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Hippo Signaling Controls NLR Family Pyrin Domain Containing 3 Activation and Governs Immunoregulation of Mesenchymal Stem Cells in Mouse Liver Injury

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The Hippo pathway, an evolutionarily conserved protein kinase cascade, tightly regulates cell growth and survival. Activation of yes-associated protein (YAP), a downstream effector of the Hippo pathway, has been shown to modulate tissue inflammation. However, it remains unknown as to whether and how the Hippo-YAP signaling may control NLR family pyrin domain containing 3 (NLRP3) activation in mesenchymal stem cell (MSC)-mediated immune regulation during liver inflammation. In a mouse model of ischemia/reperfusion (IR)-induced liver sterile inflammatory injury, we found that adoptive transfer of MSCs reduced hepatocellular damage, shifted macrophage polarization from M1 to M2 phenotype, and diminished inflammatory mediators. MSC treatment reduced mammalian Ste20-like kinase 1/2 and large tumor suppressor 1 phosphorylation but augmented YAP and β-catenin expression with increased prostaglandin E2 production in ischemic livers. However, disruption of myeloid YAP or β-catenin in MSC-transferred mice exacerbated IR-triggered liver inflammation, enhanced NLRP3/caspase-1 activity, and reduced M2 macrophage phenotype. Using MSC/macrophage coculture system, we found that MSCs increased macrophage YAP and β-catenin nuclear translocation. Importantly, YAP and β-catenin colocalize in the nucleus while YAP interacts with β-catenin and regulates its target gene X-box binding protein 1 (XBP1), leading to reduced NLRP3/caspase-1 activity after coculture. Moreover, macrophage YAP or β-catenin deficiency augmented XBP1/NLRP3 while XBP1 deletion diminished NLRP3/caspase-1 activity. Increasing NLRP3 expression reduced M2 macrophage arginase1 but augmented M1 macrophage inducible nitric oxide synthase expression accompanied by increased interleukin-1β release. *Conclusion:* MSCs promote macrophage Hippo pathway, which in turn controls NLRP3 activation through a direct interaction between YAP and β-catenin and regulates XBP1-mediated NLRP3 activation, leading to reprograming macrophage polarization toward an anti-inflammatory M2 phenotype. Moreover, YAP functions as a transcriptional coactivator of β-catenin in MSC-mediated immune regulation. Our findings suggest a therapeutic target in MSC-mediated immunotherapy of liver sterile inflammatory injury. (Hepatology 2019;70:1714-1731).

Multipotent mesenchymal stem cells (MSCs) have shown promising therapeutic potential for tissue damage and inflammation owing to their unique immunoregulatory properties. Previous studies have shown that interactions between MSCs and inflammatory cells are crucial in MSC-mediated immune modulation and tissue repair.[(1)] With the ability to regulate both the innate

Abbreviations: Arg1, arginase1; BMMs, bone marrow–derived macrophages; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9; ChIP, chromatin immunoprecipitation; ChIP-seq, ChIP sequencing; CMFDA, 5-chloromethylfluorescein diacetate; IL, interleukin; IP, immunoprecipitation; iNOS, inducible nitric oxide synthase; IR, ischemia/reperfusion; IRI, ischemia/reperfusion injury; KO, knockout; LATS, large tumor suppressor; LPS, lipopolysaccharide; MSCs, mesenchymal stem cells; MST, mammalian Ste20-like kinase; MW, molecular weight; NLRP3, NLR family pyrin domain containing 3; PGE2, prostaglandin E2; sALT, serum alanine aminotransferase; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; XBP1, X-box binding protein 1; YAP, yes-associated protein; $YAP^{FL/FL}$, floxed yes-associated protein; YAP^{M-KO}, myeloid-specific yes-associated protein knockout.

and adaptive immune systems, MSC-based therapy has been successfully applied in various immune-mediated diseases in humans.[2] However, a number of phase III clinical trials of MSC immunotherapy were unable to meet the primary endpoints because of the low immunoregulatory efficacy of engrafted cells.[3] Thus, exploring modulatory mechanisms that govern the immunosuppressive potential of MSC emerges as one of the key challenges of MSC therapy.

The Hippo signaling pathway is an evolutionarily conserved pathway that regulates mammalian organ size by controlling cell proliferation, apoptosis, and stem cell self-renewal.[4] Yes-associated protein (YAP), as a key downstream transcriptional coactivator of the Hippo signaling pathway, functions to negatively regulate the target gene activities.[5] Indeed, YAP is modulated by its upstream kinases mammalian Ste20-like kinase (MST)1 and MST2. Phosphorylation of MST1/2 activates large tumor suppressor (LATS)1 and LATS2, which in turn phosphorylates YAP, leading to the nuclear exclusion of YAP and, ultimately, their cytoplasmic degradation.[5] However, unphosphorylated YAP enter the nucleus where they interact with a variety of transcription factors to regulate gene expression that promotes cell proliferation, differentiation, and survival.[5] It was shown that YAP can control organ size, most notably in the liver.[6] Increasing YAP expression contributed to the wound repair and tissue regeneration in inflammatory injury.[7] Moreover, recent evidence has indicated that Hippo signaling regulates innate immunity through a YAP-mediated transcriptional mechanism.[8] Activation of YAP inhibited immune response, whereas YAP deficiency resulted in aggravated tissue inflammatory injury.[9]

β-catenin, a canonical Wnt signaling pathway, is key for the cell proliferation, development, and tissue homeostasis.[10] Activation of the canonical Wnt/β-catenin pathway promotes bone marrow–derived MSC differentiation and confers resistance to oxidative stress in tissue injury.[11] We have demonstrated that myeloid β-catenin can regulate NLR family pyrin domain containing 3 (NLRP3)-mediated innate immunity in liver ischemia/reperfusion injury (IRI).[12,13] Activation of β-catenin regulates cell differentiation and is associated with the Hippo-YAP pathway[14]; YAP may physically interact with β-catenin to regulate tissue homeostasis and regeneration.[15] Although several reports have shown that Hippo signaling can be integrated with other pathways to coordinately regulate biological processes,[16] and MSCs can program macrophage plasticity and modulate inflammatory response by secreting prostaglandin E2 (PGE2),[17] it remains largely unknown as to whether and how the Hippo-YAP signaling may regulate NLRP3 activation and mediate the immunoregulatory function of MSCs in liver inflammatory injury.

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In the present study, we have identified a functional role and regulatory mechanism of the Hippo signaling in MSC-mediated immune regulation. We demonstrate that MSCs promote macrophage Hippo-YAP pathway and control NLRP3 activation through a direct interaction between YAP and β-catenin, which in turn regulates their target gene X-box binding protein 1 (XBP1), leading to reprograming macrophage polarization from a proinflammatory M1 to an anti-inflammatory M2 phenotype in ischemia/reperfusion (IR)-triggered liver inflammation.

Materials and Methods

ANIMALS

The floxed YAP ($YAP^{FL/FL}$) mice and the mice expressing the Cre recombinase under the control of the Lysozyme M promoter were used to generate myeloid-specific YAP knockout ($YAP^{M\text{-}KO}$) mice (Supporting Fig. S1). The $\beta\text{-catenin}^{M\text{-}KO}$ mice were generated as described.[13] 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 University of California at Los Angeles. See Supporting Information.

MOUSE LIVER IRI MODEL AND TREATMENT

We used a well-established mouse model of warm hepatic ischemia (90 minutes) followed by reperfusion (6 hours), as described.[12] Some animals were injected through the tail vein with bone marrow–derived MSCs (1×10^6 cells in phosphate-buffered saline/mouse) or prelabeled with 5-chloromethylfluorescein diacetate (CMFDA) green fluorescent dye (Invitrogen) 24 hours before 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 AND IMMUNOFLUORESCENCE STAINING

Liver sections were stained with hematoxylin and eosin. The severity of IRI was graded using Suzuki's histological criteria.[18] Liver macrophages and bone marrow–derived macrophages (BMMs) were detected using primary goat anti-mouse CD68, rabbit anti-mouse arginase1 (Arg1), and rabbit anti-mouse β-catenin or YAP monoclonal antibodies for immunofluorescence staining. See Supporting Information.

QUANTITATIVE REAL-TIME PCR ANALYSIS

Quantitative real-time PCR was performed as described.[13] Primer sequences used for the amplification are shown in Supporting Table S1. See Supporting Information.

IMMUNOBLOT ANALYSIS

Protein was extracted from liver tissue or cell cultures as described.[13] The nuclear and cytosolic fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents ThemoFisher Scientific (Waltham, MA). Rabbit anti-mouse β-catenin, p-β-catenin, p-MST1/2, MST1/2, p-LATS1, LATS1, p-YAP, YAP, Arg1, inducible nitric oxide synthase (iNOS), XBP1s, NLRP3, cleaved caspase-1, p-Akt, Akt, Lamin B, and β-actin Abs (Cell Signaling Technology) were used. See Supporting Information.

IMMUNOPRECIPITATION ANALYSIS

BMMs from coculture were lysed in Nonidet P40 lysis buffer. The lysates were incubated with β-catenin antibody or control immunoglobulin G and protein A/G beads at 4°C overnight. After immunoprecipitation (IP), the immunocomplexes were analyzed by standard immunoblot procedures. See Supporting Information.

ISOLATION OF HEPATIC KUPFFER CELLS, BMMs, AND BONE MARROW–DERIVED MSCs

Primary liver macrophages (Kupffer cells) and BMMs were isolated, as described.[13] Bone

marrow–derived MSCs were isolated, as described.[19] See Supporting Information.

IN VITRO TRANSFECTION

BMMs (1×10^6) were transfected with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) XBP1 knockout (KO), CRISPR YAP activation, CRISPR NLRP3 activation, or CRISPR control vector (Santa Cruz Biotechnology) by using Lipofectamine 3000 according to the manufacturer's instructions (Invitrogen). See Supporting Information.

COCULTURE OF MACROPHAGES AND MSCs

Macrophages (1×10^6) were cultured in a 6-well plate. After 24 hours, MSCs (2×10^5) in a Transwell insert were placed into the 6-well plate in which macrophages were initially seeded. Cocultures were incubated for 24 hours with or without adding lipopolysaccharide (LPS; 100 ng/mL). See Supporting Information.

ELISA ASSAY

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure PGE2, tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-10, and transforming growth factor β (TGF-β) levels in murine serum and coculture supernatants. See Supporting Information.

LUCIFERASE ASSAYS

BMMs were cotransfected with β-catenin-luciferase and CRISPR YAP activation vectors and the transcriptional activity was measured using a luciferase assay system according to the manufacturer's instructions (Promega, WI). See Supporting Information.

CASPASE-1 ENZYMATIC ACTIVITY ASSAY

Caspase-1 enzymatic activity was determined by a colorimetric assay kit (R&D System), as described.[20] See Supporting Information.

ChIP AND ChIP SEQUENCING

The chromatin immunoprecipitation (ChIP) analysis was carried out using ChIP Assay Kit (Abcam) according to the manufacturer's instructions. The ChIP-DNA was amplified to generate a library for sequencing with an Illumina HiSeq3000 (Illumina) at the Technology Center for Genomics & Bioinformatics at UCLA. 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 less than 0.05 were considered statistically significant. Multiple group comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. When groups showed unequal variances, we applied Welch's ANOVA to make multiple group comparisons. All analyses were performed using SAS/STAT software, version 9.4 (SAS Institute, Cary, NC).

Results

ADOPTIVE TRANSFER OF MSCs ATTENUATES IR-INDUCED LIVER INJURY AND INHIBITS PROINFLAMMATORY MEDIATORS

We used a mouse model of hepatic warm ischemia (90 minutes) followed by reperfusion to test the effect of MSCs *in vivo*. As MSC migration and recruitment are crucial to the success of MSC-mediated immune regulation, we determined whether exogenous MSCs may respond to signals of cellular damage to the sites of injury after liver IR. To track the distribution of MSCs in ischemic livers, MSCs were labeled with 5-CMFDA and adoptively transferred into wild-type (WT) mice. Indeed, increased number of MSCs (green) were recruited to the injured livers compared with the sham controls at 6 hours of reperfusion (Fig. 1A). The hepatocellular function as measured by sALT levels (IU/L) was significantly improved after adoptive transfer of MSCs in mice compared with untreated controls (Fig. 1B; 8,861 ± 2,507.9 vs. 18,366 ± 4,386.8; $P < 0.01$). These functional data

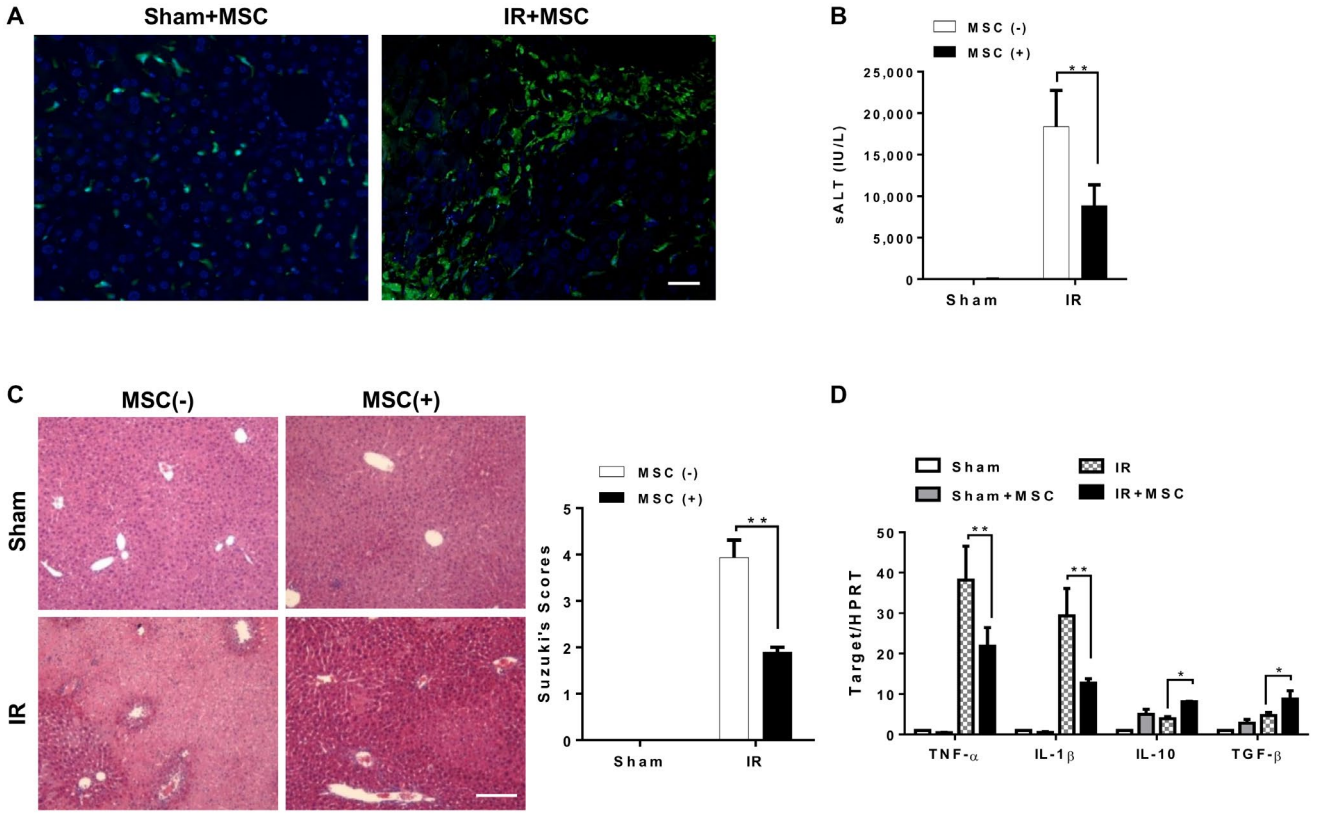


FIG. 1. Adoptive transfer of MSCs attenuates IR-induced liver injury and inhibits proinflammatory mediator program. Mice were subjected to 90 minutes of partial liver warm ischemia, followed by 6 hours of reperfusion. Some animals were injected through the tail vein with MSCs (1×10^6) 24 hours before ischemia insult. (A) MSCs were labeled with 5-CMFDA to track the distribution of MSCs in ischemic livers. Representative immunofluorescence staining for the MSCs labeled with 5-CMFDA (green) localized in IR-stressed livers after MSC treatment (n = 3-4 mice/group). 4′,6-diamidino-2-phenylindole was used to visualize nuclei (blue). Scale bars, 20 μm. (B) Hepatocellular function was evaluated by sALT levels (IU/L; n = 4-6 samples/group). (C) Representative histological staining (hematoxylin and eosin) of ischemic liver tissue (n = 4-6 mice/group) and Suzuki's histological score. Scale bars, 100 μm. (D) Real-time quantitative PCR–assisted detection of TNF-α, IL-1β, IL-10, and TGF-β in ischemic livers (n = 3-4 samples/group). Data were normalized to HPRT gene expression. All data represent the mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$. Abbreviation: HPRT, hypoxanthine-guanine phosphoribosyltransferase.

are correlated with Suzuki's histological grading of IR-mediated liver damage (Fig. 1C). Unlike in controls, which showed moderate or severe sinusoidal congestion, cytoplasmic vacuolization, and hepatocellular necrosis (Suzuki score = 3.93 ± 0.38), livers from MSC-treated mice exhibited well-preserved hepatic architecture, with minimal sinusoidal congestion and without edema, vacuolization, or necrosis (Suzuki score = 1.90 ± 0.10; $P < 0.01$). Consistent with the histopathological and hepatocellular function data, MSC treatment in mice significantly reduced the expression of proinflammatory TNF-α and IL-1β but augmented IL-10 and TGF-β compared with untreated controls (Fig. 1D).

MSCs REGULATE HIPPO SIGNALING AND β-CATENIN ACTIVATION AND CONTROL MACROPHAGE POLARIZATION IN IR-STRESSED LIVERS

We then analyzed whether MSCs may influence the Hippo signaling and β-catenin activation in IR-induced liver injury. By 6 hours of reperfusion after 90 minutes of ischemia, IR stress increased

phosphorylation of MST1/2 (p-MST1/2) and LATS1 (p-LATS1) in ischemic livers. However, MSC treatment reduced p-MST1/2, p-LATS1, and p-YAP but augmented nuclear YAP expression after liver IRI (Fig. 2A). Unlike in untreated controls, increased p-Akt, p-β-catenin at Ser552, and β-catenin were observed in MSC-treated livers (Fig. 2A). The serum PGE2 production was significantly increased after MSC treatment (Fig. 2B). Interestingly, using double immunofluorescence staining, we found that administration of MSCs increased M2 macrophage Arg1 expression (Fig. 2C), which resulted in reduced serum TNF-α and IL-1β but increased IL-10 and TGF-β production compared with the untreated controls (Fig. 2D). To determine whether MSCs specifically influence the Hippo-YAP pathway and β-catenin activation in liver macrophages, we isolated hepatic Kupffer cells from ischemic livers in WT mice. Indeed, MSC treatment augmented nuclear YAP and β-catenin expression (Fig. 2E), which was accompanied by increased Arg1 and reduced iNOS expression (Fig. 2F) in hepatic Kupffer cells.

MYELOID YAP DEFICIENCY IN MSC-TREATED LIVERS AGGRAVATES IR-INDUCED HEPATOCELLULAR DAMAGE AND PROMOTES NLRP3 INFLAMMASOME-DRIVEN INFLAMMATORY RESPONSE

As increased nuclear YAP expression was found in hepatic Kupffer cells after MSC treatment, we next determined whether YAP may play a role in the regulation of NLRP3-driven inflammatory response in IR-stressed livers after MSC intervention. The $YAP^{M\text{-}KO}$ and $YAP^{FL/FL}$ mice were treated with MSCs and subjected to liver IR. We isolated both hepatocytes and liver macrophages (Kupffer cells) from these ischemic livers. Indeed, $YAP^{M\text{-}KO}$ did not change hepatocyte YAP expression. However, the YAP expression was lacking in liver macrophages from the $YAP^{M\text{-}KO}$ mice but not from the $YAP^{FL/FL}$ mice (Fig. 3A). Unlike livers in the $YAP^{FL/FL}$ mice, which showed mild to moderate edema without necrosis (Fig. 3B; score = 1.30 ± 0.28) after MSC treatment, livers in the $YAP^{M\text{-}KO}$ mice revealed significant edema, severe sinusoidal congestion/cytoplasmic vacuolization, and extensive (30%-50%) necrosis (Fig. 3B; score = 2.43 ± 0.31; $P < 0.05$). Consistent with these data, the sALT levels were significantly increased in the $YAP^{M\text{-}KO}$ mice even with concomitant MSC treatment, compared with the MSC-treated $YAP^{FL/FL}$ mice (Fig. 3C; 7,367 ± 929.8 vs. 3,367 ± 564.2; $P < 0.05$). Moreover, increased proinflammatory TNF-α and IL-1β and decreased anti-inflammatory IL-10 and TGF-β expression profile was observed in the $YAP^{M\text{-}KO}$ but not the $YAP^{FL/FL}$ livers after MSC treatment (Fig. 3D). Strikingly, $YAP^{M\text{-}KO}$ augmented NLRP3 and cleaved caspase-1 protein expression (Fig. 3E) with increased serum IL-1β release (Fig. 3F), compared with the MSC-treated $YAP^{FL/FL}$ mice.

DISRUPTION OF MYELOID β-CATENIN IN MSC-TREATED LIVERS ACTIVATES NLRP3 AND DIMINISHES M2 MACROPHAGE POLARIZATION IN IR-STRESSED LIVERS

As MSCs promoted Akt and β-catenin phosphorylation at Ser552, which resulted in translocation of β-catenin into nucleus,[21] we then determined whether β-catenin may modulate NLRP3 function and macrophage differentiation in MSC-mediated immune regulation. The $\beta\text{-catenin}^{M\text{-}KO}$ and $\beta\text{-catenin}^{FL/FL}$ mice were treated with MSCs and subjected to liver IR. We found that the $\beta\text{-catenin}^{M\text{-}KO}$ aggravated IR-induced liver damage after MSC treatment, as evidenced by the increased Suzuki's histological score (Fig. 4A; score = 2.87 ± 0.25 vs. 1.80 ± 0.20; $P < 0.05$) and sALT levels (Fig. 4B; 7,422 ± 1,422.3 vs. 4,472 ± 1,132.3; $P < 0.05$) compared with the MSC-treated $\beta\text{-catenin}^{FL/FL}$ controls. Consistent with the histopathological and hepatocellular function data, the mRNA levels coding for TNF-α and IL-1β were significantly increased in the $\beta\text{-catenin}^{M\text{-}KO}$ whereas IL-10 and TGF-β levels were reduced in the $\beta\text{-catenin}^{M\text{-}KO}$ livers compared with the $\beta\text{-catenin}^{FL/FL}$ controls (Fig. 4C). Moreover, immunoblot analysis revealed that increased NLRP3 and cleaved caspase-1 protein levels (Fig. 4D) and IL-1β release (Fig. 4E) were found in the β-

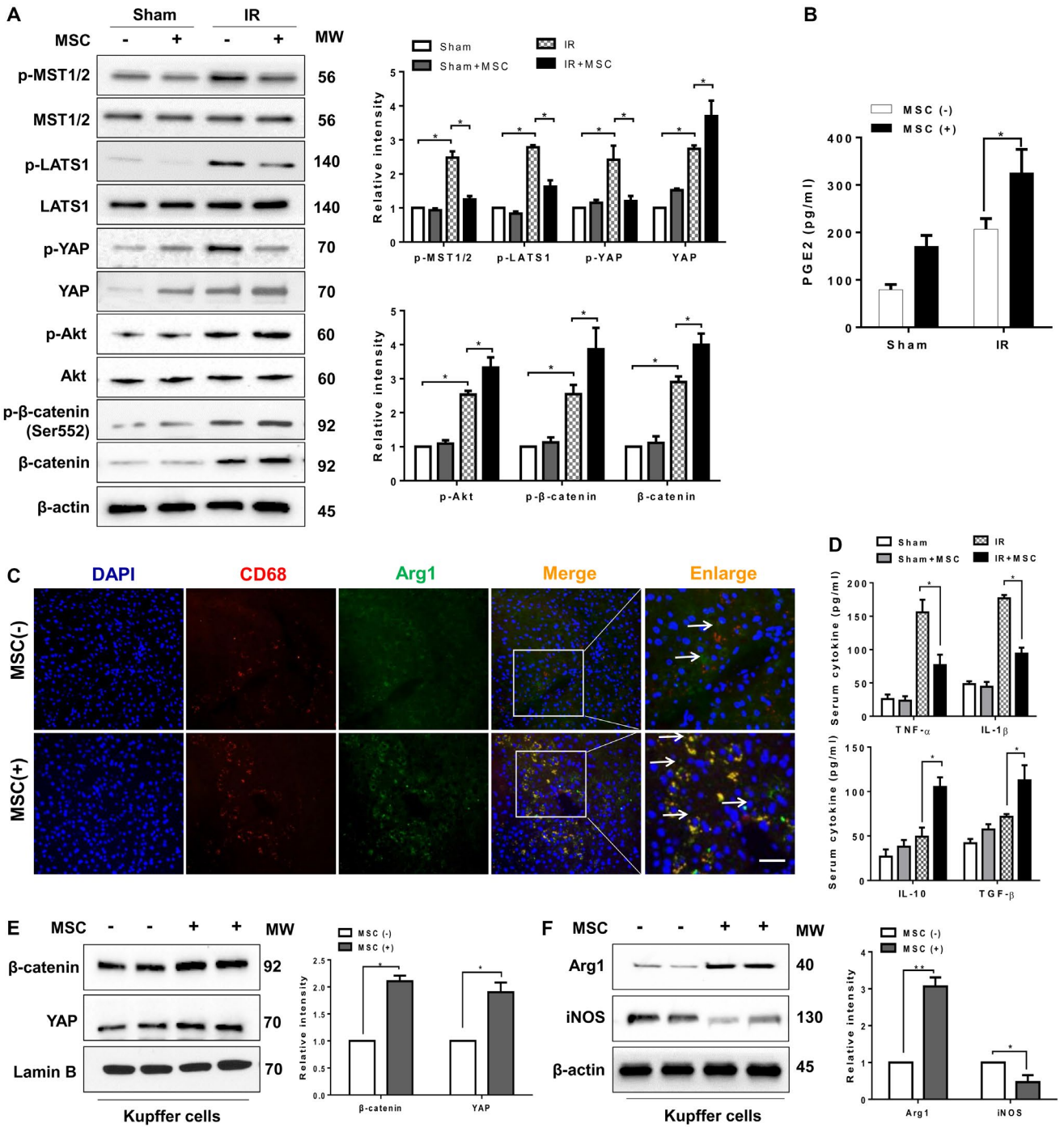


FIG. 2. MSCs regulate Hippo signaling/β-catenin activation and control macrophage polarization in IR-stressed livers. (A) Immunoblot-assisted analysis and relative density ratio of p-MST1/2, MST1/2, p-LATS1, LATS1, p-YAP, YAP, p-Akt, Akt, p-β-catenin, and β-catenin in IR-stressed livers with or without MSC treatment. Representative of three experiments. (B) ELISA analysis of PGE2 levels in animal serum (n = 3-4 samples/group). (C) Representative immunofluorescence staining for the macrophage marker CD68 (red) and Arg1 (green) colocalization in IR-stressed livers (n = 3-4 mice/group). DAPI was used to visualize nuclei (blue). Arrow indicated CD68 and Arg1 double positive macrophages (yellow). Scale bars, 20 μm. (D) ELISA analysis of TNF-α, IL-1β, IL-10, and TGF-β levels in animal serum (n = 3-4 samples/group). Immunoblot-assisted analysis and relative density ratio of nuclear (E) YAP and β-catenin and (F) Arg1 and iNOS in liver Kupffer cells. Representative of three experiments. All data represent the mean ± SD. **P* < 0.05, ***P* < 0.01. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; MW, molecular weight.

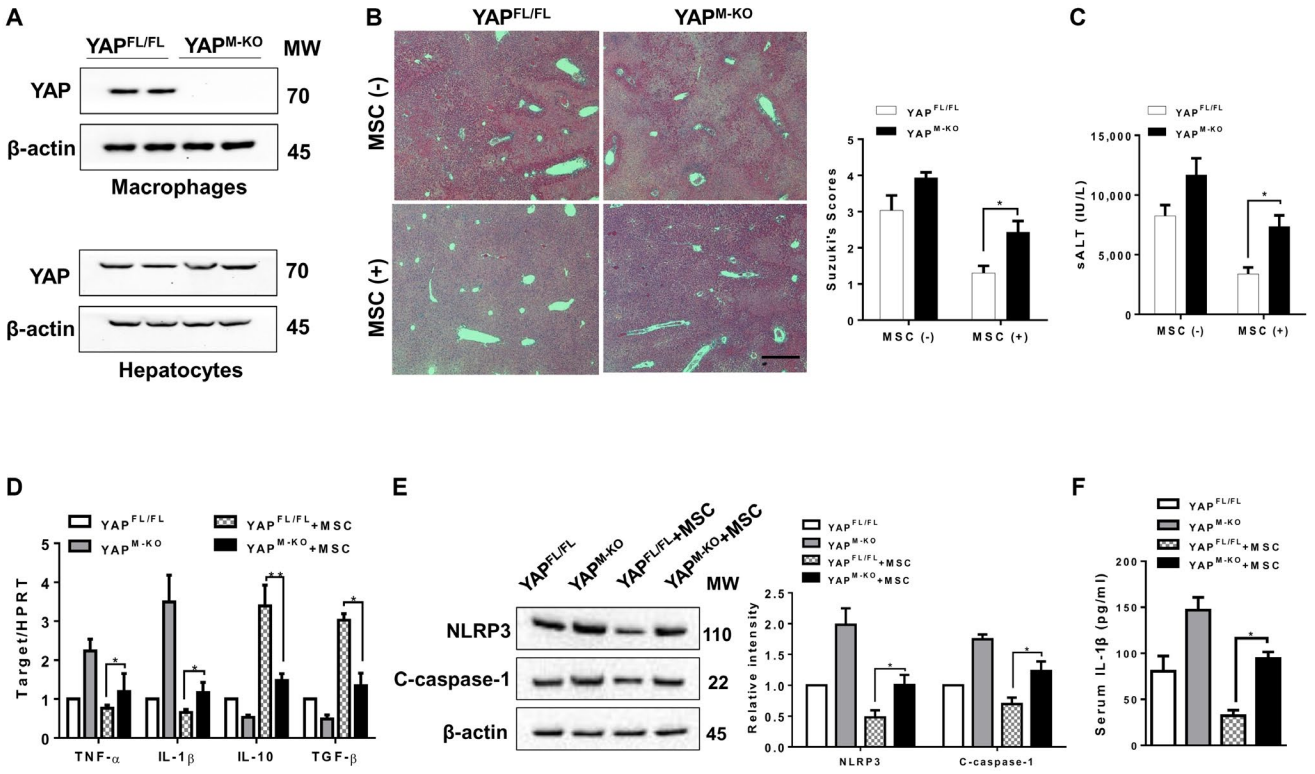
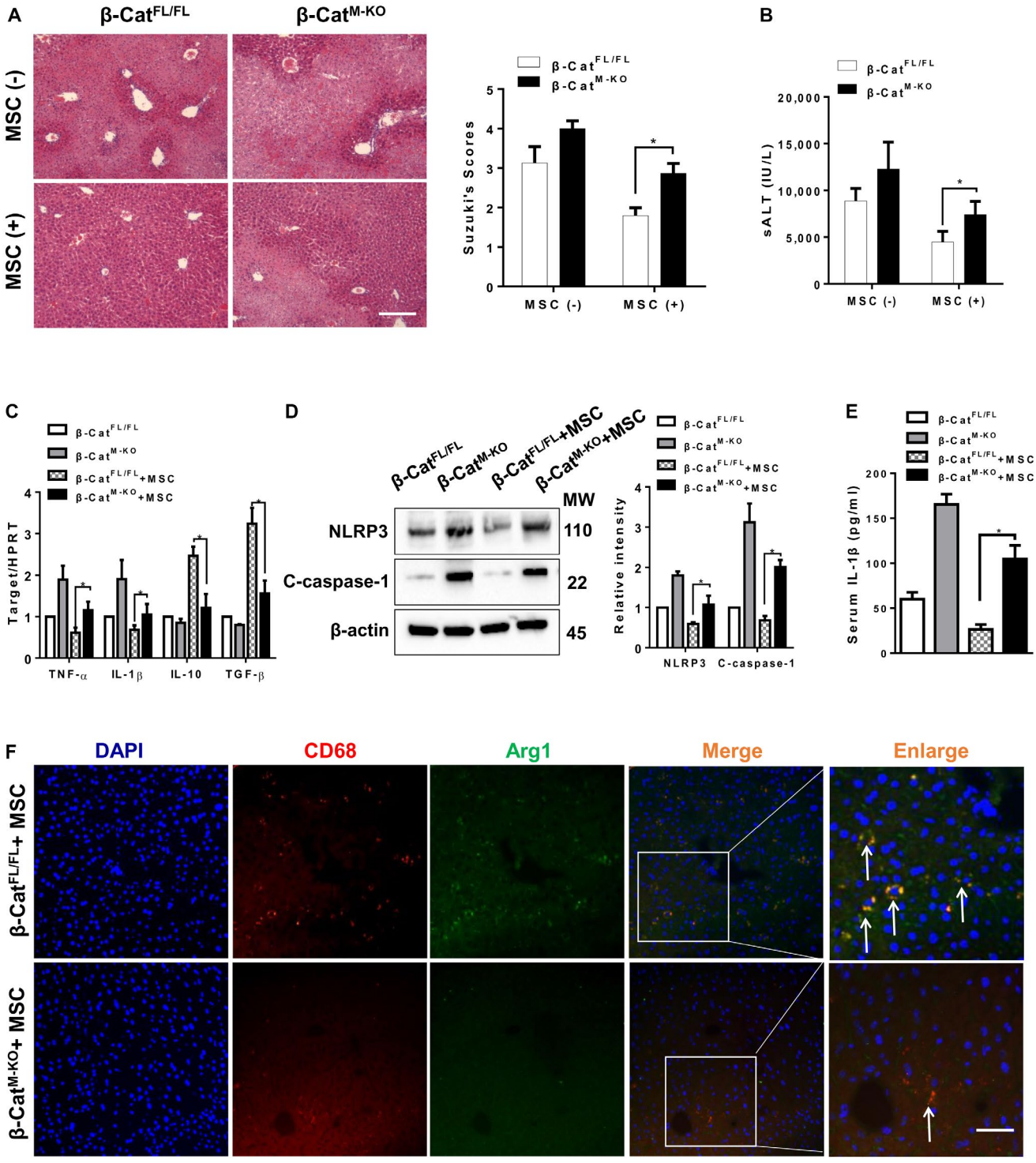


FIG. 3. Myeloid YAP deficiency in MSC-treated livers aggravates IR-induced hepatocellular damage and promotes NLRP3 inflammasome-driven inflammatory response. The $YAP^{FL/FL}$ and $YAP^{M\text{-}KO}$ mice were subjected to 90 minutes of partial liver warm ischemia, followed by 6 hours of reperfusion. Some animals were injected through the tail vein with MSCs (1×10^6) 24 hours before ischemia. (A) The YAP expression was detected in hepatocytes and liver macrophages (Kupffer cells) by western blot assay. Representative of three experiments. (B) Representative histological staining (hematoxylin and eosin) of ischemic liver tissue (n = 4-6 mice/group) and Suzuki's histological score. Scale bars, 100 μm. (C) Hepatocellular function was evaluated by sALT levels (IU/L; n = 4-6 samples/group). (D) Real-time quantitative PCR–assisted detection of TNF-α, IL-1β, IL-10, and TGF-β in ischemic livers (n = 3-4 samples/group). Data were normalized to HPRT gene expression. (E) Immunoblot-assisted analysis and relative density ratio of NLRP3 and cleaved caspase-1 in ischemic livers. Representative of three experiments. (F) ELISA analysis of IL-1β levels in animal serum (n=3-4 samples/group). All data represent the mean ± SD. *p<0.05, **p<0.01. Abbreviation: HPRT, hypoxanthine-guanine phosphoribosyltransferase.

catenin$^{M\text{-}KO}$ but not in the β-catenin$^{FL/FL}$ livers after MSC treatment. The β-catenin$^{M\text{-}KO}$ diminished macrophage Arg1 expression in MSC-treated livers compared with the β-catenin$^{FL/FL}$ controls (Fig. 4F).

YAP INTERACTS WITH β-Catenin AND REGULATES ITS TRANSCRIPTION ACTIVITY IN MSC-MEDIATED IMMUNE REGULATION

Having demonstrated the importance of both the Hippo-YAP pathway and β-catenin in the modulation of NLRP3 function *in vivo* after MSC intervention, we next tested whether there is crosstalk between the Hippo-YAP pathway and β-catenin in MSC-mediated immune regulation. Using a Transwell system, BMMs were cocultured with MSCs followed by LPS stimulation. Immunofluorescent staining revealed increased nuclear YAP (Fig. 5A) and β-catenin (Fig. 5B) expression in macrophages after coculture with MSCs. This was further confirmed by western blots, which showed increased macrophage nuclear YAP and β-catenin protein expression after coculture (Fig. 5C). Interestingly, both YAP and β-catenin were colocalized in the nucleus (Fig. 5D). Moreover, coimmunoprecipitation



assays revealed that YAP can bind to endogenous β-catenin in macrophages after coculture (Fig. 5E). YAP stimulated β-catenin transcriptional activity in a dose-dependent manner, as evidenced by consistently increased β-catenin-luciferase reporter gene (Fig. 5F).

FIG. 4. Disruption of myeloid β-catenin in MSC-treated livers activates NLRP3 and diminishes M2 macrophage polarization in IR-stressed livers. The β-catenin$^{FL/FL}$ and β-catenin^{M-KO} mice were subjected to 90 minutes of partial liver warm ischemia, followed by 6 hours of reperfusion. Some animals were injected through the tail vein with MSCs (1×10^6) 24 hours before ischemia. (A) Representative histological staining (hematoxylin and eosin) of ischemic liver tissue (n = 4-6 mice/group) and Suzuki's histological score. Scale bars, 100 μm. (B) Hepatocellular function was evaluated by sALT levels (IU/L; n = 4-6 samples/group). (C) Real-time quantitative PCR–assisted detection of TNF-α, IL-1β, IL-10, and TGF-β in ischemic livers (n = 3-4 samples/group). Data were normalized to HPRT gene expression. (D) Immunoblot-assisted analysis and relative density ratio of NLRP3 and cleaved caspase-1 in ischemic livers. Representative of three experiments. (E) ELISA analysis of IL-1β levels in animal serum (n = 3-4 samples/group). (F) Representative immunofluorescence staining for the macrophage marker CD68 (red) and Arg1 (green) colocalization in IR-stressed livers (n = 3-4 mice/group). DAPI was used to visualize nuclei (blue). Arrow indicated CD68 and Arg1 double positive macrophages (yellow). Scale bars, 20 μm. All data represent the mean ± SD. *$P < 0.05$. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

THE YAP-β-CATENIN SIGNALING TARGETS XBP1 AND INHIBITS NLRP3-DRIVEN INFLAMMATORY RESPONSE IN MSC-MEDIATED IMMUNE REGULATION

To explore the potential mechanism of the YAP-β-catenin signaling in the modulation of NLRP3 activation in MSC-mediated immune regulation, we performed β-catenin ChIP coupled to massively parallel sequencing (ChIP-seq; Fig. 6A). Clearly, β-catenin ChIP-seq peaks were identified within the Xbp1 gene. One was located in the promoter region, and others were located within the intron or exon (Fig. 6B). To validate the ChIP-seq peak located in the Xbp1 promoter region, ChIP-PCR was performed using β-catenin and YAP antibodies in MSC-treated BMMs. After ChIP with YAP or β-catenin antibody, primer was designed to detect the β-catenin/T cell factor (TCF) DNA-binding site in Xbp1 promoter by PCR analysis. To confirm that YAP is colocalized with β-catenin on the promoter of Xbp1, sequential ChIPs were performed. The first ChIP was performed with β-catenin antibody, and the second ChIP was carried out with either YAP or β-catenin antibody using the chromatin eluted from the first ChIP. Following the second ChIP, both YAP and β-catenin were still bound to the β-catenin/TCF-binding motif in the β-catenin-chromatin complex (Fig. 6C), confirming that YAP and β-catenin are present at the same promoter region of Xbp1. Hence, Xbp1 is a target gene regulated by the YAP-β-catenin complex. Moreover, MSCs reduced macrophage p-MST1/2, p-LATS1, and p-YAP but augmented nuclear YAP levels (Fig. 6D). However, increased macrophage Akt and β-catenin phosphorylation and nuclear β-catenin expression (Fig. 6E) accompanied by diminished XBP1s, NLRP3, and cleaved caspase-1 expression (Fig. 6F) were observed after coculture with MSCs. This result was confirmed by caspase-1 activity assay, which showed that MSCs significantly reduced macrophage caspase-1 activity in cocultures (Fig. 6G).

YAP-β-CATENIN SIGNALING IS ESSENTIAL FOR THE MODULATION OF XBP1 AND REPROGRAMS NLRP3-DEPENDENT MACROPHAGE POLARIZATION IN MSC-MEDIATED IMMUNE REGULATION

To elucidate the mechanistic role of the Hippo-YAP pathway and β-catenin signaling in the control of XBP1/NLRP3 activation in MSC-mediated immune regulation, BMMs were isolated from the β-catenin$^{FL/FL}$, β-catenin^{M-KO}, YAP$^{FL/FL}$, and YAP^{M-KO} mice. β-catenin or YAP deficiency augmented XBP1s accompanied by increased NLRP3 and cleaved caspase-1 expression in macrophages after coculture with MSCs (Fig. 7A,B). Overexpression of YAP by transfecting CRISPR-mediated YAP activation diminished XBP1s expression in β-catenin-proficient macrophages (Fig. 7C). However, the expression of XBP1s was not significantly changed in β-catenin-deficient macrophages after transfection of CRISPR-mediated YAP activation (Fig. 7C), suggesting that the YAP-β-catenin interaction is crucial for the regulation of XBP1 activation. Indeed, macrophage XBP1 is activated by inositol-requiring enzyme 1 alpha (IRE1α), a stress sensor, which signals innate immunity during inflammatory response.[22] Moreover, activation macrophage IRE1α

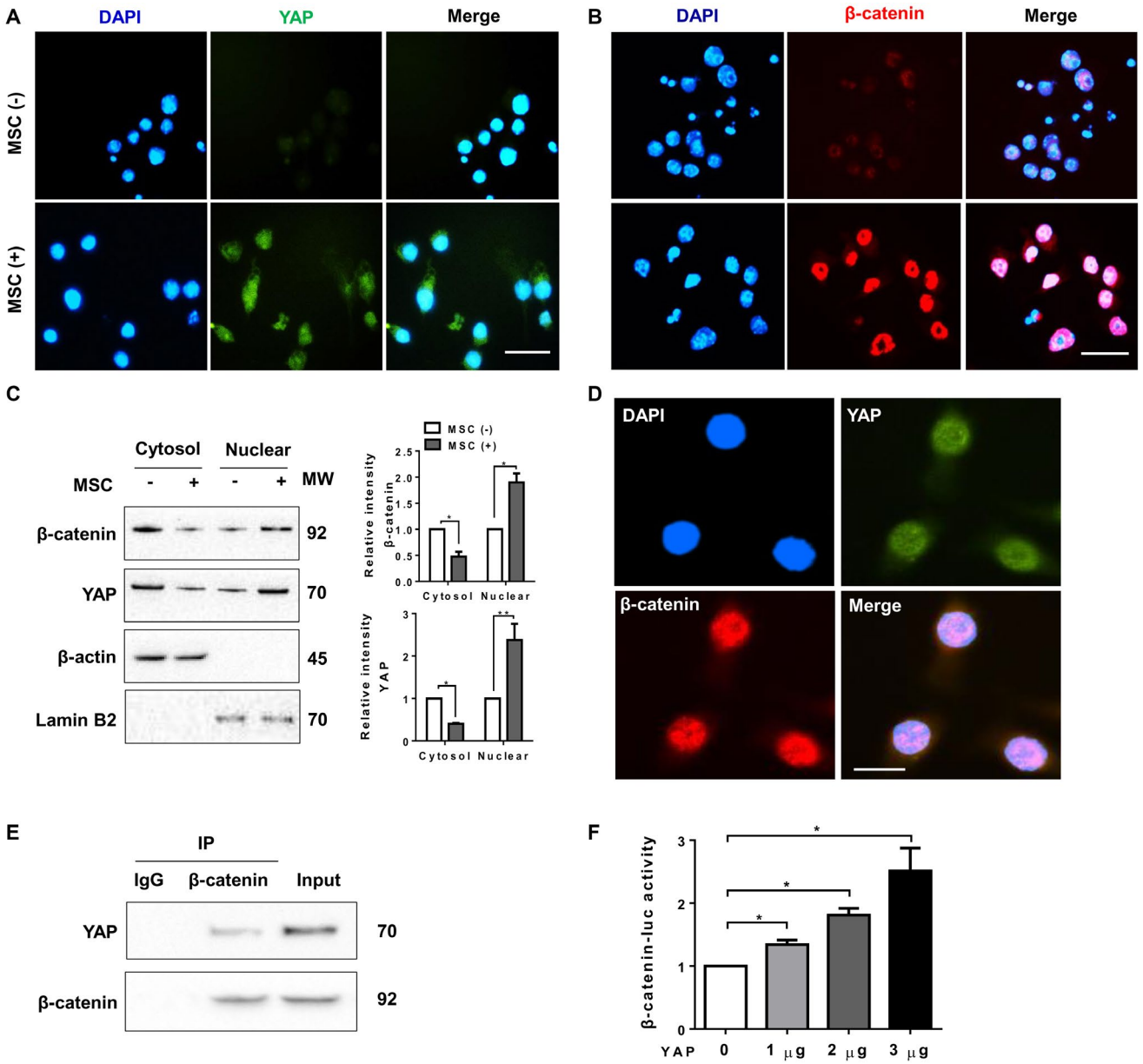
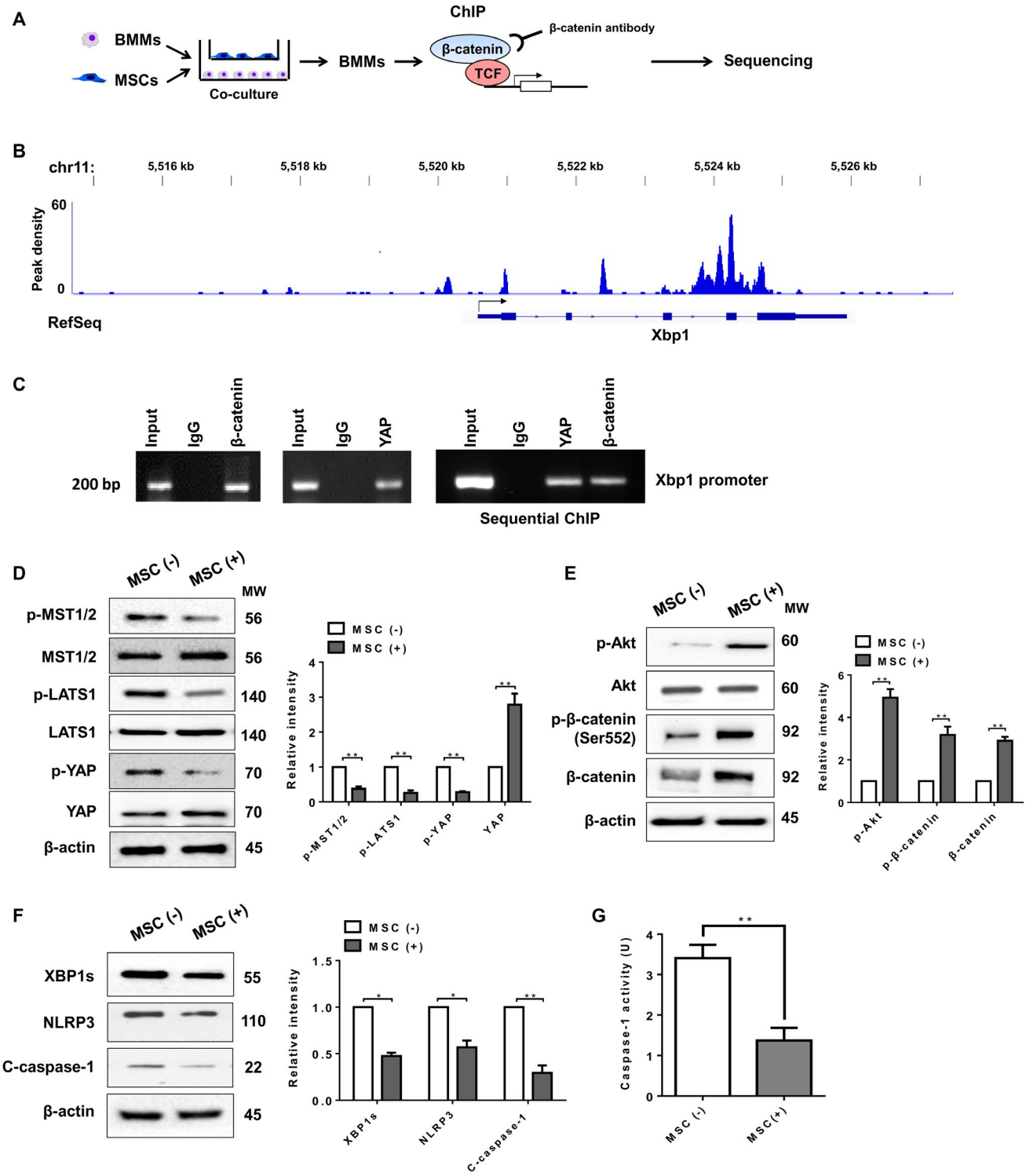


FIG. 5. YAP interacts with β-catenin and regulates its transcription activity in MSC-mediated immune regulation. BMMs (1×10^6) were cocultured with MSCs (2×10^5) for 24 hours followed by LPS (100 ng/mL) stimulation. (A,B) Immunofluorescence staining of nuclear YAP (green) and β-catenin (red) in macrophages after coculture with or without MSCs. DAPI was used to visualize nuclei (blue). Scale bars, 20 μm. (C) Immunoblot-assisted analysis of cytosol and nuclear YAP and β-catenin in macrophages after coculture with or without MSCs. Representative of three experiments. (D) Immunofluorescence staining for macrophage YAP (green) and β-catenin (red) colocalization in the nucleus after coculture with MSCs. DAPI was used to visualize nuclei (blue). Scale bars, 10 μm. (E) Immunoprecipitation analysis of YAP and β-catenin in macrophages after coculture with MSCs. Representative of three experiments. (F) BMMs were cotransfected with 1 μg β-catenin-luc and CRISPR YAP activation vectors. The luciferase activity was measured after 48 hours (n = 3-4 samples/group). Data represent the mean ± SD. **P* < 0.05. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; IgG, immunoglobulin G.

signaling is required for NLRP3 activation.[23] These reports imply that XBP1 may play a role in the regulation of NLRP3 function. To dissect the role of the β-catenin/XBP1 axis on NLRP3 activation in macrophages, BMMs from β-catenin$^{M\text{-}KO}$ mice were transfected with a CRISPR/Cas9 XBP1 knockout



vector and then cocultured with MSCs. Strikingly, XBP1 deficiency resulted in decreased NLRP3 and cleaved caspase-1 expression (Fig. 7D). To determine whether NLRP3 is associated with macrophage polarization after coculture with MSCs, we transfected BMMs with CRISPR-mediated NLRP3 activation vector or control vector followed by LPS stimulation. Clearly, compared with the control vector,

FIG. 6. The YAP-β-catenin signaling targets XBP1 and inhibits NLRP3-driven inflammatory response in MSC-mediated immune regulation. (A) Experimental design of β-catenin ChIP-seq analysis. BMMs were collected and fixed after coculture with MSCs. Following chromatin shearing and β-catenin antibody selection, the precipitated DNA fragments bound by β-catenin-containing protein complexes were used for sequencing. (B) Localization of β-catenin-binding sites on the mouse *xbp1* gene. The five exons, four introns, 3′ untranslated region (UTR), 5′ UTR, and transcription start sites (TSS) of the mouse *xbp1* gene on chromosome 11 are shown. (C) ChIP-PCR analysis of YAP and β-catenin binding to the Xbp1 promoter. Protein-bound chromatin was prepared from BMMs and immunoprecipitated with YAP or β-catenin antibodies. For sequential ChIP, the protein-bound chromatin was first immunoprecipitated with the β-catenin antibody, followed by elution with a second immunoprecipitation using YAP antibody, and then the immunoprecipitated DNA was analyzed by PCR. The normal IgG was used as a negative control. (D-F) Immunoblot-assisted analysis and relative density ratio of p-MST1/2, MST1/2, p-LATS1, LATS1, p-YAP, YAP, p-Akt, Akt, p-β-catenin, β-catenin, XBP1s, NLRP3, and cleaved caspase-1 in macrophages after coculture with or without MSCs. Representative of three experiments. (G) Caspase-1 activity (U) in macrophages after coculture (n = 3-4 samples/group). All data represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviation: IgG, immunoglobulin G.

transfection of CRISPR-mediated NLRP3 activation vector in BMMs markedly increased NLRP3, cleaved caspase-1, and iNOS but depressed Arg1 expression (Fig. 7E) accompanied by augmented IL-1β release (Fig. 7F) after coculture with MSCs. Furthermore, reduced Arg1-positive macrophages were observed in NLRP3-transfected groups compared with the control vector-transfected cells (Fig. 7G). Consistent with these results, transfection of CRISPR-mediated NLRP3 activation vector in BMMs increased TNF-α and IL-1β but reduced IL-10 and TGF-β mRNA levels compared with the control vector-transfected cells (Fig. 7H).

Discussion

This study documents the key role of Hippo signaling in controlling NLRP3-driven innate immune responses and mediating immunoregulatory properties of MSCs in a liver sterile inflammatory injury. We demonstrate that (1) adoptive transfer of MSCs alleviates IR-induced liver inflammation by modulating crosstalk between macrophage Hippo signaling downstream effector YAP and β-catenin activation; (2) YAP is colocalized and interacts with β-catenin, which in turn regulates their target gene XBP1, leading to reduced NLRP3/caspase-1 activity; and (3) YAP is crucial to mediate β-catenin transcriptional activity and reprograms NLRP3-dependent macrophage polarization in MSC-mediated immune regulation. Our results highlight the importance of macrophage YAP-β-catenin complex as a key regulator of the NLRP3 function in MSC-mediated immune regulation during liver inflammatory injury.

Although various studies have demonstrated that MSCs exert immunosuppressive effects and MSC-based therapy has shown therapeutic potential in various human diseases,[24] the greatest challenge is to overcome the low immunosuppressive efficacy of engrafted cells. Understanding immunoregulatory mechanisms of MSCs is crucial to improve immunosuppressive properties of MSCs for the next-generation stem cell therapeutics. In our current study, we revealed that adoptive transfer of MSCs reduced IR-induced hepatocellular damage and ameliorated hepatic function. Interestingly, MSC treatment promoted the Hippo pathway downstream effector YAP and activated β-catenin signaling, accompanied by augmented M2 macrophage phenotype and anti-inflammatory program in IR-stressed livers. These results imply that the Hippo pathway and β-catenin signaling play pivotal roles and that modulation of their signaling pathways may provide a possible targeting strategy in MSC-mediated immune regulation in IR-stressed liver.

As β-catenin has been indicated to regulate immune response in inflammatory diseases,[25] MSC-mediated β-catenin signaling could act as a native regulator for NLRP3 activation in macrophages. We found that myeloid β-catenin deficiency exacerbated IR-induced liver damage even with concomitant MSC treatment. Disruption of β-catenin activated NLRP3 and caspase-1, a key mediator in processing proinflammatory cytokine IL-1β from an inactive precursor to an active, secreted molecule to trigger innate immune response.[26] Notably, myeloid β-catenin-deficiency reduced M2 while increasing M1 macrophage phenotype accompanied by decreased IL-10/TGF-β and increased TNF-α/IL-1β expression in MSC-treated IR-livers, suggesting the importance of myeloid β-catenin in the modulation of macrophage polarization. Indeed, the β-catenin signaling has shown multiple regulatory functions during inflammatory

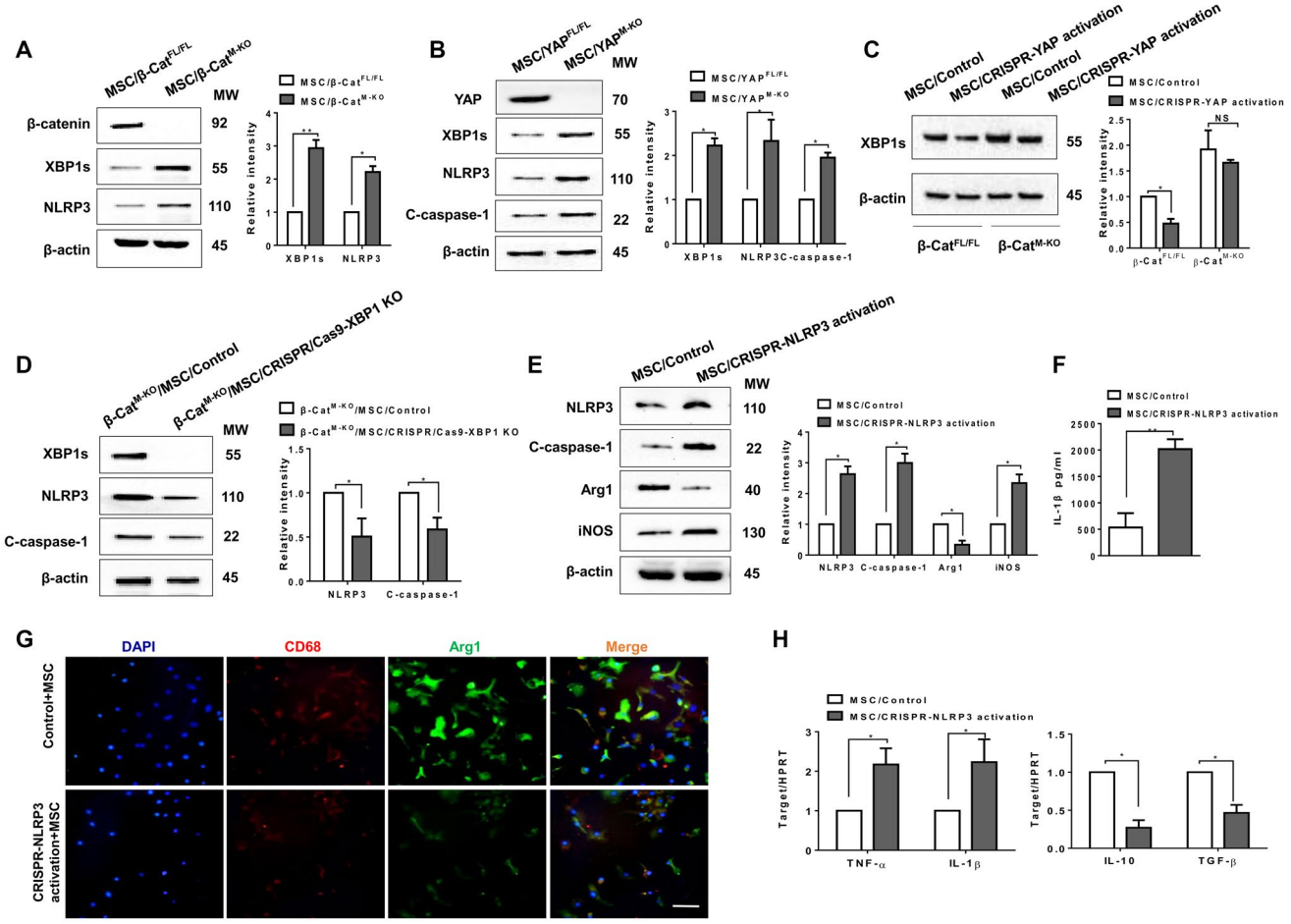


FIG. 7. YAP is crucial to mediate β-catenin activity and reprograms NLRP3-dependent macrophage polarization in MSC-mediated immune regulation. (A,B) BMMs were isolated from β-catenin$^{FL/FL}$, β-catenin$^{M\text{-}KO}$, YAP$^{FL/FL}$, YAP$^{M\text{-}KO}$ mice and then cocultured with MSCs followed by LPS stimulation (n = 3-4 samples/group). Immunoblot-assisted analysis and relative density ratio of macrophage β-catenin, YAP, XBP1s, and NLRP3, and cleaved caspase-1. Representative of three experiments. (C) BMMs were isolated from β-catenin$^{FL/FL}$ and β-catenin$^{M\text{-}KO}$ mice and transfected with CRISPR-mediated YAP activation or control vector and then cocultured with MSCs followed by LPS stimulation. Immunoblot-assisted analysis and relative density ratio of macrophage XBP1s. Representative of three experiments. (D) BMMs were isolated from β-catenin$^{M\text{-}KO}$ mice and transfected with CRISPR/Cas9-mediated XBP1 KO or control vector and then cocultured with MSCs followed by LPS stimulation. Immunoblot-assisted analysis and relative density ratio of macrophage XBP1s, NLRP3, and cleaved caspase-1. Representative of three experiments. (E-H) BMMs were isolated from β-catenin$^{FL/FL}$ mice and transfected with CRISPR-mediated NLRP3 activation or control vector and then cocultured with MSCs followed by LPS stimulation. (E) Immunoblot-assisted analysis and relative density ratio of macrophage NLRP3, cleaved caspase-1, Arg1, and iNOS. Representative of three experiments. (F) ELISA analysis of IL-1β levels in animal serum (n = 3-4 samples/group). (G) Representative immunofluorescence staining for the macrophage marker CD68 (red) and Arg1 (green) colocalization in BMMs. DAPI was used to visualize nuclei (blue). Scale bars, 20 μm. (H) Real-time quantitative PCR–assisted detection of TNF-α, IL-1β, IL-10, and TGF-β in macrophages (n = 3-4 samples/group). Data were normalized to HPRT gene expression. All data represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

response. Activation of β-catenin enhances MSC migration ability to the injury sites for the tissue repair.[11,27] Consistent with our previous reports that β-catenin regulated liver inflammatory response by controlling toll-like receptor 4 signaling to program innate and adaptive immunity,[12] our present results imply that myeloid β-catenin is essential for the modulation of NLRP3 function in MSC-mediated immune regulation.

One striking finding was that MSCs regulated macrophage Hippo pathway, especially increased YAP nuclear translocation to control liver inflammation

in IR-stressed livers. Indeed, YAP has been shown to be a major regulator of tissue growth and development.[28] Transgene-mediated YAP induction increases cell proliferation[29] while activation of YAP reprograms cell activity and facilitates efficient tissue repair during inflammatory injury.[7] Although the emerging roles of Hippo signaling in inflammation are based on different cell context and microenvironment,[30] YAP displays a distinct ability to regenerate and repair following partial liver damage.[31] We found that IR stress promoted Hippo core kinase signaling cascade (MST1/2-LATS1) and phosphorylated YAP to accomplish its cytoplasmic sequestration, which in turn triggered liver inflammation, evidenced by increased proinflammatory cytokine profile. However, MSC administration restricted the kinases MST1/2 and LATS1 phosphorylation and increased YAP nuclear translocation, leading to augmented M2 macrophage phenotype and anti-inflammatory mediators. Moreover, although the decreased inflammation was observed in MSC-treated $YAP^{FL/FL}$ mice, YAP deficiency resulted in enhanced NLRP3/caspase-1 activity and exacerbated liver inflammatory injury in $YAP^{M\text{-}KO}$ mice after MSC treatment. Our findings document the ability of the Hippo-YAP pathway to control NLRP3-driven inflammation in IR-stressed liver.

As both YAP and β-catenin signaling play important roles in the regulation of NLRP3 function in liver IRI after MSC treatment, the question arises as to what other mechanisms may confer YAP and β-catenin with their ability to selectively affect NLRP3 activation in MSC-mediated immune regulation. We found that IR stress increased PGE2 section from MSCs and activated macrophage Akt, which phosphorylated β-catenin at Ser552 and resulted in translocation of β-catenin into nucleus after MSC treatment. This is consistent with a previous report that β-catenin activity is dependent on Akt phosphorylation.[21] However, IR stress induced phosphorylation of MST1/2 and LATS1 while MSC treatment reduced MST1/2 and LATS1 phosphorylation, leading to increased YAP nuclear translocation in ischemic livers, suggesting that MSC-mediated immune regulation may be involved in both YAP and β-catenin activity. Although β-catenin activity is regulated through multiple mechanisms, we have shown that activation of β-catenin modulates innate immune response by nuclear translocation of β-catenin in macrophages and alleviates IR-induced liver injury.[12] Thus, we speculate that nuclear localization of endogenous YAP and β-catenin is essential for transcriptional activity in MSC-mediated immune regulation during liver IRI. This was confirmed by our *in vitro* MSC/macrophage coculture system. Indeed, we found that macrophage YAP and β-catenin colocalized in the nucleus and that YAP mediated β-catenin transcriptional activity. The ChIP and ChIP-seq data further revealed that YAP was colocalized with β-catenin on the promoter of Xbp1, suggesting that Xbp1 is a target gene regulated by the YAP and β-catenin complex while YAP acts as a transcriptional coactivator of β-catenin. Moreover, β-catenin deficiency promoted XBP1 and NLRP3/caspase-1 activation in macrophages after coculture with MSCs whereas XBP1 deletion augmented NLRP3/caspase-1 activity. Consistent with our previous findings that activation of XBP1 enhanced NLRP3-driven inflammatory response in liver IRI,[13] XBP1 is essential for the activation of NLRP3 in response to inflammatory stimuli. Taken together, these results reveal a crosstalk between macrophage YAP and β-catenin signaling in the modulation of NLRP3 function. YAP interacts with β-catenin, which in turn regulates its target gene XBP1, leading to reduced NLRP3/caspase-1 activity. Hence, our current findings demonstrated a fundamental role of Hippo signaling in regulating NLRP3-mediated innate immune response in MSC-mediated immune regulation.

Another important implication of our results is that the YAP-β-catenin interaction is key to shifting macrophage polarization toward M2 macrophage phenotype in MSC-mediated immune regulation. We have shown that myeloid YAP or β-catenin deficiency reduced M2 macrophage Arg1 expression while increasing proinflammatory cytokine genes in MSC-treated livers in response to IR stress. Moreover, macrophage depletion by clodronate liposome treatment in ischemic livers dampened MSC-mediated immune regulation (Supporting Fig. S2). It is less clear how the YAP-β-catenin complex regulates macrophage polarization from a proinflammatory M1 to an anti-inflammatory M2 phenotype in IR-triggered liver inflammation after MSC intervention. It is known that YAP or β-catenin deficiency enhances innate immune response[13,32] while activation of YAP or β-catenin

orchestrates immunosuppressive response following tissue injury.[9,25] In line with these findings, we found that MSC promoted YAP and β-catenin activation, which in turn inhibited NLRP3 expression, whereas disruption of YAP or β-catenin enhanced XBP1-mediated NLRP3/caspase-1 activity. Importantly, increasing NLRP3 expression inhibited M2 macrophage Arg1 but enhanced M1 macrophage iNOS expression with increased IL-1β release after coculture with MSCs. Thus, NLRP3 is key to balance M1/M2 macrophage polarization in YAP-β-catenin-mediated regulation. Our results revealed a crucial role of the YAP-β-catenin complex in controlling a dynamic crosstalk with the NLRP3 in MSC-mediated immune regulation.

It is worth noting that other regulatory molecules and pathways may also relate to NLRP3 function in sterile inflammation. It was reported that miR-223 is required for the regulation of NLRP3-driven inflammation in intestine and lung.[33,34] Under ischemic or hypoxic conditions, purinergic receptors play important roles in triggering inflammatory response and tissue injury.[35,36] The purinergic receptor signaling was crucial for the ATP-mediated NLRP3 activation in allograft rejection.[36] Interestingly, the purinergic receptors induced cell proliferation and migration in injured tissues by regulating YAP activation,[37] suggesting that there is a crosstalk between purinergic receptors and the Hippo-YAP pathway. Moreover, the hypoxia-inducible factors regulated their target genes, leading to resistance to ischemia and controlling excessive inflammation,[38,39] which may interact with YAP under hypoxic conditions.[40] These reports suggest that Hippo pathway–mediated immune regulation may be involved in multiple signaling pathways during sterile inflammatory injury.

Indeed, liver IRI represents one of the most understudied yet critical problems as it often leads to primary graft nonfunction or failure in transplant recipients. However, during surgical procedures, patients may also be threatened by other perioperative types of organ injuries.[41] Thus, it is crucial to develop therapeutic approaches for the prevention or treatment of perioperative organ injury.[41] Our current study provides mechanistic insights into liver IRI and might also provide therapeutic potential for transplant inflammatory injury and other types of organ injuries in MSC-mediated immune regulation.

Figure 8 depicts putative molecular mechanisms by which Hippo signaling may regulate NLRP3

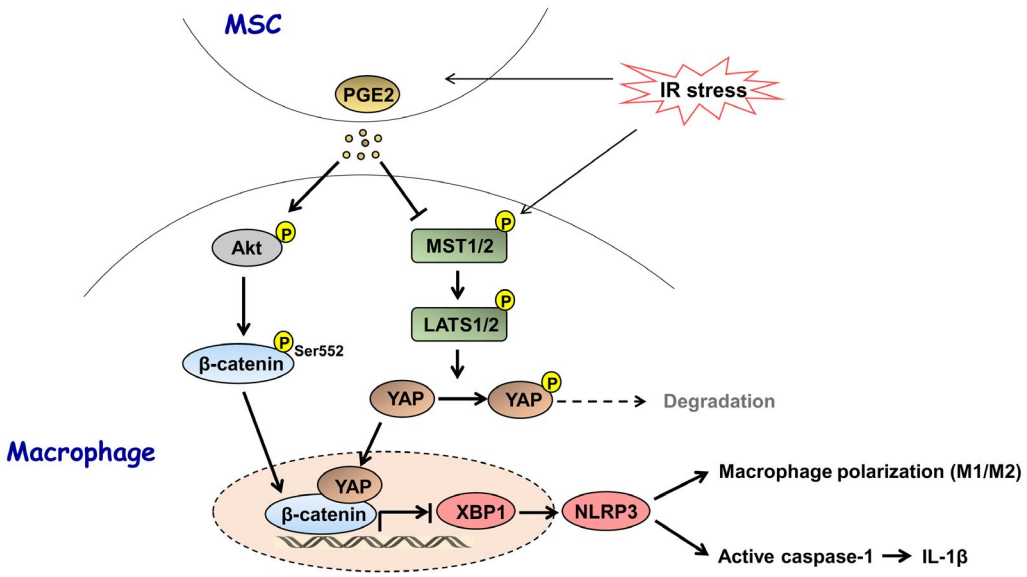


FIG. 8. Schematic illustration of how Hippo signaling may control NLRP3 activation in MSC-mediated immune regulation. IR stress increases MSC-mediated PGE2 section, which in turn activates macrophage Akt and phosphorylates β-catenin at Ser552, leading to translocation of β-catenin into nucleus. Notably, MSCs regulate macrophage Hippo-YAP pathway by depressing MST1/2 and LATS1 phosphorylation and increasing YAP translocation from cytoplasm to nucleus where YAP colocalizes and interacts with nuclear β-catenin, which in turn regulates their target gene XBP1, leading to reduced NLRP3/caspase-1 activity and IL-1β release and augmented M2 macrophage phenotype in IR-triggered liver inflammation.

activation in MSC-mediated immune regulation. IR stress increases MSC-mediated PGE2 secretion, which in turn activates macrophage Akt and phosphorylates β-catenin at Ser552, leading to translocation of β-catenin into nucleus. Notably, MSCs regulate macrophage Hippo-YAP pathway by depressing MST1/2 and LATS1 phosphorylation as well as increasing YAP translocation from cytoplasm to nucleus where YAP colocalizes and interacts with nuclear β-catenin, which in turn regulates their target gene XBP1, leading to reduced NLRP3/caspase-1 activity and IL-1β release and augmented M2 macrophage phenotype in IR-triggered liver inflammation.

In conclusion, we identify a role of Hippo signaling in controlling NLRP3 activation in MSC-mediated immune regulation. Our findings demonstrate that YAP is a coactivator of β-catenin and that the YAP-β-catenin interaction is crucial for the inhibition of XBP1-mediated NLRP3 activation and reprograms macrophage differentiation during liver sterile inflammatory injury. Indeed, as a key regulator of NLRP3-driven inflammation in liver IRI, the Hippo signaling downstream effector YAP is a therapeutic target. We may develop therapeutic strategies to treat transplant recipients using genetically modified MSC approaches or pharmacological interventions targeting YAP in the donor or in the graft before implantation or reperfusion.

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