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Inflammation mediated by JNK in myeloid cells promotes the development of hepatitis and hepatocellular carcinoma

Myoung Sook Han[1], **Tamera Barrett**[1,2], **Michael A. Brehm**[1], and **Roger J. Davis**[1,2,*]

[1]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA

[2]Howard Hughes Medical Institute, Worcester, MA 01605, USA

SUMMARY

The cJun NH_2-terminal kinase (JNK) signaling pathway is required for the development of hepatitis and hepatocellular carcinoma. A role for JNK in liver parenchymal cells has been proposed, but more recent studies have implicated non-parenchymal liver cells as the relevant site of JNK signaling. Here we tested the hypothesis that myeloid cells mediate this function of JNK. We show that mice with myeloid cell-specific JNK-deficiency exhibit reduced hepatic inflammation and suppression of both hepatitis and hepatocellular carcinoma. These data identify myeloid cells as a site of pro-inflammatory signaling by the JNK signaling pathway that can promote liver pathology. Targeting myeloid cells with a drug that inhibits JNK may therefore provide therapeutic benefit for the treatment of inflammation-related liver disease.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major cause of human cancer death (Fitzmorris et al., 2014; Mikhail et al., 2014). The world-wide incidence of HCC has increased in recent years, but the management of patients with HCC has not dramatically changed. Primary treatment options for early stage disease include surgical resection and liver transplantation. Unresectable disease is treated with loco-regional therapies and/or systemic chemotherapy and is associated with poor rates of survival. New treatment options for patients with HCC are therefore critically important.

The development of HCC appears to require hepatocyte death that triggers disease progression from hepatitis associated with a number of liver insults, including steatosis,

*Contact information: Roger.Davis@umassmed.edu.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cell.com/cell-reports.

AUTHOR CONTRIBUTIONS

M.S.H. and R.J.D. designed the study; M.A.B., M.S.H. and T.B. performed experiments; and M.S.H. and R.J.D. analyzed data and wrote the paper.

hepatotoxins, viral infection, and autoimmune disease (Luedde et al., 2014). These changes are associated with the development of inflammation, fibrosis, and cirrhosis. Recent studies have demonstrated that inflammation is a hallmark of liver disease that may represent a cause of HCC development (Sun and Karin, 2013). This insight suggests that targeting hepatic inflammation may provide therapeutic benefit for the treatment of HCC.

Inflammatory responses are frequently associated with MAP kinases, including the cJun NH_2-terminal kinase (JNK) signaling pathway that is activated by inflammatory cytokines, endotoxin, and physical-chemical stress (Davis, 2000). This signaling pathway is mediated by ubiquitously expressed JNK isoforms that are encoded by the *Mapk8* and *Mapk9* genes (also known as *Jnk1* and *Jnk2*) (Gupta et al., 1996). JNK inhibition therefore represents a potential mechanism for decreasing hepatic inflammation and preventing hepatitis and HCC.

Studies using JNK-deficient mice have confirmed that JNK plays a key role in the development of hepatitis and HCC. For example, $Mapk8^{-/-}$ and $Mapk9^{-/-}$ mice were reported to be resistant to hepatitis (Maeda et al., 2003), although subsequent studies indicated that JNK1 may play a primary role in this response (Kamata et al., 2005; Chang et al., 2006). Moreover, $Mapk8^{-/-}$ mice are resistant to the development of HCC (Sakurai et al., 2006; Hui et al., 2008). Together, these studies demonstrate that JNK plays a key role in hepatitis and the development of HCC in mice.

To test whether JNK in liver parenchymal cells (hepatocytes) is required for hepatitis and HCC development, studies have been performed using tissue-specific JNK knockout mice. These studies demonstrated that JNK in parenchymal cells is not required for the development of hepatitis or HCC (Das et al., 2009; Das et al., 2011). Nevertheless, JNK-deficiency in both parenchymal and non-parenchymal cells did protect against hepatitis and HCC (Das et al., 2009; Das et al., 2011). These data indicate that non-parenchymal cells may represent the site of JNK function that is required for the development of hepatitis and HCC. However, the identity of the relevant non-parenchymal cell population is unclear. These hepatic cells include stellate cells, endothelial cells that form blood vessels and bile ducts, and other cells that mediate innate and adaptive immune responses.

We considered it likely that myeloid cells may represent a site of JNK function during the development of hepatitis and HCC because of the association of inflammation with liver disease (Shirabe et al., 2012; Sun and Karin, 2013) and the role of JNK in the promotion of inflammation (Han et al., 2013). The purpose of this study was to test this hypothesis. We report that mice with myeloid cell-specific JNK-deficiency are resistant to hepatitis and the development of HCC. These data identify myeloid cells as a key site of JNK function in inflammation-related liver disease. This information is important for the design of potential therapies based on the use of small molecules that target JNK because the relevant cell population is now established (myeloid cells). Moreover, relevant biomarkers for dose-ranging studies (myeloid inflammatory molecules) can be defined. Together with previous studies, this analysis confirms JNK inhibition as a possible therapeutic strategy for the treatment of inflammation-related liver disease.

RESULTS

Mice with JNK-deficiency in myeloid cells

We established mice with myeloid cell-specific ablation of the *Mapk8* plus *Mapk9* genes (*LysM-cre*$^{+}$ *Mapk8*$^{LoxP/LoxP}$ *Mapk9*$^{LoxP/LoxP}$ mice) and control mice (*LysM-cre*$^{+}$). To test for *Mapk8* and *Mapk9* gene ablation, we isolated Kupffer cells, macrophages, and neutrophils from control (Ø^{WT}) and JNK-deficient (Ø^{KO}) mice. Genotype analysis demonstrated ablation of the *Mapk8* and *Mapk9* genes in each cell population (Figure S1A). Immunoblot analysis confirmed similar JNK expression in non-myeloid cells of Ø^{WT} and Ø^{KO} mice, including B cells, T cells, and hepatocytes (Figure S1A). Immunophenotyping demonstrated that similar numbers of CD45^{+} leukocytes, dendritic cells (DC), B cells, T cells, NKT cells, NK cells, and monocytes in the blood of Ø^{WT} and Ø^{KO} mice (Figure S1B). Small changes in the number of B cells, T cells, and NK cells were detected in bone marrow and lymph nodes, but no significant differences in these cell populations were observed in the spleen or liver (Figure S1B). This analysis demonstrates that myeloid cell JNK-deficiency does not cause major changes in leukocyte cell numbers. We conclude that Ø^{KO} mice represent a model for studies of JNK-deficiency in myeloid cells (Han et al., 2013).

JNK promotes infiltration of the liver by inflammatory cells

We examined the hepatotoxic response of Ø^{WT} and Ø^{KO} mice exposed to lipopolysaccharide (LPS) plus N-acetyl-galactosamine (GalN). Treatment with LPS/GalN caused a marked increase in the total number of hepatic leukocytes in Ø^{WT} mice (Figure 1). This increase in hepatic leukocytes was strongly suppressed ($p<0.001$) in LPS/GalN-treated Ø^{KO} mice (Figure 1). Flow cytometry using CD11b and F4/80 antibodies identified populations of Kupffer cells (CD11b^{low} F4/80^{hi}) and infiltrating monocytes (CD11b^{hi} F4/80^{low}) (Movita et al., 2012). No significant change in the Kupffer cell population was detected (Figure 1). In contrast, the infiltrating monocyte population was increased in LPS/GalN-treated Ø^{WT} mice and this increase was suppressed ($p<0.001$) in Ø^{KO} mice (Figure 1).

We also examined neutrophils in the Ø^{WT} and Ø^{KO} mice. Flow cytometry demonstrated that treatment with LPS/GalN caused a similar increase in the number of neutrophils (Gr-1^{hi} Cd11b^{+}) circulating in the blood of Ø^{WT} and Ø^{KO} mice (Figure 1). However, the increased hepatic neutrophil population in LPS/GalN-treated Ø^{WT} mice was suppressed in Ø^{KO} mice ($p<0.001$) (Figure 1). Together, these data indicate that while JNK-deficiency does not alter the LPS/GalN-stimulated mobilization of neutrophils from the bone marrow, JNK-deficiency does suppress the infiltration of neutrophils into the liver.

The JNK-mediated promotion of hepatic infiltration by monocytes and neutrophils (Figure 1) may be caused by chemokines (Marra and Tacke, 2014). We therefore examined whether myeloid cell JNK-deficiency disrupted chemokine signaling networks. The monocyte chemokine receptor CCR2 binds ligands (CCL2, CCL7, and CCL8) that are expressed at low levels in the liver of LPS/GalN-treated Ø^{KO} mice compared with LPS/GalN-treated Ø^{WT} mice (Figure 2A). A similar reduction in expression of ligands CCL3, CCL4, CCL5, and CCL8 that bind the chemokine receptor CCR5 (Figure 2A), the ligands CCL2, CCL4, CCL5, CCL17 and CCL22 that bind the chemokine receptor CCR4, and the ligands CXCL1,

CXCL2 and CXCL5 that bind the chemokine receptors CXCR1 and CXCR2 (Figure 2A) were detected in the liver of LPS/GalN-treated $Ø^{KO}$ mice compared with LPS/GalN-treated $Ø^{WT}$ mice. These changes in chemokine signaling may contribute to reduced hepatic infiltration by inflammatory cells detected in $Ø^{KO}$ mice compared with $Ø^{WT}$ mice.

JNK promotes the expression of inflammatory cytokines

The requirement of JNK for normal myeloid cell infiltration of the liver following treatment of mice with LPS/GalN (Figure 1) suggests that JNK plays an important role in the promotion of hepatic inflammation. To explore the role of JNK in inflammation, we examined the hepatic expression of inflammatory cytokines in mice treated with LPS/GalN. We found reduced amounts of inflammatory cytokines (IFNγ , IL1β , IL6, and TNFα) and chemokines (CCL2 and CCL5) circulating in the blood of LPS/GalN-treated $Ø^{KO}$ mice compared with $Ø^{WT}$ mice (Figure 2B). This reduction in cytokine and chemokine expression is consistent with the reduced expression of chemokine mRNA (Figure 2A) and inflammatory cytokine mRNA (Figure 2A) detected in the liver of $Ø^{KO}$ mice compared with $Ø^{WT}$ mice.

Myeloid cells represent a source of inflammatory cytokines (e.g. TNFα) in mice treated with LPS/GalN. We therefore performed intracellular staining to detect the expression of TNFα by hepatic leukocytes. We found that JNK-deficiency caused significantly reduced TNFα expression ($p<0.05$) by Kupffer cells, infiltrating monocytes, and neutrophils (Figures 2C & S2). Together, these data demonstrate that multiple myeloid cell types may contribute to the suppression of TNFα expression caused by JNK-deficiency.

JNK promotes the development fulminant hepatitis

The reduction of hepatic infiltration by myeloid cells (Figure 1) and the decreased expression of inflammatory cytokines (Figure 2) suggest that LPS/GalN-induced hepatitis may be suppressed in $Ø^{KO}$ mice compared with $Ø^{WT}$ mice. Indeed, we found that myeloid cell JNK-deficiency caused reduced hepatic hemorrhage and reduced TUNEL-staining of apoptotic cells (Figure 3A). We also examined serum aminotransferases (a marker for hepatic damage) in LPS/GalN-treated $Ø^{WT}$ and $Ø^{KO}$ mice. This analysis demonstrated that JNK-deficiency significantly ($p<0.01$) reduced the amount of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in $Ø^{KO}$ mice compared with $Ø^{WT}$ mice (Figure 3B). Immunoblot analysis of liver extracts demonstrated reduced expression of Bad, a pro-apoptotic BH3-only protein implicated in LPS/GalN-induced hepatitis (Takamura et al., 2007), in liver extracts prepared from in LPS/GalN-treated $Ø^{WT}$ and $Ø^{KO}$ mice. Moreover, we found reduced caspase 3 activation and reduced cleavage of PARP (a caspase 3 substrate) in $Ø^{KO}$ mice compared with $Ø^{WT}$ mice (Figure 3C). Consistent with these observations, the survival of $Ø^{KO}$ mice was significantly increased ($p<0.005$) compared with $Ø^{WT}$ mice following treatment with LPS/GalN (Figure 3D). Together, these data demonstrate that JNK in myeloid cells can function to promote the development of fulminant hepatitis.

It is established that the LPS/GalN model of hepatitis depends upon the interaction of soluble TNFα with TNF-R1 (Nowak et al., 2000). Our analysis suggests that reduced TNFα

expression (Figure 2) may account for the resistance of $Ø^{KO}$ mice to LPS/GalN-induced hepatitis compared with $Ø^{WT}$ mice. However, it is also possible that $Ø^{KO}$ mice exhibit a defect in TNFα signaling. To test this hypothesis, we compared the response of $Ø^{WT}$ and $Ø^{KO}$ mice to treatment with TNFα /GalN. We found that TNFα /GalN caused similar hepatic hemorrhage (Figure S3A) and similar concentrations of serum aminotransferases (ALT & AST) in $Ø^{WT}$ and $Ø^{KO}$ mice (Figure S3B). Moreover, biochemical analysis of liver extracts demonstrated similar caspase 3 activation and PARP cleavage in $Ø^{WT}$ and $Ø^{KO}$ mice (Figure S3C). No significant difference in the blood concentration of inflammatory cytokines (IL6 and TNFα) or chemokines (CCL2 and CCL5) between $Ø^{WT}$ and $Ø^{KO}$ mice was detected (Figure S3D). Flow cytometry demonstrated that TNFα /GalN caused similar hepatic infiltration by inflammatory cells in $Ø^{WT}$ and $Ø^{KO}$ mice (Figure S3E). Together, these data demonstrate that JNK-deficiency in myeloid cells suppresses TNFα expression (Figure 2), but does not suppress TNFα signaling (Figure S3) in a mouse model of hepatitis, including increased hepatic infiltration by myeloid cells (Figure S3E).

JNK in myeloid cells promotes the development of HCC

It is established that the development of HCC is increased by inflammation (Sun and Karin, 2013). Since hepatic inflammation is suppressed in $Ø^{KO}$ mice compared with $Ø^{WT}$ mice (Figures 1–3), we anticipated that JNK-deficiency in myeloid cells might protect mice against the development of HCC. To test this hypothesis, we examined the development of HCC in $Ø^{WT}$ and $Ø^{KO}$ mice using the diethlynitrosamine (DEN) model of liver cancer. The mice were exposed to the carcinogen DEN at age 2 wk and tumor development was examined at age 38 wk (Figure 4A). JNK-deficiency in myeloid cells caused significantly decreased liver mass ($p<0.05$) and decreased tumor size ($p<0.01$), but did not cause a significant change in the number of hepatic tumors detected by macroscopic examination (Figure 4B). Examination of liver sections by a pathologist demonstrated that myeloid cell JNK-deficiency decreased the development of adenoma and carcinoma (Figure S4A). Control studies indicated that HCC in $Ø^{WT}$ and $Ø^{KO}$ mice expressed similar amounts of JNK (Figure S4B). Together, these data demonstrate that JNK in myeloid cells is not required for tumor formation, but myeloid cell JNK acts to promote tumor development by increasing both tumor grade and tumor mass. To test this conclusion, we examined a different model of hepatic tumor development. We found that liver tumors caused by intrasplenic injection of B16 melanoma cells were markedly suppressed in $Ø^{KO}$ mice compared with $Ø^{WT}$ mice (Figure S4C,D). This observation confirms the conclusion that JNK in myeloid cells can promote tumor development in the liver.

Liver sections stained by TUNEL assay and an antibody to the proliferation marker PCNA demonstrated that myeloid cell JNK-deficiency caused both decreased hepatocyte cell death and decreased proliferation in the DEN model of HCC (Figure 4C). This observation is consistent with a role for JNK-mediated inflammation in damage-induced regeneration during development of HCC (Das et al., 2011). To test whether JNK in myeloid cells contributes to tumor-associated inflammation, we examined the expression of cytokines and chemokines. Blood analysis demonstrated decreased amounts of chemokines and inflammatory cytokines (Figure 4D). It is likely that the observed reduction in cytokine and chemokine expression reflects both expression by myeloid cells and also actions of myeloid

cells on expression by different cell types, including other immune cells and tumor cells. Indeed, we found decreased hepatic expression of markers of CD8 T cells (but not CD4 T cells), Tregs (*Foxp3*), and macrophages (*Emr1* (F4/80)) (Fig. 4E) together with inflammatory cytokine mRNA (*Il1β* , *Il6*, and *Tnfα*) and chemokines /chemokine receptor mRNA, including *Ccr2* (and ligands *Ccl2*, *Ccl7*, and *Ccl8*), *Ccr4* (and ligands *Ccl2*, *Ccl4*, *Ccl5*, *Ccl17* and *Ccl22*), *Ccr5* (and ligands *Ccl3*, *Ccl4*, *Ccl5* and *Ccl8*), and *Cxcr1/2* (and ligands *Cxcl1*, *Cxcl2* and *Cxcl5*) in $Ø^{KO}$ tumors compared with $Ø^{WT}$ tumors (Figure 4F). These data confirm that JNK in myeloid cells promotes tumor-associated inflammation.

Studies of cell cycle regulatory gene expression demonstrated that myeloid cell JNK-deficiency caused decreased tumor-associated expression of *Cdk1*, *cMyc*, $p15^{INK4B}$, $p21^{CIP1}$ and *Tgfβ 1* mRNA (Figure 4F). The finding that $Ø^{KO}$ tumors express decreased amounts of *Cdk1* and *cMyc* compared with $Ø^{WT}$ tumors is consistent with the larger tumor burden of control mice compared with myeloid cell JNK-deficient mice.

DISCUSSION

It is established that immunosurveillance plays a key role in tumor development (Hanahan and Weinberg, 2011) that can lead to either prevention or promotion of cancer (Schreiber et al., 2011). Pro-tumorigenic inflammation is generally associated with tumor infiltration by M2-like macrophages, myeloid-derived suppressor cells, Th2 cells, and Treg cells (Melief and Finn, 2011). In contrast, anti-tumorigenic inflammation is most often associated with tumor infiltration by M1-like macrophages, Th1 cells, and $CD8^+$ cytotoxic T cells (Melief and Finn, 2011). The balance of these inflammatory mechanisms represents one factor that controls tumor formation.

Interestingly, both M1-like and M2-like macrophages (and also mixed-phenotype macrophages) are present in tumors, although there appears to be regional localization with M1-like macrophages primarily located in the peri-tumoral stroma and M2-like macrophages within the growing tumor (Kuang et al., 2007). Tumor development is associated with the progressive replacement of M1-like macrophages during early stages by M2-like macrophages within established tumors (Capece et al., 2013). The mechanism of macrophage polarization to the M2 phenotype in tumors may involve multiple mechanisms, including IL4 produced by T cells (DeNardo et al., 2009) and lactic acid produced by tumor cells (Colegio et al., 2014), that regulate signaling pathways that control macrophage polarization (Zhou et al., 2014).

The JNK signaling pathway is implicated in the control of macrophage polarization (Zhou et al., 2014). Consequently, reduced JNK signaling causes decreased expression of the M1-associated pro-inflammatory cytokines TNFα , IL1β , and IL6 (Han et al., 2013) and reduced expression of the M2-associated chemokines CCL17 and CCL22 (Hefetz-Sela et al., 2014). These observations are significant because TNFα , IL1β , and IL6 promote hepatic inflammation, hepatitis, and HCC development (Park et al., 2010; Negash and Gale, 2015) and CCL17/CCL22 can act as chemoattractants for tumor-associated $Foxp3^+$ Tregs that regulate anti-tumor immunity (Nishikawa and Sakaguchi, 2010). Consequently, decreased expression of chemokines and inflammatory cytokines by JNK-deficient myeloid cells may

contribute to reduced hepatic infiltration by inflammatory cells and the suppression of both hepatitis and HCC detected in $Ø^{KO}$ mice compared with $Ø^{WT}$ mice (Figure 3 & 4). Moreover, JNK in myeloid cells may influence the overall inflammatory microenvironment through immunoregulatory cells (Figure 4E), including $CD8^+$ $Foxp3^+$ Tregs that are associated with high grade HCC development (Yang et al., 2010).

In conclusion, we identify myeloid cells as a site of pro-inflammatory signaling by the JNK signaling pathway that can promote liver pathology. Targeting myeloid cells with a drug that inhibits JNK may therefore provide therapeutic benefit for the treatment of inflammation-related liver disease. A challenge for the design of such therapy is the potential for altered innate and adaptive immune responses caused by defects in macrophage function.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J mice (stock number 000664) and B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J mice (stock number 004781) (Clausen et al., 1999) were obtained from The Jackson Laboratory. We have described *Mapk8*$^{LoxP/LoxP}$ mice (Das et al., 2007) and *Mapk9* $^{LoxP/LoxP}$ mice (Han et al., 2013). The mice were backcrossed to the C57BL/6J strain (ten generations) and housed in a specific pathogen free facility (accredited by the American Association for Laboratory Animal Care) at 20°C using laminar flow cages. The Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School approved all studies using animals.

Hepatitis was induced by intraperitoneal (i.p.) injection (male mice at age 8 – 10 wks.) with 35 μg/kg *E. coli* 0111:B LPS (Sigma) plus 750 mg/kg N-acetyl-galactosamine (GalN) (Sigma) or 20 μg/kg TNFα (R&D Systems) plus 750 mg/kg GalN (Galanos et al., 1979; Wallach et al., 1988). Kaplan-Meier analysis was performed using mice treated with 25 μg/kg LPS plus 750 mg/kg GalN. Carcinogen-induced HCC was induced by i.p. injection (male mice at age 2 wks.) with single dose (25 mg/kg) of DEN (Sigma) diluted in glyceryl trioctanoate (Sigma); the mice were euthanized at age 38 wks. Control mice were injected with solvent alone. Liver metastasis was examined using mice (6–7 wk old) intrasplenically injected with 1 x 10^6 B16 melanoma cells in 100μl PBS (Kitajima et al., 2008); the mice were euthanized on day 12 post-injection.

Statistical analysis

Data are expressed as the mean ± SEM to assess whether the variance of each group was similar. The statistical significance of differences between groups was examined using Student’s test (two-sided) or analysis of variance (ANOVA) with the Fisher’s test. Kaplan-Meier analysis was performed using the Log-rank test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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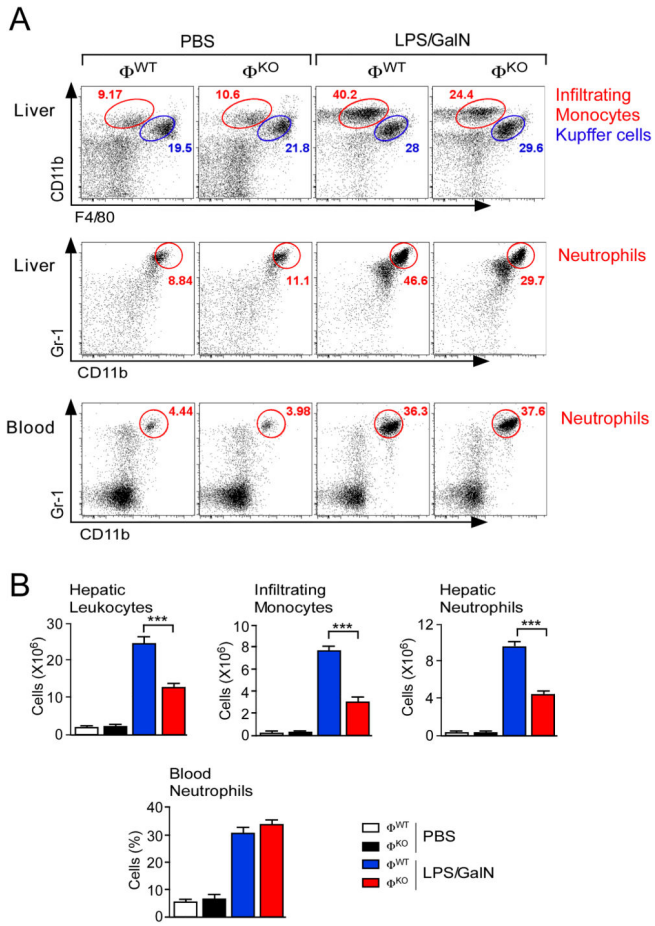

Figure 1. Myeloid JNK promotes hepatic infiltration by monocytes and neutrophils

(**A**) Mice ($Ø^{WT}$ and $Ø^{KO}$) were treated with PBS or LPS/GalN (5.5 h). Representative flow cytometry data of hepatic leukocytes stained with antibodies to CD11b and F4/80 (red, infiltrating monocytes; blue, Kupffer cells) are presented (*upper panels*). Representative flow cytometry data of neutrophils stained with antibodies to CD11b and Gr-1 (red) within total hepatic leukocytes (*middle panels*) and blood (*lower panels*) are presented.

(**B**) The total number of hepatic leukocytes is presented (mean ± SEM; PBS $Ø^{WT}$, n = 3; PBS $Ø^{KO}$, n = 4; LPS/GalN $Ø^{WT}$, n = 13; LPS/GalN $Ø^{KO}$, n = 12). The number of total hepatic leukocytes corresponding to infiltrating monocytes, infiltrating neutrophils, and Kupffer cells is presented (mean ± SEM; PBS $Ø^{WT}$, n = 5; PBS $Ø^{KO}$, n = 6; LPS/GalN $Ø^{WT}$, n = 11 (except neutrophils, n = 16); LPS/GalN $Ø^{KO}$, n = 11 (except neutrophils, n = 15)). The percentage of total blood leukocytes corresponding to neutrophils is also presented (mean ± SEM). PBS groups, n = 3; LPS/GalN groups, n = 9). Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (***, $p<0.001$).

See also Figure S1.

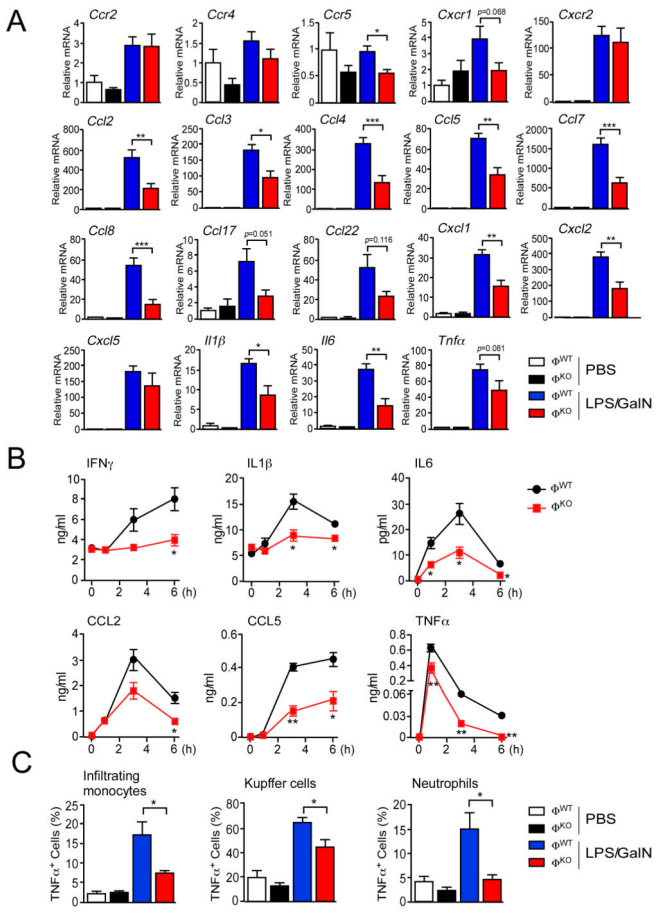

Figure 2. Myeloid JNK-deficiency suppresses expression of inflammatory cytokines

(**A**) Hepatic expression of the chemokine receptors (*Ccr2*, *Ccr4*, *Ccr5*, *Cxcr1* and *Cxcr2*), ligands (*Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl7*, *Ccl8*, *Ccl17*, *Ccl22*, *Cxcl1*, *Cxcl2* and *Cxcl5*) and inflammatory cytokines (*Il1β* , *Il6*, and *Tnfα*) is presented. The data shows relative mRNA expression (mean ± SEM; n = 8) measured by quantitative RT-PCR assays using total RNA isolated from $Ø^{WT}$ and $Ø^{KO}$ mice treated (6 h) with PBS or LPS/GalN. Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

(**B**) The concentration of inflammatory cytokines and chemokines in the blood of $Ø^{WT}$ and $Ø^{KO}$ mice treated with LPS/GalN was measured by multiplexed ELISA (mean ± SEM; n = 10). Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (*, $p<0.05$; **, $p<0.01$).

(**C**) The expression of TNFα by hepatic myeloid cells (infiltrating monocytes, Kupffer cells, and neutrophils) was measured by intracellular staining. The percentage TNFα-positive cells was quantitated (mean ± SEM; PBS, n = 3; LPS/GalN, n = 6). Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (*, $p<0.05$).

See also Figure S2.

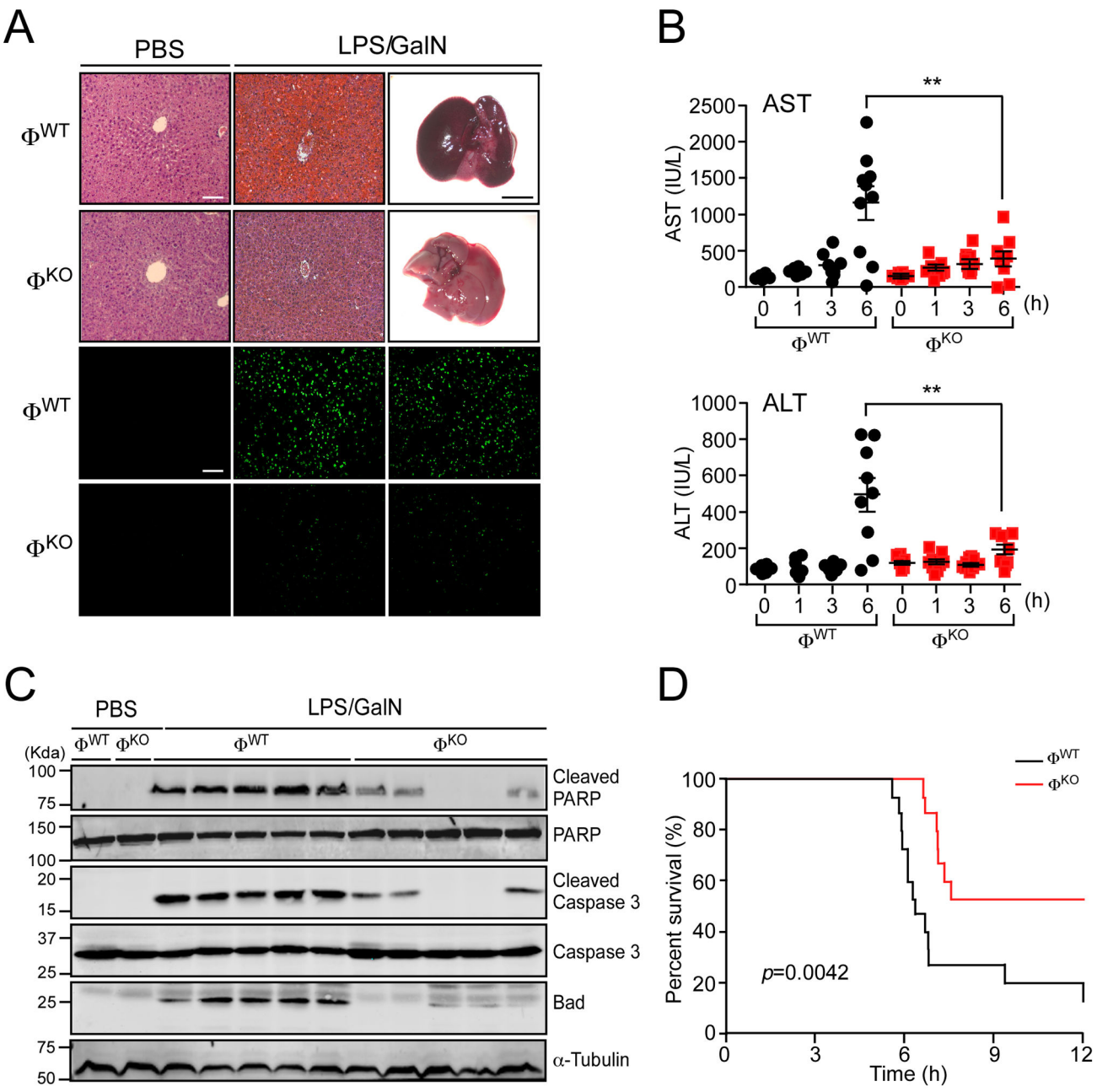

Figure 3. Myeloid JNK-deficiency suppresses the development of fulminant hepatitis

(**A**) Mice ($Ø^{WT}$ and $Ø^{KO}$) were treated with PBS or LPS/GalN (6 h). Representative images of the dissected liver (scale bar, 5 mm) and hematoxylin & eosin-stained liver sections are presented (*upper panels*). Apoptotic cells in the liver sections were examined by TUNEL assay (*lower panels*). Scale bars, 100 μm.

(**B**) The accumulation of hepatic aminotransferases (ALT and AST) in the blood of $Ø^{WT}$ and $Ø^{KO}$ mice was measured (mean ± SEM; n = 10, except ALT at 6 h, n = 9). Statistically significant differences between LPS/GalN-treated $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (**, $p<0.01$).

(**C**) Liver extracts prepared from mice treated with PBS or LPS/GalN (6 h) were examined by immunoblot analysis using antibodies to α-Tubulin, Bad, Caspase 3, cleaved Caspase 3, PARP and cleaved PARP.

(**D**) Kaplan-Meier analysis of survival following treatment of $Ø^{WT}$ and $Ø^{KO}$ mice with LPS/GalN (n = 15). See also Figure S3.

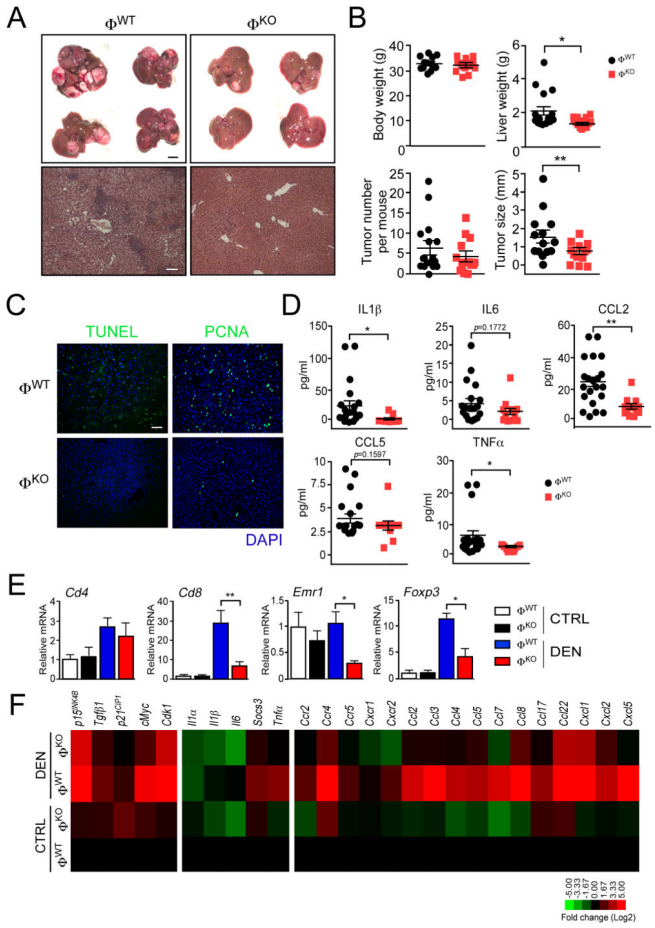

Figure 4. Myeloid JNK promotes the development of HCC

(**A**) Mice ($Ø^{WT}$ and $Ø^{KO}$) were treated with diethynitrosamine (DEN) at age 2 wks. and euthanized at age 38 wks. Representative images of livers (scale bar, 500 μm) and hematoxylin & eosin-stained liver sections (scale bar, 100 μm) are presented.

(**B**) The total body mass, liver mass, surface tumor number, and tumor size are presented (mean ± SEM). Body mass: $Ø^{WT}$, n = 17; $Ø^{KO}$, n = 14. Liver mass: $Ø^{WT}$, n = 18; $Ø^{KO}$, n = 14. Tumor number and size: $Ø^{WT}$, n = 19; $Ø^{KO}$, n = 14. Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (*, $p<0.05$; **, $p<0.01$).

(**C**) Representative sections of liver stained with DAPI (blue) and by TUNEL assay (green) or with an antibody to PCNA (green) are presented. Scale bar, 100 μm.

(**D**) The concentration of blood cytokines (IL1β , IL6, and TNFα) and chemokines (CCL2 and CCL5) was measured by multiplexed ELISA (mean ± SEM). $Ø^{WT}$, n = 21; $Ø^{KO}$, n = 13). Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (*, $p<0.05$; **, $p<0.01$).

(**E**) Hepatic mRNA associated with immune cell sub-sets (*Cd4*, *Cd8*, *Foxp3*, and *Emr1* (F4/80)) was examined by quantitative RT-PCR assays (mean ± SEM; n = 3). Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (*, $p<0.05$; **, $p<0.01$).

(**F**) The expression of hepatic mRNA for inflammatory cytokines (*Il1α* , *Il1β* , *Il6,* and *Tnfα*) and the cytokine target gene *Socs3*, the chemokine receptors (*Ccr2*, Ccr4, *Ccr5,*

Cxcr1 and *Cxcr2)*, the chemokine ligands (*Ccl2, Ccl3, Ccl4, Ccl5, Ccl7*, *Ccl8, Ccl17, Ccl22, Cxcl1, Cxcl2 and Cxcl5*) and proliferation-associated genes (*Cdk1, p15*^{INK4B}, *p21*^{CIP1}, *cMyc* and *Tgfβ 1*) are presented as a heat map of log2 transformed data normalized to control $Ø^{WT}$ mice (mean; n = 3).

See also Figure S4.