Published in final edited form as:

Hepatology. 2013 March ; 57(3): 1203–1214. doi:10.1002/hep.26100.

β-CATENIN REGULATES INNATE AND ADAPTIVE IMMUNITY IN MOUSE LIVER ISCHEMIA-REPERFUSION INJURY

Bibo Ke, Xiu-Da Shen, Naoko Kamo, Haofeng Ji, Shi Yue, Feng Gao, Ronald W. Busuttil, and Jerzy W. Kupiec-Weglinski

Dumont-UCLA Transplant Center, Department of Surgery, Division of Liver and Pancreas Transplantation, David Geffen School of Medicine at UCLA, Box 957054, Los Angeles, CA 90095

Abstract

Dendritic cells (DCs) are critical mediators of immune responses that integrate signals from the innate immune system to orchestrate adaptive host immunity. This study was designed to investigate the role and molecular mechanisms of STAT3-induced β-catenin in the regulation of DC function and inflammatory responses *in vitro* and *in vivo*. STAT3 induction in LPS-stimulated mouse bone marrow derived-DCs (BMDCs) triggered β-catenin activation via GSK-3β phosphorylation. The activation of β-catenin inhibited PTEN and promoted PI3K/Akt pathway, which in turn downregulated DC maturation and function. In contrast, knockdown of β-catenin increased PTEN/TLR4, IRF3, NF-κB activity and proinflammatory cytokine programs in response to LPS stimulation. In a mouse model of warm liver ischemia and reperfusion injury (IRI), disruption of β-catenin signaling increased the hepatocellular damage, enhanced hepatic DC maturation/function and PTEN/TLR4 local inflammation *in vivo*. Conclusion: Our novel findings underscore the role of β-catenin to modulate DC maturation and function at the innate - adaptive interface. Activation of β-catenin triggered PI3K/Akt which in turn inhibited TLR4-driven inflammatory response in a negative feedback regulatory mechanism. By identifying the molecular pathways by which β-catenin regulates DC function, our findings provide the rationale for novel therapeutic approaches to manage local inflammation and injury in IR-stressed liver.

Keywords

TLR4; PTEN; Immunity; Liver Injury

Liver ischemia and reperfusion injury (IRI), a local inflammatory response driven by innate and supported by adaptive immune responses, represents an important cause of organ dysfunction and failure in liver transplantation (1). Our group was one of the first to document the essential function of TLR4 in the mechanism of liver IRI by promoting local inflammation and hepatocellular damage via downstream IFN regulatory factor (IRF) 3 pathway (2). It soon became evident that IR-induced liver damage triggers TLR4 endogenous ligands, such as high-mobility group box 1 (HMGB1), to activate dendritic cells (DCs) and facilitate inflammatory cytokine programs that further enhance TLR4-mediated local inflammation (3-4).

Although different cell types (hepatocytes, Kupffer cells, sinusoidal endothelial cells, and infiltrating T cells) contribute to IRI pathophysiology, hepatic DCs are well-suited to

modulate local immune responses that can bridge innate and adaptive immunity in the liver (5). Indeed, immature DCs in peripheral tissues function to capture and process antigens (5-6). Upon exposure to pathogens and TLR ligands, however, DC rapidly acquire an activated phenotype and undergo maturation characterized by upregulated expression of MHC antigens, costimulatory CD80/CD86 molecules, and proinflammatory cytokines that stimulate naïve T cell differentiation (5-6). Hence, controlling DC differentiation is important to prevent hepatic innate and adaptive inflammatory development.

STAT3 is known to mediate many biological effects by regulating immune homeostasis and influencing cell proliferation/differentiation (7). Disruption of STAT3 during hematopoiesis activates innate immune response and promotes proinflammatory phenotype (8). STAT3 signaling may halt DC maturation *in vitro* (9), whereas STAT3 deficiency in IL-10^{-/-} DCs was shown to increase NF- κ B binding to the IL-12p40 promoter and to promote TLR-dependent IL-12 inflammation (10). As conditional deletion of STAT3 results in severe colitis and enhanced Th1-type activity (11), STAT3 may serve as an intrinsic negative regulator of DC function (12).

The Wnt- β -catenin pathway is an important regulator of cell development, regeneration, and carcinogenesis (13-14). In response to Wnt signaling, β -catenin is rapidly phosphorylated and enters the nucleus, where it interacts with T cell factor/lymphoid enhancer factor (TCF/LEF) family members to regulate transcription of the target genes. Inhibition of STAT3 induces translocation of β -catenin from the nucleus to the cytoplasm leading to decreased β -catenin transcription activity (15), suggesting β -catenin function might be mediated by STAT3. Moreover, activation of β -catenin was shown to regulate the local immunity and tolerance balance in murine intestinal mucosa (16). Despite its essential immunomodulatory functions, however, little is known on the molecular mechanisms by which β -catenin may regulate DC function and/or local inflammation responses in the liver.

Here, we report on the crucial regulatory function of STAT3-induced β -catenin on DC function and inflammatory responses in hepatic IRI. We demonstrate that β -catenin inhibits PTEN and promotes PI3K/Akt pathway, which in turn downregulates DC immune function and depresses TLR4-driven inflammation. Our data document β -catenin as a novel regulator of innate and adaptive immune responses in the mechanism of liver IRI.

Experimental Procedures

Animals

Male C57BL/6 wild-type (WT) mice at 6-8 weeks of age were used (The Jackson Laboratory, Bar Harbor, ME). Animals, housed in UCLA animal facility under specific pathogen-free conditions, received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23 revised 1985).

Cell isolations, *in vitro* cultures

Murine BMDCs and liver DCs were generated, as described (17, 18). In brief, bone-marrow cells from femurs of WT mice were cultured in RPMI-1640 supplemented with 10% FBS, 100 μ g/ml of penicillin/streptomycin (Life Technologies; Grand Island, NY), in 12-well plates (1 $\times$ 10⁶ cells/ml) with GM-CSF (20ng/ml, R&D System Inc., Minneapolis, MN) and IL-4 (10ng/ml, R&D System). Adherent immature DCs (purity 90% CD11c⁺) were recovered for *in vitro* experiments on day +7.

To separate hepatic DCs, mouse livers perfused with PBS followed by collagenase type IV/DNase 1 (Sigma-Aldrich). After washing, the resuspended cells were incubated with anti-mouse CD11c-coated immunomagnetic beads (STEMCELL) for 15min at 4°C, and

positively selected by using a magnetic column according to the manufacturer's instruction. For DC maturation studies, CD11c-enriched cells were cultured for 24h with lipopolysaccharide (LPS; 0.5µg/ml).

Preparation of siRNA

siRNA against β -catenin was designed using the siRNA selection program (19). The sense and antisense strands of murine β -catenin siRNA were 5'-AGCUGAUUAUGAUGGACAG-3' (sense) and 5'-CUGUCCAUCAAUAUCAGCU-3' (antisense). The non-silencing (NS) siRNA were 5'-UUCUCCGAACGUGUCACGU-3' (sense) and 5'-ACGUGACACGUUCGGAGAA-3' (antisense), served as negative controls. The generation of siRNA against STAT3 was described (20). All siRNAs were synthesized in 2'-deprotected, duplexed, desalted and purified siRNA form (Qiagen Inc., Chatsworth, CA).

In vitro transfections and treatment

On day 7, one $\times 10^6$ cells/well of immature BMDCs were transfected with 100nM of siRNA using lipofectamine 2000 reagent (Invitrogen), and incubated for 24h. Cells were then treated with 10µg/ml of cobalt protoporphyrin (CoPP; HO-1 inducer) or tin protoporphyrin (SnPP; competitive HO-1 inhibitor) (Porphyrin Products Inc., Logan, UT) or with 50ng/ml of murine recombinant IL-10 (rIL-10; R&D System Inc., Minneapolis, MN), and incubated for additional 6h (20).

ELISA assay

Murine BMDC culture supernatants were harvested for cytokine analysis. ELISA kits were used to measure IL12p40/TNF- α /IL-6 levels (eBiosciences, San Diego, CA).

Flow cytometry analysis

Murine BMDCs were stained with anti-CD11c, CD40, CD80, and CD86 PE-conjugated mAbs (eBiosciences, San Diego, CA). PE-labeled rat anti-IgG2a isotypes were used as negative controls. Measurements were performed using a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using CellQuest software.

Malachite green phosphate assay

Murine BMDC and hepatic DC protein lysates were immunoprecipitated with anti-PTEN Ab and incubated with protein A/G agarose beads. The PTEN malachite green assay was performed with beads-bound PTEN (Echelon Biosciences Inc., Salt Lake City, UT). The released phosphate was determined relative to a phosphatase standard curve.

Mouse liver IRI model and treatment

We used an established mouse model of warm hepatic ischemia followed by reperfusion (19-20). Mice were injected with heparin (100U/kg) and an atraumatic clip was used to interrupt the arterial/portal venous blood supply to the cephalad liver lobes. After 90min of ischemia, the clip was removed, and mice were sacrificed at 6h of reperfusion. Some animals were injected via tail vein with Ad-HO-1, Ad-IL-10 or Ad- β -gal (2.5×10^9 pfu) 24h prior to ischemia. β -catenin siRNA or nonspecific siRNAs (2mg/kg) was injected i.v. at 4h prior to ischemia (19-20). Consistent with others (21), >40% of i.v. infused siRNA consistently accumulate in the ischemic lobes (19).

Hepatocellular function assay

Serum glutamic-pyruvic transaminase (sGPT) levels, an indicator of hepatocellular injury, were measured with an autoanalyzer (ANTECH Diagnostics, Los Angeles, CA).

Histology, immunohistochemistry, double immunofluorescence staining

Liver sections (5- μ m) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's criteria on a scale from 0-4 (22). Liver DCs were detected using primary mAb against mouse CD11c (EMD Millipore, Billerica, MA) followed by incubation with secondary Ab, biotinylated goat anti-hamster IgG (Vector, Burlingame, CA). CD11c/ β -catenin double positive DCs were identified by immunofluorescence using hamster anti-mouse CD11c (Santa Cruz Biotechnology) and rabbit anti-mouse β -catenin (Cell Signaling Technology) mAb. After incubation with secondary goat anti-rabbit FITC-conjugated IgG (Sigma-Aldrich) and goat anti-hamster Texas Red-conjugated IgG (Vector), the samples were pre-mounted with VECTASHIELD medium with DAPI (Vector). Positive cells were counted blindly in 10 HPF/ section (x200).

Caspase-3 activity assay

Caspase-3 activity was performed and determined by an assay kit (Calbiochem, La Jolla, CA) as described (20).

TUNEL 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 (19-20). Results were scored semi-quantitatively by averaging number of apoptotic cells/microscopic field at 200 $\times$ magnification. Ten fields were evaluated/sample.

Quantitative RT-PCR analysis

Quantitative real-time PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25 μ l, the following were added: 1 $\times$ SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen, San Diego, CA) cDNA and 10 μ M of each primer. Amplification conditions were: 50 $^{\circ}$ C (2min), 95 $^{\circ}$ C (5min), followed by 40 cycles of 95 $^{\circ}$ C (15sec) and 60 $^{\circ}$ C (30sec). Primers used to amplify specific gene fragments were published (20, 23)

Western blot analysis

Proteins (30 μ g/sample) from cell cultures or liver samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Polyclonal rabbit anti-mouse TLR4 (Imgenex, San Diego, CA), phos-Stat3, Stat3, phos- β -catenin, β -catenin, phos-GSK-3 β , GSK-3 β , PTEN, phos-Akt, Akt, phos-I κ B α , I κ B α , phos-IRF3, IRF3, Bcl-2, Bcl-xl, cleaved caspase-3, and β -actin Abs (Cell Signaling Technology) were used. The relative quantities of proteins were determined by densitometer, and expressed in absorbance units (AU).

Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical comparisons between groups were analyzed by Student's t-test. Differences were considered statistically significant at the p-value of <0.05.

Results

STAT3 activates β -catenin and inhibits DC maturation/function

We have shown that STAT3 exerts potent anti-inflammatory activity both *in vitro* and *in vivo* (20). To delineate whether STAT3-induced β -catenin plays a role in DC maturation/function, mouse LPS-pulsed BMDCs were supplemented with CoPP (HO-1 inducer) or rIL-10. Western blot analysis showed that LPS slightly increased STAT3 phosphorylation (Fig. 1A, 0.5-0.7 AU), whereas addition of CoPP/rIL-10 markedly enhanced phosphorylated STAT3 (2.5-2.7 AU) in BMDCs. Interestingly, DC maturation after CoPP/rIL-10 was accompanied by up-regulation of β -catenin and GSK-3 β phosphorylation (2.1-2.4 AU and 2.2-2.4 AU, respectively), compared with LPS-matured BMDCs (0.4-0.6 AU). FACS analysis has revealed phenotypic changes in DC maturation program, as demonstrated by CoPP-/rIL-10-mediated depression of otherwise robust LPS-induced CD40, CD80, and CD86 phenotype (Fig. 1B). This data was consistent with diminished ELISA profile for IL-12p40, TNF- α and IL-6 in LPS-BMDCs after CoPP/rIL-10 treatment (Fig. 1C). Hence, STAT3-induced β -catenin inhibits BMDCs maturation and function.

β -catenin regulates DC function in STAT3-dependent manner

To elucidate the regulatory role of STAT3 and β -catenin on DC function, we disrupted STAT3 signaling in BMDCs by using a small interfering RNA (siSTAT3). This resulted in diminished CoPP-/rIL-10-mediated β -catenin expression (Fig. 2A: 0.2-0.4 AU), compared with nonspecific (NS) siRNA-transfected cells (2.5-2.8 AU). In addition, SnPP (HO-1 inhibitor) treated cells showed decreased β -catenin levels (0.2-0.6 AU). Interestingly, specific knockdown of STAT3 in CoPP-/rIL-10-treated BMDCs promoted PTEN activation (Fig. 2A, 2.2-2.4 AU) but inhibited Akt phosphorylation (0.2-0.5 AU), as compared with NS siRNA-transfected cells (0.2-0.3 AU and 2.3-2.5 AU, respectively). Furthermore, disruption of STAT3 reversed CoPP or rIL-10-mediated inhibition of LPS-triggered DC maturation, evidenced by increased CD40, CD80, and CD86 expression (Fig. 2B). Consistent with flow cytometry data, the production of IL-12p40, TNF- α and IL-6 was elevated after blockade of STAT3 in CoPP- or rIL-10-treated, but not in NS siRNA-treated BMDCs (Fig. 2C). Thus, STAT3 knockdown inhibits β -catenin signaling and triggers PTEN/PI3K and DC maturation, suggesting that β -catenin regulates DC function in STAT3-dependent manner.

Knockdown of β -catenin activates PTEN/TLR4 signaling in DCs

To further dissect putative mechanisms by which β -catenin may regulate DC function, we disrupted β -catenin signaling in BMDCs by using a small interfering RNA (si β -cat). As shown in Fig. 3A, LPS-stimulated BMDCs readily induced PTEN (2.3-2.5 AU) and TLR4 (2.6-2.8 AU). Interestingly, disruption of β -catenin in CoPP or rIL-10 pretreated BMDCs led to enhanced expression of PTEN and TLR4 (2.2-2.4 AU and 1.9-2.1 AU, respectively), compared to non-specific siRNA (siNS)-treated controls (0.3-0.7 AU and 0.2-0.4 AU, respectively). Furthermore, knockdown of β -catenin in CoPP or rIL-10 pretreated BMDCs increased the phosphorylation of IRF3 and I κ B α (Fig. 3A, 1.5-1.7 AU and 1.6-1.8 AU, respectively). Similar findings were recorded in LPS-stimulated BMDC without adjunctive CoPP/rIL-10 (supplementary Figure 3). As PTEN/PI3K signaling regulates TLR4 activation in DCs (24), we used PTEN phosphate release assay, in which β -catenin knockdown was found to increase PTEN activity (Fig. 3B) in CoPP- or rIL-10-treated LPS-stimulated DCs. These results were consistent with increased expression of CCR2, CCR5 and CXCR3 in si β -cat-treated DCs, compared with those without β -catenin-silenced cells (Fig. 3C). Thus, disruption of β -catenin activates PTEN/TLR4 signaling in DCs.

Knockdown of β -catenin increases hepatocellular damage in liver IRI

Next, we investigated whether disruption β -catenin signaling may affect local inflammatory responses in a mouse liver IRI model. The hepatocellular damage at 6h of reperfusion following 90min of partial liver warm ischemia was evaluated by Suzuki's histological grading (Fig. 4A/B). Livers in mice treated with NS siRNA (siNS) plus Ad-HO-1 or Ad-IL-10, showed mild to moderate edema without necrosis (Panel f/h; score=1.2 $\pm$ 0.42 and 1.1 $\pm$ 0.3, p <0.0001). In contrast, livers in mice after adjunctive β -catenin siRNA (si β -cat) and Ad-HO-1 or Ad-IL-10 revealed significant edema, severe sinusoidal congestion/cytoplasmic vacuolization, and extensive (30-50%) necrosis (Panel e/g; score=3.3 $\pm$ 0.48 and 3.2 $\pm$ 0.42). This data was consistent with hepatocellular function, assessed by sGPT levels (IU/L). Indeed, disruption of β -catenin in Ad-HO-1/Ad-IL-10-transfected mice increased sGPT levels, compared to NS siRNA-treated controls (Fig. 4C, 9518 $\pm$ 3797 and 9061 $\pm$ 3374 vs. 781 $\pm$ 442 and 561 $\pm$ 284, respectively, p <0.005).

In parallel experiments, we studied as to whether β -catenin modifies liver IRI under baseline conditions, i.e., in the absence of adjunctive IL-10 or HO-1. Indeed, knockdown of endogenous β -catenin in otherwise untreated WT mice exacerbated the hepatocellular damage as compared with β -catenin proficient controls, and evidenced by Suzuki's histological grading (Figure 4A/B, Panel b/d: Suzuki's score=2.8 $\pm$ 0.42 and 3.6 $\pm$ 0.7, respectively, p <0.05) and sGPT levels (Figure 4C: 7162 $\pm$ 2657 IU/L in β -catenin proficient and 13604 $\pm$ 6971 IU/L in β -catenin-deficient WT, p <0.05).

Knockdown of β -catenin enhances DC activation and PTEN/TLR4-inflammation in liver IRI

To investigate the regulatory role of β -catenin in DC function, we analyzed CD11c⁺ DC in the ischemic liver lobes by immunohistochemistry (Fig. 5A and B). Indeed, disruption of β -catenin in Ad-HO-1 or Ad-IL-10-transfected livers increased CD11c⁺ DC infiltration (Panel c and e; 25.3 $\pm$ 6.9 and 23.6 $\pm$ 7.3) compared to NS siRNA-group (Panel d and f: 11.6 $\pm$ 3.4 and 9.5 $\pm$ 4.3, p <0.005). Moreover, knockdown of β -catenin in Ad-HO-1/Ad-IL-10-treated livers increased mRNA levels coding for IL-12p40, TNF- α , IL-6, and CXCL-10, as compared with NS siRNA controls (Fig. 5C). These results were supported by Western analysis, in which β -catenin knockdown in mice subjected to Ad-HO-1 or Ad-IL-10 diminished the expression of β -catenin (Fig. 5D, 0.2-0.5 AU) in the ischemic liver lobes, whereas NS siRNA followed by Ad-HO-1 or Ad-IL-10 did not affect β -catenin levels (2.0-2.3 AU). Interestingly, the expression of PTEN, TLR4 and phosphorylated I κ B α markedly increased after disruption of β -catenin in Ad-HO-1 or Ad-IL-10 treated (2.2-2.4 AU, 2.1-2.3 AU and 2.0-2.2 AU, respectively) but not in NS siRNA-treated (0.5-0.7 AU, 0.2-0.4 AU and 0.2-0.5 AU, respectively) groups (Fig. 5D).

We used immunofluorescence staining to identify and quantify β -catenin (green) and CD11c (red) double-positive cells in IR-stressed livers (Fig. 6A,B). Knockdown of β -catenin decreased (p <0.005) the frequency of hepatic β -catenin⁺ DCs in Ad-HO-1/Ad-IL-10-treated mice (Fig. 6A, panel c/e; mean=1.8-2.3 cells/HPF) as compared with non-specific siRNA-conditioned controls (Fig. 6A, panel d/f; mean=12.2-15.3 cells/HPF). We observed marginal β -catenin expression in hepatic SEC or hepatocytes after Ad-HO-1/Ad-IL-10 gene transfer. To detect whether knockdown of endogenous β -catenin affected DC function, we isolated DCs from ischemic liver lobes subjected to β -catenin siRNA vs. NS siRNA pretreatment. Although disruption of β -catenin signaling did not affect frequency of CD4⁺ DC vs. CD8 α ⁺ DC populations in the liver (supplementary Fig. 4), it did increase (p <0.005) PTEN activity (Fig. 6C) and IL-12p40 mRNA expression (Fig. 6D) in hepatic DCs, as compared with NS siRNA controls.

Knockdown of β -catenin increases apoptosis in IR-stressed liver

We investigated the regulatory role of β -catenin on apoptosis pathways by Western blots. By 6h of reperfusion after 90min of ischemia, knockdown of β -catenin in Ad-HO-1 or Ad-IL-10-transfected livers downregulated Bcl-2/Bcl-xL (0.1-0.3 AU and 0.3-0.6 AU, respectively), yet upregulated cleaved caspase-3 (2.4-2.7 AU) (Fig. 7A). In contrast, the expression of Bcl-2/Bcl-xL strongly up-regulated in NS siRNA-treated livers after Ad-HO-1 or Ad-IL-10 (2.0-2.2 AU and 2.1-2.3 AU, respectively), whereas the expression of cleaved caspase-3 was inhibited in NS siRNA-treated controls (0.3-0.5 AU). These results were confirmed by increased caspase-3 activity in si β -cat but not NS siRNA-treated mice (Fig. 7B: 4.12 ± 0.42 and 4.01 ± 0.4 vs. 1.19 ± 0.29 and 1.08 ± 0.32 , respectively, $p < 0.001$). We further analyzed IR-induced hepatic oncotic necrosis/apoptosis by TUNEL staining (Fig. 7C,D). Livers in mice treated with si β -cat showed increased frequency of TUNEL+ cells (Fig. 7C, panel c/e: 28.6 ± 10.8 and 26.1 ± 11.1 , respectively), compared with NS siRNA controls (Fig. 7C, panel d/f: 6.5 ± 3.6 and 5.5 ± 3.2 , respectively, $p < 0.0001$).

Discussion

Both, innate and adaptive immune responses are essential in the mechanism of liver IRI (1). By regulating the initial response in damaged/necrotic cells via TLR4 signaling, DCs are key mediators of immune homeostasis (25), yet by amplifying innate responses they may also promote the development of adaptive immunity (5-6). Our results highlight the regulatory role of β -catenin to orchestrate local inflammation, PTEN/PI3K and TLR4 signaling in IR-stressed liver. Our *in vitro* data support the regulatory function of STAT3-induced β -catenin in DC activation and PTEN/TLR4 signaling. Previous studies have implicated STAT3-mediated antiinflammatory phenotype in LPS-stimulated DCs (26). We found that CoPP- or rIL-10-induced STAT3 triggered translocation of β -catenin from the cytoplasm to the nucleus, and transcription of its target genes in BMDCs. Activation of β -catenin inhibited IL-12p40, TNF- α and IL-6 expression, as well as DC maturation by downregulating costimulatory CD40, CD80 and CD86. In addition, our findings suggest that GSK-3 β may play a role in β -catenin activation and DC maturation. Interestingly, STAT3 knockdown in LPS-stimulated BMDCs depressed β -catenin and Akt but enhanced PTEN expression, leading to increased DC expression of proinflammatory mediators and costimulatory molecules, suggesting STAT3 can mediate β -catenin activation to program DC functions. We found that knockdown of β -catenin in DCs augmented proinflammatory mediators, and enhanced PTEN/TLR4, to initiate downstream increased CCR2, CCR5, and CXCR3 chemokine program. Moreover, β -catenin knockdown promoted IRF3 activation and phosphorylated I κ B to enhance NF- κ B activity. Thus, DC proinflammatory phenotype arose from direct control of β -catenin - TLR4 axis.

Next, we determined whether β -catenin signaling is essential for hepatic homeostasis. Although Wnt transcription regulates the cellular redox balance and hepatocytes that overexpress β -catenin were found resistant to IR-damage via hypoxia inducible factor (HIF)-1 α (27), the cross talk between β -catenin and host immune responses, pivotal in the mechanism of hepatic IR, remains to be elucidated. We have shown that HO-1-induced STAT3 is required for regulating innate immunity in hepatic IRI (20). In the current study, we used a mouse model of partial liver warm IRI to demonstrate that siRNA-induced β -catenin deficiency exacerbated the hepatocellular damage, assessed by sGPT levels and Suzuki's liver histological grading, in Ad-HO-1/Ad-IL-10-pretreated as well as at baseline conditions in otherwise untreated WT mice. In addition, β -catenin knockdown increased local CD11c+ DC infiltration, implicating β -catenin as a key regulator of inflammatory responses in IR-stressed hepatic DCs. Several factors may contribute to the regulatory function of β -catenin signaling. First, although myeloid/conventional DC (mDC/cDC) become activated in liver IRI by hepatocyte DNA via TLR9 (28), this DC subset can also

cross-link TLR4 ligand to promote adaptive immune activation (5-6). Indeed, β -catenin knockdown in Ad-HO-1/Ad-IL-10-treated livers enhanced local inflammation by augmenting PTEN/TLR4, IRF3 and NF- κ B expression. Thus, β -catenin downregulates hepatic DC function and downstream signaling that control inflammation in the liver. Second, during IRI, DCs rapidly enter hepatic parenchyma in response to endogenous TLR ligands (4), resulting in TLR4/NF- κ B activation and increased production of IL-12, the key cytokine at the innate - adaptive immune interface (29). Indeed, DCs are one of the major IL-12 producers (5,30). Our results show that β -catenin knockdown in Ad-HO-1/Ad-IL-10-treated livers increased DC-mediated IL-12p40 expression, which further enhanced intrahepatic adaptive immune cascades. Hence, β -catenin is a crucial regulator of TLR4-mediated IL-12 production in IR-stressed liver.

Consistent with our *in vitro* data, we found that disruption of β -catenin signaling enhanced PTEN activation but inhibited Akt phosphorylation, suggesting PTEN/PI3K/Akt pathway as an important regulatory mechanism in β -catenin function. Indeed, β -catenin knockdown promoted I κ B phosphorylation and increased TLR4-driven proinflammatory gene program, suggesting that β -catenin may affect TLR4 signaling via a negative feedback regulatory mechanism. Furthermore, Akt known to act as an anti-apoptotic molecule that promotes cell survival, can also inhibit caspase-mediated cell death through phosphorylation of Bcl-2/Bcl-xL-associated death promoter (BAD), releasing Bcl-2 family members, and directly phosphorylating caspase protease (31). Our *in vivo* results further support the role of β -catenin-mediated PI3K/Akt in the regulation of hepatic oncotic necrosis/apoptosis. Thus, defective β -catenin downregulated Bcl-2/Bcl-xL but upregulated cleaved caspase-3 and its activity, which in turn enhanced apoptotic cell death in IR-stressed livers. Thus, our results highlight the function of β -catenin to trigger PI3K/Akt signaling and ameliorate liver cell death in IRI pathology.

Figure 8 depicts putative molecular mechanisms by which β -catenin signaling may regulate immune responses in the mechanism of liver IRI. STAT3 triggers β -catenin activation via GSK-3 β phosphorylation. After translocating to the nucleus, β -catenin activates transcription of its target genes, depresses PTEN activity, and promotes PI3K/Akt signaling, to provide a negative TLR4 regulatory feedback to inhibit NF- κ B/IRF3 activity, and ultimately suppress pro-inflammatory gene programs in the liver. Furthermore, PI3K/Akt inhibits IL-12 production and promotes antiapoptotic Bcl-2/Bcl-xL function, which may also limit the hepatocyte death.

In conclusion, this study extends our recent findings on the role of Akt/ β -catenin/Foxo1 axis in the mechanism of macrophage innate activation (32), by demonstrating that β -catenin may program DC development and regulate innate - adaptive interface in IR-stressed liver. By identifying molecular pathways critical for β -catenin function, our study provides the rationale for novel therapeutic approaches to ameliorate IR-triggered liver inflammation and damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support: NIH Grants DK 062357; The Diann Kim and The Dumont Research Foundations.

Abbreviations

Ad-β-gal	recombinant adenovirus β-galactosidase reporter gene
DC	dendritic cell
BMDCs	bone marrow derived-dendritic cells
GSK-3β	glycogen synthase kinase 3β
HO-1	hemeoxygenase-1
IRF3	interferon regulatory factor-3
LPS	lipopolysaccharide
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homolog delete on chromosome 10
sGPT	serum glutamic-pyruvic transaminase
siRNA	small interfering RNA
TLR4	Toll-like receptor 4
TUNEL	terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-digoxigenin Nick End Labeling

References

1. Zhai Y, Busuttil RW, Kupiec-Weglinski JW. Liver ischemia and reperfusion injury: New insights into mechanisms of innate–adaptive immune-mediated tissue inflammation. *Am J Transplant.* 2011; 11:1563–1569. [PubMed: 21668640]
2. Zhai Y, Shen XD, O'Connell R, Gao F, Lassman C, Busuttil RW, Cheng G, et al. Cutting edge: TLR4 activation mediates liver ischemia/reperfusion inflammatory response via IRF3-dependent, MyD88-independent pathway. *J Immunol.* 2004; 173:115–119.
3. Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med.* 2005; 201:1135–1143. [PubMed: 15795240]
4. Tsung A, Zheng N, Jeyabalan G, Izuishi K, Klune JR, Geller DA, Lotze MT, et al. Increasing numbers of hepatic dendritic cells promote HMGB1-mediated ischemia-reperfusion injury. *J Leukoc Biol.* 2007; 81:119–128. [PubMed: 17062605]
5. Sumpter TL, Abe M, Tokita D, Thomson AW. Dendritic cells, the liver, and transplantation. *Hepatology.* 2007; 46:2021–31. [PubMed: 18027875]
6. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature.* 1998; 392:245–52. [PubMed: 9521319]
7. Darnell JE Jr. STATs and gene regulation. *Science.* 1997; 277:1630–1635. [PubMed: 9287210]
8. Welte T, Zhang SS, Wang T, Zhang Z, Hesslein DG, Yin Z, Kano A, et al. STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: A critical role of STAT3 in innate immunity. *Proc Natl Acad Sci U S A.* 2003; 100:1879–1884. [PubMed: 12571365]
9. Cheng F, Wang H-W, Cuenca A, Huang M, Ghansah T, Brayer J, Kerr WG, et al. A critical role for Stat3 signaling in immune tolerance. *Immunity.* 2003; 19:425–436. [PubMed: 14499117]
10. Hoentjen F, Sartor RB, Ozak M, Jobin C. STAT3 regulates NF-κB recruitment to the IL-12p40 promoter in dendritic cells. *Blood.* 2005; 105:689–696. [PubMed: 15251981]
11. Kobayashi M, Kweon MN, Kuwata H, Schreiber RD, Kiyono H, Takeda K, Akira S. Toll-like receptor-dependent production of IL-12p40 causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. *J Clin Invest.* 2003; 111:1297–1308. [PubMed: 12727921]

12. Melillo JA, Song L, Bhagat G, Blazquez AB, Plumlee CR, Lee C, Berin C, et al. Dendritic Cell (DC)-Specific Targeting Reveals Stat3 as a Negative Regulator of DC Function. *J Immunol.* 2010; 184:2638–2645. [PubMed: 20124100]
13. Staal FJT, Tiago C, Luis TC, Tiemessen MM. WNT signalling in the immune system: WNT is spreading its wings. *Nature Rev Immunol.* 2008; 8:581–593. [PubMed: 18617885]
14. Moon RT, Kohn AD, Deferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet.* 2004; 5:691–701. [PubMed: 15372092]
15. Kawada M, Seno H, Uenoyama Y, Tateo Sawabu T, Kanda N, Fukui H, Shimahara Y, et al. Signal transducers and activators of transcription 3 activation is involved in nuclear accumulation of β -catenin in colorectal cancer. *Cancer Res.* 2006; 66:2913–2917. [PubMed: 16540637]
16. Manicassamy S, Reizis B, Ravindran R, Nakaya H, Salazar-Gonzalez RM, Wang YC, Pulendran B. Activation of β -catenin in dendritic cells regulate immunity versus tolerance in the intestine. *Science.* 2010; 329:849–853. [PubMed: 20705860]
17. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med.* 1992; 176:1693–1702. [PubMed: 1460426]
18. Johansson C, Wick MJ. Liver dendritic cells present bacterial antigens and produce cytokines upon *salmonella* encounter. *J Immunol.* 2004; 172:2496–2503. [PubMed: 14764722]
19. Ke B, Shen XD, Gao F, Qiao B, Ji H, Busuttil RW, Volk HD, et al. Small interfering RNA targeting heme oxygenase-1 (HO-1) reinforces liver apoptosis induced by ischemia-reperfusion injury in mice: HO-1 is necessary for cytoprotection. *Hum Gene Ther.* 2009; 20:1133–1142. [PubMed: 19534599]
20. Ke B, Shen XD, Ji H, Kamo N, Gao F, Freitas MC, Busuttil RW, 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. [PubMed: 21756853]
21. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature.* 2004; 432:173–178. [PubMed: 15538359]
22. 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. [PubMed: 7685932]
23. Ke B, Shen XD, Gao F, Ji H, Qiao B, Zhai Y, et al. Adoptive transfer of ex vivo HO-1 modified bone marrow-derived macrophages prevents liver ischemia and reperfusion injury. *Mol Ther.* 2010; 18:1019–1025. [PubMed: 20029397]
24. Fukao T, Koyasu S. PI3K and negative regulation of TLR signaling. *Trends in Immunol.* 2003; 24:358–363. [PubMed: 12860525]
25. Kaisho T, Akira S. Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends in Immunol.* 2001; 22:78–83. [PubMed: 11286707]
26. Samarasinghe R, Tailor P, Tamura T, Kaisho T, Akira S, Ozato K. Induction of an anti-inflammatory cytokine, IL-10, in dendritic cells after toll-like receptor signaling. *J Interferon & Cytokine Res.* 2006; 26:893–900. [PubMed: 17238832]
27. Lehwald N, Tao GZ, Jang KY, Sorkin M, Knoefel WT, Sylvester KG. Wnt- β -catenin signaling protects against hepatic ischemia and reperfusion injury in mice. *Gastroenterology.* 2011; 141:707–718. [PubMed: 21679710]
28. Bamboat ZM, Lee M, Ocuin LM, Balachandran VP, Obaid H, Plitas G, DeMatteo RP. Conventional DCs reduce liver ischemia/reperfusion injury in mice via IL-10 secretion. *J Clin Invest.* 2010; 120:559–569. [PubMed: 20093775]
29. Fukao T, Tanabe M, Terauchi Y, Ota T, Matsuda S, Asano T, Kadowaki T, et al. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat Immunol.* 2002; 3:295–304. [PubMed: 11850627]
30. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol.* 1995; 13:251–276. [PubMed: 7612223]

31. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*. 1997; 91:231. [PubMed: 9346240]
32. Kamo N, Ke B, Busuttil RW, Kupiec-Weglinski JW. PTEN-mediated AKT/ β -catenin/FOXO1 signaling regulates innate immune responses in mouse liver ischemia/reperfusion injury. *Hepatology*. (In press).

Figure 1.

STAT3 activates β -catenin and inhibits DC maturation and function in murine LPS-stimulated BMDCs. Cells were incubated with CoPP (10 μ g/ml) or rIL-10 (50ng/ml) for 6h, and stimulated with/without LPS (0.5 μ g/ml) for 24h. (A) Protein was extracted from BMDCs and the expression of STAT3, β -catenin, and GSK-3 β was evaluated by Western blots. Shown are representative of three separate experiments. (B). BMDC phenotypic changes were evaluated by flow cytometry. Representative of three separate experiments. (C) The production of IL-12p40, TNF- α and IL-6 was measured in cell culture supernatants by ELISA. Mean $\pm$ SD (n=3-4 samples/group). *p< 0.0005, **p< 0.0001.

Figure 2.

β-catenin-mediated regulation of DC function in murine LPS-stimulated BMDCs is STAT3-dependent. Cells were transfected with Stat3 siRNA/NS siRNA (100nM) and incubated for 24h. After washing, cells were treated with CoPP (10μg/ml) or rIL-10 (50ng/ml) for 6h, and stimulated with/without LPS (0.5μg/ml) for 24h. (A) Western-assisted expression of β-catenin, PTEN, and Akt. Results are representative of three separate experiments. (B) BMDC phenotypic changes were evaluated by flow cytometry. Representative of three separate experiments. (C) ELISA-assisted production of IL-12p40, TNF-α and IL-6 in cell culture supernatants. Mean±SD (n=3-4 samples/group). *p< 0.0001.

Figure 3.

Knockdown of β -catenin in LPS-stimulated BMDCs activates PTEN/TLR4 signaling. Cells transfected with β -catenin siRNA/NS siRNA (100nM) were treated with CoPP (10 μ g/ml) or rIL-10 (50ng/ml), and then stimulated with or without LPS (0.5 μ g/ml). (A) Western-assisted expression of β -catenin, PTEN, TLR4, IRF3 and I κ B α . Representative of three experiments. (B) PTEN activity was measured by malachite green phosphate assay. Mean $\pm$ SD; n=4-6 samples/group. *p<0.05. (C) Quantitative RT-PCR-assisted chemokine expression in LPS-stimulated BMDCs. Each column represents mean $\pm$ SD (n=3-4 samples/group). *p<0.05.

Figure 4.

Knockdown of β -catenin increases hepatocellular damage after liver IR. Mice were subjected to 90min of partial liver warm ischemia, followed by 6h of reperfusion. (A/B) The severity of liver IRI was evaluated by the Suzuki's histological grading. (a) Sham control; (b) WT (2.8 ± 0.42); (c) Ad- β -gal (3.5 ± 0.53); (d) WT+si β -cat (3.6 ± 0.7); (e) si β -cat+Ad-HO-1 (3.3 ± 0.48); (f) nonspecific siRNA+Ad-HO-1 (1.2 ± 0.42); (g) si β -cat+Ad-IL-10 (3.2 ± 0.42); (h) nonspecific siRNA+Ad-IL-10 (1.1 ± 0.3). Representative of n=6 mice/group (* $p < 0.05$; ** $p < 0.0001$); original magnification x200. (C). Hepatocellular function in serum samples was evaluated by sGPT levels (IU/L). Results expressed as mean $\pm$ SD (n=6 samples/group). * $p < 0.05$; ** $p < 0.005$.

Figure 5.

Knockdown of β -catenin enhances hepatic DC activation and PTEN/TLR4-mediated inflammation in liver IRI. Liver DCs were detected by immunohistochemical staining using mAb against mouse CD11c (A/B). (a) Sham control; (b) Ad- β -gal; (c) si β -cat+Ad-HO-1; (d) nonspecific siRNA+Ad-HO-1; (e) si β -cat+Ad-IL-10; (f) nonspecific siRNA+Ad-IL-10. Results scored semi-quantitatively by averaging number of positively-stained cells (mean $\pm$ SD)/field at 200 $\times$ magnification. Representative of 4-6 mice/group (* p <0.005). (C) Western-assisted detection of β -catenin, Akt, PTEN, TLR4, and I κ B α . Representative of three experiments. (D). Quantitative RT-PCR-assisted detection of cytokines in mouse livers. Each column represents the mean $\pm$ SD (n=3-4 samples/group). * p <0.05.

Figure 6.

Immunofluorescence staining of β -catenin and CD11c double-positive cells in ischemic liver lobes (A/B). Goat anti-hamster CD11c (stained red) and rabbit anti-mouse β -catenin (stained green) mAbs were used. (a) Sham control; (b) Ad- β -gal; (c) si β -cat+Ad-HO-1; (d) nonspecific siRNA+Ad-HO-1; (e) si β -cat+Ad-IL-10; (f) nonspecific siRNA+Ad-IL-10. Results scored semi-quantitatively by averaging frequency of double-positive cells. Mean $\pm$ SD/ HPF (200 $\times$ magnification). Representative of 4/group (* p <0.005). (C) PTEN activity measured by malachite green phosphate assay. Mean $\pm$ SD; n=4/group. * p <0.05. (D) Quantitative RT-PCR-assisted IL-12p40 expression in LPS-stimulated DCs. Mean $\pm$ SD (n=4 /group). * p <0.05.

Figure 7.

Knockdown of β -catenin increases IR-induced apoptosis. Mice were subjected to 90min of partial liver warm ischemia, followed by 6h reperfusion. (A) Western-assisted analysis of Bcl-2, Bcl-xl and cleaved caspase-3. Representative of three experiments. (B). Caspase-3 activity in mouse livers. Results expressed as mean $\pm$ SD; n=4-6 samples/group. * p <0.001. (C/D). Liver apoptosis by TUNEL staining. (a) Sham control; (b) Ad- β -gal; (c) si β -cat+Ad-HO-1; (d) nonspecific siRNA+Ad-HO-1; (e) si β -cat+Ad-IL-10; (f) nonspecific siRNA+Ad-IL-10. Results scored semi-quantitatively by averaging the number of apoptotic cells (mean $\pm$ SD) per field at 200 $\times$ magnification. Representative of 4-6 mice/group (** p <0.0001).

Figure 8. Schematic representation of signaling pathway by which β-catenin may regulate intricate inflammatory responses in liver IRI. See text for details.