Inflammation Mediated by JNK in Myeloid Cells Promotes the Development of Hepatitis and Hepatocellular Carcinoma

Graphical Abstract

Highlights

- JNK in myeloid cells promotes hepatic infiltration by inflammatory cells
- Myeloid cell JNK deficiency suppresses development of fulminant hepatitis
- JNK in myeloid cells promotes the development of hepatocellular carcinoma

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In Brief

Han et al. examine the role of JNK in inflammation-associated liver disease. Myeloid cell JNK deficiency reduces the expression of inflammatory cytokines and chemokines, reduces hepatic infiltration by inflammatory cells, and suppresses the development of hepatitis and hepatocellular carcinoma. JNK in myeloid cells is therefore a key regulator of hepatic inflammation.
Inflammation Mediated by JNK in Myeloid Cells Promotes the Development of Hepatitis and Hepatocellular Carcinoma

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SUMMARY

The cJun NH2-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 JNK 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 ( Fitzmorrises et al., 2015 ; Mikhail et al., 2014 ). The worldwide 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, 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 mitogen-activated protein (MAP) kinases, including the cJun NH2-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 , 2011 ). Nevertheless, JNK deficiency in both parenchymal and non-parenchymal cells did protect against hepatitis and HCC (Das et al., 2009 , 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+ Mapk8LoxP/LoxP Mapk9LoxP/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 (DCs), B cells, T cells, natural killer T (NKT) cells, natural killer (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 ØKO 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 ØKO mice (Figure 1).

We also examined neutrophils in the ØWT 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 ØWT and ØKO mice (Figure 1). However, the increased hepatic neutrophil population in LPS/GalN-treated ØWT mice was suppressed in ØKO 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 ØKO mice compared with LPS/GalN-treated ØWT 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 ØKO mice compared with LPS/GalN-treated ØWT mice. These changes in chemokine signaling may contribute to reduced hepatic infiltration by inflammatory cells detected in ØKO mice compared with ØWT 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 (interferon γ [IFN-γ], interleukin-1β [IL-1β], IL-6, and tumor necrosis factor α [TNF-α]) and chemokines (CCL2 and CCL5) circulating in the blood of LPS/GalN-treated ØKO mice compared with ØWT 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 ØKO mice compared with ØWT mice.

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

JNK Promotes the Development of 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 ØKO mice compared with ØWT 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 ØWT and ØKO mice. This analysis demonstrated that JNK deficiency significantly (p < 0.01) reduced the amount of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in ØKO mice compared with ØWT 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), and aspartate aminotransferase (AST) in ØKO mice compared with ØWT mice (Figure 3B). Consistent with these observations, the survival of ØKO mice was significantly increased (p < 0.005) compared with ØWT 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 TNF-α with TNF-R1 (Nowak et al., 2000). Our analysis suggests that reduced TNF-α expression (Figure 2) may account for the resistance of ØKO mice to LPS/GalN-induced hepatitis compared with ØWT mice. However, it is also possible that ØKO mice exhibit a defect in TNF-α signaling.
To test this hypothesis, we compared the response of ØWT and ØKO mice to treatment with TNF-α/GalN. We found that TNF-α/GalN caused similar hepatic hemorrhage (Figure S3A) and similar concentrations of serum aminotransferases (ALT and AST) in ØWT and ØKO mice (Figure S3B). Moreover, biochemical analysis of liver extracts demonstrated similar caspase-3 activation and PARP cleavage in ØWT and ØKO mice (Figure S3C). No significant difference in the blood concentration of inflammatory cytokines (IL-6 and TNF-α) or chemokines (CCL2 and CCL5) between ØWT and ØKO mice was detected (Figure S3D). Flow cytometry demonstrated that TNF-α/GalN caused similar hepatic infiltration by inflammatory cells in ØWT and ØKO mice (Figure S3E). Together, these data demonstrate that JNK-deficiency in myeloid cells suppresses TNF-α expression (Figure 2), but does not suppress TNF-α 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 ØKO mice compared with ØWT mice (Figures 1, 2, and 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 ØWT and ØKO mice using the diethylnitrosamine (DEN) model of liver cancer. The mice were exposed to the carcinogen DEN at age 2 weeks, and tumor development was examined at age 38 weeks (Figure 4A). JNK deficiency in myeloid cells caused significantly decreased liver mass (p < 0.05) and decreased tumor size (p < 0.01), but it 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 ØWT and ØKO 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 ØKO mice compared with ØWT mice (Figures S4C and S4D). 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), regulatory T cells (Tregs; Foxp3), and macrophages (Emr1; F4/80) (Figure 4E) together with inflammatory cytokine mRNA (Il13, 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 Cxc1f1, Cxc1f2, and Cxc1f5) in ØKO tumors compared with ØWT tumors (Figure 4F). These data confirm that JNK in myeloid cells promotes tumor-associated inflammation.

**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 (Melleif 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 (Melleif 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 (Capce 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 (Colecgi 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 TNF-α, IL-1β, and IL-6 (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 TNF-α, IL-1β, and IL-6 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 ØKO mice compared with Ø WT mice (Figures 3 and 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 no. 000664) and B6.129P2-Lyz2tm1(cre)ifo/J mice (stock no. 004781) (Clausen et al., 1999) were obtained from The Jackson Laboratory. We have previously described Mapk8LoxP/LoxP mice (Das et al., 2007) and Mapk9LoxP/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 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 weeks) with 35 μg/kg E. coli O111:B LPS (Sigma-Aldrich) plus 750 mg/kg GalN (Sigma-Aldrich) or 20 μg/kg TNF-α (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 weeks) with a single dose (25 mg/kg) of DEN (Sigma) diluted in glycercyl trictanoate (Sigma); the mice were euthanized at age 38 weeks. Control mice were injected with solvent alone. Liver metastasis was examined using mice (6–7 weeks old) intrasplenically injected with 1 x 10^6 B16 melanoma cells in 100 μl 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 t-test (two-sided) or ANOVA with the Fisher’s test. Kaplan-Meier analysis was performed using the log-rank test.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.03.008.

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

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