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mTOR signaling disruption from myeloid-derived suppressive cells protects against immune-mediated hepatic injury through the HIF1α-dependent glycolytic pathway

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ABSTRACT

The mechanistic target of rapamycin (mTOR) pathway integrates diverse environmental inputs, including immune signals and metabolic cues, to direct innate and adaptive immune responses. Myeloid-derived suppressive cells (MDSCs) are a heterogeneous cell population that plays a crucial regulatory effect in immune-related diseases. However, whether mTOR signaling affects the functions of MDSCs remains largely unexplored. Here, we show that mTOR signaling is a pivotal, negative determinant of MDSC function in immune-mediated hepatic injury (IMH) diseases. In the context of IMH, the blocking of mTOR with rapamycin or mTOR-deficient CD11b$^+$Gr1$^+$ MDSCs mediates the protection against IMH; mTOR with rapamycin and mTOR-deficient CD11b$^+$Gr1$^+$ MDSCs are suppressive immune modulators that result in less IFN-γ-producing T_H1 cells and more Foxp3$^+$ T_{regs}. Mechanistically, mTOR activity down-regulation in MDSCs induced iNOS expressions and NO productions. Pharmacologic inhibitions of iNOS completely eliminate MDSC-suppressive function and lose their inducible effects on T cell differentiation. Importantly, HIF1α-dependent glycolytic activity is responsible for mTOR-deficient, increased MDSC functional changes in IMH inflammation. Thus, these data demonstrate that mTOR acts as a fundamental "rheostat" in MDSCs to link immunologic signals to glycolytic pathways and functional fitness and highlights a central role of metabolic programming of MDSC-suppressive activity in protecting against immune hepatic injuries. *J. Leukoc. Biol.* 100: 1349–1362; 2016.

Abbreviations: 2-DG = 2-deoxy-D-glucose, ALT = aminotransferase, Arg1 = arginase 1, BM = bone marrow, CD62L = cluster of differentiation 62 ligand, CIH = Con A-induced immune-mediated hepatic injuries, DC = dendritic cell, Eno1 = enolase 1, ER = estrogen receptor, FAO = fatty acid β-oxidation, FCM = flow cytometry, FKBP12 = FK506-binding protein 12, Foxp3 = forkhead box p3, Glut1 = glucose transport 1, GPI = glucose-6-phosphate isomerase

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The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

MDSCs are a heterogeneous cell population [1, 2] that suppresses T cell proliferation and function, blocks NK cell cytotoxicity, and promotes the development of T_{regs} in tumor-bearing hosts [1, 3–5]. MDSCs have been detected in the blood of cancer patients and in the BM, spleen, and peripheral blood of tumor-bearing mice [6, 7]. Murine MDSCs are defined as CD11b$^+$Gr1$^+$ myeloid cells that suppress T cell proliferation. Although much has been learned about the immunosuppressive function of MDSCs in cancers, less is known about liver MDSCs, especially with respect to their specific regulatory mechanisms in T cell-mediated IMH.

The mTOR is an evolutionarily conserved and ubiquitously expressed controller of cell growth, proliferation, and survival [8]. mTOR senses growth or metabolic signals (e.g., ATP, oxygen, amino acids, glucose, reactive oxidant species) and exerts anabolic effects by stimulating protein synthesis and ribosomal biogenesis, enhancing cell proliferation, and promoting cell survival [9]. Rapamycin [i.e., sirolimus (Rapamune)] is a specific inhibitor of mTOR and is a commonly used pharmacologic tool for the study of mTOR biology. In addition, rapamycin is approved by the U.S. Food and Drug Administration for immunosuppression in transplant patients, cancer chemotherapy, and local prevention of coronary artery stent thrombosis [10–12].

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It has been shown that the mTOR pathway acts to modulate the function of DCs in a manner that is dependent on the activation or maturation state and DC type studied. Administration of rapamycin in vivo or in vitro can impair DC maturation, such that DCs remain tolerogenic, failing to up-regulate costimulatory molecules [13]. Moreover, mTOR signaling is required from DCs to gain T cell-stimulatory capacity during differentiation, whereas mTOR signaling can also limit the proinflammatory capacity of mature DCs [14]. However, whether mTOR signaling affects the functions of $CD11b^+Gr1^+$ MDSCs remains largely unexplored.

In the present study, we have sought to determine whether mTOR signaling takes critical effects on MDSC functional activities in IMH. mTOR signaling was significantly up-regulated in immune-mediated hepatic injuries. The blocking of mTOR with rapamycin or mTOR deficiency in $CD11b^+Gr1^+$ MDSCs significantly potentiates its suppressive activities, and mTOR with rapamycin or mTOR deficiency is required for protection against the IMH. Thus, our current study uncovers mTOR signaling as a pivotal, negative determinant of MDSC function in mediating protection of immune-mediated liver diseases.

MATERIALS AND METHODS

Mice

All animal experiments were performed in accordance with the approval of the Animal Ethics Committee of Fudan University (Shanghai, China) or Beijing Normal University (Beijing, China). Rosa26-Cre-ER^{T2}, $HIF1\alpha^{flox/flox}$, and $mTOR^{flox/flox}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). $CD45.1^+C57BL/6$ mice were obtained from the Center of Model Animal Research at Nanjing University (Nanjing, China). C57BL/6 ($CD45.2^+$) mice were obtained from Fudan University Experimental Animal Center or Weitonglihua Experimental Animal Limited Company (Beijing, China). All mice were bred and maintained in specific pathogen-free conditions. Sex-matched littermates at 6–8 wk of age were used in the experiments described in this study.

CIH

Age-matched mice received an intravenous injection of Con A (C2010; Sigma-Aldrich, St. Louis, MO, USA) at a dose of 15 or 25 mg/kg body weight, as indicated. The higher lethal dose was used to generate survival curves, and the lower dose was permitted assessment of liver pathology and other parameters following Con A challenge. Liver samples were fixed in 4% paraformaldehyde, embedded in paraffin, and stained by H&E. ALT enzyme activity was determined in serum samples using a kit from Cayman Chemical (Ann Arbor, MI, USA)

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HIF1α = hypoxia-inducible factor-1α, IMH = immune-mediated hepatic injury, iT_{reg} = induced forkhead box $p3^+$ regulatory T cell, L-NMMA = NG-monomethyl-L-arginine, LDHα = lactate dehydrogenase α, MCT4 = monocarboxylic acid transporter member 4, MDSC = myeloid-derived suppressive cell, mTOR = mechanistic target of rapamycin, mTORC1/2 = mechanistic target of rapamycin complex 1/2, PD-L1 = programmed death ligand 1, PIH = picryl chloride, RORγt = retinoic acid receptor-related orphan receptor γt, shRNA = short hairpin RNA, T_{reg} = regulatory T cell, WT = wild-type

PIH

Age-matched mice were sensitized twice by painting 0.1 ml of 1% PIH (Nacalai Tesque, Kyoto, Japan) in ethanol on the skin of their abdomens with an interval of 5 d (inductive phase), as described previously [15]. Five days after the second sensitization, their livers were injected with 10 μl of 0.5% PIH in olive oil. After 18 h (effector phase), mice were bled, and livers were removed under anesthesia. Liver samples were analyzed by histologic H&E staining, and ALT enzyme activity was determined in serum samples.

Cell purification and culture

Following cardiac perfusion with PBS, the liver was aseptically removed and mechanically disrupted between sterile-frosted microscope slides, as described before [16, 17]. Liver $CD11b^+$ cells were isolated using anti-CD11b magnetic beads and positive selection columns (Miltenyi Biotec, Bergisch Gladbach, Germany). $Gr1^+$ cells were isolated using anti-Gr1-PE mAb (RB6-8C5; eBioscience, San Diego, CA, USA) and positive immunomagnetic separation using a selection kit (Stem Cell Technologies, Vancouver, BC, Canada) or sorted on a FACSAria II (BD Biosciences, San Jose, CA, USA). FCM verified that all isolated cells yielded above a 90% pure population.

Lymphocytes were isolated from the lymph nodes, and naïve T cells were sorted on a FACSAria II. Sorted, naïve T cells were used for in vitro culture in RPMI-1640 medium, supplemented with 10% FBS and 1% penicillin-streptomycin, as described [18].

mAb and FCM

For FCM analysis of cell-surface markers, cells were stained with antibodies in PBS containing 0.1% (wt/vol) BSA and 0.1% NaN_3. The following antibodies were obtained from eBioscience: anti-CD11b (M1/70), anti-F4/80 (BM8), anti-Gr-1 (RB6-8C5), anti-CD4 (GK1.5), anti-CXCR4 (2B11), anti-PD-L1 (MIH5), anti-CD86 (GL1), anti-CD62L (MEL-14), and anti-CD44 (IM7). The following antibodies were obtained from BD Biosciences: anti-CD45 (TU116) and anti-CD8 (53-6.7). The following antibodies were obtained from Miltenyi Biotec: anti-CD3 (145-2C11) and anti-CD19 (6D5). Anti-CD124 (1015F8) was obtained from BioLegend (San Diego, CA, USA).

Intracellular staining and FCM assays

For detection of phosphorylated signaling proteins, purified cells were activated with LPS (Sigma-Aldrich), were immediately fixed with Phosflow Perm buffer (BD Biosciences), and were stained with PE or allophycocyanin, directly conjugated to S6, phosphorylated at Ser235 and Ser236 (D57.2.2E; Cell Signaling Technology, Danvers, MA, USA). For intracellular staining, IFN-γ (XMG1.2; eBioscience), Foxp3 (FJK-16s; eBioscience), and HIF1α (MAB1935; R&D Systems, Minneapolis, MN, USA) were analyzed by FCM, according to the manufacturer's instructions. FCM data were acquired on a FACSCaliber (BD Biosciences), and data were analyzed with FlowJo (Tree Star, San Carlos, CA, USA).

Glycolysis flux assay

Glycolysis of MDSCs were determined by measuring the detritiation of [3-^{3}H] glucose, as described previously [19]. In brief, the assay was initiated by adding 1 μCi [3-^{3}H]glucose (Perkin Elmer), and 2 h later, medium was transferred to microcentrifuge tubes containing 50 μl 5 N HCl. The microcentrifuge tubes were then placed in 20 ml scintillation vials containing 0.5 ml water and the vials capped and sealed. ^3H_2O was separated from unmetabolized [3-^{3}H]glucose by evaporation diffusion for 24 h at room temperature. Cell-free samples containing 1 μCi [3-^{3}H]glucose were included as a background control.

FAO flux

FAO flux was determined by measuring the detritiation of [9,10-^{3}H]palmitic acid, as described previously [19]. MDSCs were stimulated with LPS (10 ng/ml).

After 24 h, 1 million MDSCs were suspended in 0.5 ml fresh medium. The experiment was initiated by adding 3 μCi [9,10-^{3}H]palmitic acid complexed to 5% BSA (lipids free; Sigma-Aldrich), and 2 h later, media were transferred to a 1.5 ml microcentrifuge tube containing 50 μl 5 N HCl. The microcentrifuge tubes were then placed in 20 ml scintillation vials containing 0.5 ml water, with the vials capped and sealed. ^3H_2O was separated from unmetabolized [9,10-^{3}H]palmitic acid by evaporation diffusion for 24 h at room temperature. The mitochondria-dependent β-oxidation rate was determined.

Quantitative RT-PCR

RNA was extracted with an RNeasy kit (Qiagen, Dusseldorf, Germany), and cDNA was synthesized using SuperScript III RT (Thermo Fisher Scientific, Waltham, MA, USA). An ABI 7900 Real-Time PCR system was used for quantitative PCR, with primer and probe sets obtained from Thermo Fisher Scientific (**Table 1**). Results were analyzed using SDS 2.1 software (Thermo Fisher Scientific). The expression of each target gene is presented as the "fold change" relative to that of control samples, as described previously [18].

In vivo and in vitro functional assay of CD11b$^+$Gr1$^+$ cells

To deplete CD11b$^+$Gr1$^+$cells in vivo, 0.25 mg depleting anti-Gr1 mAb (RB6-8C5) was administered intraperitoneally into recipient mice on d −1 and 3 after Con A treatment. To detect immunosuppression of liver CD11b$^+$Gr1$^+$ cells in vivo, C57BL/6 CIH was first induced by intravenous injection of low-dose Con A (15 mg/kg), and at 40–60 h, a total of 1×10^6 CD11b$^+$Gr1$^+$ cells was sorted from liver tissue of CIH mice and transferred into C57BL/6 mice via intravenous injection. At 10–12 h after cell transfer, all of these groups were injected with a high dose of Con A (25 mg/kg), and mouse survival was observed.

The in vitro-suppressive function of MDSCs was assessed by determining their ability to inhibit T cell activation and as described previously [20]. Purified T cells were plated at a density of 2×10^5 cells/well in 1 μg/ml anti-CD3 antibody-coated plates in the presence of 1 μg/ml anti-CD28 antibody. Isolated MDSCs were added to the wells at different ratios. Cell proliferation was determined 72 h later after incubation with [^{3}H]thymidine for the last 16 h of culture.

NO production assay

After incubating equal volumes of culture supernatant or serum (100 μl) with Griess reagent, the absorbance at 550 nm was measured using at a wavelength of 550 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA), as described before [20].

NOS2 knockdown with RNA interference

With NOS2 knockdown with RNA interference, as described previously [21], a gene-knockdown lentiviral construct was generated by subcloning gene-specific shRNA sequences into lentiviral shRNA expression plasmids (pLL3.7). Lentiviruses were harvested from culture supernatant of 293T cells transfected with shRNA vector. Sorted liver CD11b$^+$Gr1$^+$ cells from CIH mice were infected with recombinant lentivirus, and GFP-expressing cells were isolated using fluorescence sorting 48 h later. The NOS2 expression was confirmed using quantitative PCR. The sorted MDSCs with control or shRNA vectors were used for functional assay.

Statistical analyses

All data are presented as the means ± SD. Student's unpaired *t* test was applied for comparison of means and used to compare differences between groups. Comparison of the survival curves was performed using the Log-Rank (Mantel-Cox) test. $P < 0.05$ (α-value) was considered to be statistically significant.

RESULTS

mTOR signaling from the CD11b$^+$Gr1$^+$ cell is constitutively active in fatal immune-mediated hepatic injuries

The CIH murine model was used in this study. The evolutionarily conserved mTOR signaling pathway couples cell growth and proliferation to nutrient availability and metabolic cues. To investigate the function of mTOR in CD11b$^+$Gr1$^+$ cells, we determined the mTOR activity of liver CD11b$^+$Gr1$^+$ cells in the mouse CIH model treated with or without rapamycin (an inhibitor of mTOR, 3 mg/kg). CD11b$^+$Gr1$^+$ cells had relatively increased phosphorylation of S6, a major mTOR downstream target, with the time-dependent manner in CIH, and rapamycin treatment significantly down-regulated the phosphorylation of S6 (**Fig. 1A**). This finding reveals that mTOR activity might be involved in CD11b$^+$Gr1$^+$ cell function in immune hepatic injuries.

CD11b$^+$Gr1$^+$ cell mTOR signaling is required for protection against immunologic hepatic injury

We firstly investigated the effects of rapamycin on CIH mortality. As shown in Fig. 1A and Supplemental Fig. 1A and B, the daily treatment of rapamycin significantly blocked the mTOR signals and reduced mortality compared with the control CIH group. The serum ALT activity (data not shown) was significantly lower in the rapamycin-treated group than the control group. Furthermore, hepatic T cells displayed lower autoimmune

TABLE 1. Primer sequences used for real-time PCR assays

Genes	Forward primer	Reverse primer
Arg1	5′-CCAGAAGAATGGAAGAGTCAGTGT-3′	5′-GCAGATATGCAGGGAGTCACC-3′
Foxp3	5′-GGCCCTTCTCCAGGACAGA-3′	5′-GGCATGGGCATCCACAGT-3′
iNOS	5′-CACCAAGCTGAACTTGAGCG-3′	5′-CGTGGCTTTGGGCTCCTC-3′
RORγt	5′-CCGCTGAGAGGGCTTCAC-3′	5′-TGCAGGAGTAGGCCACATTACA-3′
T-bet	5′-AGCAAGGACGGCGAATGTT-3′	5′-GGGTGGACATATAAGCGGTTC-3′
HPRT	5′-CCTAAGATGAGCGCAAGTTGAA-3′	5′-CCACAGGACTAGAACACCTGCTAA-3′
GATA3	5′-GAATCCTCTGCATCAACAAGC-3′	5′-GGGCAAGGGTTCTGAGGT-3′
GAPDH	5′-GACTTCAACAGCAACTCCCAC-3′	5′-TCCACCACCCTGTTGCTGTA-3′
Glut1/Slc2a1	5′-CAGTTCGGCTATAACACTGGTG-3′	5′-GCCCCCGACAGAGAAGATG-3′

HPRT, Hypoxanthine phosphoribosyltransferase.

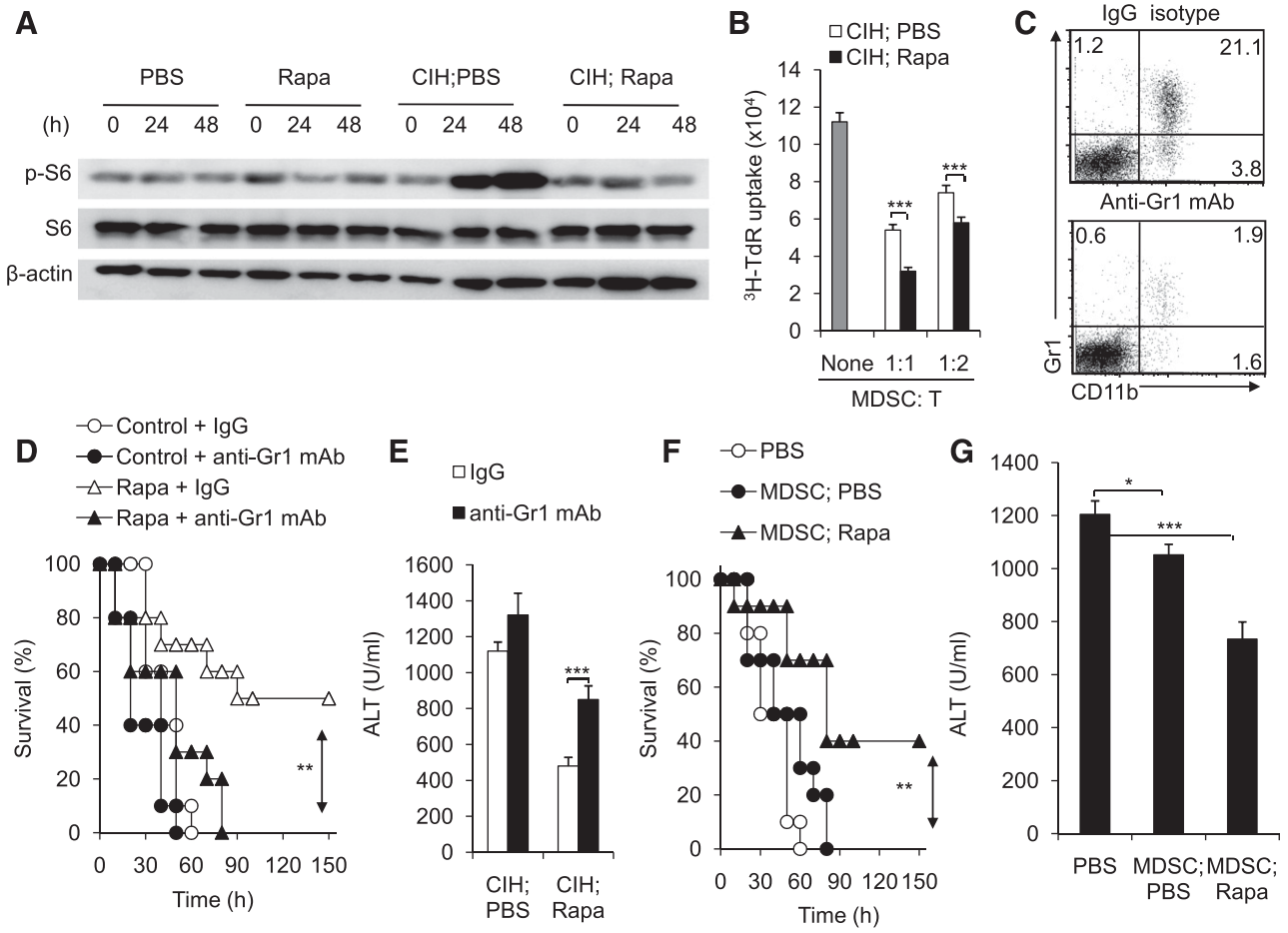

Figure 1. mTOR signaling is constitutively active in CD11b$^+$Gr1$^+$ cells, and its disruption protects against fatal immune-mediated hepatic injuries. (A) C57BL/6 control or C57BL/6 CIH mice were induced by intravenous injection of Con A (15 mg/kg). CD11b$^+$Gr1$^+$ cells were sorted from liver tissue of rapamycin (Rapa; 3 mg/kg; an inhibitor of mTOR), vehicle (PBS)-treated CIH, or control mice at the different time points, and the phosphorylated S6 (p-S6) and S6 expressions were determined with Western blot. (B) The liver CD11b$^+$Gr1$^+$ MDSCs treated with rapamycin (3 mg/kg) in the CIH mice showed increased suppressive activity. T Cells were stimulated with anti-CD3/CD28 antibodies in the presence of liver MDSCs that were isolated from Con A-injected PBS control or rapamycin-treated mice. T cell proliferation was determined with [^{3}H]thymidine incorporation. (C–E) The depletion of Gr1$^+$ cells in CIH (Con A injection, 25 mg/kg) mice with mAb (RB6-8C5). The depletion of Gr1$^+$ cells was confirmed with FCM and typical figure shown in C. The depletion of Gr1$^+$ cells in rapamycin-treated CIH (Con A injection, 25 mg/kg) mice with mAb (RB6-8C5) significantly aggravated the mortality seen in CIH mice (D; $n = 10$). (E) The depletion of Gr1$^+$ cells in rapamycin-treated CIH (Con A injection, 15 mg/kg) mice with mAb (RB6-8C5) and the serum ALT levels were assessed at 12 h after Con A injection. (F) Adoptive transfer of CD11b$^+$Gr1$^+$ cells from rapamycin-treated mice significantly ameliorated the mortality of CIH mice ($n = 10$). C57BL/6 CIH was first induced by intravenous injection of low-dose Con A (15 mg/kg), and at 40–60 h, a total of 1×10^6 CD11b$^+$Gr1$^+$ cells was sorted from liver tissue of rapamycin-treated or control mice and transferred into C57BL/6 mice via intravenous injection. At 10–12 h after cell transfer, both groups were injected with a high dose of Con A (25 mg/kg), and the mouse survival curve was followed daily (10 mice/group). (G) C57BL/6 CIH was first induced by intravenous injection of low-dose Con A (15 mg/kg), and at 40–60 h, a total of 1×10^6 CD11b$^+$Gr1$^+$ cells was sorted from liver tissue of rapamycin- or vehicle-treated control mice and transferred into C57BL/6 mice via intravenous injection. At 10–12 h after cell transfer, both groups were injected with a low-dose Con A (15 mg/kg), and the serum ALT levels were assessed at 12 h after Con A injection. The data were shown as means ± SD ($n = 4$–10) from 1 of 3–4 independent experiments performed. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for comparisons made among the indicated groups.

phenotypes and proliferation abilities in rapamycin-treated CIH mice than did control mice (Supplemental Fig. 1C and D).

Consistently, the effects of rapamycin on PIH-induced hepatic injury was studied. Rapamycin-treated mice displayed significantly fewer hepatocytic necrosis than did their control counterparts (Supplemental Fig. 1E). The serum ALT activity of the inductive phase and effector phase was significantly lower in the rapamycin-treated group than the control group. Furthermore, hepatic T cells displayed a lower autoimmune phenotype in rapamycin-treated PIH mice than did control mice (data not shown).

How does mTOR signaling from CD11b$^+$Gr1$^+$ cells affects immune-mediated hepatic injury inflammation? We examined whether these CD11b$^+$Gr1$^+$ cells, following rapamycin treatment, can behave as immune suppressors, as described for MDSCs [4, 5, 22]. With the comparison of cells isolated from

the control group, CD11b$^+$Gr1$^+$ cells isolated from the rapamycin-treated CIH liver (Fig. 1B) and spleen (data not shown) displayed significantly enhanced immune-suppressive activities on CD4$^+$ T cell proliferation than that seen in controls in vitro. We further depleted these cells by injecting anti-Gr1 mAb, as described previously [20]. Of note, an efficient depletion of CD11b$^+$Gr1$^+$ cells was achieved in these mice, as evidenced by FCM (Fig. 1C). Strikingly, the depletion of Gr1$^+$ cells significantly reduced rapamycin-mediated protection against CIH mice, indicated by the changes of mortality (Fig. 1D) and serum ALT level (Fig. 1E). Importantly, adoptive transfer of sorted CD11b$^+$Gr1$^+$ cells, from rapamycin-treated CIH but not the control group, into WT C57BL/6 recipients exhibited potent and sustained down-regulation of phosphorylation of S6 (Supplemental Fig. 2A) and significantly prolonged the percentage of CIH mouse survival (Fig. 1F) and reduced serum ALT level (Fig. 1G).

Similar results were observed in PIH mice (data not shown). Rapamycin treatment significantly potentiated suppressive activity of CD11b$^+$Gr1$^+$ cells compared with control. Furthermore, the depletion of Gr1$^+$ cells significantly reduced rapamycin-mediated protection against PIH mice, confirmed by the changes of hepatic pathology and serum ALT level. Thus, these observations suggested that mTOR signaling was critically required for CD11b$^+$Gr1$^+$ MDSC-mediated protective effects against immune-mediated hepatic injuries.

CD11b$^+$Gr1$^+$ cell mTOR signaling modulates reciprocal T_H1 and T_{reg} differentiation during immune-mediated hepatic injuries

With the comparison of control CIH mice, rapamycin treatment resulted in a significantly lower level of IFN-γ in blood (**Fig. 2A**). In addition, a reciprocal decrease of IFN-γ-producing T_H1 cells and an increase of iT_{regs} were observed

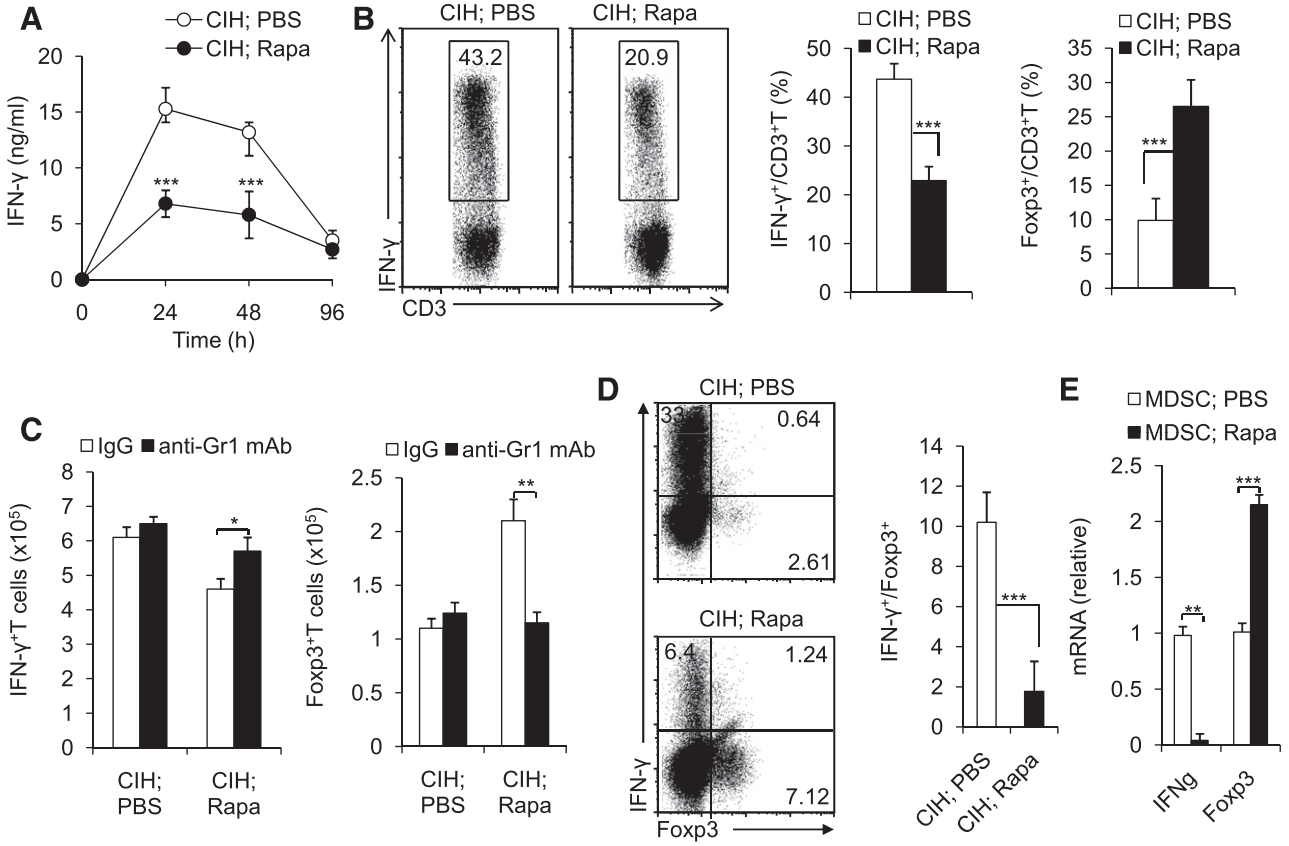

Figure 2. CD11b$^+$Gr1$^+$ MDSC mTOR down-regulation promotes reciprocal differentiation of T_H1 to T_{regs} in IMH. (A) Serum levels of IFN-γ were determined at different time points after Con A (15 mg/kg) injection to mice. (B) mTOR activity down-regulation decreased the frequency of IFN-γ-producing T cells, as determined 40 h after Con A injection by intracellular cytokine staining. The typical FCM figures are shown. (Right) The frequencies of IFN-γ$^+$/CD3$^+$ T cells and Foxp3$^+$/CD3$^+$ T cells were summarized. (C) Absolute cell numbers of liver IFN-γ$^+$CD3$^+$ T cells and Foxp3$^+$CD3$^+$ T cells from rapamycin-treated CIH mice, PBS-treated control CIH mice or mice pretreated with isotype IgG1 or rat-anti-mouse Gr1 mAb are summarized. (D) CIH was induced by low-dose Con A (15 mg/kg) intravenous injection. At 40–60 h after Con A injection, liver CD11b$^+$Gr1$^+$ MDSCs were isolated from PBS-treated control or rapamycin-treated CIH mice. MDSCs (1×10^6) were transferred into C57BL/6 mice that were pretreated with Gr1 mAb (RB6-8C5) on d −1. Subsequently, CIH were induced by Con A (15 mg/kg). At 40–50 h after cell transfer, liver T cells were isolated. The intracellular expression of IFN-γ and Foxp3 and the proportion between IFN-γ$^+$ and Foxp3$^+$ among T cells (right) after PMA + ionomycin stimulation were determined. (E) Analysis of IFN-γ and Foxp3 mRNA expression in liver-derived T cells restimulated with α-CD3 for 5 h. The data were shown as means ± SD (n = 3–5) from 1 of 3 independent experiments performed. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for comparisons made among the indicated groups.

in the livers of CIH following rapamycin treatment (Fig. 2B). Likewise, the mRNA level of IFN-γ and Foxp3 increased and decreased following rapamycin treatment, respectively. T-bet showed consistent tendency with IFN-γ expression, but the RORγt and GATA3 are comparable in both groups (Supplemental Fig. 2B). Furthermore, the depletion of $Gr1^+$ cells in the rapamycin-treated but not the control group clearly restored the changes of $Foxp3^+$ T cells and IFN-γ^+ T cells in liver tissue of CIH (Fig. 2C). Consistently, a reciprocal decrease of IFN-γ-producing T_H1 cells and an increase of $Foxp3^+$ T_{regs} were observed in the livers of PIH following rapamycin treatment. Furthermore, the depletion of $Gr1^+$ cells in the rapamycin-treated but not the control group clearly restored the changes of $Foxp3^+$ T cells and IFN-γ^+ T cells in liver tissue of PIH (data not shown). Collectively, these data reveal that mTOR down-regulation in MDSCs potentiates the reciprocal T cell differentiation of T_H1 and iT_{reg} and consequently, contributes to protect against hepatic injuries.

To investigate directly whether MDSCs regulate the reciprocal T cell differentiation in vivo and whether the aspect of regulating T cell differentiation contributes to protection against immune-mediated hepatic injuries, we first isolated the $CD11b^+Gr1^+$ cells from rapamycin-treated CIH mice and PBS-treated control mice. Then, we transferred isolated $CD11b^+Gr1^+$ cells into WT C57BL/6 recipient mice, following the induction of CIH, by injecting low-dose Con A (15 mg/kg). Rapamycin-treated MDSC-transferred groups displayed a lower ALT level in CIH mice compared with the control group (data not shown). At 40–50 h after Con A injection, liver T cells were isolated. In liver $CD3^+$ cells of CIH mice, rapamycin-treated MDSC induced significantly higher $Foxp3^+$ but lower IFN-γ^+ expression compared with the control group (Fig. 2D). Similar Foxp3 and IFN-γ mRNA expression patterns were observed in the cotransferred donor T cells (Fig. 2E). To differentiate further the role of mTORC1 and mTORC2, we found that rapamycin-treated MDSCs showed the down-regulated phosphorylation of S6, indicative of mTORC1 activation. However, the phosphorylation of AKT Ser473, indicative of mTORC2 activation, was largely comparable between controls or rapamycin-treated MDSC donor cells (Supplemental Fig. 3A and B). These results suggest that the mTORC1 is responsible for mediating the protective role of MDSCs in IMH. Collectively, these data suggest that in CIH, mTOR disruption-altered MDSCs can reciprocally direct the differentiation of T_H1 and iT_{regs} and consequently, offer the protection against immune hepatic injury.

mTOR signaling negatively controls the functions of $CD11b^+Gr1^+$ MDSCs via NO production

NO production has been suggested as a critical component mediating immunosuppressive activity of MDSCs [23]. Thus, we measured the NO levels following Con A injection. In agreement, the average NO level was greatly higher in rapamycin-treated CIH compared with control in a dose-dependent manner (**Fig. 3A**). In addition, the mRNA level of iNOS, a NO-producing metabolic enzyme, was significantly induced, whereas the mRNA level of arginase, the metabolic enzyme sharing the same substrate of iNOS, was reduced in $CD11b^+Gr1^+$ MDSCs following rapamycin treatment in CIH mice (Fig. 3A). To determine whether NO production is an essential component in MDSC-mediated protection against hepatic injury, we applied L-NMMA, an inhibitor of iNOS, to the in vivo function assay. L-NMMA significantly reduced NO production and expression of iNOS (Fig. 3B) and efficiently relieved immunosuppressive activities of MDSC isolated from rapamycin-treated CIH mice, indicated by the T cell proliferation assay (Fig. 3C).

To investigate directly whether NO signaling is critical for MDSCs regulating the reciprocal T cell differentiation in vivo, we first isolated the $CD11b^+Gr1^+$ cells from rapamycin and L-NMMA-treated CIH and control mice. Then, we transferred isolated $CD11b^+Gr1^+$ cells into WT C57BL/6 recipient mice, following the induction of CIH, by injecting low-dose Con A (15 mg/kg). Rapamycin-treated MDSC-transferred groups displayed a lower ALT level (Fig. 3D) and histologic injuries (Fig. 3E) in CIH mice compared with the control group. At 40–50 h after Con A injection, liver T cells were isolated. In the liver $CD3^+$ cell of CIH mice, rapamycin-treated MDSC induced significantly higher $Foxp3^+$ but lower IFN-γ^+ expression compared with control group (Fig. 3F). Similar Foxp3 and IFN-γ mRNA expression patterns were observed in the cotransferred donor T cells (data not shown). Importantly, the blocking of NO signals with L-NMMA treatment (Supplemental Fig. 3C) can almost completely reverse the rapamycin-induced MDSC changes and protective effects in CIH (Fig. 3C–F). Consistent with the above results, iNOS (NOS2) small interfering RNA efficiently silenced NOS2 expressions in MDSCs and recovered the rapamycin-altered NO production, MDSC-suppressive activities, and reciprocal T_H cell differentiation (Fig. 3G–J). Collectively, these data reveal that in CIH, mTOR disruption in MDSCs can promote its suppressive activities, reciprocally directs the differentiation of T_H1 and iT_{regs} and offers the protection against tissue injury via a NO-dependent mechanism.

mTOR signaling controls MDSC activity associated with the glycolytic metabolism mechanism

Although it has been well appreciated that immune cell activation is accompanied by a metabolic switch glycolysis, how glycolytic activity is regulated during MDSC function is poorly understood. WT liver MDSCs isolated in CIH mice and the glycolytic activity of MDSCs were measured by the generation of $[^3H]$-labeled H_2O from [3-^{3}H]glucose. Results showed that MDSCs contained increased glycolytic activity in a time-dependent manner (**Fig. 4A**). Glycose use depends on a chain of reactions catalyzed by multiple enzymes, eventually leading to the generation of lactate and net production of 2 ATP molecules as the energy source. Transcriptional mRNA expression of Glut1, GPI, Eno1, MCT4, and LDHα in liver MDSCs isolated from CIH mice showed consistent results (Fig. 4B). Collectively, these data indicate strong up-regulation of glucose metabolism in CIH.

We next tested whether mTOR signaling contributes to the differential activity of glycosis of MDSCs in CIH. We purified liver MDSCs from rapamycin- or PBS-treated control CIH mice, and the blocking of mTOR with

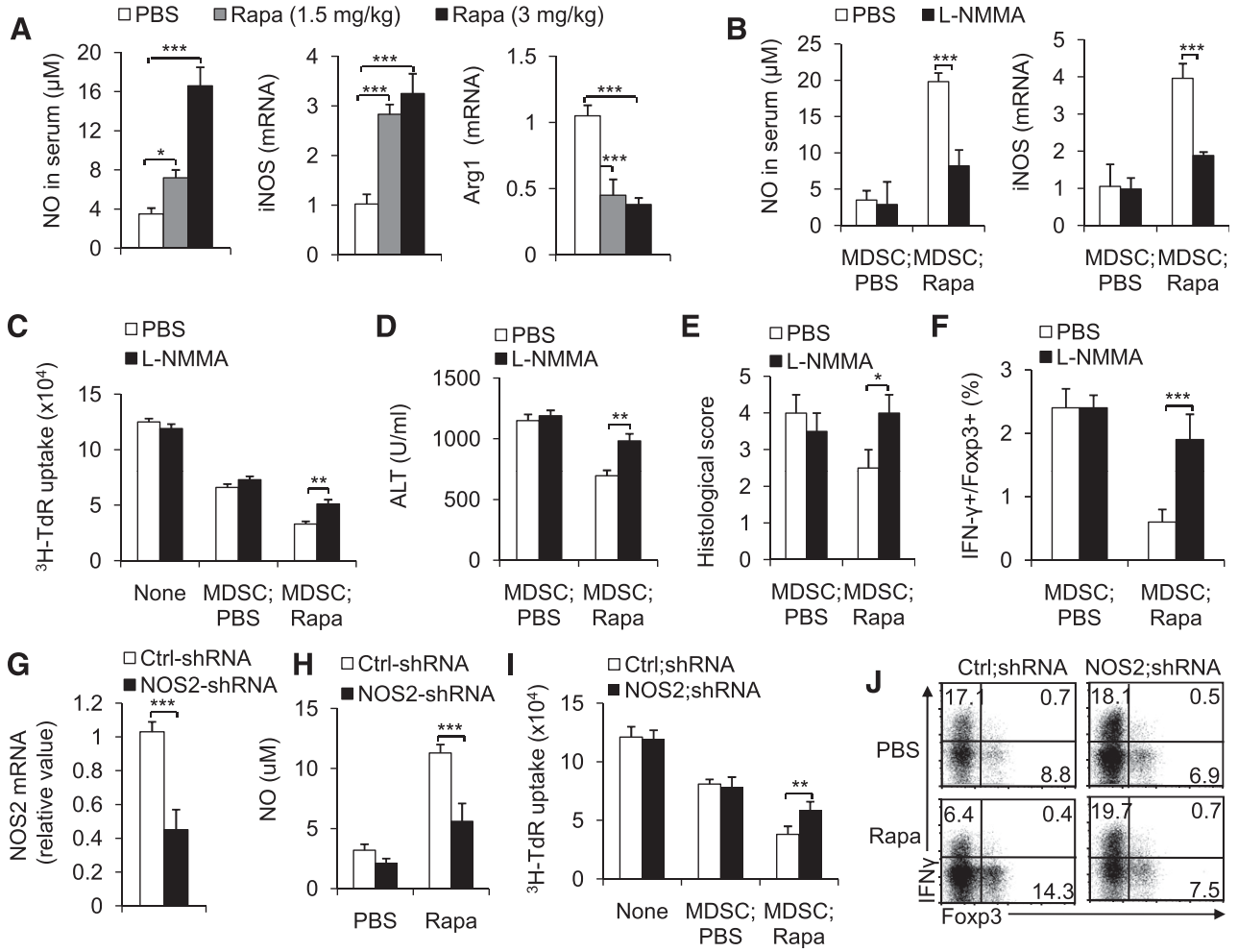

Figure 3. mTOR down-regulation potentiates CD11b$^+$Gr1$^+$ MDSC functions and promotes reciprocal differentiation of T_H1 to T_{regs} in protecting against hepatic injury through a NO-dependent pathway. (A) Significant higher NO levels in serum and higher iNOS mRNA and lower Arg1 mRNA in liver CD11b$^+$Gr1$^+$ cells from rapamycin-treated CIH mice compared with vehicle-treated control CIH mice. (B) CIH was induced by low-dose Con A (15 mg/kg) intravenous injection in C57BL/6 mice. Experimental mice were treated by vehicle (PBS), rapamycin (3 mg/kg), or rapamycin in combination with L-NMMA (80 mg/kg; gavage daily). The serum NO levels and iNOS mRNA in liver CD11b$^+$Gr1$^+$ cells were analyzed. (C) The blocking of NO production significantly decreased the inhibitory ability of rapamycin-treated CD11b$^+$Gr1$^+$ MDSCs, isolated from liver tissue of CIH mice. (D) The blocking of NO production significantly recovers the serum ALT level and liver histologic score (E) from PBS and rapamycin in combination with L-NMMA treatment groups, respectively. (F) CIH was induced by low-dose Con A (15 mg/kg) intravenous injection in C57BL/6 mice. Experimental mice were treated by PBS, rapamycin (3 mg/kg), or rapamycin in combination with L-NMMA (80 mg/kg; gavage daily). At 40–60 h after Con A injection, liver CD11b$^+$Gr1$^+$ MDSCs were isolated from indicated groups. MDSCs (1×10^6) were transferred into C57BL/6 mice that were pretreated with Gr1 mAb (RB6-8C5) on d −1. Subsequently, CIH were induced by Con A (15 mg/kg). At 40–50 h after cell transfer, liver T cells were isolated. The intracellular expression of IFN-γ and Foxp3 and the proportion between IFN-γ$^+$ and Foxp3$^+$ among T cells (right panel) after PMA + ionomycin stimulation were determined. (G) Expression of iNOS (NOS2) in MDSCs from CIH mice transfected with control shRNA (Ctrl-shRNA) or NOS2-specific shRNA (NOS2-shRNA). (H) Indicated MDSCs as in G were stimulated by LPS (10 ng/ml) for 12 h, and NO production in supernatant was determined with ELISA. (I) The suppressive activities of indicated MDSCs as in G. (J) Indicated MDSCs cocultured with naive CD4$^+$CD62L^{high}CD44^{low}CD25$^-$ T cells under T_H 0 conditions (anti-CD3, 2 mg/ml; anti-CD28, 2 mg/ml; human IL-2) for 5 d. The percentage of indicated CD4$^+$ T cells was determined by FACS at d 5, and typical figures were shown. Data are representative of 3 independent experiments. Data represent the means ± SD, $n = 4$. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the indicated groups.

rapamycin results in greatly down-regulated glycolytic activity in liver MDSCs (Fig. 4C). This showed a consistent pattern with blocking the glycolytic metabolism with 2-DG (Fig. 4C). Therefore, these data indicate an important role of mTOR signaling to promote glycolytic metabolism in MDSC functional activities. FAO also was shown as a major driver of the suppressive activities of MDSCs. So, the FAO flux of MDSCs was measured by the detritiation of [9,10-^{3}H]palmitic acid. Although FAO showed a reduced level in MDSCs in CIH, mTOR disruption treated by rapamycin cannot alter the FAO level of liver MDSCs in CIH (Fig. 4D). These data suggest that mTOR signaling probably is not related to FAO in MDSC functional activities in CIH.

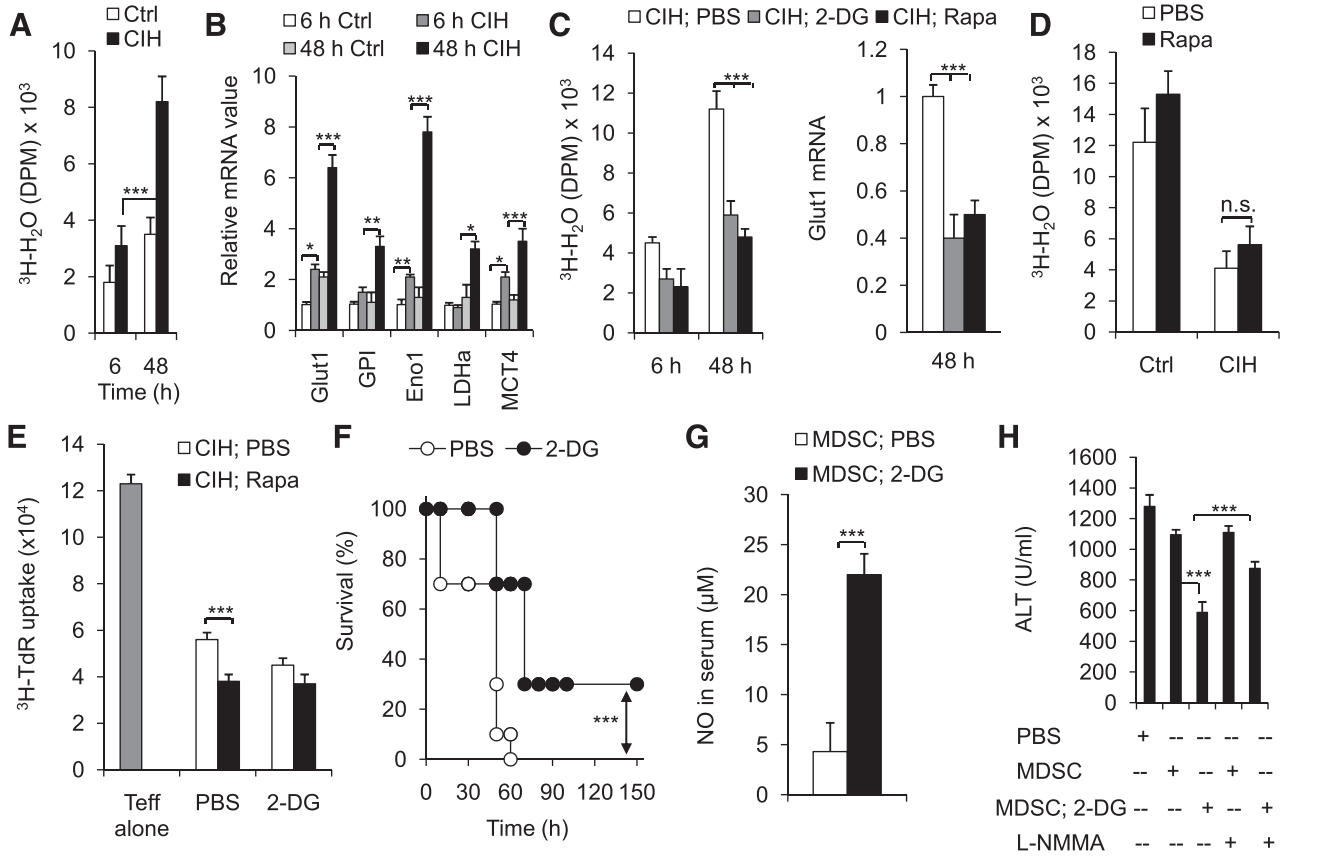

Figure 4. mTOR coordinates MDSC functional activities by orchestrating glycolytic metabolism in protecting against hepatic injury. (A) MDSCs isolated from liver tissue in nontreated control or CIH mice and the glycolytic activity of these cells were measured by the generation of [^{3}H]-labeled H_2O from [3-^{3}H]glucose. (B) All liver tissue was subjected to the same enzymatic digestions. Two indicated time points were selected. RNA was isolated from liver MDSCs in nontreated control or CIH mice from an indicated time point and used for real-time PCR analyses of glycolytic molecules. (C) 2-DG (1 mmol/l) and rapamycin (50 nmol/l) treatment can significantly decrease the generation of [^{3}H]-labeled H_2O from [3-^{3}H] glucose (left) and mRNA expression of glycolytic molecules (right) in liver MDSC of CIH mice. (D) MDSCs isolated from liver tissue in nontreated control or CIH mice and the FAO flux of these cells were measured by the detritiation of [9,10-^{3}H]palmitic acid. (E) Rapamycin (50 nmol/l) treatment increases the suppressive activity of MDSCs through decreasing the level of the glycolytic metabolism. Teff, Effector T cell. (F) Mice were injected intraperitoneally with 2-DG (2 g/kg body weight) or solvent alone (PBS) daily. 2-DG treatment increases the mouse survival in the CIH model, and the serum NO level (G) and ALT level (H) were analyzed. Data are representative of 3 independent experiments. Data represent the means ± SD, $n = 4$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the indicated groups.

Importantly, the blocking of the glycolytic metabolism with 2-DG significantly promoted the suppressive activities of MDSCs isolated from liver in CIH mice (Fig. 4E), prolonged the CIH mouse-survival percentage (Fig. 4F), reduced the serum ALT level (data not shown), and increased the NO production (Fig. 4G). Therefore, these data indicate that mTOR signaling controls MDSC functional activities associated with glycolytic metabolism changes. Whereas 2-DG treatment can significantly reduce the serum ALT level in CIH mice, L-NMMA treatment can significantly rescue these changes (Fig. 4H). Altogether, these data suggest that mTOR glycolytic signaling controls MDSC function changes via NO production in the CIH model.

mTOR signaling from MDSCs suppresses T cell activation and modulates T cell differentiation by targeting the HIF1α-dependent glycolytic pathway

How does mTOR signaling regulate glycolytic activity in directing MDSC functions in IMH? To study the mechanisms that mediate mTOR function and liver MDSCs isolated from rapamycin- or PBS-treated control CIH mice and the downstream signal of mTOR, HIF1α expressions were analyzed by intracellular staining analysis. Rapamycin-treated MDSCs showed a lower activation of transcriptional factor HIF1α (**Fig. 5A**). Thus, mTOR signaling is associated with the HIF1α pathway.

To investigate directly whether HIF1α signaling is required for MDSC functions in vivo in IMH, we crossed mice bearing *loxP*-flanked alleles encoding HIF1α (*HIF1*α$^{fl/fl}$) with Rosa26-Cre-ER^{T2} mice (in which sequence encoding a fusion of Cre recombinase and the ER was recombined into the ubiquitously expressed Rosa26 locus) to generate HIF1α$^{fl/fl}$ Rosa26-Cre-ER^{T2} mice (called "HIF1α^{CreER} mice" here). We treated HIF1α^{CreER} mice with tamoxifen daily for 3 d before CIH and isolated liver CD11b$^+$Gr1$^+$ cells from HIF1α^{CreER} and WT CIH mice. The HIF1α^{CreER} mouse MDSC phenotype showed Ly6C^{high}, CD206^{high}, PD-L1^{high},

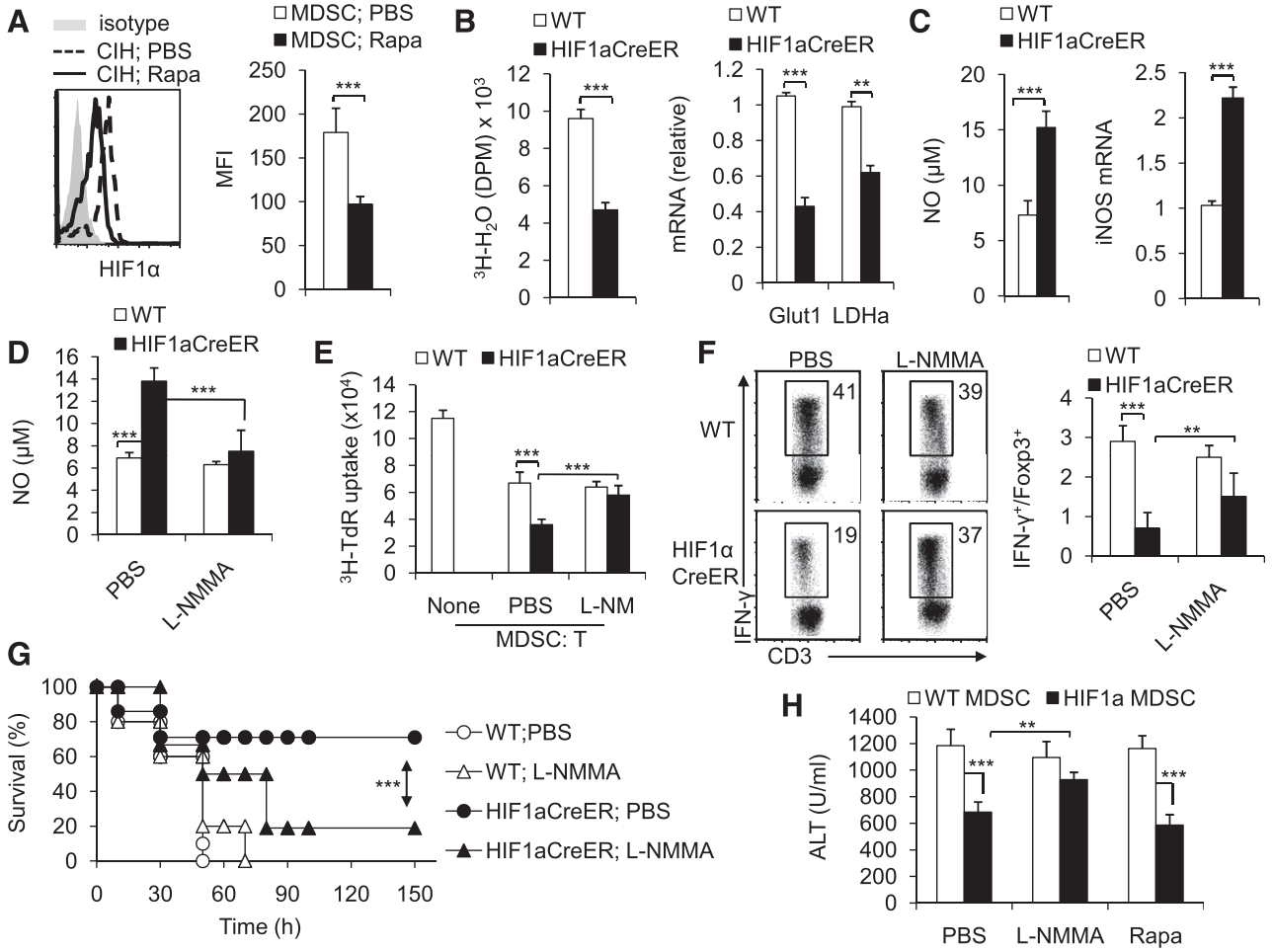

Figure 5. HIF1α-dependent glycolytic metabolism is responsible for MDSC mTOR activity in IMH. (A) HIF1α expression of liver MDSC in PBS or rapamycin-treated CIH mice. MFI, Mean fluorescence intensity. (B) HIF1α^{CreER} mice treated with 2 mg tamoxifen daily for 3 d before Con A injection (15 mg/kg) for CIH induction. MDSCs (1×10^6) isolated from liver tissue of CIH and transferred into C57BL/6 mice that were pretreated with Gr1 mAb (RB6-8C5) on d −1. Subsequently, CIH were induced by Con A (15 mg/kg). At 40 h, the glycolytic activity of these cells was measured by the generation of [^{3}H]-labeled H_2O from [3-^{3}H]glucose, and the mRNA expression of glycolytic molecules was analyzed (right). All liver tissue was subjected to the same enzymatic digestions, and serum NO production and the mRNA expression of liver MDSC were analyzed (C). (D–H) HIF1α^{CreER} mice treated with 2 mg tamoxifen daily for 3 d before CIH induction and L-NMMA (L-NM) treatment (80 mg/kg; gavage daily). MDSCs (1×10^6) isolated from liver tissue of CIH and transferred into C57BL/6 mice that were pretreated with Gr1 mAb (RB6-8C5) on d −1. Subsequently, CIH were induced by Con A (15 mg/kg). (D) At 40–50 h, the serum NO production was analyzed. (E) Liver CD11b$^+$Gr1$^+$ MDSCs isolated from CIH mice. T cells were stimulated with anti-CD3/CD28 antibodies in the presence of liver MDSCs. T cell proliferation was determined with [^{3}H]thymidine incorporation. (F) At 40–50 h after cell transfer, liver T cells were isolated. The intracellular expression of IFN-γ and Foxp3 and the proportion between IFN-γ$^+$ and Foxp3$^+$ among T cells (right) after PMA + ionomycin stimulation were determined. (G) The survival percentage of CIH mice at the indicated group was summarized, and serum ALT level was analyzed (H). Data are representative of 3–4 independent experiments. Data represent the means ± SD, $n = 4$. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the indicated groups.

and CD86^{low} but identical CD124 expressions compared with WT control (Supplemental Fig. 4A and B). Then, we transferred isolated CD11b$^+$Gr1$^+$cells into WT C57BL/6 recipient mice, followed the induction of CIH by injecting low-dose Con A (15 mg/kg). HIF1α^{CreER} MDSC-transferred groups displayed a lower ALT level and histologic injuries in CIH mice compared with the control group (data not shown). The MDSC isolated from HIF1α^{CreER} CIH mice showed lower glycolytic activities (Fig. 5B) and higher NO production (Fig. 5C) compared with WT control. The blocking of iNOS with L-NMMA in vivo significantly reversed the NO production (Fig. 5D) and MDSC-suppressive activities (Fig. 5E). Moreover, at 40–50 h after Con A injection, liver T cells were isolated. In the liver CD3$^+$ cell of CIH mice, L-NMMA treatment could significantly rescue HIF1α^{CreER} MDSC and induce higher Foxp3$^+$ but lower IFN-γ$^+$ expression compared with control (Fig. 5F). Finally, L-NMMA treatment eliminated the HIF1α^{CreER} MDSC-prolonged CIH mouse survival (Fig. 5G) and serum ALT level (Fig. 5H). Altogether, these data reveal that mTOR

signaling from MDSC suppresses T cell activation and modulates T cell differentiation by targeting the HIF1α-dependent glycolytic pathway.

mTOR signals are intrinsic in MDSCs in protecting against immune hepatic injury diseases

To ascertain the intrinsic effects of MDSC mTOR signals in protecting against immune hepatic injury diseases, we observed the effects of BM-derived MDSCs treated with rapamycin compared with vehicle-treated control in vitro. Rapamycin-treated MDSCs showed increased suppressive activities, decreased glycolysis, higher iNOS expression, and down-regulation of HIF1α (**Fig. 6A–E**). Moreover, nuclear translocation decreases of HIF1α in rapamycin-treated MDSCs also support that HIF1α is involved in this modulation. Importantly, adoptive transfer of rapamycin-treated, BM-derived MDSCs can effectively attenuate mouse survival in IMH (Fig. 6G). These data suggest that mTOR signaling is intrinsic in MDSCs in protecting against IMH.

Effects of mTOR signaling in the local infiltrating MDSCs in immune hepatic injury diseases

During CIH, liver MDSCs are recruited into the liver tissue, where they inhibit the activity of T cells and polarize the local T_H1 responses. We crossed mice bearing *loxP*-flanked alleles encoding mTOR ($mTOR^{fl/fl}$) with Rosa26-Cre-ER^{T2} mice to generate $mTOR^{fl/fl}$ Rosa26-Cre-ER^{T2} mice (called "$mTOR^{CreER}$ mice" here). To determine if liver local-infiltrating MDSCs require mTOR signaling to inhibit actively the T cell activity and the proinflammatory T_H1 responses and promote anti-inflammatory iT_{reg} effects, we treated $mTOR^{CreER}$ mice with tamoxifen daily for 3 d before CIH. At 40–50 h, the liver MDSCs isolates and the downstream pathways of mTOR, including S6 and HIF1α, were determined by intracellular staining analysis of phosphorylated proteins or transcriptional factors. As expected, $mTOR^{CreER}$ MDSC showed a lower activation of phosphorylation of S6 and transcriptional factor HIF1α (**Fig. 7A**). Consistently, the $mTOR^{CreER}$ mouse MDSC phenotype copied $HIF1α^{CreER}$ mouse changes (Supplemental Fig. 4C). Thus, these indicate that mTOR-HIF1α signaling is associated with MDSC activities.

To investigate directly whether mTOR signaling required for MDSC functions in vivo, we treated $mTOR^{CreER}$ mice with tamoxifen daily for 3 d before CIH and isolated liver $CD11b^+Gr1^+$ cells from $mTOR^{CreER}$ and WT CIH mice. Then, we transferred isolated $CD11b^+Gr1^+$ cells into WT C57BL/6 recipient mice, following the induction of CIH, by injecting low-dose Con A (15 mg/kg). As expected, $mTOR^{CreER}$ MDSC-transferred groups

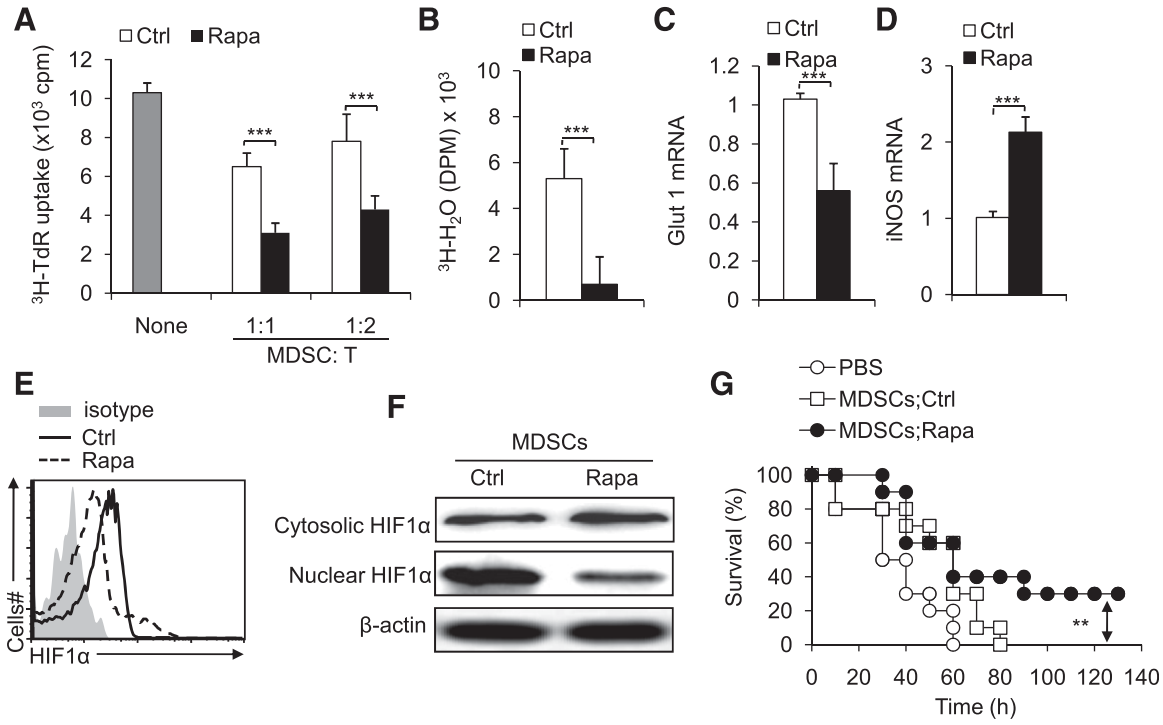

Figure 6. mTOR signaling negatively controls MDSC functional activities and protects against hepatic injury. (A) BM cells were cultured for 4 d in the presence of 40 ng/ml mouse recombinant GM-CSF and vehicle (PBS) or rapamycin (1 μm; Sigma-Aldrich), and the immunosuppressive activities of sorted $CD11b^+Gr1^+$ MDSCs were analyzed as described above. The glycolytic activity was measured by the generation of [^{3}H]-labeled H_2O from [3-^{3}H]glucose (B); Glut1 mRNA expressions (C) and iNOS mRNA expressions (D) were detected by quantitative PCR from sorted MDSCs as in A. (E) HIF1α expressions were determined with FCM. (F) HIF1α nuclear or cytosolic expressions were determined with Western blot. (G) Total of 1×10^6 sorted indicated that BM-derived MDSCs were transferred into C57BL/6 mice via intravenous injection. At 10–12 h after cell transfer, both groups were injected with a low-dose Con A (15 mg/kg), and the mouse survival curve was plotted. Values in panels are shown as means ± SD (n = 5–20) from 4 independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the Con A-alone group.

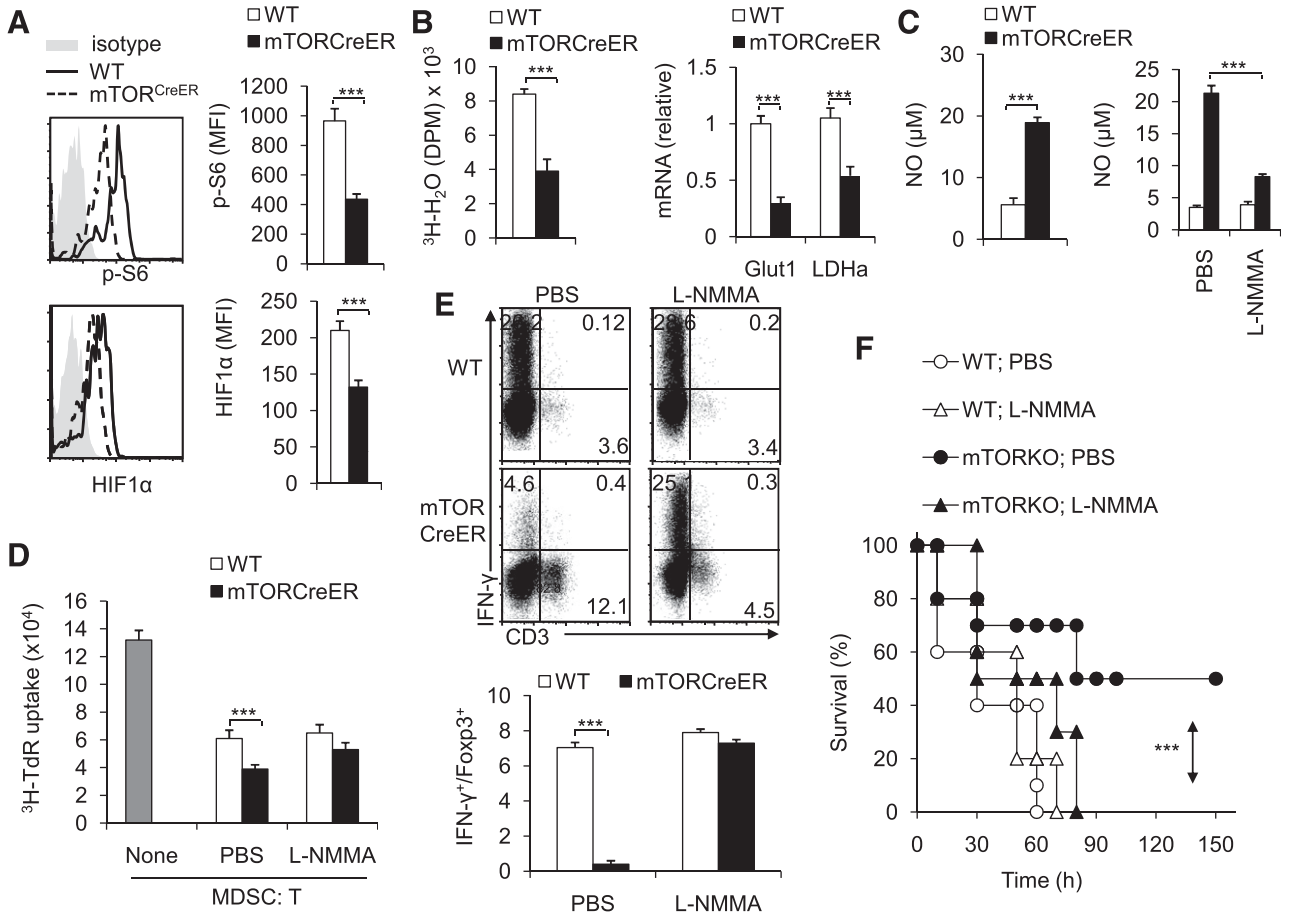

Figure 7. MDSC mTOR activity is required for protecting against immune-mediated hepatic injuries by the HIF1α-dependent glycolytic pathway. (A) mTOR^{CreER} mice treated with 2 mg tamoxifen daily for 3 d before Con A injection (15 mg/kg) for CIH induction. Expression of phosphorylated S6 and HIF1α in liver MDSC isolated from WT or mTOR^{CreER} CIH mice. (B and C) mTOR^{CreER} mice treated with 2 mg tamoxifen daily for 3 d before Con A injection (15 mg/kg) for CIH induction. MDSCs (1×10^6) isolated from liver tissue of CIH and transferred into C57BL/6 mice that were pretreated with Gr1 mAb (RB6-8C5) on d −1. Subsequently, CIH were induced by Con A (15 mg/kg). At 40 h, the glycolytic activity of these cells was measured by the generation of [^{3}H]-labeled H_2O from [3-^{3}H]glucose, and the mRNA expression of glycolytic molecules was analyzed (right). All liver tissue was subjected to the same enzymatic digestions, and serum NO production of liver MDSC was analyzed (C). (D–F) mTOR^{CreER} mice treated with 2 mg tamoxifen daily for 3 d before CIH induction and L-NMMA treatment (80 mg/kg; gavage daily). MDSCs (1×10^6) isolated from liver tissue of CIH and transferred into C57BL/6 mice that were pretreated with Gr1 mAb (RB6-8C5) on d −1. Subsequently, CIH were induced by Con A (15 mg/kg). (D) At 40–50 h, liver CD11b$^+$Gr1$^+$ MDSCs isolated from CIH mice. T cells were stimulated with anti-CD3/CD28 antibodies in the presence of liver MDSCs. T cell proliferation was determined with [^{3}H]thymidine incorporation. (E) At 40–50 h after cell transfer, liver T cells were isolated. The intracellular expression of IFN-γ and Foxp3 and the proportion between IFN-γ$^+$ and Foxp3$^+$ among T cells (lower) after PMA + ionomycin stimulation were determined. (F) The survival percentage of CIH mice at the indicated group was summarized, and serum ALT level was analyzed. mTORKO, mTOR knockout. Data are representative of 2–3 independent experiments. Data represent the means ± SD, $n = 4$. ***$P < 0.001$ compared with the indicated groups.

displayed a lower ALT level in CIH mice compared with the control group (data not shown). Importantly, the MDSC isolated from mTOR^{CreER} CIH mice showed lower glycolytic activities (Fig. 7B) and higher NO production (Fig. 7C) and suppressive activities (Fig. 7D) compared with WT control. The blocking of iNOS with L-NMMA in vivo, significantly reversed the NO production (Fig. 7C) and MDSC-suppressive activities (Fig. 7D). Moreover, at 40–50 h after Con A injection, liver T cells were isolated. In the liver CD3$^+$ cell of CIH mice, L-NMMA treatment could significantly rescue mTOR^{CreER} MDSC and induce higher Foxp3$^+$ but lower IFN-γ$^+$ expression compared with control (Fig. 7E). Finally, L-NMMA treatment eliminated the mTOR^{CreER} MDSC-prolonged CIH mouse survival (Fig. 7F) and serum ALT level (data not shown). Taken together, these data reveal that mTOR signaling from MDSC suppresses T cell activation and modulates T cell differentiation by targeting the HIF1α-dependent glycolytic pathway.

DISCUSSION

Although emerging evidence indicates that blocking mTOR signaling with rapamycin could negatively regulate immune-mediated liver diseases [24–27], the potential mechanism remains unclear. Here, we show that MDSCs are essential for

rapamycin-protected CIH and PIH—2 kinds of typical immunologic hepatic injuries—by blocking mTOR signaling. We further provided mechanistic insights by showing direct T cell-inhibitory effects of MDSCs. This is inconsistent with the previously established role of MDSC in limiting the autoimmune hepatic inflammatory injury [28]. Our studies reveal a previously unknown feature of the MDSC function in immune homeostasis, i.e., the reprogramming of T cell differentiation from T_H1 to iT_{regs}, which represents a novel mechanism of mTOR signaling and down-regulation of mTOR signaling-mediated protection against immune-mediated tissue injuries. mTOR negatively regulates MDSC functions in IMH, and mTOR activities in MDSCs mediate T_H1 and T_{reg} conversion during hepatic injury. Importantly, depletion of MDSCs significantly recovered the conversion between T_H1 and T_{regs} in IMH. However, adoptive transfer of MDSCs effectively promoted the conversion between T_H1 and T_{regs} in protecting against IMH. These data collectively suggest that MDSCs are critical for protecting against T cell-mediated IMH by inducing the T cell subset conversions. Otherwise, as well known and showed in this study, mTOR also directs T cell activation and differentiation in T cell-mediated IMH. Therefore, mTOR signals might play double roles in T cell-mediated hepatic injury through MDSC-dependent or -independent regulatory mechanisms.

In yeast and mammalian cells, mTOR integrates environmental cues in terms of nutrients, energy, and growth factors and directs cell growth and proliferation. Hyperactivated mTOR is observed in many types of cancers and autoimmune diseases [29, 30]. Downstream, mTOR signaling proceeds via 2 distinct complexes: mTORC1 and mTORC2 [31, 32]. There are pharmacologic consequences as well. Rapamycin and other rapalogs bind to FKBP12 and by binding to the FKBP12/rapamycin-binding site on mTOR, are believed to block the ability of regulatory-associated protein of mTOR to bind to mTOR, thus inhibiting mTORC1. With downstream mTORC1 and mTORC2 signaling from mTORC1, the phosphorylation of S6 is a standard marker of mTORC1 activity [32]. S6 plays a critical role in promoting translation of mRNA and is activated by mTORC1, and its phosphorylation by mTOR leads to its inactivation [8]. In the present study, we reported that $CD11b^+Gr1^+$ MDSCs had relatively increased phosphorylation of S6, a major downstream target of mTOR (mTORC1), with the time- and dose-dependent manner in IMH. The blocking of mTOR with rapamycin or mTOR deficiency can efficiently increase the suppressive activities of $CD11b^+Gr1^+$ MDSCs and redirected the reciprocal differentiation of T_H1 and T_{reg} in a murine immune-mediated, hepatic-injury murine model, whereas it reverses the phosphorylation level of S6. These findings reveal mTOR (mTORC1) activity is significantly required in $CD11b^+Gr1^+$ MDSC function in protecting against immune-mediated hepatic injuries.

It has been shown that the mTOR pathway acts to modulate the function of DCs in a manner that is dependent on the activation or maturation state and DC type studied [8, 33–36]. Administration of rapamycin in vivo or in vitro can impair DC maturation, such that DCs remain tolerogenic, failing to up-regulate costimulatory molecules [13]. Researchers also studied the impact of rapamycin on TLR-induced responses of freshly isolated myeloid classical DCs and in vitro-differentiated monocyte-derived DCs [37]. They found that rapamycin treatment could act to inhibit or potentiate cytokine secretion in a manner that was dependent on the cell type, the nature of the stimulus, and the specific cytokine studied [38]. Moreover, mTOR signaling is required for DCs to gain T cell-stimulatory capacity during differentiation, whereas mTOR signaling can also limit the proinflammatory capacity of mature DCs [14]. However, whether mTOR signaling affects the functions of $CD11b^+Gr1^+$ MDSCs remains largely unexplored. In the present study, we directly showed that mTOR signaling negatively controls the suppressive activity of MDSCs in a mouse hepatic injury model and redirects the differentiation of T_H1 and iT_{regs} for protecting against immune-mediated hepatic injuries.

Recent observations also indicate that HIF1α, the regulated subunit of the transcription factor HIF1, is a novel "command and control" protein in activities of myeloid leukocytes [39, 40]. HIF1α is controlled by mTORC1 in a 4E-BP1-dependent manner [41, 42]. Data are emerging that HIF1α plays critical roles in regulating glucose metabolism. HIF1α exclusively regulates ATP generation and contributes to cytokine production, as well as myeloid cell function in acute inflammation [39, 40, 43]. HIF1α is essential for the regulation of glycolytic capacity in myeloid cells or T_H9 cells: when HIF1α is absent, the cellular ATP pool is drastically reduced [19, 39, 44]. Consistently, our results showed that the HIF1α-deficient MDSC had a diminished glycolytic activity and increased suppressive functions in a murine IMH model.

Much emphasis has been placed on the transcriptional mechanisms that orchestrate MDSC-suppressive activity, but how adaptive immunity, including T cell functions, is integrated and controlled by MDSCs for its functional activation remains obscure. We show that mTOR-HIF1α-dependent metabolic programming is a central mechanism to control the functional activities of MDSCs and programming the T cell lineage differentiation in making decisions about immunity and tolerance in hepatic immune homeostasis modulation. The mTOR (mTORC1)-HIF1α axis promotes the glycolytic metabolism and negatively controls the MDSC functions by NO production. Thus, the mTOR-dependent glycolytic metabolism provides a novel link between innate immunity and adaptive immunity, and mTOR signaling from MDSC controls its function and reprogramming adaptive T cell lineage differentiation to protect against IMH inflammation. Notably, mTOR-dependent metabolic programming operates under immune stimulation, and this provides important mechanistic insights into the immune defense to integrating the innate immunity and adaptive immunity. We propose that MDSCs adopt the evolutionarily mTOR signaling pathway to link the innate immunity and adaptive immunity to metabolic activity and functional fitness, therefore implicating mTOR as a functional rheostat to program MDSC-suppressive activity through a nonconventional mechanism.

To investigate directly whether HIF1α or mTOR signaling is required for MDSC functions in vivo in IMH, we used $HIF1\alpha^{CreER}$ mice and $mTOR^{CreER}$ mice in this investigation. Rosa26-Cre is not a myeloid-specific Cre to deplete mTOR and HIF1α

specifically in MDSCs; therefore, the adoptive transfer experiments are the most used methods to observe the effects of mTOR-HIF1α deficiency in MDSCs in IMH. Consistently, BM-derived MDSCs were used in the present study, and further supporting roles of mTOR and HIFα signaling are intrinsic in regulating MDSC functions and redirecting T cell subset differentiation in protecting against immune hepatic injuries.

In summary, our results reveal mTOR negatively controls MDSC-suppressive functions and programming the reciprocal differentiation of T_H1 and iT_{reg} in immune-mediated hepatic inflammatory diseases by the HIF1α-dependent glycolytic mechanism, which collectively demonstrates that mTOR acts as a fundamental rheostat in MDSCs to link both innate and adaptive immune cell components to glycolytic pathways and functional fitness and highlights a central role of metabolic programming of MDSC-suppressive activity in immunity and tolerance. The pathway in MDSCs may be explored for the development of new therapeutics for autoimmunity, cancer, and infection.

AUTHORSHIP

X.C., Z.Z., Y.B. and Z.F. designed and conducted the experiments with cells and mice and analyzed data; P.G. and Y.L. did the experiments with cells; Q.Y., A.J., and J.W. did the experiments with mice and analyzed histological data; L.X. and H.Y. did the experiments with mice and wrote the manuscript; G.L. developed the concept, designed and conducted the experiments with cells and mice, analyzed data, wrote the manuscript and provided overall direction.

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
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