gupta, Y., et al. (2012b). MyD88 inhibition amplifies dendritic cell capacity to promote pancreatic carcinogenesis via Th2 cells. J. Exp. Med. *209*, 1671–1687.

Paik, Y.H., Schwabe, R.F., Bataller, R., Russo, M.P., Jobin, C., and Brenner, D.A. (2003). Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. Hepatology *37*, 1043–1055.

Peri, F., and Piazza, M. (2012). Therapeutic targeting of innate immunity with Toll-like receptor 4 (TLR4) antagonists. Biotechnol. Adv. *30*, 251–260.

Preisser, L., Miot, C., Le Guillou-Guillemette, H., Beaumont, E., Foucher, E.D., Garo, E., Blanchard, S., Fremaux, I., Croue, A., Fouchard, I., et al. (2014). IL-34 and M-CSF are overexpressed in HCV fibrosis and induce pro-fibrotic macrophages which promote collagen synthesis by hepatic stellate cells. Hepatology *60*, 1879–1890.

Rao, R., Graffeo, C.S., Gulati, R., Jamal, M., Narayan, S., Zambirinis, C.P., Barilla, R., Deutsch, M., Greco, S.H., Ochi, A., et al. (2014). Interleukin 17-producing gammadeltaT cells promote hepatic regeneration in mice. Gastroenterology *147*, 473–484.

Rehman, A., Hemmert, K.C., Ochi, A., Jamal, M., Henning, J.R., Barilla, R., Quesada, J.P., Zambirinis, C.P., Tang, K., Ego-Osuala, M., et al. (2013). Role of fatty-acid synthesis in dendritic cell generation and function. J. Immunol. *190*, 4640–4649.

Ren, X., Hu, B., and Colletti, L.M. (2010). IL-22 is involved in liver regeneration after hepatectomy. Am. J. Physiol. Gastrointest. Liver Physiol. *298*, G74–G80.

Saijo, S., Fujikado, N., Furuta, T., Chung, S.H., Kotaki, H., Seki, K., Sudo, K., Akira, S., Adachi, Y., Ohno, N., et al. (2007). Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. Nat. Immunol. *8*, 39–46.

Seki, E., De Minicis, S., Osterreicher, C.H., Kluwe, J., Osawa, Y., Brenner, D.A., and Schwabe, R.F. (2007). TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat. Med. *13*, 1324–1332.

Strasser, D., Neumann, K., Bergmann, H., Marakalala, M.J., Guler, R., Rojowska, A., Hopfner, K.P., Brombacher, F., Urlaub, H., Baier, G., et al. (2012). Syk kinase-coupled C-type lectin receptors engage protein kinase C-σ to elicit Card9 adaptor-mediated innate immunity. Immunity *36*, 32–42.

Tsung, A., Sahai, R., Tanaka, H., Nakao, A., Fink, M.P., Lotze, M.T., Yang, H., Li, J., Tracey, K.J., Geller, D.A., and Billiar, T.R. (2005). The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J. Exp. Med. *201*, 1135–1143.

Vasel, M., Rutz, R., Bersch, C., Feick, P., Singer, M.V., Kirschfink, M., and Nakchbandi, I.A. (2014). Complement activation correlates with liver necrosis and fibrosis in chronic hepatitis C. Clin. Immunol. *150*, 149–156.

Vautier, S., MacCallum, D.M., and Brown, G.D. (2012). C-type lectin receptors and cytokines in fungal immunity. Cytokine *58*, 89–99.

Wang, L., Gordon, R.A., Huynh, L., Su, X., Park Min, K.H., Han, J., Arthur, J.S., Kalliolias, G.D., and Ivashkiv, L.B. (2010). Indirect inhibition of Toll-like receptor and type I interferon responses by ITAM-coupled receptors and integrins. Immunity *32*, 518–530.

Whetton, A.D., Heyworth, C.M., Nicholls, S.E., Evans, C.A., Lord, J.M., Dexter, T.M., and Owen-Lynch, P.J. (1994). Cytokine-mediated protein kinase C activation is a signal for lineage determination in bipotential granulocyte macrophage colony-forming cells. J. Cell Biol. *125*, 651–659.

Yee, N.K., and Hamerman, J.A. (2013). β(2) integrins inhibit TLR responses by regulating NF-κB pathway and p38 MAPK activation. Eur. J. Immunol. *43*, 779–792.

Yoshimura, A., Ohishi, H.M., Aki, D., and Hanada, T. (2004). Regulation of TLR signaling and inflammation by SOCS family proteins. J. Leukoc. Biol. *75*, 422–427.

Yuk, J.M., Shin, D.M., Lee, H.M., Kim, J.J., Kim, S.W., Jin, H.S., Yang, C.S., Park, K.A., Chanda, D., Kim, D.K., et al. (2011). The orphan nuclear receptor SHP acts as a negative regulator in inflammatory signaling triggered by Toll-like receptors. Nat. Immunol. *12*, 742–751.

Zanoni, I., Ostuni, R., Capuano, G., Collini, M., Caccia, M., Ronchi, A.E., Rocchetti, M., Mingozzi, F., Foti, M., Chirico, G., et al. (2009). CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. Nature *460*, 264–268.