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Phosphatase and Tensin Homolog- β -Catenin Signaling Modulates Regulatory T Cells and Inflammatory Responses in Mouse Liver Ischemia/Reperfusion Injury

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The phosphatase and tensin homolog (PTEN) deleted on chromosome 10 plays an important role in regulating T cell activation during inflammatory response. Activation of β -catenin is crucial for maintaining immune homeostasis. This study investigates the functional roles and molecular mechanisms by which PTEN- β -catenin signaling promotes regulatory T cell (Treg) induction in a mouse model of liver ischemia/reperfusion injury (IRI). We found that mice with myeloid-specific phosphatase and tensin homolog knockout (PTEN^{M-KO}) exhibited reduced liver damage as evidenced by decreased levels of serum alanine aminotransferase, intrahepatic macrophage trafficking, and proinflammatory mediators compared with the PTEN-proficient (floxed phosphatase and tensin homolog [PTEN^{FL/FL}]) controls. Disruption of myeloid PTEN-activated β -catenin promoted peroxisome proliferator-activated receptor gamma (PPAR γ)-mediated Jagged-1/Notch signaling and induced forkhead box P3 (FOXP3)⁺ Tregs while inhibiting T helper 17 cells. However, blocking of Notch signaling by inhibiting γ -secretase reversed myeloid PTEN deficiency-mediated protection in ischemia/reperfusion-triggered liver inflammation with reduced FOXP3⁺ and increased retinoid A receptor-related orphan receptor gamma t-mediated interleukin 17A expression in ischemic livers. Moreover, knockdown of β -catenin or PPAR γ in PTEN-deficient macrophages inhibited Jagged-1/Notch activation and reduced FOXP3⁺ Treg induction, leading to increased proinflammatory mediators in macrophage/T cell cocultures. In conclusion, our findings demonstrate that PTEN- β -catenin signaling is a novel regulator involved in modulating Treg development and provides a potential therapeutic target in liver IRI.

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Abbreviations: ARG1, arginase 1; BMM, bone marrow-derived macrophage; Cre, cyclization recombination; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DMSO, dimethyl sulfoxide; FOXP3, forkhead box P3; H & E, hematoxylin-eosin; Hes1, hairy and enhancer of split-1; HPF, high-power field; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IL, interleukin; IL2R, interleukin 2 receptor; iNOS, inducible nitric oxide synthase; IR, ischemia/reperfusion; IRI, ischemia/reperfusion injury; LPS, lipopolysaccharide; LysM, lysozyme M; mAb, monoclonal antibody; mRNA, messenger RNA; NICD, Notch intracellular domain; NLRP3, NLR family, pyrin domain-containing 3; NS, nonspecific; PE, phycoerythrin; PI3K, phosphoinositide 3-kinase; PPAR γ , peroxisome proliferator-activated receptor gamma; PTEN, phosphatase and

Liver inflammatory injury induced by ischemia/reperfusion injury (IRI) is the major pathogenesis to cause hepatic dysfunction and failure following liver transplantation.⁽¹⁾ Macrophages (Kupffer cells) play a pivotal role in triggering innate immune response and developing liver inflammation during liver IRI.⁽²⁾ Activated macrophages release tumor necrosis factor α (TNF- α) and interleukin (IL) 1, which initiate a complex inflammatory cascade that leads to activate CD4⁺ T cells.^(3,4) Moreover, the retinoid A receptor-related orphan receptor gamma t-expressing (ROR γ t⁺) T cells are the main source of T helper 17 (T_H17)-

producing effector cells during the early phase of liver IRI. Inhibition of $\text{ROR}\gamma\text{t}^+$ activity resulted in reduced ischemia/reperfusion (IR)-induced liver damage.⁽⁵⁾

Phosphatase and tensin homolog (PTEN) has been known to play an important role during inflammatory response. PTEN positively regulates lipopolysaccharide (LPS)-induced toll-like receptor 4 (TLR4) signaling and proinflammatory cytokine secretion by antagonizing phosphoinositide 3-kinase (PI3K) signaling.⁽⁶⁾ Increased PTEN activity promotes tissue inflammation, whereas deletion of PTEN activates PI3K/Akt signaling and reduces inflammatory response.⁽⁷⁾ Our previous studies demonstrated that PTEN/PI3K regulated innate TLR4-driven inflammatory response, which was mediated by activating β -catenin signaling in IR-triggered liver inflammation.⁽⁸⁾

Macrophage polarization plays an important role in the regulation of inflammatory responses. The innate

and adaptive immunity regulated by macrophage PTEN/PI3K signaling might be involved in the expression of arginase 1 (ARG1).⁽⁹⁾ Activation of Wnt/ β -catenin signaling promoted tissue damage repair by a signal transducer and activator of transcription 6-mediated M2 polarization.⁽¹⁰⁾ Moreover, disruption of peroxisome proliferator-activated receptor gamma (PPAR γ) in myeloid cells impairs alternative macrophage activation,⁽¹¹⁾ suggesting PPAR γ is required for the acquisition and maintenance of the anti-inflammatory macrophage phenotype.

Regulatory T cells (Tregs) are essential for the maintenance of immune homeostasis in liver inflammatory injury.⁽¹²⁾ Tregs constitutively express the transcription factor forkhead box P3 (FOXP3), which is a key for Treg development and function.⁽¹³⁾ Increasing PTEN activity inhibits the development of $\text{CD4}^+\text{CD25}^+$ Tregs via a distinct interleukin 2 receptor (IL2R) signaling, which is associated with downstream mediators of PI3K.⁽¹⁴⁾ PTEN deficiency increases nuclear accumulation of β -catenin⁽¹⁵⁾ and promotes PI3K, leading to activation of downstream Akt and induction of Tregs.⁽¹⁶⁾ Moreover, stabilization of β -catenin enhances Treg survival and controls inflammatory responses.⁽¹⁷⁾ Activation of Jagged-1/Notch signaling instructs Treg differentiation.⁽¹⁸⁾ Thus, PTEN- β -catenin signaling might play an important role in promoting Treg induction during inflammatory response. However, it is still unclear how PTEN- β -catenin signaling may regulate Treg induction during liver inflammatory injury. Here, we report a novel regulatory mechanism of PTEN- β -catenin signaling on inflammatory response in liver injury. We have demonstrated that myeloid PTEN deficiency ameliorates IR-induced liver injury through the activation of β -catenin, which in turn promotes PPAR γ -mediated Jagged-1/Notch signaling to induce FOXP3^+ Tregs while inhibiting $\text{T}_\text{H}17$ cells. Our data document that PTEN- β -catenin signaling is crucial for the modulation of innate and adaptive immunity in the mechanism of IR-induced liver injury.

Materials and Methods

ANIMALS

Floxed phosphatase and tensin homolog (PTEN^{FL/FL}) mice (The Jackson Laboratory, Bar Harbor, ME) and the mice expressing the cyclization recombination (Cre) recombinase under the control of the lysozyme M (LysM) promoter (LysM-Cre; The Jackson Laboratory) were used to generate myeloid-specific

tensin homolog; PTEN^{FL/FL}, floxed phosphatase and tensin homolog; PTEN^{M-KO}, myeloid-specific phosphatase and tensin homolog knock-out; RBP-J, recombination signal sequence-binding protein jk; $\text{ROR}\gamma\text{t}$, retinoid A receptor-related orphan receptor gamma t; $\text{ROR}\gamma\text{t}^+$, retinoid A receptor-related orphan receptor gamma t-expressing; RT-PCR, real-time polymerase chain reaction; *SALT*, serum alanine aminotransferase; SD, standard deviation; si β -cat, β -catenin small interfering RNA; siJagged-1, Jagged-1 small interfering RNA; siPPAR γ , proliferator-activated receptor gamma small interfering RNA; siRNA, small interfering RNA; TGF- β , transforming growth factor β ; $\text{T}_\text{H}17$, T helper 17; TLR4, toll-like receptor 4; TNF- α , tumor necrosis factor α ; Treg, regulatory T cell; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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FIG. 1. Myeloid PTEN deficiency ameliorates hepatocellular damage and reduces macrophage trafficking in IR-induced liver injury. (A) Representative histological staining (H & E) of ischemic liver tissue (n = 4-5/group, magnification × 100). (B) Liver damage, evaluated by Suzuki's score. ***P < 0.001. (C) Hepatocellular function, assessed by sALT levels (IU/L). Results expressed as mean ± SD (n = 4-5/group), ***P < 0.001. (D) Liver apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining. Results expressed as mean ± SD (n = 4-6/group, magnification × 400), ***P < 0.001. (E) Immunofluorescence staining of CD68⁺ macrophages (short arrow) in ischemic liver lobes. Results expressed as mean ± SD (n = 4/group, magnification × 400), ***P < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]

phosphatase and tensin homolog knockout (PTEN^{M-KO}) mice, as described.⁽¹⁹⁾ All animals received humane care according to the criteria outlined in *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 86-23, 1985 revision) and were maintained under specific pathogen-free conditions. The study protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

MOUSE LIVER IRI MODEL

We used an established mouse model of warm hepatic ischemia followed by reperfusion, as described.⁽²⁰⁾ Some animals were injected via tail vein with Notch1 small

interfering RNA (siRNA) or nonspecific (NS) siRNAs (2 mg/kg, Santa Cruz Biotechnology, Dallas, TX) at 4 hours prior to ischemia,⁽⁸⁾ or with γ -secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (10 mg/kg, Sigma-Aldrich) and dimethyl sulfoxide (DMSO) vehicle at 30 minutes prior to ischemia (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, AND IMMUNOFLUORESCENCE STAINING

Liver sections were stained with hematoxylin-eosin (H & E). The IRI severity was graded using Suzuki's criteria.⁽²¹⁾ Liver CD11b⁺ macrophages were detected by immunohistochemistry staining with a rat anti-mouse CD11b monoclonal antibody (mAb; BD Biosciences, San Jose, CA). Immunofluorescence staining was used to identify CD68⁺ macrophages with a goat anti-mouse CD68 mAb (Santa Cruz Biotechnology; see Supporting Information).

TUNEL ASSAY

The Klenow-FragEL DNA Fragmentation Detection Kit (EMD Chemicals, Billerica, MA) was used to detect the DNA fragmentation characteristic of oncotic necrosis/apoptosis in formalin-fixed paraffin-embedded liver sections.⁽⁸⁾ Results were scored semi-quantitatively by averaging the number of apoptotic cells/microscopic field at 400 $\times$ magnification. Ten fields were evaluated per sample.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS

Quantitative real-time polymerase chain reaction (RT-PCR) was performed as previously described.⁽²²⁾ The primer sequences used for the amplification are shown in Supporting Table 1.

WESTERN BLOT ANALYSIS

Protein was extracted from liver tissue or cell cultures, as described.⁽²²⁾ Monoclonal rabbit anti-mouse β -catenin, phos-Akt, Akt, PPAR γ , Jagged-1, cleaved Notch1, hairy and enhancer of split-1 (Hes1), and β -actin Abs (Cell Signaling Technology, Danvers, MA) were used. The relative quantities of proteins were determined by densitometer and expressed in absorbance units.

BONE MARROW-DERIVED MACROPHAGE ISOLATION AND IN VITRO TRANSFECTION

Murine marrow-derived macrophages (BMMs) were generated as previously described.⁽²²⁾ Cells (1×10^6 /

well) were cultured for 7 days and then transfected with 100 nM of siRNA (β -catenin small interfering RNA [si β -cat], proliferator-activated receptor gamma small interfering RNA [siPPAR γ], or Jagged-1 small interfering RNA [siJagged-1]; Santa Cruz Biotechnology; see Supporting Information).

SPLEEN T CELL ISOLATION

The spleen T cells were purified using the EasySep mouse T cell isolation kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. T cells were then stimulated with anti-CD3 (1 μ g/mL) and anti-CD28 (2 μ g/mL; eBioscience).

MACROPHAGE/T CELL COCULTURE

The PTEN^{FL/FL}, PTEN^{M-KO}, si β -cat, siPPAR γ , or siJagged-1-transfected macrophages (5×10^5 cells/mL) were cultured and stimulated with LPS (100 ng/mL) for 6 hours. Splenic T cells were added at a ratio of 1:10 (macrophage:T cell). The cocultured cells were incubated for 24 hours.

FLOW CYTOMETRY ANALYSIS

Spleen T cells from DMSO or DAPT-treated PTEN^{M-KO} mice were stained with anti-mouse CD4-phycoerythrin (PE)–Cyanine5, CD25-PE, retinoid A receptor-related orphan receptor gamma t (ROR γ t)–PE, and FOXP3–fluorescein isothiocyanate mAbs (eBioscience) according to the manufacturer's instructions. PE-labeled rat anti-mouse immunoglobulin G2a isotypes were used as negative controls. Measurements were performed using a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using CellQuest software.

STATISTICAL ANALYSIS

Data are expressed as mean $\pm$ standard deviation (SD) and analyzed by Student *t* tests. Per comparison, 2-sided *P* values < 0.05 were considered statistically significant. Multiple group comparisons were performed using 1-way analysis of variance with a post hoc test.

FIG. 2. Myeloid PTEN deficiency promotes β -catenin activation and Treg induction in IR-induced liver injury. (A) Western blot analysis and relative density ratio of β -catenin, P -Akt, and Akt in ischemic livers. * P < 0.05, ** P < 0.01. Quantitative RT-PCR-assisted detection of (B) TNF- α , IL1 β , IL6, TGF- β , (C) ARG1, iNOS, and (D) FOXP3, ROR γ t, IL17A in ischemic livers or (E) Notch1, Hes1, RBP-J and (F) FOXP3, ROR γ t, IL17A in spleen T cells from PTEN^{FL/FL} and PTEN^{M-KO} mice. Each column represents the mean $\pm$ SD (n = 3-4/group). * P < 0.05, ** P < 0.01.

Results

MYELOID PTEN DEFICIENCY AMELIORATES HEPATOCELLULAR DAMAGE AND REDUCES MACROPHAGE TRAFFICKING IN IR-INDUCED LIVER INJURY

The hepatocellular damage was evaluated in mouse livers subjected to 90 minutes of warm ischemia followed by 6 hours of reperfusion. Livers in PTEN^{FL/FL} mice showed severe edema, sinusoidal congestion, and necrosis (Fig. 1A,B; score = 3.4 ± 0.5). However, livers in PTEN^{M-KO} mice showed mild to moderate edema and sinusoidal congestion (Fig. 1A,B;

score = 1.3 ± 0.2 ; P < 0.001). Consistent with the histopathological data, the serum ALT levels (IU/L) in PTEN^{M-KO} mice were significantly lower than those in the PTEN^{FL/FL} controls (Fig. 1C; 6905 ± 1852 versus $26,265 \pm 2610$; P < 0.001). Moreover, PTEN^{M-KO} reduced the frequency of TUNEL⁺ cells in ischemic livers compared with the PTEN^{FL/FL} controls (Fig. 1D; 29.5 ± 3.5 versus 67.9 ± 5.6 ; P < 0.001). PTEN deficiency in PTEN^{M-KO} livers decreased CD68⁺ macrophage infiltration (Fig. 1E; 14.5 ± 2.5) compared with the PTEN^{FL/FL} controls (26.5 ± 4.5 ; P < 0.001). To confirm inflammatory cell recruitment in ischemic livers, CD11b⁺ macrophages were detected by immunohistochemistry staining. Indeed, reduced CD11b⁺ macrophages were observed in PTEN^{M-KO} but not PTEN^{FL/FL} mice (Supporting Fig. 1; 12.3 ± 2.2 versus 31.5 ± 4.6 ; P < 0.001).

FIG. 3. PTEN- β -catenin axis activates PPAR γ and Jagged-1/Notch signaling pathway and induces FOXP3⁺Tregs in vitro. BMMs were transfected with si β -cat, and then cocultured with spleen T cells after LPS stimulation for 6 hours. (A) Western blot analysis and relative density ratio of β -catenin, PPAR γ , and Jagged-1 in LPS-stimulated macrophages. * P < 0.05, ** P < 0.01. Quantitative RT-PCR-assisted detection of (B) TNF- α , IL1 β , IL6, TGF- β , (C) ARG1, iNOS in LPS-stimulated macrophages. Each column represents the mean $\pm$ SD (n = 3-4/group). * P < 0.05, ** P < 0.01. (D) Western blot analysis and relative density ratio of cleaved Notch1 in spleen T cells after coculture * P < 0.05, ** P < 0.01. Quantitative RT-PCR-assisted detection of (E) Notch1, Hes1, RBP-J and (F) FOXP3, ROR γ t, IL17A in spleen T cells after coculture. Each column represents the mean $\pm$ SD (n = 3-4/group). * P < 0.05, ** P < 0.01.

MYELOID PTEN DEFICIENCY PROMOTES β -CATENIN ACTIVATION AND TREG INDUCTION IN IR-INDUCED LIVER INJURY

We found that by 6 hours of reperfusion after 90 minutes of ischemia, the protein expression of β -catenin (P = 0.006) and phosphorylated Akt (P = 0.03) was up-regulated in PTEN^{M-KO} but not in PTEN^{FL/FL} livers (Fig. 2A). The messenger RNA (mRNA) levels of proinflammatory genes coding for TNF- α (P = 0.008), IL1 β (P = 0.02), and IL6 (P = 0.03) were decreased, whereas transforming growth factor β (TGF- β ; P = 0.03) expression was

increased in PTEN^{M-KO} livers compared with the PTEN^{FL/FL} controls (Fig. 2B). Moreover, PTEN^{M-KO} promoted M2 macrophage differentiation as evidenced by the increased ARG1 (P = 0.03) and reduced M1 macrophage inducible nitric oxide synthase (iNOS; P = 0.006) expression in ischemic livers compared with the PTEN^{FL/FL} controls (Fig. 2C). Interestingly, the expression of FOXP3 (P = 0.02), a master regulator of Treg cells, was increased while the expression of ROR γ t (P = 0.006) and IL17A (P = 0.01) was decreased in PTEN^{M-KO} but not in PTEN^{FL/FL} livers (Fig. 2D). Consistent with these data, we found that PTEN^{M-KO} activated Notch signaling by increased expression of Notch1 (P = 0.03) and its downstream target gene, Hes1 (P = 0.005),

and transcription factor recombination signal sequence-binding protein $\text{j}\kappa$ (RBP-J; $P = 0.04$; Fig. 2E), which was accompanied by augmented FOXP3 ($P = 0.01$) and reduced ROR γ t ($P = 0.005$) and IL17A ($P = 0.02$) in spleen T cells (Fig. 2F).

PTEN- β -CATENIN AXIS ACTIVATES PPAR γ AND JAGGED- 1/NOTCH SIGNALING PATHWAY AND INDUCES FOXP3⁺TREGS IN VITRO

To elucidate the putative mechanisms by which PTEN-mediated β -catenin regulates Notch signaling and adaptive Treg development, we disrupted β -catenin in BMMs from PTEN^{M-KO} mice by using a si β -cat. Indeed, PTEN^{M-KO} increased the expression of β -catenin ($P = 0.004$), PPAR γ ($P = 0.04$), and Jagged-1 ($P = 0.02$) in LPS-stimulated BMMs after NS siRNA treatment. However, knockdown of β -catenin with si β -cat pretreatment in PTEN^{M-KO}-BMMs resulted in reduced PPAR γ ($P = 0.03$) and Jagged-1 ($P = 0.008$) expression after LPS stimulation (Fig. 3A). Moreover, si β -cat treatment in PTEN^{M-KO}-BMMs augmented the mRNA levels of TNF- α ($P = 0.02$), IL1 β ($P = 0.02$), and IL6 ($P = 0.01$) but reduced levels of TGF- β ($P = 0.04$) in response to LPS stimulation compared with the NS siRNA-treated controls (Fig. 3B). The decreased ARG1 ($P = 0.008$) and increased iNOS ($P = 0.03$) expression was observed in si β -cat-treated PTEN^{M-KO}-BMMs but not in NS siRNA-treated cells (Fig. 3C). Furthermore, we used si β -cat-pretreated BMMs from PTEN^{M-KO} mice and then cocultured them with spleen T cells after LPS stimulation. Indeed, knockdown of β -catenin in PTEN^{M-KO}-BMMs decreased cleaved Notch1 ($P = 0.006$) protein expression (Fig. 3D) and mRNA levels coding for Notch1 ($P = 0.04$), Hes1 ($P = 0.04$), and RBP-J ($P = 0.02$; Fig. 3E). This was accompanied by reduced FOXP3 ($P = 0.04$) but augmented ROR γ t ($P = 0.04$) and IL17A ($P = 0.03$) in spleen T cells (Fig. 3F).

PPAR γ MEDIATES JAGGED-1/ NOTCH SIGNALING PATHWAY IN VITRO

Using a macrophage (BMM)/spleen T cell coculture system, we found that myeloid PTEN deficiency increased PPAR γ ($P = 0.009$) and Jagged-1 ($P = 0.02$) expression in NS siRNA-treated macrophages after

LPS stimulation (Fig. 4A). However, knockdown of PPAR γ with siPPAR γ treatment resulted in reduced Jagged-1 ($P = 0.007$) expression in PTEN-deficient cells (Fig. 4A), with increased expression of TNF- α ($P = 0.03$), IL1 β ($P = 0.04$), and IL6 ($P = 0.02$) and reduced levels of TGF- β ($P = 0.03$) in response to LPS stimulation compared with the NS siRNA-treated controls (Fig. 4B). siPPAR γ treatment decreased ARG1 ($P = 0.005$) but increased iNOS ($P = 0.01$) expression in PTEN-deficient macrophages compared with the NS siRNA-treated cells (Fig. 4C). Moreover, PPAR γ knockdown in macrophages reduced cleaved Notch1 ($P = 0.03$) protein expression (Fig. 4D) and Notch1 ($P = 0.04$) and RBP-J ($P = 0.04$) mRNA levels (Fig. 4E), with reduced FOXP3 ($P = 0.04$) but increased ROR γ t ($P = 0.03$) and IL17A ($P = 0.008$; Fig. 4F) in spleen T cells after coculture.

JAGGED-1/NOTCH SIGNALING IS ESSENTIAL FOR THE FOXP3⁺TREG INDUCTION IN THE PTEN- β -CATENIN SIGNALING-MEDIATED IMMUNE REGULATION IN VITRO

We disrupted Jagged-1/Notch signaling in BMMs from PTEN^{M-KO} mice with siJagged-1, and then cocultured with spleen T cells. Pretreatment of LPS-stimulated BMMs with siJagged-1 diminished Jagged-1 expression compared with the NS siRNA-treated cells (Fig. 5A; $P = 0.005$). Moreover, unlike NS siRNA-treated controls, siJagged-1 pretreatment inhibited cleaved Notch1 ($P = 0.008$) and Hes1 ($P = 0.006$) protein expression in spleen T cells after coculture (Fig. 5B). The mRNA levels coding for Notch1 ($P = 0.03$) and RBP-J ($P = 0.04$) were reduced in siJagged-1 but not NS siRNA-treated cells (Fig. 5C). Consistent with these data, knockdown of Jagged-1 resulted in reduced FOXP3 ($P = 0.03$) while increasing ROR γ t ($P = 0.02$) and IL17A ($P = 0.04$) expression in T cells (Fig. 5D).

BLOCKING JAGGED-1/NOTCH SIGNALING PATHWAY AGGRAVATES IR-INDUCED LIVER DAMAGE AND INHIBITS FOXP3⁺TREG INDUCTION IN VIVO

We next investigated whether disruption of Notch signaling may affect local inflammatory responses in mouse liver IRI. DAPT, a γ -secretase inhibitor, can

FIG. 4. PPAR γ mediates Jagged-1/Notch signaling pathway in vitro. BMMs were transfected with siPPAR γ , and then cocultured with spleen T cells after LPS stimulation for 6 hours. (A) Western blot analysis and relative density ratio of PPAR γ and Jagged-1 in LPS-stimulated macrophages. * P < 0.05, ** P < 0.01. Quantitative RT-PCR-assisted detection of (B) TNF- α , IL1 β , IL6, TGF- β , (C) ARG1, iNOS in LPS-stimulated macrophages. Each column represents the mean $\pm$ SD (n = 3-4/group). * P < 0.05, ** P < 0.01. (D) Western blot analysis and relative density ratio of cleaved Notch1 in spleen T cells after coculture. * P < 0.05. Quantitative RT-PCR-assisted detection of (E) Notch1, RBP-J and (F) FOXP3, ROR γ t, IL17A in spleen T cells after coculture. Each column represents the mean $\pm$ SD (n = 3-4/group). * P < 0.05, ** P < 0.01.

prevent the final cleavage step of the precursor form of Notch1 to the active Notch intracellular domain (NICD).⁽²³⁾ At 90 minutes of partial liver warm ischemia followed by 6 hours of reperfusion, livers in PTEN^{M-KO} mice treated with vehicle DMSO controls showed mild to moderate edema without necrosis (Fig. 6A,B; score = 1.1 $\pm$ 0.2). In contrast, livers in mice after receiving DAPT revealed significant edema, severe sinusoidal congestion/cytoplasmic vacuolization, and extensive (30%-50%) necrosis (score = 3.2 $\pm$ 0.5; P < 0.001). These data were consistent with hepatocellular function, which showed that DAPT treatment in PTEN^{M-KO} mice increased sALT levels compared with the DMSO-treated controls (Fig. 6C; 22,274 $\pm$ 3340 versus 4914 $\pm$ 1941; P < 0.001). Liver

cell apoptosis was analyzed by TUNEL staining. DAPT treatment in PTEN^{M-KO} mice increased the frequency of apoptotic TUNEL⁺ cells in ischemic livers compared with the DMSO-treated controls (Fig. 6D; 67.5 $\pm$ 7.8 versus 30.9 $\pm$ 4.2; P < 0.001). To further confirm the role of Notch1 signaling in liver IRI, we disrupted Notch signaling in PTEN^{M-KO} mice using Notch1 siRNA or NS siRNA. Livers in mice treated with NS siRNA showed mild to moderate edema without necrosis (Supporting Fig. 2; score = 1.1 $\pm$ 0.2). In contrast, Notch1 siRNA-treated livers revealed significant edema, severe sinusoidal congestion/cytoplasmic vacuolization, and extensive (30%-50%) necrosis (score = 3.3 $\pm$ 0.7; P < 0.001). PTEN deficiency increased Hes1 expression in DMSO-treated PTEN^{M-}

FIG. 5. Jagged-1/Notch signaling is essential for the FOXP3⁺Treg induction in the PTEN-β-catenin signaling-mediated immune regulation in vitro. BMMs were transfected with siJagged-1, and then cocultured with spleen T cells after LPS stimulation for 6 hours. (A) Western blot analysis and relative density ratio of Jagged-1 in LPS-stimulated macrophages. **P* < 0.05, ***P* < 0.01. Quantitative RT-PCR-assisted detection of (B) Western blot analysis and relative density ratio of cleaved Notch1 in spleen T cells after coculture. **P* < 0.05, ***P* < 0.01. Quantitative RT-PCR-assisted detection of (C) Notch1, RBP-J and (D) FOXP3, RORγt, IL17A in spleen T cells after coculture. Each column represents the mean ± SD (*n* = 3-4/group). **P* < 0.05.

KO mice (Fig. 6E; *P* = 0.006). However, blocking Notch signaling in PTEN^{M-KO} mice resulted in reduced Hes1 expression (Fig. 6E; *P* = 0.03) after DAPT treatment. The expression of proinflammatory TNF-α (*P* = 0.03), IL1β (*P* = 0.03), and IL6 (*P* = 0.008) was increased in DAPT but not in DMSO-treated PTEN^{M-KO} mice (Fig. 6F). Consistent with these data, the mRNA level of RBP-J was decreased (Fig. 6G; *P* = 0.007), leading to reduced FOXP3 (*P* = 0.04) while augmented RORγt (*P* = 0.02) and IL17A (*P* = 0.008) expression in DAPT-treated PTEN^{M-KO} mice, as compared with that in DMSO-treated controls (Fig. 6H). We then analyzed FOXP3 or RORγt expression in spleen T cells by flow cytometry analysis. As a result, we observed a significantly reduced percentage of CD4⁺CD25⁺

FOXP3⁺ Tregs in DAPT-treated PTEN^{M-KO} mice but not in DMSO-treated controls (Fig. 6I; 3.9 ± 0.4 versus 6.8 ± 0.6 ; *P* < 0.001). In contrast, DAPT treatment resulted in an increased percentage of RORγt⁺ T cells (IL17A-producing T cells) compared with the control groups (Fig. 6I; 3.6 ± 0.5 versus 1.5 ± 0.4 ; *P* < 0.001).

Discussion

This study documents for the first time that PTEN-β-catenin signaling is crucial for orchestrating inflammatory responses, PPARγ activation, Jagged-1/Notch signaling, and Treg induction in liver inflammatory injury due to IR. First, PTEN, a key negative regulator

FIG. 6. Blocking Jagged-1/Notch signaling pathway aggravates IR-induced liver damage and inhibits FOXP3⁺Treg induction in vivo. (A) Representative histological staining (H & E) of ischemic livers. (n = 4-5/group, magnification × 100). (B) The severity of liver IRI was evaluated by the Suzuki's histological grading. ****P* < 0.001. (C) Hepatocellular function was evaluated by sALT levels (IU/L). Results expressed as mean ± SD (n = 4-5/group). ****P* < 0.001. (D) Liver apoptosis by TUNEL staining. Results expressed as mean ± SD (n = 4-6/group, magnification × 400), ****P* < 0.001. (E) Western blot analysis and relative density ratio of Hes1 in ischemic livers. **P* < 0.05, ***P* < 0.01. Quantitative RT-PCR-assisted detection of (F) TNF-α, IL1β, IL6, (G) RBP-J, and (H) FOXP3, RORγt, IL17A in ischemic livers. Each column represents the mean ± SD (n = 3-4/group). **P* < 0.05, ***P* < 0.01. (I) FOXP3 and RORγt expression in spleen T cells were evaluated by flow cytometry. Results expressed as mean ± SD (n = 3/group, *P* < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]

of the PI3K/Akt signaling pathway, is essential for IR-induced liver injury. PTEN^{M-KO} reduced liver damage and macrophage trafficking in ischemic livers. Second, PTEN^{M-KO} promoted β-catenin activation, which in turn induced PPARγ activation, increased anti-inflammatory M2 macrophage differentiation, and decreased proinflammatory cytokines. Third, PTEN-β-catenin axis activated PPARγ-mediated Jagged-1/Notch signaling, leading to increased FOXP3⁺ Tregs. Fourth, disruption of the Jagged-1/Notch signaling pathway resulted in aggravated IR-induced liver damage and reduced FOXP3⁺ Treg induction. Our results highlight the role of PTEN-β-catenin signaling in

regulating innate and adaptive immune responses during IR-triggered liver inflammation.

The molecular mechanisms of IR-induced liver damage involved in the activation of innate and adaptive immunity may be through multiple cellular and molecular signaling pathways. PTEN, a multifunctional phosphatase, is shown to be essential for controlling innate immunity in liver injury through regulation of its downstream PI3K/Akt signaling.^(24,25) Activation of Akt increases β-catenin activity and inhibits TLR4 and NLR family, pyrin domain-containing 3 (NLRP3)-mediated innate immune response during liver IRI.^(8,22) Consistent with the role of Akt/β-catenin signaling

FIG. 7. Schematic illustration of molecular mechanisms of PTEN- β -catenin axis in the regulation of Tregs and inflammatory responses in liver IRI.

cascade in the inflammatory response, our current in vivo study has shown that myeloid-specific PTEN deficiency promoted β -catenin activation and diminished inflammatory injury, as evidenced by ameliorated IR-induced liver damage, reduced macrophage activation, and proinflammatory cytokines while increasing anti-inflammatory M2 macrophage differentiation. Interestingly, myeloid-specific PTEN deficiency augmented liver FOXP3 and reduced ROR γ t/IL17A expression, implying the important role of PTEN- β -catenin signaling in the regulation of FOXP3⁺ Treg induction during liver IRI.

The transcription factor FOXP3 is crucial for the ability of Treg cells to inhibit inflammatory response.⁽²⁶⁾ Using the macrophage and T cell coculture system, we found that myeloid-specific PTEN deficiency activated β -catenin, PPAR γ , Jagged-1/Notch signaling, and increased FOXP3⁺ Treg induction. However, knockdown of β -catenin in PTEN-deficient macrophages inhibited PPAR γ and Jagged-1/Notch, which led to reduced FOXP3⁺ Tregs. Indeed, β -catenin signaling was required for the control of innate and adaptive immunity during inflammatory response.⁽⁸⁾ Activation of β -catenin increased anti-inflammatory mediators and Treg induction while inhibiting inflammatory effect T cells.⁽²⁷⁾ PPAR γ was shown to transcriptionally regulate macrophage activation and function.⁽²⁸⁾ Ligand of PPAR γ inhibited T cell and proinflammatory cytokine

activation during the regulation of inflammatory activities.⁽²⁹⁾ In agreement with these findings, we found that PPAR γ knockdown inhibited M2 macrophage differentiation as evidenced by reduced ARG1 and augmented iNOS expression. Moreover, PPAR γ knockdown in PTEN-deficient macrophages resulted in reduced Jagged-1/Notch1 activation. This may imply the mechanistic links between PTEN- β -catenin axis and the PPAR γ -mediated Jagged-1/Notch signaling pathway in the regulation of FOXP3⁺ Treg induction in liver inflammation.

To further elucidate the regulatory network by which PTEN- β -catenin signaling may regulate FOXP3⁺ Treg induction through a Jagged-1/Notch pathway-dependent manner in live inflammation, we knocked down Jagged-1 in PTEN-deficient macrophages and then cocultured with T cells. We found that knockdown of Jagged-1 inhibited Notch1 activation, which resulted in reducing its target gene Hes1 and transcription factor RBP-J expression in T cells. Hes1 is a basic helix-loop-helix-type transcriptional repressor and negatively regulates gene transcription. It is known for cell proliferation and differentiation.⁽³⁰⁾ Hes1 may regulate innate TLR4 activation by targeting RBP-J via a feedback inhibitory loop.⁽³¹⁾ Moreover, Hes1 inhibits inflammatory response by modulating transcription elongation.⁽³²⁾ Loss of Hes1 gene results in impairing T cell development in the selective expansion of early T cell precursors.⁽³³⁾ Notch signaling might directly bind to FOXP3 promoter in Hes1- and RBP-J-dependent mechanisms.⁽³⁴⁾ Activation of Notch-Hes1 may mediate Treg immunosuppressive functions via a TGF- β signaling,⁽³⁵⁾ which is known to be crucial for Treg development.⁽³⁶⁾ Consistent with these results, we found that PTEN^{M-KO} increased Notch1 and Hes1 expression in T cells. Blocking Jagged-1/Notch signaling suppressed Hes1, leading to reduced FOXP3⁺ Tregs while increasing ROR γ t/IL17A expression. This indicates that Hes1 is a key mediator for the induction of FOXP3⁺ Tregs during liver IRI.

Further evidence of Jagged-1/Notch signaling-mediated modulation of Tregs in liver IRI was obtained from PTEN^{M-KO} mice. We found that PTEN^{M-KO} mice treated with DAPT or Notch1 siRNA (Supporting Fig. 2) exacerbated liver damage. Indeed, DAPT is a γ -secretase inhibitor. γ -secretase mediates cleavage of the Notch receptor and releases NICD, which translocates into the nucleus and directly participates in a transcriptional complex with the DNA-binding proteins to activate transcription of target genes.⁽²³⁾ DAPT has been shown to inhibit Notch signaling in studies of autoimmune and

lymphoproliferative diseases.⁽³⁷⁾ Inhibition of γ -secretase activity by DAPT treatment increased LPS-induced inflammation by activating NF- κ B signaling.⁽³⁸⁾ In agreement with these findings, we found that blocking Notch signaling by DAPT treatment suppressed Hes1 expression and increased proinflammatory mediators. Notably, DAPT treatment resulted in reduced CD4⁺CD25⁺FOXP3⁺ Tregs and increased ROR γ t-mediated IL17A-producing T cells in PTEN^{M-KO} livers, which suggests that Jagged-1/Notch signaling most likely contributed to the CD4⁺CD25⁺FOXP3⁺ Treg induction during liver IRI.

Figure 7 depicts putative molecular mechanisms by which PTEN- β -catenin signaling may regulate Treg development in liver IRI. Myeloid-specific PTEN deficiency promotes β -catenin activation via Akt phosphorylation. After translocating to the nucleus, β -catenin activates PPAR γ -mediated Jagged-1/Notch signaling pathway by γ -secretase cleavage of Notch to NICD, which interacts with Hes1 and RBP-J, leading to promote FOXP3⁺ Treg induction and inhibit inflammatory response in the liver.

In conclusion, we demonstrate that PTEN- β -catenin signaling regulates FOXP3⁺ Treg induction via activating a PPARc-mediated Jagged-1/Notch signaling pathway in liver IRI. Myeloid-specific PTEN ablation promotes β -catenin, which in turn activates PPARc and Jagged-1/Notch signaling, leading to augmented FOXP3⁺ Treg induction while inhibiting ROR γ t/IL17A in IR-triggered liver inflammation. By identifying molecular mechanisms of PTEN- β -catenin signaling in the modulation of innate immune response and adaptive Treg development, our study provides potential therapeutic targets in liver IRI followed by liver transplantation.

REFERENCES

- 1) Peralta C, Jiménez-Castro MB, Gracia-Sancho J. Hepatic ischemia and reperfusion injury: effects on the liver sinusoidal milieu. *J Hepatol* 2013;59:1094-1106.
- 2) Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cellular Mol Immunol* 2016;13:316-327.
- 3) Colletti LM, Remick DG, Burtch GD, Kunkel SL, Strieter RM, Campbell DA Jr. Role of tumor necrosis factor- α in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. *J Clin Invest* 1990;85:1936-1943.
- 4) Zwacka RM, Zhang Y, Halldorson J, Schlossberg H, Dudus L, Engelhardt JF. CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver. *J Clin Invest* 1997;100:279-289.
- 5) Eggenhofer E, Rovira J, Sabet-Baktach M, Groell A, Scherer MN, Dahlke MH, et al. Unconventional ROR γ t + T cells drive hepatic ischemia reperfusion injury. *J Immunol* 2013;191:480-487.
- 6) Cao X, Wei G, Fang H, Guo J, Weinstein M, Marsh CB, et al. The inositol 3-phosphatase PTEN negatively regulates Fc gamma receptor signaling, but supports Toll-like receptor 4 signaling in murine peritoneal macrophages. *J Immunol* 2004;172:4851-4857.
- 7) Schabbauer G, Matt U, Günzl P, Warszawska J, Furtner T, Hainzl E, et al. Myeloid PTEN promotes inflammation but impairs bactericidal activities during murine pneumococcal pneumonia. *J Immunol* 2010;185:468-476.
- 8) Ke B, Shen XD, Kamo N, Ji H, Yue S, Gao F, et al. β -catenin regulates innate and adaptive immunity in mouse liver ischemia-reperfusion injury. *Hepatology* 2013;57:1203-1214.
- 9) Sahin E, Haubenwallner S, Kuttke M, Kollmann I, Halfmann A, Dohnal AM, et al. Macrophage PTEN regulates expression and secretion of arginase I modulating innate and adaptive immune responses. *J Immunol* 2014;193:1717-1727.
- 10) Cosín-Roger J, Ortiz-Masiá D, Calatayud S, Hernández C, Esplugues JV, Barrachina MD. The activation of Wnt signaling by a STAT6-dependent macrophage phenotype promotes mucosal repair in murine IBD. *Mucosal Immunol* 2016;9:986-998.
- 11) Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, et al. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 2007;447:1116-1120.
- 12) Ma X, Hua J, Mohamood AR, Hamad AR, Ravi R, Li Z. A high-fat diet and regulatory T cells influence susceptibility to endotoxin-induced liver injury. *Hepatology* 2007;46:1519-1529.
- 13) Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057-1061.
- 14) Bensinger SJ, Walsh PT, Zhang J, Carroll M, Parsons R, Rathmell JC, et al. Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. *J Immunol* 2004;172:5287-5296.
- 15) Persad S, Troussard AA, McPhee TR, Mulholland DJ, Dedhar S. Tumor suppressor PTEN inhibits nuclear accumulation of beta-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation. *J Cell Biol* 2001;153:1161-1174.
- 16) Pierau M, Engelmann S, Reinhold D, Lapp T, Schraven B, Bommhardt UH. Protein kinase B/Akt signals impair Th17 differentiation and support natural regulatory T cell function and induced regulatory T cell formation. *J Immunol* 2009;183:6124-6134.
- 17) Ding Y, Shen S, Lino AC, Curotto de Lafaille MA, Lafaille JJ. Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells. *Nat Med* 2008;14:162-169.
- 18) Asano N, Watanabe T, Kitani A, Fuss IJ, Strober W. Notch1 signaling and regulatory T cell function. *J Immunol* 2008;180:2796-2804.
- 19) Yue S, Rao J, Zhu J, Busuttill RW, Kupiec-Weglinski JW, Lu L, et al. Myeloid PTEN deficiency protects livers from ischemia reperfusion injury by facilitating M2 macrophage differentiation. *J Immunol* 2014;192:5343-5353.
- 20) Shen XD, Ke B, Zhai Y, Amersi F, Gao F, Anselmo DM, et al. CD154-CD40 T-cell costimulation pathway is required in the mechanism of hepatic ischemia/reperfusion injury, and its blockade facilitates and depends on heme oxygenase-1 mediated cytoprotection. *Transplantation* 2002;74:315-319.

- 21) Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. *Transplantation* 1993;55:1265-1272.
- 22) Yue S, Zhu J, Zhang M, Li C, Zhou X, Zhou M, et al. The myeloid heat shock transcription factor 1/ β -catenin axis regulates NLR family, pyrin domain-containing 3 inflammasome activation in mouse liver ischemia/reperfusion injury. *Hepatology* 2016; 64:1683-1698.
- 23) Guruharsha KG, Kankel MW, Artavanis-Tsakonas S. The Notch signalling system: recent insights into the complexity of a conserved pathway. *Nat Rev Genet* 2012;13:654-666.
- 24) Ke B, Shen XD, Ji H, Kamo N, Gao F, Freitas MC, et al. HO-1-STAT3 axis in mouse liver ischemia/reperfusion injury: regulation of TLR4 innate responses through PI3K/PTEN signaling. *J Hepatol* 2012;56:359-366.
- 25) Kamo N, Ke B, Busuttill RW, Kupiec-Weglinski JW. PTEN-mediated Akt/ β -catenin/Foxo1 signaling regulates innate immune responses in mouse liver ischemia/reperfusion injury. *Hepatology* 2013;57:289-298.
- 26) Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity* 2013;38:414-423.
- 27) Manicassamy S, Reizis B, Ravindran R, Nakaya H, Salazar-Gonzalez RM, Wang YC, Pulendran B. Activation of β -catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science* 2010;329:849-853.
- 28) Chawla A. Control of macrophage activation and function by PPARs. *Circ Res* 2010;106:1559-1569.
- 29) Clark RB, Bishop-Bailey D, Estrada-Hernandez T, Hla T, Puddington L, Padula SJ. The nuclear receptor PPAR gamma and immunoregulation: PPAR gamma mediates inhibition of helper T cell responses. *J Immunol* 2000;164:1364-1371.
- 30) Kageyama R, Ohtsuka T, Kobayashi T. The Hes gene family: repressors and oscillators that orchestrate embryogenesis. *Development* 2007;134:1243-1251.
- 31) Hu X, Chung AY, Wu I, Foldi J, Chen J, Ji JD, et al. Integrated regulation of Toll-like receptor responses by Notch and interferon-gamma pathways. *Immunity* 2008;29:691-703.
- 32) Shang Y, Coppo M, He T, Ning F, Yu L, Kang L, et al. The transcriptional repressor Hes1 attenuates inflammation by regulating transcription elongation. *Nat Immunol* 2016;17:930-937.
- 33) Tomita K, Hattori M, Nakamura E, Nakanishi S, Minato N, Kageyama R. The bHLH gene Hes1 is essential for expansion of early T cell precursors. *Genes Dev* 1999;13:1203-1210.
- 34) Ou-Yang HF, Zhang HW, Wu CG, Zhang P, Zhang J, Li JC, et al. Notch signaling regulates the FOXP3 promoter through RBP-J- and Hes1-dependent mechanisms. *Mol Cell Biochem* 2009;320:109-114.
- 35) Ostroukhova M, Qi Z, Oriss TB, Dixon-McCarthy B, Ray P, Ray A. Treg-mediated immunosuppression involves activation of the Notch-HES1 axis by membrane-bound TGF- β . *J Clin Invest* 2006;116:996-1004.
- 36) Lu L, Wang J, Zhang F, Chai Y, Brand D, Wang X, et al. Role of SMAD and non-SMAD signals in the development of Th17 and regulatory T cells. *J Immunol* 2010;184:4295-4306.
- 37) Teachey DT, Seif AE, Brown VI, Bruno M, Bunte RM, Chang YJ, et al. Targeting Notch signaling in autoimmune and lymphoproliferative disease. *Blood* 2008;111:705-714.
- 38) Nasoohi S, Hemmati AA, Moradi F, Ahmadiani A. The γ -secretase blocker DAPT impairs recovery from lipopolysaccharide-induced inflammation in rat brain. *Neuroscience* 2012;210:99-109.