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Protein 4.1R regulates M1 macrophages polarization via glycolysis, alleviating sepsis-induced liver injury in mice

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ABSTRACT

Acute liver injury (ALI) is a common clinical disease caused by sepsis, metabolic syndrome, hepatitis virus. Macrophage plays an important role in the development of ALI, which is characterized by polarization and inflammatory regulation. The polarization process of macrophages is related to membrane binding proteins and adaptors. Protein 4.1R acts as an adaptor, linking membrane proteins to the cytoskeleton, and is involved in cell activation and cytokine secretion. However, whether protein 4.1R is involved in regulating macrophage polarization and inflammation-induced liver injury remains unknown. In this study, protein 4.1R is identified with the special effect on macrophage M1 polarization. And it is further demonstrated that protein 4.1R deficiency significantly enhance glycolytic metabolism. Mechanistically, the regulation of protein 4.1R on pyruvate kinase M2 (PKM2) plays a key role in glycolysis metabolism. In addition, we found that protein 4.1R directly interacts with toll-like receptor 4 (TLR4), inhibits the activation of the AKT/HIF-1α signaling pathway. In conclusion, protein 4.1R targets HIF-1α mediated glycolysis regulates M1 macrophage polarization, indicating that protein 4.1R is a candidate for regulating macrophage mediated inflammatory response. In conclusion, we have revealed a novel function of protein 4.1R in macrophage polarization and ALI, providing important insights into the metabolic reprogramming, which is important for ALI therapy. We have revealed a novel function of protein 4.1R in macrophage polarization and ALI, providing important insights into the metabolic reprogramming, which is important for ALI therapy.

1. Introduction

Sepsis was latest defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection [1–3]. About 8 million people die from sepsis every year due to the high mortality [4,5]. Many organs eventually be affected, liver injury occurs earlier in sepsis. As a lymphoid organ, the liver has important physiological functions such as detoxification, energy production, nutrient conversion and plays a central role in metabolic and immunological homeostasis [6–9]. Kupffer cells, the resident macrophages in the liver, are the largest group of innate macrophages in the human body, accounting for about 80 %-90 % of the total number of innate macrophages. Under pathological conditions, Kupffer cells can be activated by endotoxin and other substances, and release a variety of inflammatory mediators, which are involved in the occurrence and development of liver infection [10,11].

The characteristics of macrophages are diversity and plasticity. Macrophages can differentiate into distinct subtypes due to their plasticity and have distinct metabolic characteristics [12]. Under the stimulation of TLR ligands and interferon-γ (IFN-γ), activated M1 macrophages secrete interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and other inflammatory factors promote inflammatory response [12,13]. M2 macrophages, stimulated by IL-4/IL-13, is involved in anti-inflammatory response and tissue remodeling [10,14,15]. Metabolic reprogramming plays a crucial role in the signal involved in macrophage polarization. The main metabolic characteristics of M1 macrophage polarization are increased glycolysis to rapidly generate energy [12]. E. M. Palsson-McDermott et al. reported that pyruvate kinase M2 (PKM2) is a key determinant of macrophage glycolytic reprogramming [16]. Thus,

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regulation of macrophage polarization and metabolism are potential strategies for ALI therapeutic strategies [17]. The polarization process of macrophages is related to membrane binding proteins and adaptors. Protein 4.1R belongs to the protein 4.1 family, which is the main component of the cell membrane [18,19]. As a membrane skeleton protein, protein 4.1R can connect a variety of transmembrane proteins and blood shadow protein actin [20]. It plays an important role in the body's physiological activities [21–23]. Research shows that protein 4.1R regulates T cell activation and affects the body's immune response [24]. It plays a negative role in regulating $CD8^+$ T cells [25]. It can also prevent pathogenic autoimmunity in the progression of experimental autoimmune encephalomyelitis (EAE) by inhibiting $CD4^+$ T cell activation [26]. However, the function and mechanism of protein 4.1R in macrophages are still unclear, especially the immune effect and mechanism of protein 4.1R in sepsis-induced liver injury have not been reported. Here, we report the role of 4.1R protein in lipopolysaccharide (LPS)-treated macrophage polarization and metabolism, and knockout of 4.1R aggravates LPS-induced acute liver injury in mice. Furthermore, protein 4.1R plays a protective role in the liver by negatively regulating TLR4/AKT/HIF-1α-mediated glycolysis and regulating M1 macrophage polarization. Further mechanism studies have shown that protein 4.1R protects liver by negatively regulating TLR4/AKT/HIF-1α-mediated glycolysis and regulating M1 macrophage polarization.

2. Materials and methods

2.1. Animals

Wild C57BL/6 mice aged 6–8 weeks (Wide-type, $4.1R^{+/+}$) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. The 4.1R knockout C57BL/6 mice (Knock out, $4.1R^{-/-}$) with specific pathogen-free (SPF) level were donated by the New York Blood Center. The mice of both genotypes were raised in the SPF animal room, the light cycle was 12/12 h, and the diet of the mice was not restricted during the experiment. All experiments were compliant with the study was conducted in accordance with the Declaration of Helsinki and authorized by the Institute of College of Life Sciences of Zhengzhou University (permit No. SYXK 2019–0002). The mouse sepsis model was established by intraperitoneal injection of 10 mg/kg lipopolysaccharide (LPS) (L8274, Sigma-Aldrich, USA) into $4.1R^{+/+}$ and $4.1R^{-/-}$ mice, respectively, as described previously [27].

2.2. Macrophage extraction and culture

Mouse bone marrow-derived macrophages (BMDM) was induced by wild-type and $4.1R^{-/-}$ mice with 6–8 weeks, weight of about 25 g and good growth status. The mouse macrophage strain RAW264.7 with 4.1R stably knocked out by CRISPR/Cas 9 system previously preserved was used in the experiment. Briefly, mechanically separate bilateral femurs of mice and extract femur cells, and then adjust the concentration of isolated cells to 2×10^6 cultured in dulbecco's modified eagle medium (DMEM) (SH30022.01, Hyclone, USA) containing 10 % fetal bovine serum (FBS) (10099141C, Gibco, USA) and 40 ng/mL macrophage colony stimulating factor (M-CSF) (315-03, PeproTech, USA). On the third and fifth days, half of the medium was replaced, and on the seventh day, the cells had matured into BMDM. These cells were incubated with LPS (250 ng/mL) for 24 h to establish an inflammatory model for further experiments.

2.3. Serum biochemical markers determination

After intraperitoneal injection of LPS into mice, mouse eyeballs were removed for blood collection at 3 and 24 h. Place the collected blood in a refrigerator at 4 °C for approximately 4 h. Subsequently, centrifuge the serum at a speed of 3000 r/min in a precooled centrifuge at 4 °C for 10 min. Gently aspirate the serum and place it in a refrigerator at −80 °C for later use. The levels of serum aspartate aminotransferase (AST) (C010-2-1, Nanjing Jiancheng Bioengineering Institute, China), alanine aminotransferase (ALT) (C009-2-1, Nanjing Jiancheng Bioengineering Institute, China), lactate dehydrogenase (LDH) (A020-2-2, Nanjing Jiancheng Bioengineering Institute, China) were detected at 3 h and 24 h after intraperitoneal injection of LPS in mice according to the kit instructions.

2.4. Flow cytometry

The experimental operation was performed according to the flow cytometry staining scheme of the cell surface of Biogene Company of the United States. Simply put, cells were washed in a phosphate buffer containing (PBS) 2 % FBS, and then stained with 2.5 μL PerCP/Cyanine5.5 anti-mouse F4/80 antibody (123127, Biolegend, USA) and 1 μL APC anti-mouse CD11c antibody (117309, Biolegend, USA) in 100 μL PBS containing 2 % FBS for 30 min. Then wash twice in phosphate buffer containing 2 % FBS (10099-141, Gibco, USA). Finally, cells were counted by BD LSRF or Tessa flow cytometry, and FlowJo7.6.1 software was used to analyze the data.

2.5. Analyses of mRNA expression

Mice were sacrificed by injection of pentobarbital sodium anesthesia. Small pieces of liver tissue of equal weight were weighed and placed in tissue homogenate tubes for subsequent RNA extraction. RNA was extracted from liver tissue and BMDM using TRIzol reagent (15596026, Invitrogen, USA), and the first strand cDNA was synthesized using RNA as a template (K1622, Thermo Fisher, USA). Quantitative Real-time PCR reaction was performed using Light Cycle® 480 (Roche, Switzerland), and the reaction condition was 95 °C, 5 min; 95 °C, 10 s, 60 °C, 10 s, 72 °C, 30 s, 40 cycles; the fluorescence value of each cycle was captured at 60 °C. The primer sequence is shown in Table 1.

2.6. Western blot analysis

RIPA lysate (P0100, Solarbio, China) was used to lyse cells, extract total protein, detect protein concentration with BCA protein determination kit (ZJ101L, Epizyme, China). Blots were incubated overnight at 4 °C with the following primary antibodies: PKM2-specific monoclonal antibody (60268-1-Ig, 1:500, Proteintech, China), AKT antibody (9272,

Table 1
q-PCR primer sequences.

Primer name	Primer sequence (5′-3′)
TNF-α	Forward: ATGAGCACTGAAAGCATGATC
	Reverse: TCACAGGGCAATGATCCCAAAGTAGACCTGCCC
IL-6	Forward: GAGGATACCACTCCCAACAGACC
	Reverse: AAGTGCATCATCGTTGTTCATACA
CCL-2	Forward: TTCACAGTTGCCGGCTGG
	Reverse: TGAATGAGTAGCAGCAGGTGAGTG
iNOS	Forward: GAGATTGGAGGCCTTGTG
	Reverse: TCAAGCACCTCCAGGAACGT
PFKFB3	Forward: GTGGAAGGCACTCAACGAGA
	Reverse: CATGTTTTGTCCGGGCAGC
IL-1β	Forward: ATGGCAGAAGTACCTAAGCTC
	Reverse: TTAGGAAGACACAAATTGCATGGTGAACTCAGT
HK2	Forward: TTTTAGGTCAGTCGGCGTTTCAG
	Reverse: ACATTGGTGTCTTCCCGTTCTTC
TLR4	Forward: AGATCTGAGCTTCAACCCCTTG
	Reverse: GCAGAAACATTCGCCAAGCA
PKM2	Forward: TCCCCTCCCCTATCCTTTCCATT
	Reverse: GGGCCAGAAGTCGTCATCTACACT
LDH	Forward: TGTCTCCAGCAAAGACTAACTGT
	Reverse: GACTGTACTTGACAATGTTGGGA
HIF-1α	Forward: CGTGTTATCTGTCGCTTTGAGTC
	Reverse: GTCTGGCTGCTGTAATAATGTTCC
18s RNA	Forward: CTTAGAGGGACAAGTGGCG
	Reverse: ACGCTGAGCCAGTCAGTGTA

1:1000, Cell Signaling Technology, USA), phospho-AKT-Ser473 antibody (9271, 1:1000, Cell Signaling Technology, USA), HIF-1α antibody (36169, 1:1000, Cell Signaling Technology, USA), mouse anti-4.1R antibody (B-11) (sc-166759, 1:100, Santa Cruz Biotechnology, UAS), rabbit anti-mouse 4.1R antibody (13014-1-AP, 1:2000, Proteintech, China), TLR4 antibody (19811-1-AP, 1:1000, Proteintech, China), GAPDH polyclonal antibody (10494-1-AP, 1:10000, Proteintech, China), β-Actin antibody (81115-1-RR, 1:10000, Proteintech, China). Next, the membrane was incubated with horseradish peroxidase coupled with secondary antibodies (SA00001-2, 1:5000, Proteintech, China). Under the condition of avoiding light, the ECL super sensitive luminous solution A and solution B (R0020, Thermo Fisher, USA) are mixed by the same volume. The PVDF transfer membranes (88518, Thermo Fisher, USA) is immersed in the prepared luminous solution and detected by the Azure c600 multi-function molecular imager.

2.7. Co-immunoprecipitation assay

First, the protein concentration was adjusted to 2 μg/μL. Then, incubation with primary antibodies rabbit anti-mouse 4.1R antibody (13014-1-AP, 1:2000, Proteintech, China) or mouse IgG (sc-2025, 1:100, Santa Cruz Biotechnology, UAS), gentle rocking overnight at 4 °C. Then, 30 μL protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology, USA) was added to each immunoprecipitation mixture incubated in oscillation incubator at 4 °C and 75 rpm for 4 h. Centrifuge at 3000 rpm for 5 min in a 4 °C centrifuge and wash with RIPA. Boil the sample in boiling water for 5 min for further western blot analysis.

2.8. Immunofluorescence microscopy

After macrophage climbing tablets were made, 1 μg/mL of LPS was added to the stimulation group and an equal volume of PBS was added to the control group. Cells were fixed with 4 % PFA (P0099, Beyotime, China) for 10 min and permeabilized with 0.1 % Triton X-100 (P0096, Beyotime, China) in PBS, then incubated in 5 % BSA with PBS for 30 min to minimize nonspecific antibody binding. Cells were then incubated with mouse anti-4.1R antibody (sc-166759, 1:100 Santa Cruz Biotechnology, USA), Rabbit anti-TLR4 antibody (19811-1-AP, 1:1000, Proteintech,China) for 12 h at 4 °C refrigerator followed by 2 h of incubation at room temperature with Goat anti-Rabbit IgG labeled with Alexa 488 (SA00013-2, 1:100, Proteintech, China) and Goat anti-mouse IgG labeled with Alexa 594 (SA00013-3, 1:100, Proteintech, China). DAPI (C1005, Beyotime, China) was used to stain the nuclear. The images were obtained using the LSM 510 META confocal microscope (Carl Zeiss, USA).

2.9. Determination of hexokinase (HK) activity and lactate secretion

The extraction and cultivation of cells are described earlier. Ultrasound fragmentation of cells (ice bath, power 200 W, ultrasound for 3 s, interval of 10 s, repeated 30 times). Place the obtained supernatant on ice for later use. Detect the activity of hexokinase (BC0745, Solarbio, China) and lactate secretion (A019-2-1, Nanjing Jiancheng Bioengineering Institute, China) in the supernatant according to the instructions of the reagent kit.

2.10. Statistical analysis

All data are expressed as the mean ± SEM. The experimental data were statistically processed with GraphPad Prism 9.0 software. The relative expression between multiple groups was analyzed by single factor ANOVA and comparisons between two groups was analyzed by an independent sample T test. The difference was statistically significant ($P < 0.05$).

3. Results

3.1. R knockout aggravates LPS-induced acute liver injury

To explore the effect of protein 4.1R on sepsis, we evaluated the survival rate of LPS-induced sepsis mice within 72 h. As presented in (Fig. 1A), the survival rate of wild-type sepsis mice was higher than that of $4.1R^{-/-}$sepsis mice ($p = 0.0046$). The LPS-induced $4.1R^{-/-}$ septic mice did not survival in 46 h. However, the survival rate of $4.1R^{+/+}$ sepsis mice in 72 h was still 37 %. This result shows that $4.1R^{-/-}$ mice have a higher incidence rate and are more prone to sepsis than wild type mice ($p = 0.0032$). Recent advances have revealed that sepsis liver injury is one of the important factors of multiple organ dysfunction and sepsis induced death. H&E staining was performed to explore the effect of protein 4.1R on liver injury. As shown in (Fig. 1B), the hepatic tissue of LPS-induced $4.1R^{+/+}$ septic model group displayed extensively swollen, the cytoplasm was vacuolated (black arrow), and some small extramedullary hematopoiesis lesions (yellow arrow) were seen in the lobules. Nevertheless, the $4.1R^{-/-}$ septic model group displayed extensive degeneration of liver cells, more small vacuoles (black arrows) in cytoplasm, several small extramedullary hematopoiesis lesions (yellow arrows) in liver sinuses, occasional necrosis of liver cells, and cytolysis (green arrows). This result shows that $4.1R^{-/-}$ mice are more susceptible to sepsis, and $4.1R^{-/-}$ mice have more severe liver tissue damage than $4.1R^{+/+}$ mice. The levels of AST, ALT and LDH in serum were used to analyze the liver function of mice (Fig. 1C). The serum AST, ALT and LDH levels in the sepsis model group were higher than those in the control group in varying degrees, and the $4.1R^{-/-}$ sepsis model group was higher than the $4.1R^{+/+}$ sepsis model group. The above results indicate that protein 4.1R knockout aggravates the death of septic mice and liver damage was more serious.

3.2. Increased macrophage infiltration in $4.1R^{-/-}$ Septic mice

Recruitment of in situ mature BMDM increases macrophages in the liver macrophage pool, which contributes to the development and regression of liver tissue inflammation. Therefore, we discussed the effect of protein 4.1R on macrophage infiltration. As shown in (Fig. 2A-B), there had higher positive expression rate of F4/80 in liver tissue of septic mice, and more infiltration amounts of macrophages. More macrophages were found in the $4.1R^{-/-}$ septic mice group. The results showed that the absence of protein 4.1R could lead to more macrophage infiltration in the process of inflammatory response.

3.3. Increased inflammatory cytokine production in $4.1R^{-/-}$ septic mice

After infection with LPS, the body triggers a cytokine storm and rapidly produces a variety of cytokines. IL-1β is an important indicator of proinflammatory response after infection, so the expression of IL-1β in liver tissue was detected. Results are as shown in (Fig. 3A-B), the positive IL-1β expression rate in $4.1R^{-/-}$ septic mice was significantly higher than that in $4.1R^{+/+}$ septic group. Secondly, we detected IL-1β and other proinflammatory cytokines by q-PCR (Fig. 3C). The secretion of inflammatory factors and monocyte chemokines in liver tissue of $4.1R^{-/-}$ group were significantly higher than that of $4.1R^{+/+}$ group. The above results indicate that 4.1R deletion causes more significant up-regulation of pro-inflammatory cytokine gene expression in LPS-induced mouse liver tissue.

3.4. Protein 4.1R deficiency enhance M1 polarization

Flow cytometry was used to detect the expression of F4/80 and CD11c in $4.1R^{+/+}$ and $4.1R^{-/-}$ macrophages (Fig. 4A-B). The results showed that the proportion of M1 macrophages of both genotypes increased after LPS stimulation. After 6 h of LPS stimulation, the proportion of M1 macrophages in $4.1R^{-/-}$ BMDM was significantly higher

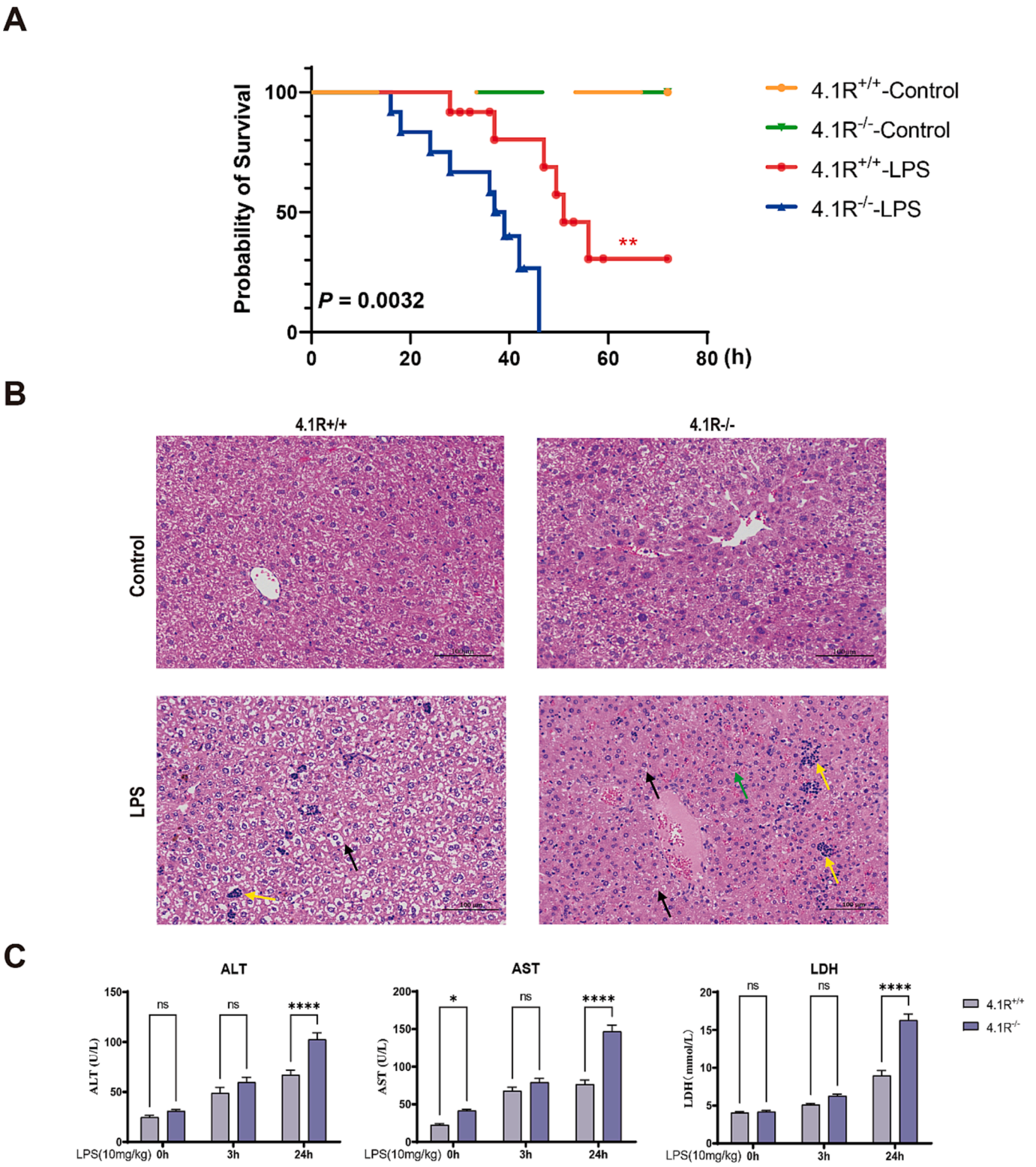

Fig. 1. Effect of 4.1R on Survival rate and liver function in LPS-induced sepsis mice. (A) Log-rank test for trend analysis for 72 h survival proportions of sepsis in the wild type and $4.1R^{-/-}$ mice, **, $P = 0.0046$, $4.1R^{+/+}$-LPS group compared with $4.1R^{-/-}$ -LPS group (n = 3 mice per control group, n = 9 mice per model group). **(B)** Effect of 4.1R on liver injury of sepsis mouse. Representative images of H&E staining of mouse liver sections. The black, yellow, and green arrows indicated hepatocyte swelling and cytoplasmic vacuolization, extraosseous hematopoietic lesions, hepatocyte necrosis and nuclear dissolution, respectively. Scale bar: 100 μm. **(C)** Serum ALT, AST and LDH levels were measured in $4.1R^{+/+}$ and $4.1R^{-/-}$ mice treated with 0, 3, or 24 h LPS (10 mg/kg). Data are expressed as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

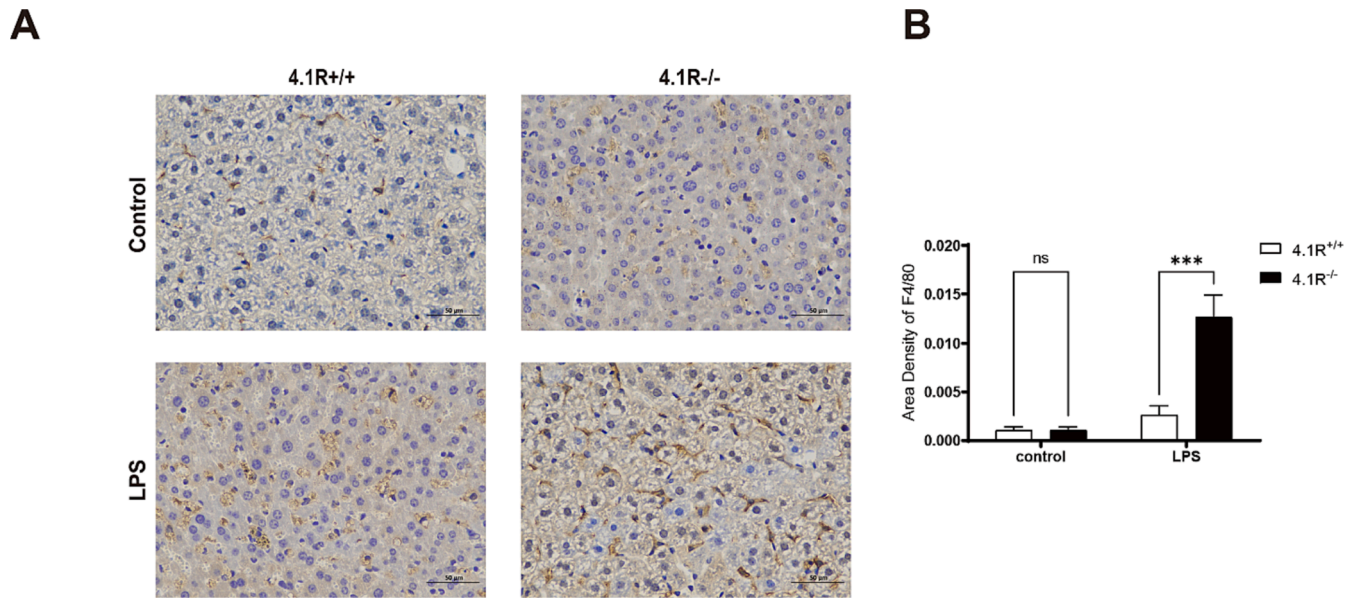

Fig. 2. Effect of protein 4.1R on macrophage infiltration. (A) representative images of immunohistochemistry for F4/80 in the liver. **(B)** Area density of F4/80-positive in the liver tissue of control group and LPS-treated group data are expressed as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$.

than that in wild type. Subsequently, we detected the expression of M1 markers (IL-6, TNF-α, CCL-2, iNOS) by q-PCR. As shown in (Fig. 4C), the expression of M1 markers in 4.1R$^{-/-}$ BMDM were more significant than that of wild type at different time points. The results indicate that protein 4.1R deficiency enhance M1 polarization.

3.5. Protein 4.1R regulates glycolysis in macrophages

To explore the effect of protein 4.1R on glycolysis of macrophages stimulated by LPS, we firstly detected the contents of key enzymes hexokinase (HK) by using the micro method (Fig. 5A). Then the PKM2 protein expression was detected by western blot (Fig. 5B-C). The results showed that in M1 macrophages, HK and PKM2 were significantly higher than those of their wild-type macrophages. In addition, the effect of protein 4.1R deficiency on the expression of key enzymes for glycolysis of LPS stimulated macrophages was detected by q-PCR (Fig. 5D). The results showed that the mRNA expression of glycolytic pathway related metabolic enzymes in 4.1R$^{-/-}$ macrophages was significantly higher than that in their wild-type. The above results showed that 4.1R$^{-/-}$ could enhance the expression of metabolic enzymes related to the glycolysis pathway of LPS stimulated macrophages. Finally, lactate secretion was detected by micro method. As shown in (Fig. 5E), the lactic acid secretion of 4.1R$^{-/-}$ macrophages were significantly higher than their wild-type counterparts, indicating that 4.1R$^{-/-}$ promote the lactic acid secretion of LPS stimulated macrophages. The above results indicate that protein 4.1R is involved in the regulation of LPS-induced macrophage glycolysis pathway.

3.6. Protein 4.1R regulates AKT/HIF-1α signaling pathway of M1 macrophages

It has been shown that the activation energy of AKT/HIF-1α signaling pathway regulates cell metabolism and mediating cytokine expression to participate in immune regulation. Western blot (Fig. 6A-B) and quantitative PCR (Fig. 6C) detect the expression level of HIF-1α. The phosphorylation of AKT protein was detected by western blot. As shown in (Fig. 6), protein 4.1R can negatively regulate HIF-1α after LPS stimulation of macrophages. Protein 4.1R did not affect the expression of AKT, but inhibited the phosphorylation level of AKT at different time points. The above results indicate that protein 4.1R regulates AKT/HIF-1α signal pathway of M1 macrophages.

3.7. Co-localization of 4.1R and TLR4 in M1 macrophages

The effects of protein 4.1R on AKT/HIF-1α signal pathway of M1 macrophages may be related to membrane receptors. TLR4 can recognize LPS of Gram-negative bacteria and initiate intracellular signal transduction. We detected the expression of TLR4 in macrophages (Fig. 7A-C). The results showed that after LPS stimulation, the expression of TLR4 protein in protein 4.1R knockout cells increased sharply in a short period of time, which is necessary for enhanced polarization phenotype and AKT/HIF-1α signal pathway. As a membrane skeleton protein, protein 4.1R may participate in cell regulation by interacting with Toll-like receptors on the membrane. Therefore, the interaction between 4.1R and TLR4 was detected by co-inmunoprecipitation and immunofluorescence (Fig. 7D-F). As shown in Fig. 7E, there was a weak correlation between TLR4 and 4.1R at rest ($0.3 < R = 0.4410 < 0.5$), and a strong correlation between TLR4 and 4.1R after LPS stimulation ($0.5 < R = 0.8089$). The above results indicate that protein 4.1R negatively regulates the inflammatory response of macrophages by inhibiting the AKT / HIF-1α signaling pathway through interaction with TLR4.

4. Discussion

The multifunctional structural protein 4.1R can connect various transmembrane proteins and actin, which is critical to the structural integrity of the skeleton and its attachment to the membrane [28]. Previous studies have shown that protein 4.1R plays an important role in multiple immune cells activation by regulate signal transduction, such as T cell [24], B cell [29] and mast cell [30]. In cancer cells, protein 4.1R down-regulates VEGFA in M2 macrophages and inhibits colon cancer metastasis [31]. However, protein 4.1R is rarely associated with inflammation in the body, especially sepsis. In this study, we demonstrated that the crucial role of protein 4.1R in the development of sepsis and identified the mechanism of protein 4.1R in macrophage activation and glycolysis.

LPS-induced sepsis is a recognized animal model, which has been used to study the mechanism of septic liver injury [32]. In the present study, we documented the survival rate of the 4.1R$^{-/-}$ sepsis mice group was significantly lower than that the wild-type sepsis mice group.

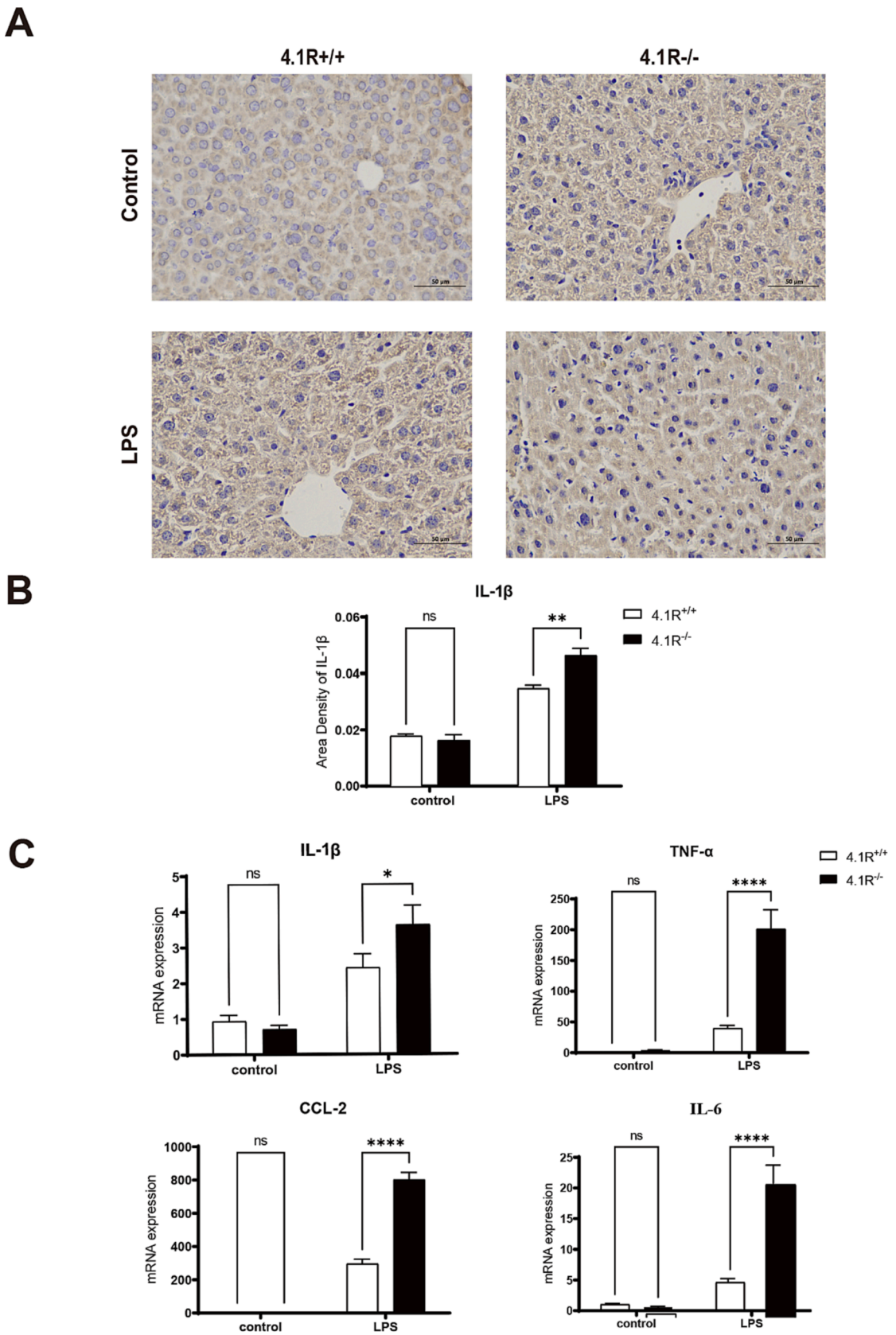

Fig. 3. Effect of protein 4.1R on inflammatory cytokine production in $4.1R^{-/-}$ **septic mice. (A)** Representative images of immunohistochemistry for IL-1β in the liver. **(B)** Area density of IL-1β-positive in the liver tissue of control group and LPS-treated group. **(C)** 4.1R regulates cytokine production in the liver tissue of sepsis mouse. Real-time PCR analysis for inflammatory cytokines from liver. Data are expressed as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$.

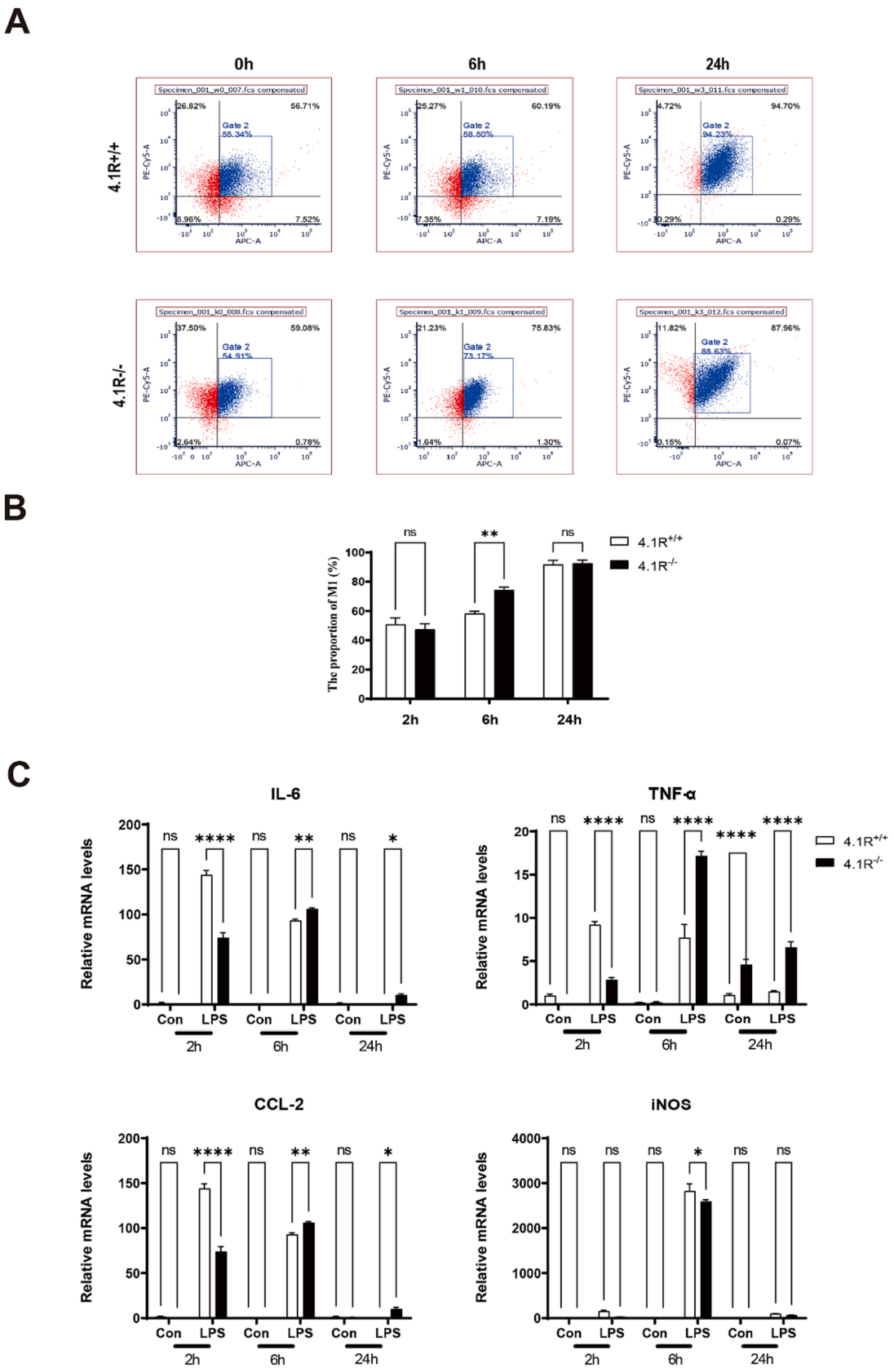

Fig. 4. Effect of 4.1R on LPS-stimulated BMDM polarization. (A) Representative flow cytometer data chart of $4.1R^{+/+}$ BMDM and $4.1R^{-/-}$ BMDM. BMDM was conjugated with PerCP/Cyanine5.5 anti-mouse F4/80 antibody and APC anti-mouse CD11c antibody staining. **(B)** Flow cytometry statistics of the percentage of M1 macrophages after LPS stimulation (n = 3). **(C)** $4.1R^{+/+}$ and $4.1R^{-/-}$ bone marrow derived macrophages (BMDM) treated with LPS for 2 h, 6 h, 24 h. Data are expressed as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$.

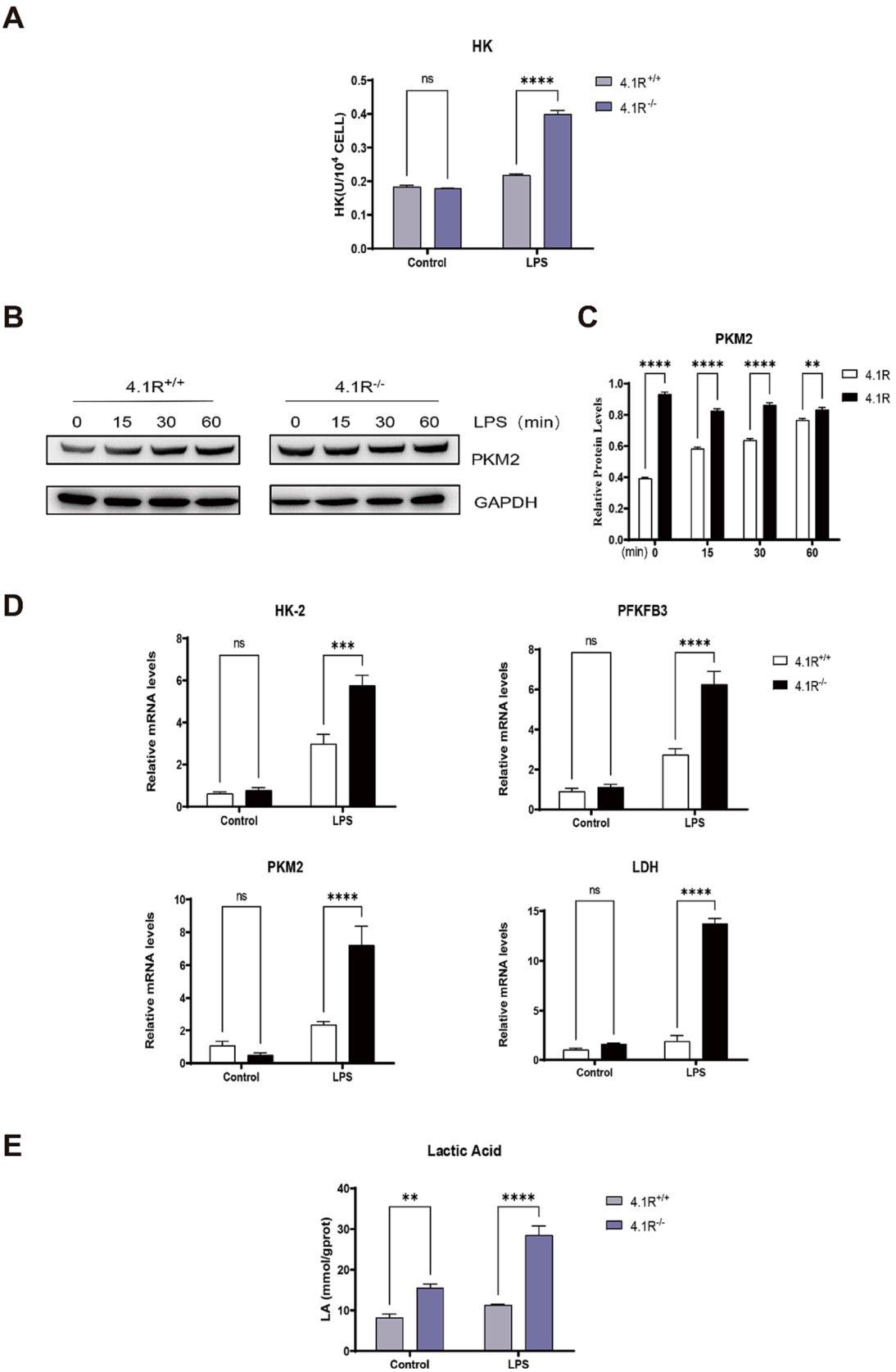

Fig. 5. The 4.1R deficiency up-regulates glycolysis-related molecule expression in LPS-stimulated BMDM. (A) Ultraviolet spectrophotometric analysis for HK expression of $4.1R^{+/+}$ BMDM and $4.1R^{-/-}$ BMDM. **(B)** Western blot was used to detect levels of PKM2 in BMDM after treated with LPS. (**C**) Quantification of PKM2 protein expression. **(D)** Real-time PCR analysis for inflammatory cytokine expression in LPS-stimulated BMDM. **(E)** Ultraviolet spectrophotometric analysis for lactate secretion of $4.1R^{+/+}$ BMDM and $4.1R^{-/-}$ BMDM. Data are expressed as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$.

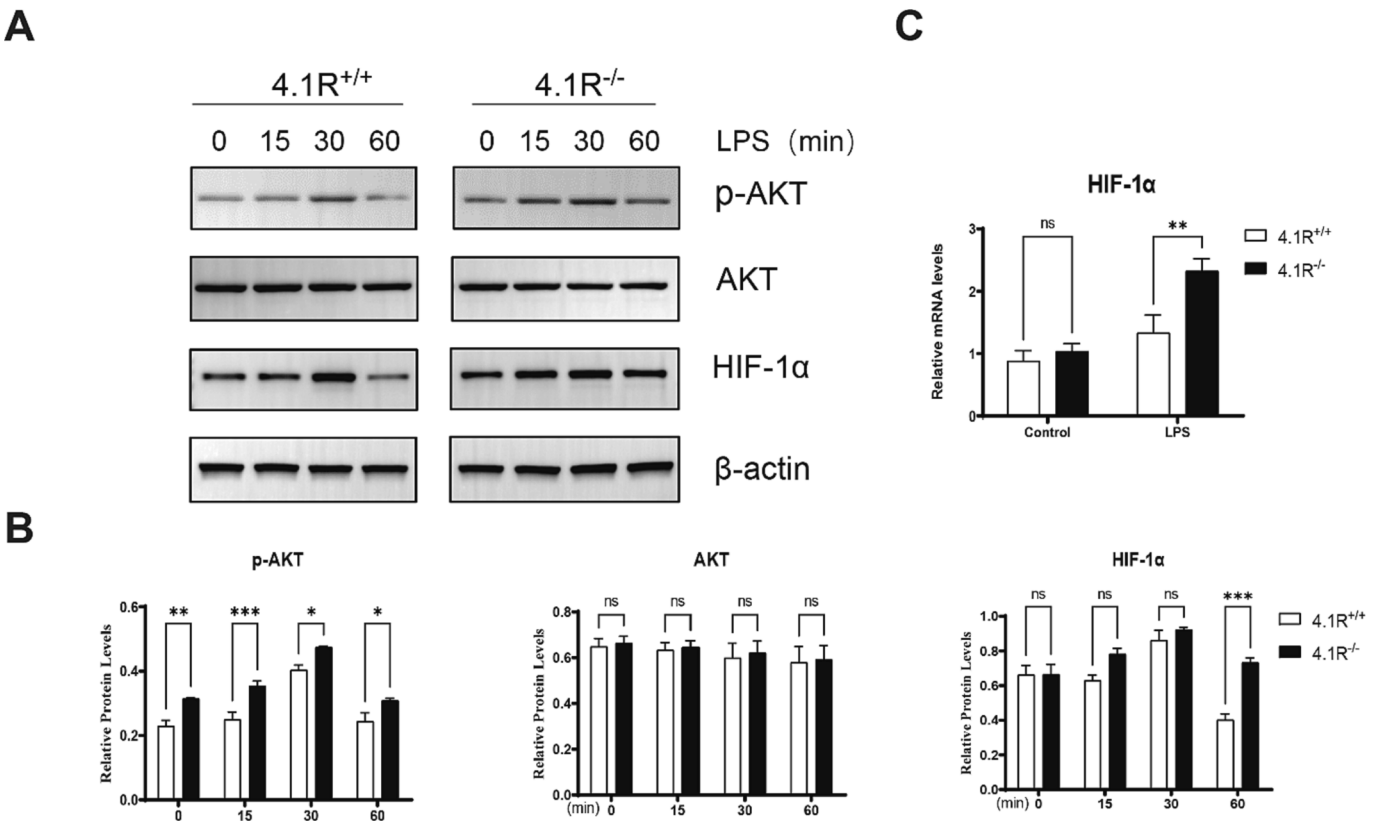

Fig. 6. The 4.1R deficiency up-regulates AKT/HIF-1α signaling pathway. (A) 4.1R regulates HIF-1α, P-AKT and AKT expression in LPS-stimulated BMDM. **(B)** Quantification of AKT, Phosphorylated-AKT, and HIF-1α protein expression. **(C)** mRNA of HIF-1α were harvested from cells and gene expression was analyzed quantitatively using q-PCR. Data are expressed as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$.

Moreover, it was found that the levels of ALT, AST and LDH in serum of $4.1R^{-/-}$ sepsis mice were significantly higher than those of wild-type sepsis mice, and more serious liver injury occurred. It is known that during sepsis, the imbalance between the defense and immunosuppressive triggers inflammation [6,8,33,34]. Thus, implying that 4.1R knockout could account for inflammatory increased.

Inflammatory mediators such as IFN, IL-1β, IL-6 and TNF produced by macrophages are involved in the immune activation of liver tissue [35]. Our study showed that the deletion of 4.1R significantly enhanced macrophage infiltration in liver tissue. The expression of pro-inflammatory cytokines such as IL-1β and IL-6 significantly increased in the $4.1R^{-/-}$ BMDM and the liver of $4.1R^{-/-}$ septic mice, indicating that 4.1R may negatively regulated macrophage M1 Polarization. Interestingly, we found that the deletion of 4.1R down-regulated the expression of pro-inflammatory cytokines in BMDM cells after 2 h of LPS stimulation. This may be due to the fact that in the early stage of bacterial infection, 4.1R promotes the recognition of pattern recognition receptor (PRR) on the surface of macrophages to the related molecules on the surface of pathogenic microorganisms, releases pro-inflammatory mediators, then kills the invading bacteria and initiates adaptive immunity [36]. Protein 4.1R plays a role in preventing cytokine storms and reducing sepsis during long-term and large-scale bacterial infection. These results suggest that protein 4.1R may act as an inflammatory checkpoint. Once protein 4.1R is damaged or missing, it will lead to cytokine storms and metabolic reprogramming, eventually leading to multiple organ damage and even death.

M1 macrophages induced by LPS showed that metabolism of anaerobic glycolysis pathway was enhanced to meet energy needs and pro-inflammatory cytokine precursor synthesis needs [37,38]. The transcription and translation of HIF-1α is enhanced by the activation of AKT-MTORC1 signaling pathway and interacts with PKM2 to regulate glycolysis [39–42]. Our results suggest that protein 4.1R may inhibit the production of proinflammatory cytokines by negatively regulating LPS induced glycolysis of macrophages.

TLR4 is a member of the Toll like receptor (TLR) family, participates in innate immunity and mediates inflammation by recognizing LPS or bacterial endotoxin [43]. In immune cells, TLR4 are universally acknowledged as one of members of synapse, our previously study has shown that 4.1R were colocalization with TLR4 in B cell synapse to regulate B cell activation. In this study we verified this discovery by IF and IP assay, shows 4.1R colocalized with TLR4 in macrophage, especially after LPS stimulate. This result indicates that protein 4.1R negatively regulates the inflammatory response of macrophages by inhibiting the AKT / HIF-1α signaling pathway through interaction with TLR4.

To sum up, our study reveals that protein 4.1R may alleviate acute liver injury in mice by regulating macrophage polarization and reprogramming metabolism. We also demonstrated that protein 4.1R inhibits M1 polarization and proinflammatory factor production of macrophages by inhibiting LPS stimulated glycolysis of macrophages. Further experiments proved that protein 4.1R, as the cytoskeleton protein of cell membrane, interacts with TLR4 to regulate the phosphorylation of AKT and HIF-1α Activation. We revealed a novel function of protein 4.1R in macrophage polarization and LPS-induced liver injury, providing insights into the metabolic reprogramming of hepatic macrophages, which is important for the progression and treatment of acute liver injury.

5. Informed consent statement

Not applicable.

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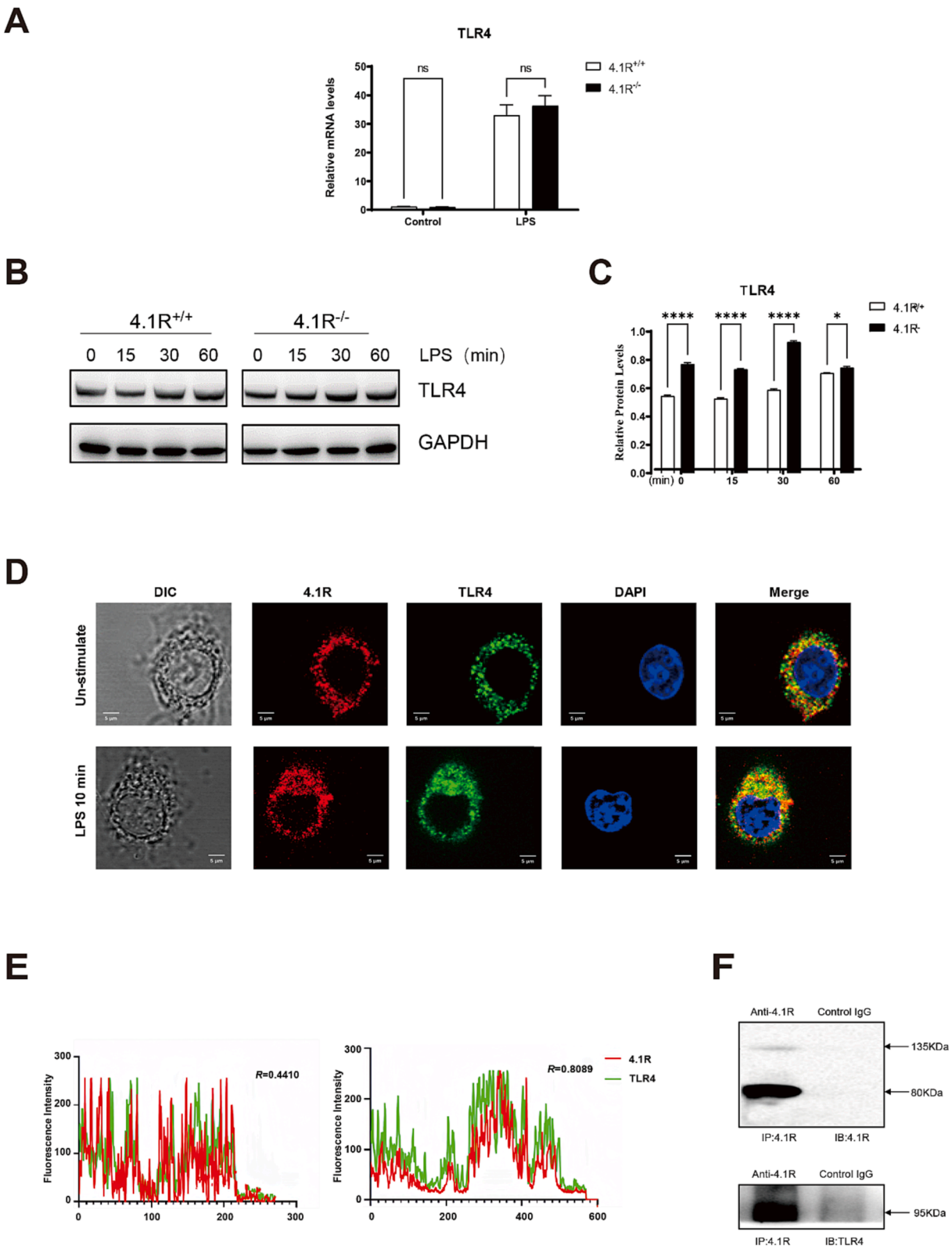

Fig. 7. Protein 4.1R interacts with TLR4 in macrophages. (A) Gene expression of TLR4 was analyzed quantitatively using q-PCR and normalized to the housekeeping gene 18S rRNA. **(B)** Western blot was used to detect levels of TLR4. **(C)** Quantification of TLR4 protein expression. **(D)** Co-localization of the protein 4.1R with the cell surface receptors TLR4 in BMDM examined by laser confocal microscopy. BMDM were fixed and stained with fluorescence-labeled antibodies against protein 4.1R (red), TLR4 (green). Simultaneously, cell nucleus was counterstained with DAPI. Scale bar, 5 μm. (**E**) Pearson correlation coefficient of 4.1R and TLR4 was generated using the Prism7. **(F)** Co-immunoprecipitation of TLR4 with 4.1R in RAW264.7. Data are expressed as the mean ± SEM. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$.

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CRediT authorship contribution statement

Si-Yao Sang: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Yuan-Jiao Wang:** Writing – original draft, Visualization, Validation, Formal analysis. **Taotao Liang:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Yan Liu:** Validation, Investigation, Data curation. **Jiao-jiao Liu:** Writing – review & editing, Data curation. **Hui Li:** Writing – review & editing. **Xin Liu:** Writing – review & editing. **Qiao-Zhen Kang:** Writing – review & editing, Supervision, Funding acquisition. **Ting Wang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

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