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Myeloid Notch1 Deficiency Activates the RhoA/ROCK Pathway and Aggravates Hepatocellular Damage in Mouse Ischemic Livers

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Notch signaling plays an emerging role in the regulation of immune cell development and function during inflammatory response. Activation of the ras homolog gene family member A/Rho-associated protein kinase (ROCK) pathway promotes leukocyte accumulation in tissue injury. However, it remains unknown whether Notch signaling regulates ras homolog gene family member A/ROCK–mediated immune responses in liver ischemia and reperfusion (IR) injury. This study investigated intracellular signaling pathways regulated by Notch receptors in the IR-stressed liver and *in vitro*. In a mouse model of IR-induced liver inflammatory injury, we found that mice with myeloid-specific Notch1 knockout showed aggravated hepatocellular damage, with increased serum alanine aminotransferase levels, hepatocellular apoptosis, macrophage/neutrophil trafficking, and proinflammatory mediators compared to Notch1-proficient controls. Unlike in the controls, myeloid Notch1 ablation diminished hairy and enhancer of split-1 (Hes1) and augmented c-Jun N-terminal kinase (JNK)/stress-activated protein kinase–associated protein 1 (JSAP1), JNK, ROCK1, and phosphatase and tensin homolog (PTEN) activation in ischemic livers. Disruption of JSAP1 in myeloid-specific Notch1 knockout livers improved hepatocellular function and reduced JNK, ROCK1, PTEN, and toll-like receptor 4 activation. Moreover, ROCK1 knockdown inhibited PTEN and promoted Akt, leading to depressed toll-like receptor 4. In parallel *in vitro* studies, transfection of lentivirus-expressing Notch1 intracellular domain promoted Hes1 and inhibited JSAP1 in lipopolysaccharide-stimulated bone marrow–derived macrophages. Hes1 deletion enhanced JSAP1/JNK activation, whereas clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9–mediated JSAP1 knockout diminished ROCK1/PTEN and toll-like receptor 4 signaling. *Conclusion:* Myeloid Notch1 deficiency activates the ras homolog gene family member A/ROCK pathway and exacerbates hepatocellular injury by inhibiting transcriptional repressor Hes1 and inducing scaffold protein JSAP1 in IR-triggered liver inflammation; our findings underscore the crucial role of the Notch–Hes1 axis as a novel regulator of innate immunity-mediated inflammation and imply the therapeutic potential for the management of organ IR injury in transplant recipients. (HEPATOLOGY 2018;67:1041-1055).

Ischemia and reperfusion (IR) injury (IRI) is an innate immunity-dominated local inflammation response. It remains the major cause of organ dysfunction and failure in liver transplantation.[1] Innate immune cells and signaling pathways recognize exogenous danger signals such as pathogen-derived molecular patterns or danger-associated molecular patterns that are released from stressed, injured, or dying cells.[2-4] Macrophages are key components of the innate immune system and contribute to liver

Abbreviations: BMM, bone marrow–derived macrophage; Cas9, CRISPR-associated protein 9; CD, cluster of differentiation; Cre, cyclization recombination; CRISPR, clustered regularly interspaced short palindromic repeats; FL, floxed; Hes1, hairy and enhancer of split-1; IL, interleukin; IR, ischemia and reperfusion; IRI, IR injury; JNK, c-Jun N-terminal kinase; JSAP1, JNK/stress-activated protein kinase–associated protein 1; KO, knockout; LPS, lipopolysaccharide; Lyz2, lysozyme 2; MCP1, monocyte chemoattractant protein 1; M-KO, myeloid-specific knockout; NF-κB, nuclear factor kappa B; NICD, Notch intracellular domain; NS, nonspecific; p-, phosphorylated; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RhoA, ras homolog gene family, member A; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; sALT, serum alanine aminotransferase; siRNA, small interfering RNA; TLR, toll-like receptor 4; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxyribonucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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inflammatory response.[5] We have demonstrated that hepatic IR activates liver macrophages (Kupffer cells) and triggers toll-like receptor 4 (TLR4) or NLRP3-driven inflammation.[6-8] Indeed, macrophage activation increases the release of oxygen free radicals and different types of cytokines such as tumor necrosis factor (TNF-α), which triggers the apoptotic pathway leading to the death of hepatocytes.[9,10]

Recent evidence suggests that ras homolog gene family member A (RhoA)/Rho kinase (ROCK) may act as a "molecular switch" in the activation and synthesis of lipopolysaccharide (LPS)–mediated monocyte proinflammatory response.[11] Specific inhibition of the ROCK pathway prevents nuclear factor kappa B (NF-κB) activation and inflammatory response in different inflammatory diseases.[12,13] Activation of RhoA/ROCK signaling increases hepatic stellate cell susceptibility in steatotic livers to IRI.[14] Moreover, activation of the RhoA downstream effector ROCK might initiate phosphatase and tensin homolog (PTEN) activity to promote leukocyte migration during inflammation.[15] We have shown that PTEN/phosphoinositide 3-kinase signaling plays an important role in the regulation of TLR4-mediated innate immune response in hepatic IRI.[6] Thus, RhoA/ROCK activation may be critical for triggering IR-induced liver inflammation.

Notch signaling is highly conserved and critically involved in cell growth, differentiation, and survival.[16] Four distinct Notch receptors (Notch 1-4) and five Notch ligands (Jagged1, Jagged2, Delta-like 1, Delta-like 3, and Delta-like 4) have been identified in mammalian cells.[17,18] The interaction between Notch receptors and their ligands leads to two proteolytic cleavage steps by a disintegrin and metalloprotease family protease and by the intracellular γ-secretase complex that releases the Notch intracellular domain (NICD). The NICD then translocates to the nucleus and binds to the recombinant recognition sequence binding protein at the Jκ site, a potent DNA-binding transcription factor that is associated with a large number of chromatin regulators, corepressors, and coactivators.[19] This interaction results in the activation of Notch target genes.[20] In the immune system, Notch signaling controls the homeostasis of several innate cell populations and regulates immune cell development and function.[21] Activation of Notch1 and its ligand Jagged-1 increases cell growth and differentiation during liver regeneration.[22] Disruption of the transcription factor recombinant recognition sequence binding protein at the Jκ site increases cell apoptosis/necrosis and inflammatory response, leading to aggravated liver injury.[23] Although these studies have shown that hepatocellular protection is correlated with Notch

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signaling during liver inflammation, the molecular mechanisms and crosstalk between transcription targets and signaling pathways of Notch-mediated regulation in liver inflammation remain largely unknown.

Materials and Methods

ANIMALS

Floxed Notch1 (Notch1$^{FL/FL}$; catalog number 007181) mice (The Jackson Laboratory, Bar Harbor, ME) and mice expressing cyclization recombination (Cre) recombinase under the control of the lysozyme 2 (Lyz2) promoter (LysM-Cre; catalog number 004781; The Jackson Laboratory) were used to generate myeloid-specific Notch1 knockout (Notch1$^{M\text{-}KO}$) mice (Supporting Fig. S3). Two steps were used to generate Notch1$^{M\text{-}KO}$ mice. First, a homozygous loxP-flanked Notch1 mouse was mated with a homozygous Lyz2-Cre mouse to generate the F1 mice that were heterozygous for a loxP-flanked Notch1 allele and heterozygous for the Lyz2-Cre. Next, these F1 mice were backcrossed to the homozygous loxP-flanked Notch1 mice, resulting in the generation of Notch1$^{M\text{-}KO}$ (25% of the offspring), which were homozygous for the loxP-flanked Notch1 allele and heterozygous for the Lyz2-Cre allele (Supporting Fig. S4). This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. The study protocols were approved by the Institutional Animal Care and Use Committee of The University of California at Los Angeles, University of Southern California, and Nanjing Medical University in China. See Supporting Information.

MOUSE LIVER IRI MODEL AND TREATMENT

We used an established mouse model of warm hepatic ischemia followed by reperfusion, as described.[24] Some animals were injected in the tail vein with c-Jun N-terminal kinase (JNK)/stress-activated protein kinase–associated protein 1 (JSAP1) small interfering RNAs (siRNAs), ROCK1 siRNA, or nonspecific (NS; control) siRNA (2 mg/kg) (Santa Cruz Biotechnology, Santa Cruz, CA) mixed with mannose-conjugated polymers at a ratio according to the manufacturer's instructions 4 hours prior to ischemia as described.[8] See Supporting Information.

HEPATOCELLULAR FUNCTION ASSAY

Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured by IDEXX Laboratories (Westbrook, ME).

HISTOLOGY, IMMUNOHISTOCHEMISTRY, IMMUNOFLUORESCENCE STAINING

Liver sections were stained with hematoxylin and eosin. The severity of IRI was graded using Suzuki's criteria.[25] Liver macrophages and neutrophils were detected using primary rat antimouse cluster of differentiation 11b–positive (CD11b^{+}) and Ly6G monoclonal antibodies for immunofluorescence or immunohistochemical staining. See Supporting Information.

TERMINAL DEOXYRIBONUCLEOTIDYL TRANSFERASE–MEDIATED DEOXYURIDINE TRIPHOSPHATE NICK-END LABELING ASSAY

The Klenow-FragEL DNA Fragmentation Detection Kit (EMD Chemicals, Gibbstown, NJ) was used to detect the DNA fragmentation characteristic of oncotic necrosis/apoptosis in formalin-fixed paraffin-embedded liver sections.[7] See Supporting Information.

CASPASE-3 ACTIVITY ASSAY

Caspase-3 activity was performed and determined by an assay kit (Calbiochem, La Jolla, CA) as described.[26] See Supporting Information.

QUANTITATIVE RT-PCR ANALYSIS

Quantitative real-time PCR was performed as described.[27] Primer sequences used for the amplification are shown in Supporting Table S1. See Supporting Information.

WESTERN BLOT ANALYSIS

Protein was extracted from liver tissue or cell cultures as described.[27] The monoclonal rabbit antimouse Notch1, NICD, hairy and enhancer of split-1

(Hes1), phosphorylated JNK (p-JNK), JNK, ROCK1, PTEN, p-Akt, Akt, TLR4, cleaved caspase-3, p-IκBα, and β-actin antibodies (Cell Signaling Technology, MA) and mouse monoclonal antibody JSAP1 (Santa Cruz Biotechnology) were used. See Supporting Information.

LENTIVIRAL VECTOR CONSTRUCTION

The pSin-NICD vector, which expresses the NICD that contains EF2 promoter and puromycin gene, was constructed. psPAX2 and pCMV-VSV-G are lentiviral packaging plasmids. We cotransfected 293T cells with pSin-NICD, psPAX2, and pCMV-VSV-G using lipofectamine LTX Plus reagent to package lentiviruses according to the manufacturer's instructions. See Supporting Information.

The lentiviral clustered regularly interspaced short palindromic repeats (CRISPR) Hes1 knockout (KO) or JSAP1 KO vector was constructed by the first cloning of Hes1 or JSAP1 single guide RNA sequences into the site of *BsmB*I of LentiCRISPRv2 vector as described.[28] Lentiviral vectors were produced as described above. The Lenti-CRISPRv2-Hes1 KO (LV-Hes1 KO) or Lenti-CRISPRv2-JSAP1 KO (LV-JSAP1 KO), psPAX2, and pCMV-VSV-G were used for packaging viruses. Lenti-CRISPRv2 without guide RNA virus was used as a control. See Supporting Information.

ISOLATION OF HEPATOCYTE AND LIVER MACROPHAGES

Primary hepatocytes and liver macrophages (Kupffer cells) from Notch1$^{FL/FL}$ and Notch1$^{M\text{-}KO}$ mice were isolated, as described.[27,29] The purity of macrophages in ischemic livers was 80% as assessed by immunofluorescence staining for CD11b^{+}. See Supporting Information.

BONE MARROW–DERIVED MACROPHAGE ISOLATION AND *IN VITRO* TRANSFECTION

Murine bone marrow–derived macrophages (BMMs) were generated, as described.[26] Cells (1×10^6/well) were cultured for 7 days and then transduced with lentivirus-expressing NICD, CRISPR/CRISPR-associated protein 9 (Cas9)-Hes1 KO, CRISPR/Cas9-JSAP1 KO, or control vector. See Supporting Information.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Murine serum and BMM culture supernatants were harvested for cytokine analysis. Enzyme-linked immunosorbent assay kits were used to measure TNF-α, interleukin 1 beta (IL-1β), monocyte chemoattractant protein 1 (MCP1), and IL-6 levels. See Supporting Information.

REACTIVE OXYGEN SPECIES ASSAY

Reactive oxygen species (ROS) production in BMMs was measured using the carboxy-2′,7′-difluorodihydrofluorescein diacetate kit as described.[8] ROS produced by BMMs were analyzed and quantified by fluorescence microscopy according to the manufacturer's instructions. See Supporting Information.

STATISTICAL ANALYSIS

Data are expressed as mean ± SD and analyzed by permutation *t* test and Pearson correlation. Per comparison, two-sided *P* values <0.05 were considered statistically significant. Multiple group comparisons were made using one-way analysis of variance followed by Bonferroni's *post hoc* test. When groups showed unequal variances, we applied Welch's analysis of variance to make multiple group comparisons. All analyses used SAS/STAT software, version 9.4.

Results

MYELOID-SPECIFIC NOTCH1 DEFICIENCY AGGRAVATES IR-INDUCED HEPATOCELLULAR DAMAGE

Notch1$^{M\text{-}KO}$ and Notch1$^{FL/FL}$ mice were subjected to 90 minutes of warm ischemia followed by 6 hours or 24 hours of reperfusion. We isolated both hepatocytes and liver macrophages (Kupffer cells) from these ischemic livers. Indeed, Notch1$^{M\text{-}KO}$ mice did not change hepatocyte Notch1 expression. However, Notch1 expression was lacking in liver macrophages from the Notch1$^{M\text{-}KO}$ mice but not from Notch1$^{FL/FL}$ mice (Fig. 1A). Hepatocellular functions were evaluated by

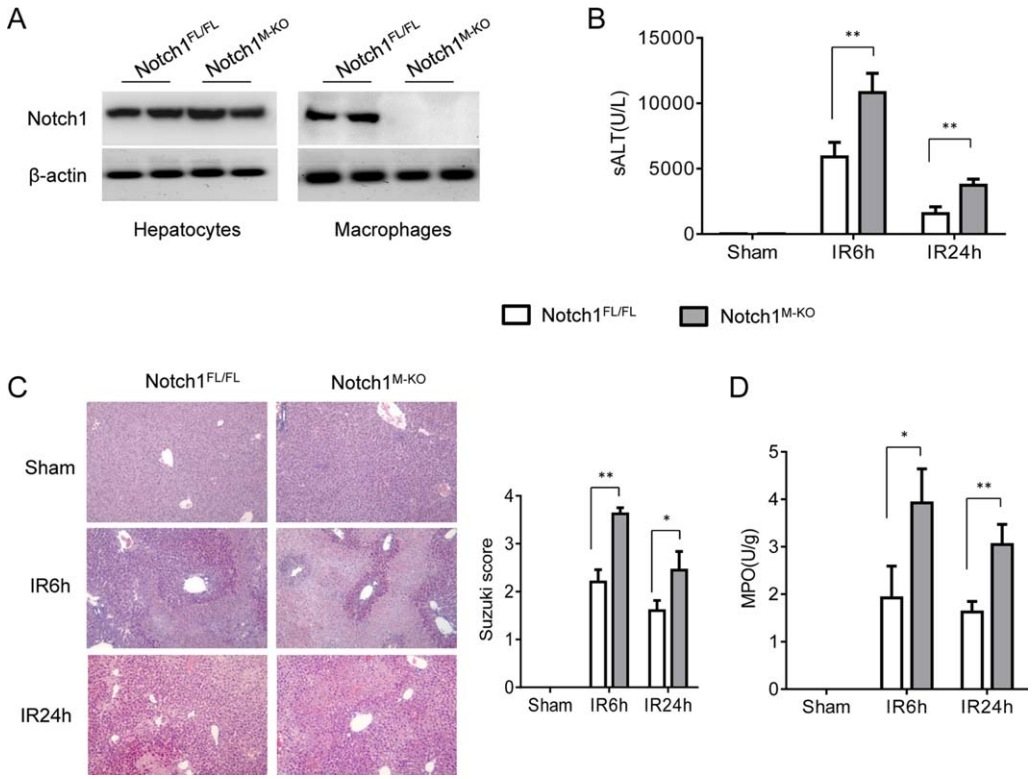

FIG. 1. Myeloid-specific Notch1 deficiency aggravates IR-induced hepatocellular damage. Mice were subjected to 90 minutes of partial liver warm ischemia, followed by 6 hours or 24 hours of reperfusion. (A) Notch1 expression was detected in hepatocytes and liver macrophages by western blot assay. Representative of three experiments. (B) Liver function in serum samples was evaluated by sALT levels. Results expressed as mean ± SD (n = 4-6 samples/group). $^{**}P < 0.01$. (C) Representative histological staining (hematoxylin and eosin) of ischemic liver tissue. Results representative of 4-6 mice/group; original magnification ×100. Liver damage evaluated by Suzuki's histological score. $^{**}P < 0.01$. (D) Liver neutrophil accumulation, analyzed by myeloperoxidase activity (U/g). Mean ± SD (n = 4-6 samples/group). $^{*}P < 0.05$. Abbreviation: MPO, myeloperoxidase.

measuring sALT levels (international units per liter) (Fig. 1B). Disruption of myeloid Notch1 increased sALT levels at 6 hours and 24 hours post–liver reperfusion in Notch1$^{M\text{-}KO}$ mice compared to Notch1$^{FL/FL}$ controls (6 hours, 10,824 ± 1,473 versus 5,911 ± 1,109, respectively; $P < 0.01$; 24 hours, 3,733 ± 469 versus 1,555 ± 530, respectively; $P < 0.01$). These data correlated with Suzuki's histological grading of liver IRI (Fig. 1C). Unlike the Notch1$^{FL/FL}$ controls, which showed mild to moderate edema, sinusoidal congestion, and mild necrosis (6 hours, score = 2.2 ± 0.25; 24 hours, score = 1.6 ± 0.21), Notch1$^{M\text{-}KO}$ mouse livers displayed severe edema, sinusoidal congestion, and extensive hepatocellular necrosis (6 hours, score = 3.62 ± 1.23, $P < 0.01$; 24 hours, score = 2.45 ± 0.38, $P < 0.05$). Consistent with the histopathological and hepatocellular function data, the myeloperoxidase levels, which reflect liver neutrophil activity (units per gram), were significantly elevated in the Notch1$^{M\text{-}KO}$ group but not in the Notch1$^{FL/FL}$ group (Fig. 1D; 6 hours, 3.93 ± 0.72 versus 1.92 ± 0.67, respectively; $P < 0.05$; 24 hours, 3.03 ± 0.42 versus 1.65 ± 0.22, respectively; $P < 0.01$).

MYELOID-SPECIFIC NOTCH1 DEFICIENCY INCREASES MACROPHAGE/NEUTROPHIL INFILTRATION AND PROINFLAMMATORY MEDIATORS IN LIVER IRI

Having shown that myeloid Notch1 deficiency enhances hepatocellular damage, we then analyzed macrophage and neutrophil accumulation in IR-stressed livers at 6 hours of reperfusion by immunofluorescence or immunohistochemical staining. The Notch1$^{M\text{-}KO}$ ischemic livers showed increased CD11b^{+} macrophage

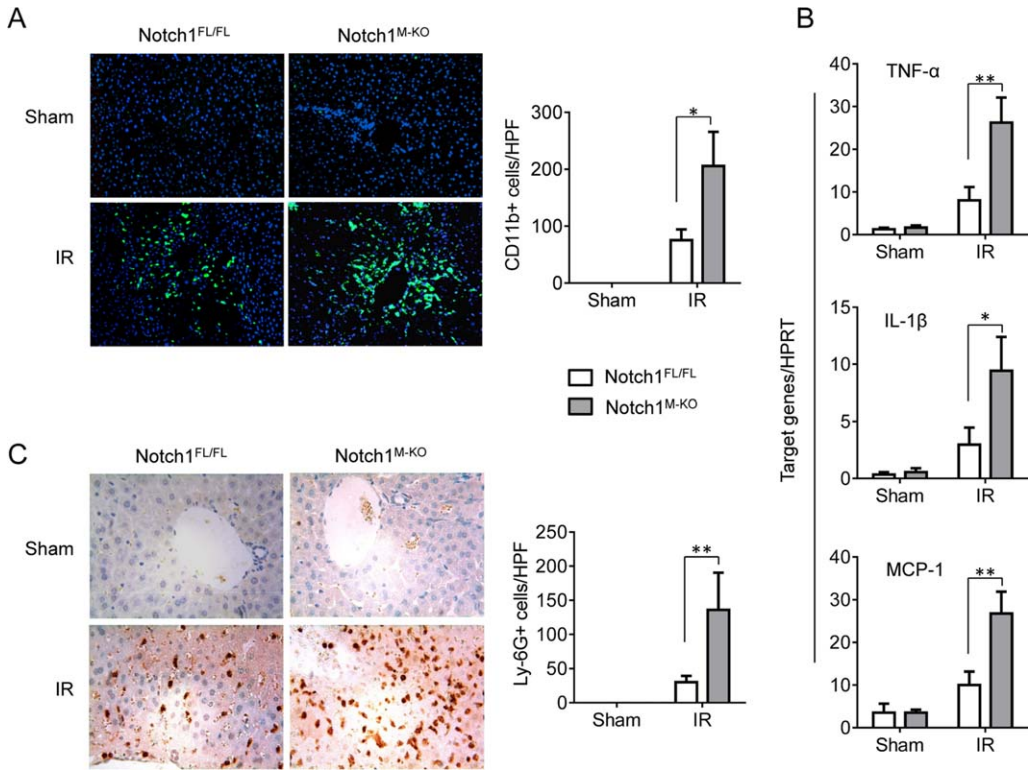

FIG. 2. Myeloid-specific Notch1 deficiency increases macrophage/neutrophil infiltration and proinflammatory mediators in liver IRI. By 6 hours of reperfusion after 90 minutes of ischemia, liver macrophages and neutrophils were detected by immunofluorescence and immunohistochemical staining using monoclonal antibodies against mouse $CD11b^+$ and Ly6G in $Notch1^{FL/FL}$ (white bars) and $Notch1^{M\text{-}KO}$ (shaded bars) mice. (A) Immunofluorescence staining of $CD11b^+$ macrophages in ischemic livers. Quantification of $CD11b^+$ macrophages per high power field. Results scored semiquantitatively by averaging number of positively stained cells (mean ± SD)/field at ×200 magnification. Representative of 4-6 mice/group. $^*P < 0.05$. (B) Quantitative RT-PCR-assisted detection of TNF-α, IL-1β, and MCP1 in mouse livers. Each column represents the mean ± SD (n = 3-4 samples/group). $^*P < 0.05$, $^{**}P < 0.01$. (C) Immunohistochemical staining of $Ly6G^+$ neutrophils in ischemic livers. Quantification of $Ly6G^+$ neutrophils per high-power field (original magnification ×200). Representative of 4-6 mice/group. $^{**}P < 0.01$. Abbreviations: HPF, high-power field; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

infiltration compared to $Notch1^{FL/FL}$ controls (Fig. 2A; 205 ± 60 versus 75 ± 19, $P < 0.05$). To evaluate the phenotype of macrophages, liver macrophages (Kupffer cells) were isolated from $Notch1^{M\text{-}KO}$ and $Notch1^{FL/FL}$ livers at 6 hours of reperfusion after 90 minutes of ischemia. Notch1-deficient macrophages from $Notch1^{M\text{-}KO}$ mice increased S100A9 while reducing arginase-1 and CD206 expression compared to the Notch1-proficient macrophages from $Notch1^{FL/FL}$ mice (Supporting Fig. S1A). Consistent with these data, the mRNA levels coding for TNF-α, IL-1β, and MCP1 were significantly increased in $Notch1^{M\text{-}KO}$, but not in $Notch1^{FL/FL}$, livers (Fig. 2B). Moreover, the $Notch1^{M\text{-}KO}$ ischemic livers exhibited increased neutrophil accumulation compared to $Notch1^{FL/FL}$ controls (Fig. 2C; 136 ± 47 versus 30.0 ± 8.16, $P < 0.01$).

MYELOID-SPECIFIC NOTCH1 DEFICIENCY DEPRESSES HES1 BUT INDUCES RhoA/ROCK ACTIVATION IN IR-STRESSED LIVER

Next, we analyzed whether Notch1 may influence its target gene Hes1 and the RhoA/ROCK pathway in IR-induced liver injury. By 6 hours of reperfusion after 90 minutes of ischemia, $Notch1^{M\text{-}KO}$ diminished Hes1 and increased RhoA mRNA expression in the ischemic livers compared to the $Notch1^{FL/FL}$ controls (Fig. 3A). Western blotting analysis revealed reduced NICD and Hes1 and augmented JSAP1, p-JNK, and ROCK1 protein levels in $Notch1^{M\text{-}KO}$, but not in $Notch1^{FL/FL}$ livers (Fig. 3B). Moreover, $Notch1^{M\text{-}KO}$

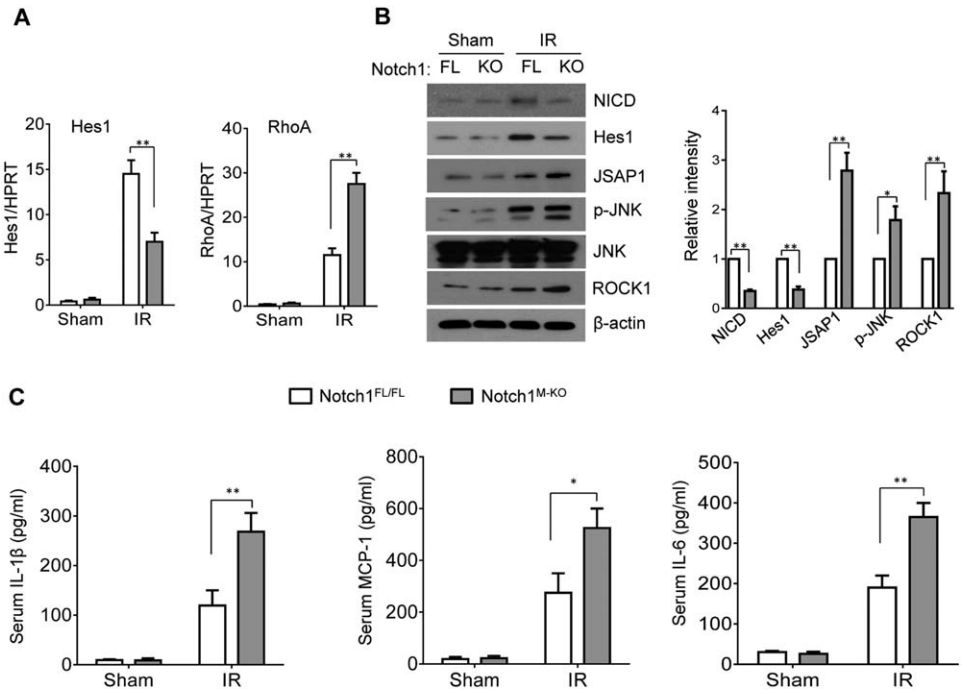

FIG. 3. Myeloid-specific Notch1 deficiency depresses Hes1 but induces RhoA/ROCK activation in IR-stressed liver. (A) Quantitative RT-PCR-assisted detection of mRNA coding for Hes1 and RhoA in mouse livers at 6 hours of reperfusion followed by 90 minutes of ischemia. Each column represents the mean ± SD (n = 3-4 samples/group). $^{**}P < 0.01$. (B) Western-assisted analysis and relative density ratio of NICD, Hes1, JSAP1, p-JNK, and ROCK1. Representative of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (C) Enzyme-linked immunosorbent assay of IL-1β, MCP1, and IL-6 levels in animal serum. Mean ± SD (n = 3-4 samples/group), $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviation: HPRT, hypoxanthine-guanine phosphoribosyltransferase.

increased IL-1β, MCP1, and IL-6 production compared to Notch1$^{FL/FL}$ controls after hepatic IR (Fig. 3C).

MYELOID-SPECIFIC NOTCH1 DEFICIENCY INCREASES HEPATOCELLULAR APOPTOSIS IN IR-STRESSED LIVER

To determine whether disruption of myeloid Notch1 may affect hepatic IR-induced apoptosis, we analyzed hepatocellular apoptosis/necrosis in ischemic livers by terminal deoxyribonucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining. By 6 hours of reperfusion after 90 minutes of ischemia, livers in Notch1$^{M\text{-}KO}$ mice revealed an increased frequency of apoptotic TUNEL^{+} cells compared to Notch1$^{FL/FL}$ livers after IR (Fig. 4A; 52.5 ± 17.7 versus 25 ± 7, $P < 0.05$). These data was confirmed by increased caspase-3 activity in Notch1$^{M\text{-}KO}$ mice compared to Notch1$^{FL/FL}$ mice (Fig. 4B; 8.25 ± 1.8 versus 4.1 ± 1.41, $P < 0.01$). Western blot analysis showed that Notch1$^{M\text{-}KO}$ up-regulated PTEN and TLR4 expression but down-regulated Akt phosphorylation compared to Notch1$^{FL/FL}$ controls (Fig. 4C). Moreover, increased TNF-α production was observed in Notch1$^{M\text{-}KO}$ mice but not in Notch1$^{FL/FL}$ mice (Fig. 4D; 128.8 ± 19.6 versus 67.4 ± 12.1, $P < 0.01$).

ACTIVATION OF THE RhoA/ROCK PATHWAY TRIGGERS INNATE IMMUNE RESPONSE AND IR-INDUCED INFLAMMATORY INJURY IN MYELOID NOTCH1-DEFICIENT LIVER

To evaluate whether activation of the RhoA/ROCK pathway in Notch1$^{M\text{-}KO}$ livers may triggers liver innate immune response and IR-induced inflammatory injury, we disrupted ROCK1 in the Notch1$^{M\text{-}KO}$ livers with an *in vivo* mannose-mediated ROCK1 siRNA delivery system that specifically delivers to macrophages by expressing a mannose-specific membrane receptor, as described.[8,30] The mannose receptor is a

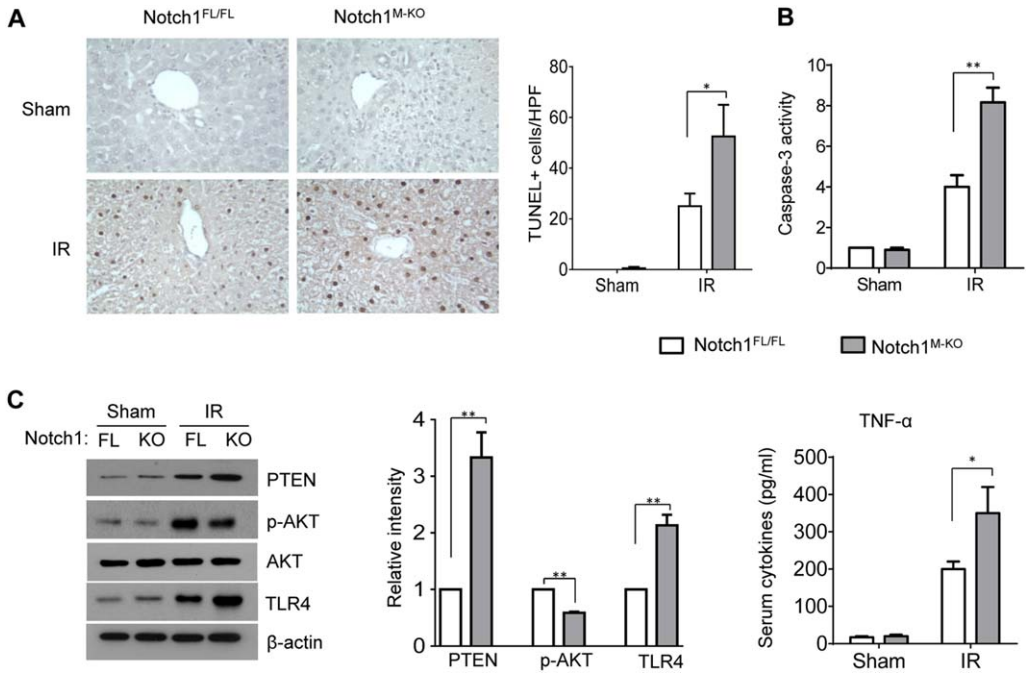

FIG. 4. Myeloid-specific Notch1 deficiency increases hepatocellular apoptosis in IR-stressed liver. (A) Liver apoptosis by TUNEL staining in mouse liver at 6 hours of reperfusion followed by 90 minutes of ischemia. Results scored semiquantitatively by averaging the number of apoptotic cells (mean ± SD) per field at ×200 magnification. Representative of 4-6 mice/group, $^{*}P < 0.05$. (B) Caspase-3 activity. Mean ± SD (n = 4-6 samples/group), $^{**}P < 0.01$. (C) Western-assisted analysis and relative density ratio of PTEN, p-Akt, and TLR4. Representative of three experiments. $^{**}P < 0.01$ (C) Enzyme-linked immunosorbent assay of TNF-α levels in animal serum. Mean ± SD (n = 3-4 samples/group), $^{**}P < 0.01$. Abbreviation: HPF, high-power field.

C-type lectin primarily present on the surface of macrophages. Indeed, knockdown of ROCK1 with the mannose-mediated siRNA treatment in Notch1$^{M\text{-}KO}$ mice reduced IR-induced liver damage as evidenced by the decreased Suzuki's histological score (Fig. 5A; score = 1.475 ± 0.55 versus 3.275 ± 0.88, $P < 0.05$) and sALT levels (Fig. 5B; 4,875.5 ± 1,444.9 versus 9,310.3 ± 1,982, $P < 0.01$) compared to the NS siRNA-treated controls. Moreover, ROCK1 siRNA treatment in Notch1$^{M\text{-}KO}$ ischemic livers decreased CD11b^{+} macrophage (Fig. 5C; 52.3 ± 22.6 versus 180.7 ± 37.1, $P < 0.01$) and neutrophil (Fig. 5D; 40.67 ± 10.2 versus 120 ± 30.3, $P < 0.05$) accumulation compared to the NS siRNA-treated controls. Moreover, liver macrophages from ROCK1 siRNA-treated-Notch1$^{M\text{-}KO}$ mice diminished S100A9 and increased arginase-1 and CD206 expression compared to the NS siRNA-treated controls at 6 hours of reperfusion after 90 minutes of ischemia (Supporting Fig. S1B). Consistent with these data, ROCK1 knockdown reduced PTEN and TLR4 and increased Akt expression in Notch1$^{M\text{-}KO}$ mice (Fig. 5E), which was accompanied by reduced liver TNF-α, IL-1β, and MCP1 mRNA levels compared to controls (Fig. 5F).

MYELOID-SPECIFIC NOTCH1 DEFICIENCY ACTIVATES THE RhoA/ROCK PATHWAY IN A JSAP1-DEPENDENT MANNER IN IR-STRESSED LIVER

Because myeloid-specific Notch1 deficiency promoted JSAP1, which is a JNK-binding protein and functions as a scaffold factor in the JNK signaling pathway,[31] we examined whether JSAP1 is required for activation of the RhoA/ROCK pathway in IR-stressed livers. We disrupted JSAP1 in Notch1$^{M\text{-}KO}$ livers using a mannose-mediated JSAP1 siRNA *in vivo*. Livers in Notch1$^{M\text{-}KO}$ mice with NS siRNA treatment revealed significant edema, severe sinusoidal congestion/cytoplasmic vacuolization, and extensive (30%-50%) necrosis (Fig. 6A; score = 3.67 ± 1.5). In contrast, livers in mice treated with mannose-mediated

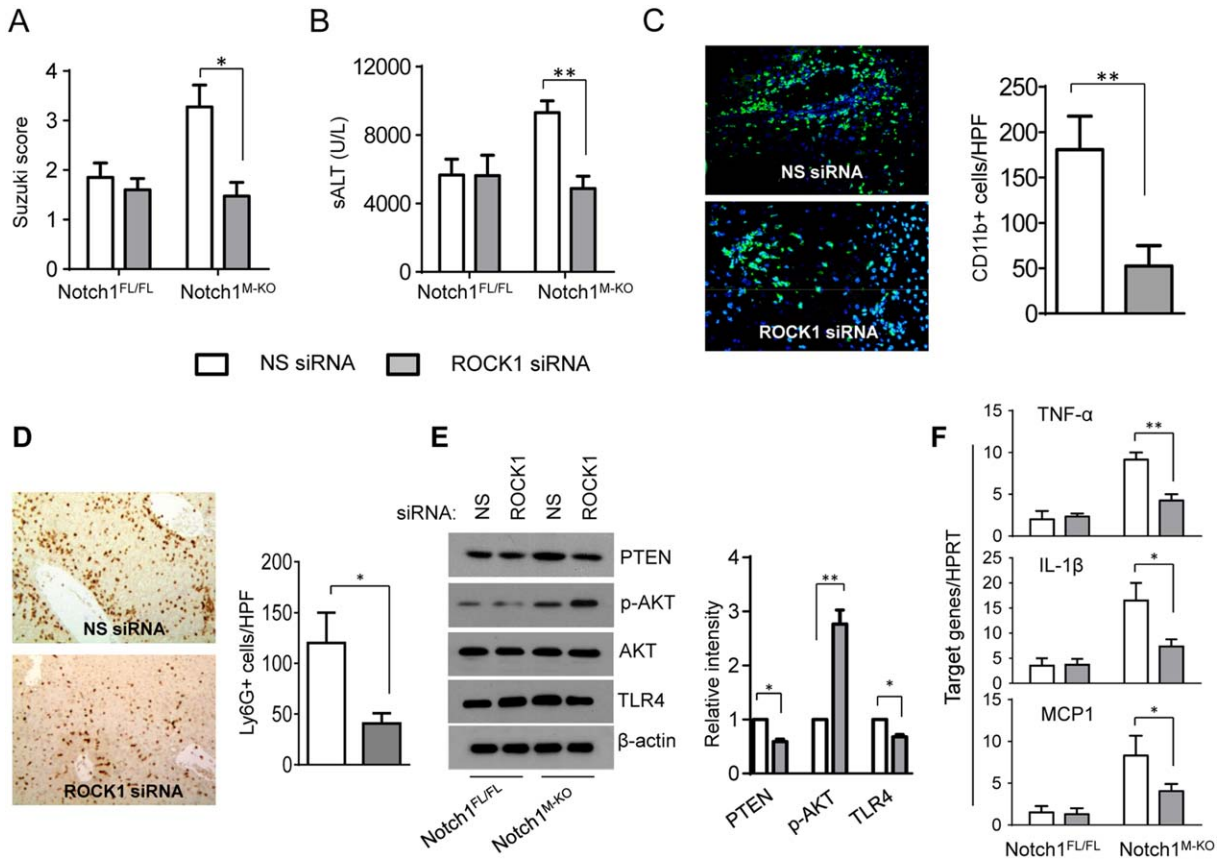

FIG. 5. Activation of the RhoA/ROCK pathway triggers innate immune response and IR-induced inflammatory injury in myeloid Notch1-deficient liver. Notch1$^{M\text{-}KO}$ and Notch1$^{FL/FL}$ mice were injected in the tail vein with NS control siRNAs (white bars) or ROCK1 siRNA (shaded bars) (2 mg/kg) mixed with mannose-conjugated polymers at 4 hours prior to ischemia. (A) The severity of liver IRI was evaluated by the Suzuki histological grading at 6 hours of reperfusion followed by 90 minutes of ischemia. $^{*}P < 0.05$. (B) Hepatocellular function was evaluated by sALT levels. Results expressed as mean ± SD (n = 4-6 samples/group), $^{**}P < 0.01$. (C) Immunofluorescence staining of CD11b^{+} macrophages in ischemic livers. Quantification of CD11b^{+} macrophages per high-power field. Results scored semiquantitatively by averaging number of positively stained cells (mean ± SD)/field at ×200 magnification. Representative of 4-6 mice/group. $^{**}P < 0.01$. (D) Immunohistochemical staining of Ly6G^{+} neutrophils in ischemic livers. Quantification of Ly6G^{+} neutrophils per high-power field (original magnification ×200). Representative of 4-6 mice/group. $^{*}P < 0.05$. (E) Western blot analysis and relative density ratio of PTEN, p-Akt, and TLR4. Representative of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (F) Quantitative RT-PCR-assisted detection of mRNA coding for TNF-α, IL-1β, and MCP1. Each column represents the mean ± SD (n = 3-4 samples/group). $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: HPF, high-power field; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

JSAP1 siRNA showed mild to moderate edema without necrosis (Fig. 6A; score = 2.11 ± 0.27, $P < 0.01$). Consistent with these data, sALT levels were significantly decreased in the JSAP1 siRNA knockdown mice compared to NS siRNA-treated controls (Fig. 6B; 5,375 ± 843 versus 11,060 ± 1,473, $P < 0.01$). Moreover, JSAP1 siRNA treatment in Notch1$^{M\text{-}KO}$ livers reduced serum TNF-α release (Fig. 6C; 210 ± 74 versus 450 ± 91, $P < 0.01$) and p-JNK, ROCK1, PTEN, TLR4, and cleaved caspase-3 protein expression (Fig. 6D), which led to decreased RhoA, IL-1β, and MCP1 mRNA expression compared to the NS siRNA-treated group (Fig. 6E).

MYELOID NOTCH1–HES1 AXIS IS CRUCIAL IN THE REGULATION OF JSAP1-DEPENDENT RhoA/ROCK ACTIVATION IN MACROPHAGES

To elucidate the mechanisms of Notch signaling in regulating RhoA/ROCK-mediated immune response, we cultured BMMs from Notch1$^{M\text{-}KO}$ mice and then transfected them with the lentivirus expressing NICD (LV-pSIN-NICD) or the control vector (LV-control), followed by LPS stimulation. Clearly,

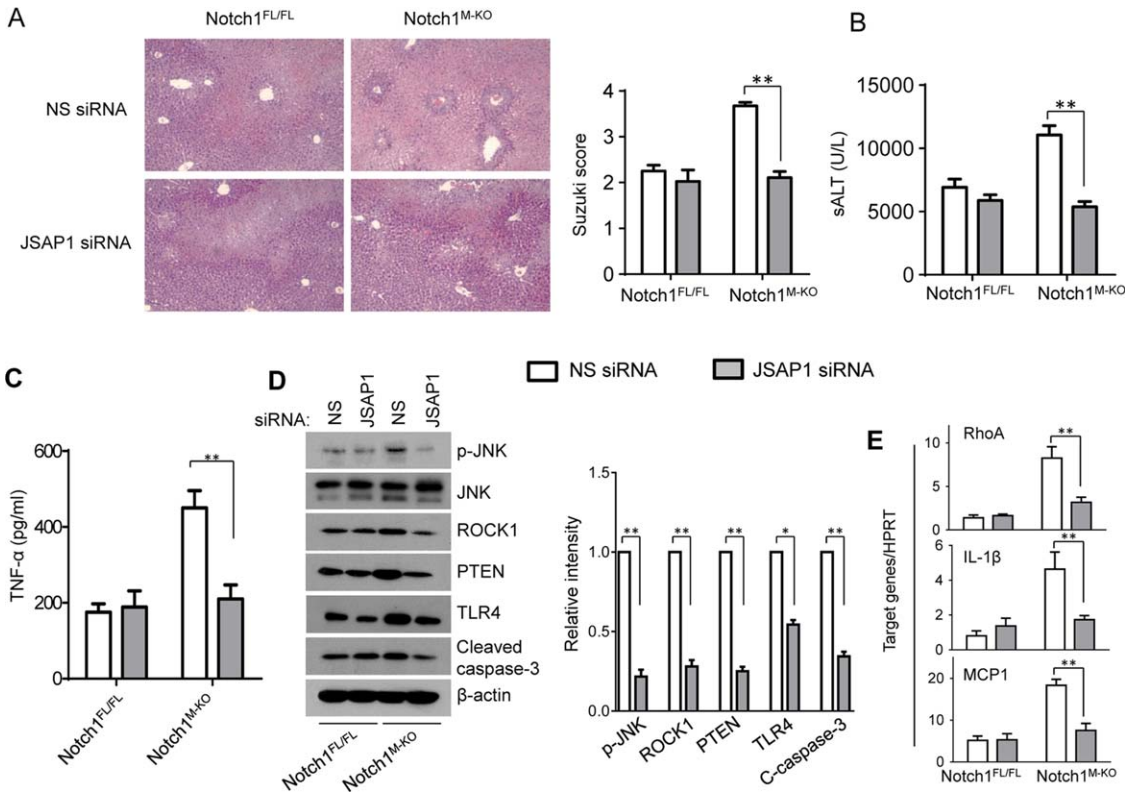

FIG. 6. Myeloid-specific Notch1 deficiency activates the RhoA/ROCK pathway in a JSAP1-dependent manner in IR-stressed livers. Notch1$^{M\text{-}KO}$ and Notch1$^{FL/FL}$ mice were injected in the tail vein with NS control siRNAs (white bars) or JSAP1 siRNA (shaded bars) (2 mg/kg) mixed with mannose-conjugated polymers at 4 hours prior to ischemia. (A) Representative histological staining (hematoxylin and eosin) of ischemic liver tissue at 6 hours of reperfusion followed by 90 minutes of ischemia. Results representative of 4-6 mice/group; original magnification ×100. The severity of liver IRI was evaluated by the Suzuki histological grading. $^{**}P < 0.01$. (B) Hepatocellular function was evaluated by sALT levels. Results expressed as mean ± SD (n = 4-6 samples/group). $^{**}P < 0.01$. (C) Enzyme-linked immunosorbent assay of TNF-α levels in animal serum. Mean ± SD (n = 3-4 samples/group), $^{**}P < 0.01$. (D) Western blot analysis and relative density ratio of p-JNK, ROCK1, PTEN, TLR4, and cleaved caspase-3. Representative of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (E) Quantitative RT-PCR-assisted detection of mRNA coding for RhoA, IL-1β, and MCP1. Each column represents the mean ± SD (n = 3-4 samples/group). $^{**}P < 0.01$. Abbreviation: HPRT, hypoxanthine-guanine phosphoribosyltransferase.

LV-pSin-NICD transfection in Notch1$^{M\text{-}KO}$ cells markedly increased Hes1 and reduced JSAP1 expression compared to the LV-control-transfected cells (Fig. 7A). In contrast to the LV-controls, transfection of lentivirus CRISPR/Cas9-mediated Hes1 knockout (LV-Hes1 KO) enhanced JSAP1, ROCK1, and PTEN (Fig. 7B), resulting in augmented TNF-α release (Fig. 7C) and IL-1β and MCP1 expression (Fig. 7D) in LPS-stimulated Notch1$^{FL/FL}$ macrophages. To determine the crosstalk between JSAP1 and RhoA/ROCK activation in Notch signaling–mediated immune regulation, we disrupted JSAP1 using a CRISPR/Cas9 JSAP1 knockout vector (LV-JSAP1 KO) in Notch1$^{M\text{-}KO}$ macrophages. Interestingly, JSAP1 deficiency in LV-JSAP1 KO–treated cells led to decreased ROCK1, PTEN, TLR4, and p-IκBα (Fig. 7E), which were accompanied by reduced ROS production (Fig. 7F; 51.5 ± 9.82 versus 265.0 ± 40.4, $P < 0.01$) and mRNA levels coding for TNF-α, IL-1β, and MCP1 (Fig. 7G) in LPS-stimulated macrophages compared with the control groups.

Discussion

This study documents the key role of myeloid Notch signaling in regulating RhoA/ROCK-mediated innate immune responses in sterile inflammatory liver injury. First, myeloid Notch1 deficiency promotes liver inflammation through the depression of its target gene Hes1. Second, inhibition of Hes1 induces the scaffold protein JSAP1, which is required for activation of the

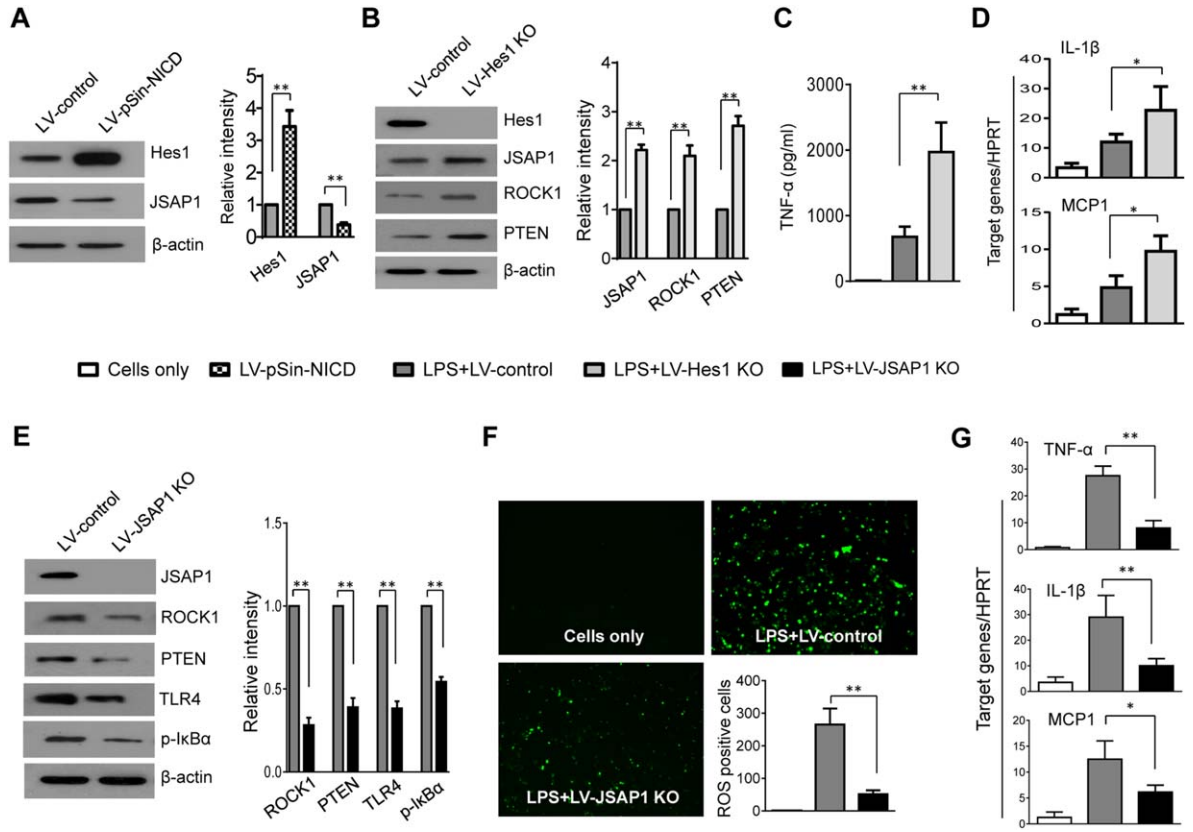

FIG. 7. Myeloid Notch1–Hes1 axis is crucial in the regulation of JSAP1-dependent RhoA/ROCK activation in macrophages. (A) BMMs from Notch1$^{M\text{-}KO}$ mice were transfected with the lentivirus expressing NICD (LV-pSIN-NICD) or the control vector (LV-control) followed by LPS (100 ng/mL) stimulation. Western-assisted analysis and relative density ratio of Hes1 and JSAP1. Representative of three experiments. $^{**}P < 0.01$ (B) BMMs from Notch1$^{FL/FL}$ mice were transfected with the lentivirus-mediated CRISPR/Cas9-mediated Hes1 knockout (LV-Hes1 KO) or the LentiCRISPRv2 vector without guide RNA sequence control (LV-control) followed by LPS (100 ng/mL) stimulation. Western-assisted analysis and relative density ratio of Hes1, JSAP1, ROCK1, and PTEN. Representative of three experiments. $^{**}P < 0.01$ (C) Enzyme-linked immunosorbent assay–assisted production of TNF-α in cell culture supernatants. Mean ± SD (n = 3-4 samples/group). $^{**}P < 0.01$. (D) Quantitative RT-PCR-assisted detection of mRNA coding for IL-1β and MCP1. Each column represents mean ± SD (n = 3-4 samples/group). $^{*}P < 0.05$. (E) BMMs from Notch1$^{M\text{-}KO}$ mice were transfected with the lentivirus-mediated CRISPR/Cas9-mediated JSAP1 knockout (LV-JSAP1 KO) or the LentiCRISPRv2 vector without guide RNA sequence control (LV-control) followed by LPS (100 ng/mL) stimulation. Western-assisted analysis and relative density ratio of JSAP1, ROCK1, PTEN, TLR4, and p-IκBα. Representative of three experiments. $^{**}P < 0.01$ (F) ROS production was detected by carboxy-2′,7′-difluorodihydrofluorescein diacetate in LPS-stimulated BMMs from Notch1$^{M\text{-}KO}$ mice. Positive green fluorescence-labeled cells were counted blindly in 10 high-power fields/section (×200). Quantification of ROS-producing BMMs (green) per high-power field (×200). $^{**}P < 0.01$. (G) Quantitative RT-PCR-assisted detection of mRNA coding for TNF-α, IL-1β, and MCP1. Each column represents mean ± SD (n = 3-4 samples/group). $^{*}P < 0.05$, $^{**}P < 0.01$. White bars, cells only; Black and white square bars, LV-pSin-NICD; light gray bars, LPS + LV-Hes1 KO; dark gray bars, LPS + LV-control; black bars, LPS + LV-JSAP1 KO. Abbreviation: HPRT, hypoxanthine-guanine phosphoribosyltransferase.

RhoA/ROCK pathway. Third, activated RhoA downstream effector ROCK1 is crucial for triggering the TLR4-driven inflammatory response. Our results highlight the importance of the myeloid Notch–Hes1 axis as a key regulator of RhoA/ROCK function in IR-triggered liver inflammation.

Notch signaling has varied roles in regulating inflammatory response and tissue homeostasis. Under inflammatory conditions, it is conceivable that Notch signaling in myeloid cells could be promoted by various stimuli, such as exogenous pathogens and/or endogenous mediators. Notch signaling can be activated through the TLR signaling cascade, which is involved in proinflammatory response.[32] However, Notch1 signaling exerts an immunoregulatory effect by inducing regulatory T-cell production both *in vitro* and *in vivo*.[33,34] Overexpression of NICD reduced TLR4-mediated proinflammatory cytokine production

in vitro.[35] Notch and TLR pathways cooperate to activate canonical Notch target genes, including transcriptional repressor Hes1, which can regulate proinflammatory cytokines through an inhibitory feedback loop.[36] The suppressive function of Hes1 in inflammatory response is associated with transcription regulation.[37] Thus, Notch signaling serves as a dual-functional regulator of inflammatory response in various animal models. In our current study, we analyzed the myeloid-specific Notch1 function in mediating its immunomodulation on RhoA/ROCK activation during liver IRI. We found that myeloid Notch1 deficiency promoted JNK-binding protein JSAP1, RhoA/ROCK, and PTEN activation by inhibiting Hes1 expression, which led to increased IR-triggered liver inflammation. Our findings demonstrate the ability of myeloid Notch signaling in modulating innate immunity and inflammation cascades in IR-stressed livers.

Attenuation of RhoA/ROCK activation by inhibiting Rho kinase has indicated that the RhoA/ROCK pathway is an important mediator during T cell–mediated inflammatory response.[38] Inhibition of Rho kinase prevents NF-κB activation and proinflammatory cytokine production in intestinal inflammation.[12] However, it is unknown how myeloid Notch signaling influences the RhoA/ROCK-mediated innate immune response in IR-induced liver injury. Based on the findings from the present study, the transcriptional repressor Hes1 is a key determinant of Notch signaling–mediated immune regulation. We found that induction of Hes1 by NICD overexpression selectively inhibited the expression of RhoA downstream effector ROCK1, resulting in reduced PTEN and increased Akt, which in turn regulated TLR4 signaling through a negative feedback mechanism.[6] Notably, CRISPR/Cas9-mediated Hes1 knockout activated JSAP1, whereas knockdown of JSAP1 reduced JNK phosphorylation, inflammatory cytokine expression, and caspase-3 activation. In addition, although Notch signaling can also be activated in liver macrophages after liver IR, disruption of myeloid Notch1 or macrophage Hes1 promoted the RhoA/ROCK pathway both *in vitro* and *in vivo*. These results suggest an important regulatory role of the myeloid Notch1–Hes1 axis on RhoA/ROCK function during liver IRI.

One striking finding was that RhoA/ROCK activation was inhibited by disrupting JSAP1. JSAP1 did not interrupt myeloid Notch–Hes1 signaling (data not shown) but instead triggered RhoA/ROCK activation. Because RhoA/ROCK-mediated PTEN activity was required for inflammatory cell accumulation[15] and myeloid PTEN promoted tissue inflammation,[39] suppression of the RhoA/ROCK pathway may provide a new mechanism by which Notch1–Hes1 signaling modulates TLR responses by specifically suppressing JSAP1 activation in IR-stressed livers. Furthermore, our data demonstrated the ability of JSAP1 to mediate JNK activation and increase TLR-induced production of MCP1. Indeed, inflammatory cell migration is critical for tissue inflammation. During liver IRI, the infiltration of monocytes/macrophages plays an important role in the initiation of local inflammatory injury.[40] MCP1 is a potent chemoattractant for monocytes/macrophages and has been shown to be involved in macrophage recruitment in acute liver injury.[41] We found that myeloid Notch1 deficiency increased macrophage infiltration, whereas ROCK1 disruption blunted macrophage infiltration and MCP1 expression in ischemic livers. This suggests that inhibition of macrophage accumulation was due to suppression of MCP1. In addition, ROCK1 knockdown significantly reduced the gene expression of TNF-α and IL-1β in myeloid Notch1-deficent livers. These data indicate that activation of the JSAP1-mediated RhoA/ROCK pathway may be crucial for macrophage accumulation in IR-triggered liver inflammation and that inhibition of this cell-specific signaling pathway may provide a possible targeting strategy.

Although inhibition of the RhoA/ROCK pathway ameliorates liver injury, the role of RhoA/ROCK in mediating innate immune response is less clear. Studies have reported that Rho kinase is involved in activation of the NF-κB pathway.[42,43] Our results showed that JSAP1 was involved in ROCK-dependent TLR4 activation during inflammatory response and that use of the CRISPR/Cas9-mediated JSAP1 knockout can inhibit RhoA/ROCK-mediated TLR4 signaling, which was accompanied by decreased NF-κB-induced inflammatory cytokines and chemokines. The involvement of JSAP1 and RhoA/ROCK in TLR4 activation indicated the existence of mechanistic links between the JSAP1-mediated RhoA/ROCK pathway and innate immunity during liver IRI. Further evidence revealed that disruption of ROCK1 suppressed PTEN and TLR4 activation, resulting in reduced liver inflammatory injury. The inhibitory effect of ROCK1 on TLR4 activation is associated with increased Akt activity. Although there could be many causes for TLR4 activation, it is clear that the JSAP1-mediated RhoA/ROCK pathway is an important mechanism for activating TLR4 in liver IRI. This was further supported by the *in vitro* study, which showed that reduced

TLR4 expression occurred after JSAP1 KO in Notch1-deficient macrophages. Thus, our findings reveal an essential role for JSAP1 in activating the RhoA/ROCK pathway and TLR4 signaling during liver inflammation.

It is worth noting that the myeloid Notch–Hes1 axis, identified here as a likely novel player in cytoprotection, could be involved in apoptotic pathways during liver IRI. Indeed, both programmed cell death (apoptosis) and necrosis are known to occur following liver IR. Studies suggest that the Rho/ROCK pathway is activated during the execution phase of apoptosis to stimulate apoptotic membrane blebbing.[44] ROCK1 is a direct target of caspase activity, whereby caspase 3 cleavage of ROCK1 occurs in early apoptosis.[45] ROCK1 cleavage also increases caspase-3 activity, which coincides with the activation of PTEN and the subsequent inhibition of Akt activity.[45] Consistent with this report, we examined apoptotic liver cell death by TUNEL staining. The percentage of apoptotic TUNEL^{+} cells was markedly increased in Notch1$^{M\text{-}KO}$, livers but not in Notch1$^{FL/FL}$, livers after IR. Further evidence was supported by caspase-3 activity assay, which showed that Notch1$^{M\text{-}KO}$ significantly increased caspase-3 activity in ischemic livers compared to Notch1$^{FL/FL}$ controls. Our findings demonstrate an unexpected role for the myeloid Notch–Hes1 axis in negatively modulating IR-induced hepatocellular apoptosis/necrosis. The regulation of apoptosis/necrosis by myeloid Notch signaling in ischemic livers may depend on several factors. First, macrophage Notch1 deficiency increases TNF-α release, which leads to increased hepatocellular apoptosis through a JNK-dependent pathway. Because JSAP1 is a scaffold protein that interacts with specific components of the JNK signaling pathway,[31] JSAP1 appears to be involved in TNF-α-mediated apoptosis in liver IRI. Indeed, knockdown of JSAP1 inhibited JNK activation and reduced proapoptotic caspase-3 expression, which was accompanied by decreased TNF-α release in Notch1$^{M\text{-}KO}$ livers. This result suggests that JSAP1 may represent a key target in strategies to limit cell apoptosis. Second, ROS production and oxidant stress are the most invoked disease mechanisms in liver IRI. ROS formation by Kupffer cells initiates cellular injury and activates a cascade of mediators leading to increased apoptosis/necrosis and acute inflammatory response.[46] Rho guanosine triphosphatases are key components of activated reduced nicotinamide adenine dinucleotide phosphate oxidase complexes and the subsequent generation of ROS.[47] Increased ROS production activates the RhoA/ROCK pathway.[48] Thus, crosstalk between ROS and Rho/ROCK signaling plays a pivotal role in activating apoptosis. Our results showed that increased NICD expression promoted Hes1 and inhibited JSAP1, whereas disruption of Hes1 augmented JSAP1 and ROCK1 with increased TNF-α release from macrophages. This finding was strengthened by our observations of *in vivo* mannose-mediated JSAP1 knockdown or *in vitro* CRISPR/Cas9-mediated JSAP1 knockout, which showed a reduction of ROCK1, cleaved caspase-3, and macrophage ROS production. Taken together, these findings suggest that the antiapoptotic effect of myeloid Notch–Hes1 signaling is likely through regulation of JSAP1-mediated ROCK signaling in liver IRI.

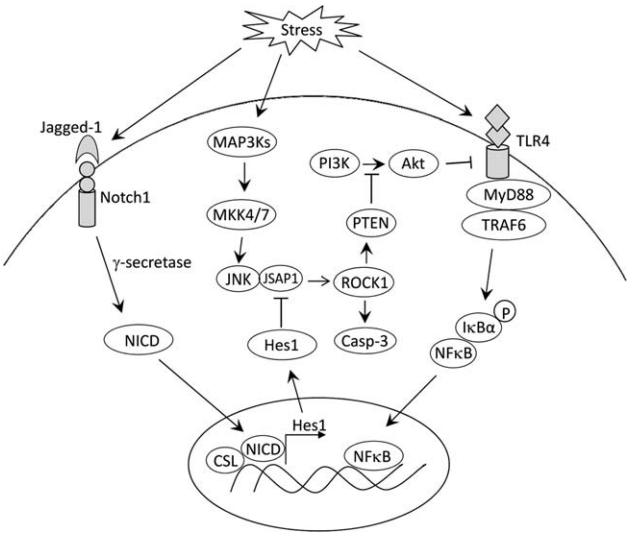

FIG. 8. Schematic illustration of myeloid Notch1 signaling in the regulation of innate immune response in IR-triggered liver inflammation. Notch1 can be activated in IR-stressed livers. Upon ligand binding, Notch1 is cleaved by γ-secretase, leading to a release of the intracellular domain (NICD), which translocates into the nucleus and forms a complex with the CSL DNA-binding protein and activates its target gene, Hes1. Induction of Hes1 inhibits JNK binding protein JSAP1-mediated ROCK1 activation. Blockade of ROCK1 reduces PTEN and augments Akt activity, leading to suppressed TLR4 signaling in liver IRI. In addition, the Notch–Hes1 axis inhibits JSAP1-dependent ROCK1 and caspase-3 activity, resulting in reduced hepatocellular apoptosis/necrosis in IR-triggered liver inflammation. Abbreviations: MAP3K, mitogen-activated protein kinase kinase kinase; MKK4, mitogen-activated protein kinase kinase 4; PI3K, phosphoinositide 3-kinase.

Another important implication of our results is that myeloid Notch1 deficiency may result in cholangiocyte injury, as evidenced by increased serum levels of alkaline phosphatase and direct bilirubin after liver IRI

(Supporting Fig. S2). It is known that Notch and Wnt signaling are required for hepatic progenitor cell differentiation and proliferation.[49,50] Hepatic progenitor cells are activated and able to differentiate into hepatic parenchymal cells, hepatocytes, and/or bile ductular epithelial cells after liver IRI. In the injured liver, different cell types such as hepatocytes, cholangiocytes, endothelial cells, macrophages, and other inflammatory cells can potentially interact with hepatic progenitor cells. Macrophages are important cell components of the hepatic progenitor cell niche in stimulating and initiating a liver regenerative response.[49] Hence, we speculate that macrophage Notch signaling may be involved in hepatic progenitor cell–mediated regeneration in response to IR-induced liver damage.

Figure 8 depicts putative molecular mechanisms by which myeloid Notch1 signaling may regulate RhoA/ROCK-mediated innate immune response in liver IRI. Notch1 can be activated in IR-stressed livers. Upon ligand binding, Notch1 is cleaved by γ-secretase, releasing the intracellular domain (NICD), which translocates into the nucleus and forms a complex with the CSL DNA-binding protein (known as RBPJ in mouse), and activates its target gene Hes1. Induction of Hes1 inhibits JSAP1-mediated ROCK1 activation. Blockade of ROCK1 reduces PTEN and augments Akt activity, leading to suppressed TLR4 signaling in liver IRI. Moreover, the Notch–Hes1 axis inhibits JSAP1-dependent ROCK1 and caspase-3 activity, resulting in reduced hepatocellular apoptosis/necrosis in IR-triggered liver inflammation.

It should be stressed that the results of this study are from a whole-animal myeloid knockout. It is possible that extrahepatic events may drive this response. Indeed, activation of the innate immune system in response to hepatic IR represents a key process determining the development of liver damage. Hepatic IR triggers inflammatory response and recruitment of infiltrating cells (macrophages, neutrophils, etc.) of extrahepatic origin, leading to different influences on the organization of the hepatic immune cascade.

In conclusion, we demonstrate that myeloid Notch1 deficiency promotes the JSAP1-mediated RhoA/ROCK signaling pathway and exacerbates liver damage by depressing its target gene Hes1 in IR-stressed livers. By identifying molecular pathways by which myeloid Notch–Hes1 signaling regulates RhoA/ROCK-mediated innate immunity, our findings provide a rationale for novel therapeutic approaches in ameliorating sterile inflammatory liver injury.

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Supporting Information

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