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BCL6 Alleviates Hepatic Ischemia/Reperfusion Injury Via Recruiting SIRT1 to Repress the NF-κB/NLRP3 Pathway

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Background. Hepatic ischemia/reperfusion (I/R) injury (HIRI) is an intrinsic phenomenon observed in the process of various liver surgeries. Unfortunately, there are currently few options available to prevent HIRI. Accordingly, we aim to explore the role and key downstream effects of B-cell lymphoma 6 (BCL6) in hepatic I/R (HIR). **Methods.** BCL6 expression levels were measured in I/R liver tissue and primary hepatocytes stimulated by hypoxia/reoxygenation (H/R). Moreover, we ascertained the BCL6 effect on HIR in vivo using liver-specific BCL6 knockout mice and adenovirus-BCL6-infected mice. RNA-sequencing, luciferase, chromatin immunoprecipitation, and interactome analysis were combined to identify the direct target and corresponding molecular events contributing to BCL6 function. DNA pull-down was applied to identify upstream of BCL6 in the H/R challenge. **Results.** HIR represses BCL6 expression in vivo and in vitro. Hepatic BCL6 overexpression attenuates inflammation and apoptosis after I/R injury, whereas BCL6 deficiency aggravates I/R-induced liver injury. RNA-sequencing showed that BCL6 modulated nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3 inflammasome signaling in HIRI. Mechanistically, BCL6 deacetylated nuclear factor kappa-B p65 lysine 310 by recruiting sirtuin 1 (SIRT1), thereby inhibiting the nuclear factor kappa-B/nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3 pathway. Moreover, overexpression of SIRT1 blocked the detrimental effects of BCL6 depletion. Moreover, EX 527, a SIRT1 inhibitor, vanished protection from BCL6 overexpression. Furthermore, transcription factor 7 was found to mediate the transcription regulation of BCL6 on H/R challenge. **Conclusions.** Our results provide the first evidence supporting BCL6 as an important protective agent of HIR. This suggests a potential therapeutic approach for HIR.

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INTRODUCTION

Ischemia/reperfusion (I/R) injury (IRI) represents an unavoidable consequence of various liver surgeries characterized by aseptic inflammation that endangers the liver.[1] As the number of patients with malignant tumors and substantial liver disease is expected to increase, the demand for liver surgery is also likely to increase.[2,3] As a result, the incidence of postoperative hepatic IRIs (HIRIs) is likely to become more prevalent. Unfortunately, few options are currently available for preventing or treating HIRI.[4,5] Hence, studying the hepatic I/R (HIR) mechanism and developing intervention strategies in a more effective and appropriate direction are crucial.

SIRT1, a member of the sirtuin (SIRT) protein family, is an important regulator of many biological processes.[6] SIRT1 can inhibit inflammation, regulate immune cell activation, and act as a key immunomodulator. For example, SIRT1 activation ameliorates lipopolysaccharide-induced lung injury.[7] In addition, recent studies have highlighted that SIRT1 activation can inhibit hepatic inflammation and ameliorate liver fibrosis.[8] Many studies have demonstrated that SIRT1 downregulates nuclear factor kappa-B (NF-κB)-mediated inflammatory cytokines by impeding p65 acetylation through the deacetylation of lysine 310, thus playing an anti-inflammatory role.[9-11]

B-cell lymphoma 6 (BCL6) was first reported to be a proto-oncogene and transcription suppressor essential for lymphoid neoplasms and innate and adaptive components of the immune system.[12,13] Myocarditis is observed in mice with whole-body BCL6 deficiency,[14,15] indicating its broader physiological roles. BCL6 has multiple roles in the liver. BCL6 acts as a novel host restriction factor, suppressing hepatitis B virus (HBV) replication and enhancing immune responses to promote HBV clearance.[16] Due to the early mortality of these mice (within 5–9 wk of birth),[14] liver-specific BCL6 knockout mice (BCL6-LKO mice) have been developed to study BCL6's role in the adult liver. Research using BCL6-LKO mice has demonstrated that BCL6 regulates the expression of cytochrome P450 metabolic enzymes in the liver, BCL6 deletion leads to the expression of cytochrome P450 enzymes in male livers resembling that of female livers, suggesting that BCL6 plays a crucial role in the sex-specific regulation of drug metabolism.[17] Additionally, Sommars et al[18] recently highlighted the importance of BCL6 in regulating β-oxidation in the liver. Nikkanen et al[19] discovered that hepatic BCL6 deletion significantly reduces male mice survival during infection. Despite these insights, the role and mechanism of BCL6 in HIRI remain poorly understood.

BCL6 plays a critical role in various disease models, such as modulating immune responses in myocarditis, regulating metabolic enzymes in the liver, and serving as a restriction factor in HBV infection. These findings highlight BCL6's broad regulatory capacity in inflammation and cellular stress responses. Building on this, we aim to explore whether anti-inflammatory properties of BCL6 and its regulatory role in immune pathways extend to protecting the liver against IRI. Specifically, we hypothesize that BCL6 can alleviate hepatic IRI by modulating the NF-κB/NLRP3 pathway, which is critical for driving inflammatory and apoptotic processes in this context. This study will thus clarify BCL6's mechanism of action in liver IRI and its potential as a therapeutic target.

MATERIALS AND METHODS

See **Supplemental Materials and Methods** (**SDC,** http://links.lww.com/TP/D222).

The short hairpin RNA sequences used are listed in **Table S1** (**SDC,** http://links.lww.com/TP/D222).

The RT-PCR primer sequences are listed in **Table S2** (**SDC,** http://links.lww.com/TP/D222).

The chromatin immunoprecipitation primer sequences are listed in **Table S3** (**SDC,** http://links.lww.com/TP/D222).

RESULTS

Downregulation of BCL6 is Associated With IRI

An HIR animal model was constructed using wild-type C57BL/6J mice (**Figure S1A, SDC,** http://links.lww.com/TP/D222). As shown in **Figure S1B** (**SDC,** http://links.lww.com/TP/D222), the hematoxylin and eosin staining showed there were partially necrotic areas of the liver after I/R in comparison with the sham group. A mouse model of HIRI was successfully established, examining BCL6 expression in the mouse liver after HIR. The results showed that both BCL6 protein and mRNA levels were downregulated at 12 h post-I/R (Figure 1A and B). Moreover, immunohistochemical staining further verified that BCL6 was significantly downregulated in the liver tissues of the I/R group mice (Figure 1C) compared with the sham group. The in vitro results showed that BCL6 protein and mRNA expression significantly decreased in the hypoxia/reoxygenation (H/R) group hepatocytes (Figure 1D and E). In this study, we compared BCL6 expression in liver biopsies of patients who received liver transplantation for HIRI, demonstrating significantly lower BCL6 protein in liver samples after I/R when comparing each individual with their baseline levels (Figure 1F). Immunohistochemical staining confirmed that BCL6 was downregulated in the liver after reperfusion (Figure 1G). In conclusion, the expression pattern of BCL6 after I/R suggests its involvement in HIRI.

Hepatic BCL6 Alleviates Inflammation and Apoptosis in IRI

To identify the probable role of BCL6 in HIRI, hepatocyte-specific BCL6 adenovirus was injected into the tails of C57BL/6J male mice (**Figure S2A, SDC,** http://links.lww.com/TP/D222). Specific BCL6 overexpression in liver tissues was proved by Western blot (**Figure S2B, SDC,** http://links.lww.com/TP/D222). The Ad BCL6 group displayed significantly lower liver tissue necrosis area than in the Ad control group post-IRI (Figure 2A), and the necrotic area was reduced by >10% (Figure 2B). Serum glutamic pyruvic transaminase and glutamic oxaloacetic transaminase levels in Ad BCL6 mice were significantly lower (Figure 2C and D). Immunohistochemical staining showed that compared with Ad control mice, F4/80-positive inflammatory cells were significantly decreased in the liver of Ad BCL6 mice at 12 h post-HIR surgery (Figure 2E).

Additionally, the Ad BCL6 group exhibited significantly lower tumor necrosis factor alpha, interleukin (IL)-6,

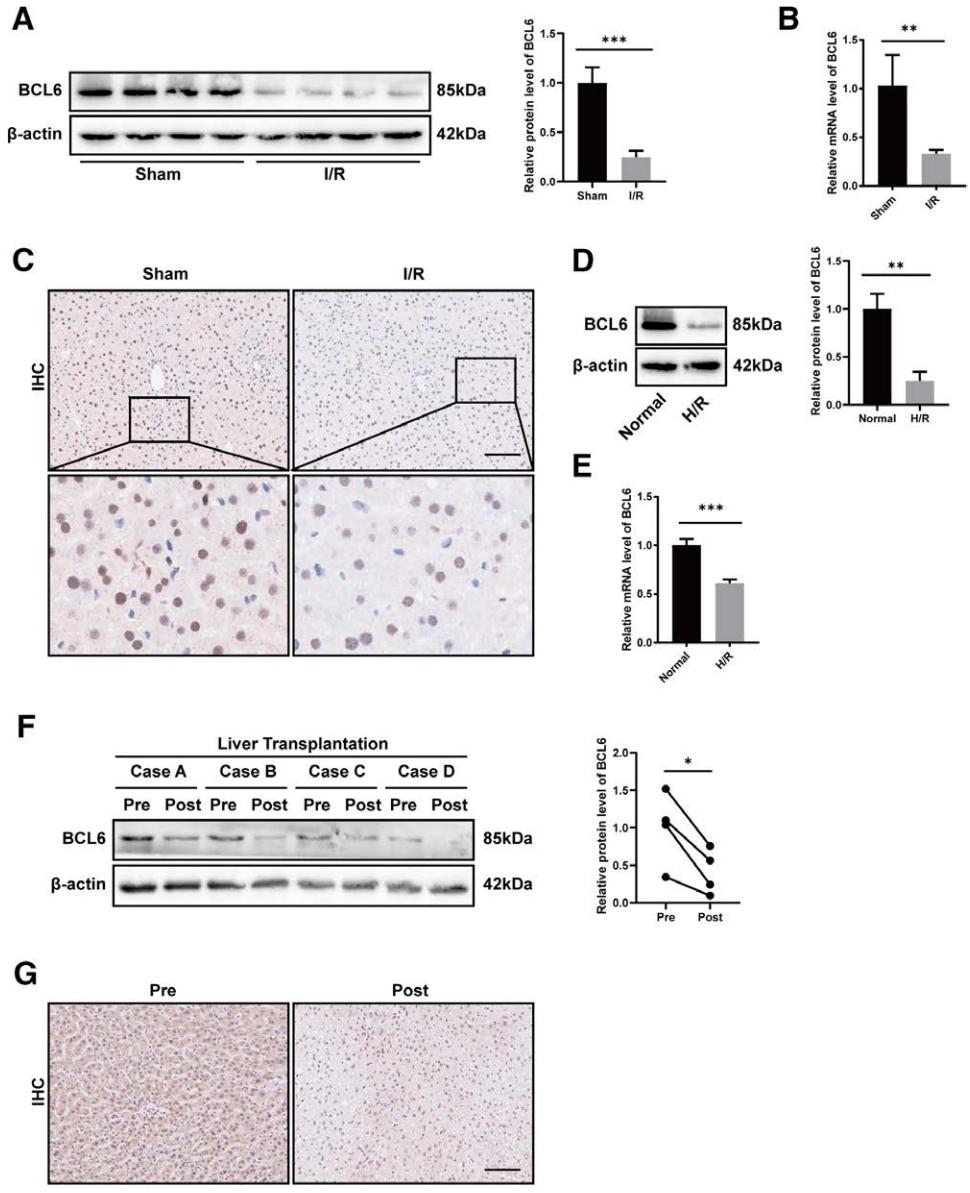

FIGURE 1. BCL6 expression is downregulated after hepatic I/R. A and B, The expression of BCL6 in the liver tissues of WT mice following I/R surgery 12 h and the sham group were tested by Western blot and RT-PCR (n = 4 per group). C, Representative immunohistochemical staining of BCL6 in ischemic liver from WT mice 12 h after I/R injury. Scale bar, 100 μm. D and E, The expression of BCL6 in mouse primary hepatocytes exposed to the H/R condition was tested by Western blot and RT-PCR (n = 3 per group). F, BCL6 protein expression in the liver of liver transplantation patients. β-actin severed as the loading control (n = 4). G, Representative immunohistochemical staining of BCL6 in the liver of liver transplantation patients. Scale bar, 100 μm. Statistical significance is indicated as $^{**}P < 0.01$, $^{***}P < 0.001$, and ns = not significant. The Student *t* test, ANOVA, or Kruskal-Wallis nonparametric statistical tests were used for statistical analysis. BCL6, B-cell lymphoma 6; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; RT-PCR, reverse transcription polymerase chain reaction; WT, wild type.

and C-C motif chemokine ligand 2 mRNA levels in liver tissue than in the control (Figure 2F). Overexpression of BCL6 reduced the serum concentrations of inflammatory cytokines significantly (**Figure S3A–C, SDC,** http://links.lww.com/TP/D222). Accordingly, in comparison with the Ad control group, the NF-κB signaling pathway was blunted in the livers of Ad BCL6 mice post-IRI (**Figure S3D, SDC,** http://links.lww.com/TP/D222). Moreover, excessive inflammation may lead to apoptosis in the liver, thus aggravating the severity of liver damage and affecting the prognosis. The Ad BCL6 group exhibited fewer C-caspase3-positive (an apoptosis marker) cells than the Ad control group (Figure 2E). As shown in Figure 2G, Western blot data demonstrated that I/R-induced apoptosis was significantly diminished in the Ad BCL6 mice livers, as evidenced by the upregulated expression of BCL2 (a survival marker) and the downregulated expression of BCL2-associated X (an apoptosis marker). These results demonstrated that BCL6 overexpression ameliorated cell death, apoptosis, and inflammation during HIRI. BCL6 is a sex-related gene; its expression is lower in adult female mice than in male mice.[19,20] We overexpressed BCL6 in C57BL/6J female mice and found that it could also inhibit hepatocyte necrosis caused by IR (**Figure S4A–D, SDC,** http://links.lww.com/TP/D222).

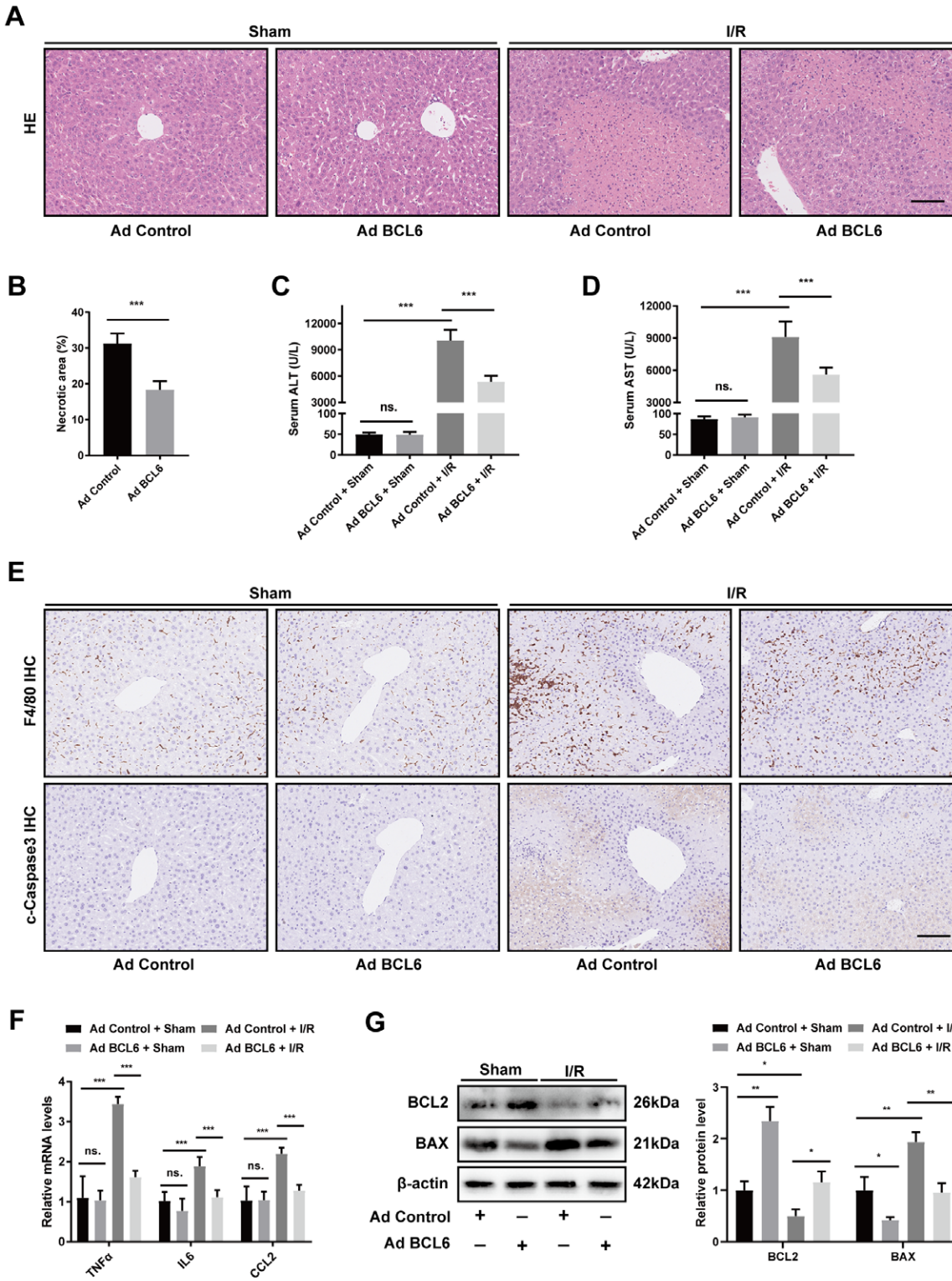

FIGURE 2. BCL6 overexpression alleviates liver damage, inflammatory responses, and apoptosis during hepatic I/R injury. A and B, Representative histological H&E-stained images and statistics showing necrotic areas in liver tissue of Ad control and Ad BCL6 male mice at 12 h after hepatic I/R surgery (n = 6 per group). Scale bar, 100 μm. C and D, Serum ALT/AST activities in Ad control and Ad BCL6 male mice at 12 h after hepatic I/R surgery (n = 6 per group). E, Representative immunohistochemical staining of F4/80 and c-Caspase3 in the liver of Ad control and Ad BCL6 male mice at 12 h after hepatic I/R surgery (n = 6 per group). Scale bar, 100 μm. F, The mRNA levels of inflammatory factors TNF-α, IL-6, and CCL2 in the liver of Ad control and Ad BCL6 male mice at 12 h after hepatic I/R surgery (n = 6 per group). G, The protein levels of BCL2 and BAX in the liver of Ad control and Ad BCL6 male mice at 12 h after hepatic I/R surgery. β-actin served as the loading control (n = 3 per group). Statistical significance is indicated as ***$P < 0.001$ and ns = not significant. Student *t* test, ANOVA, or Kruskal-Wallis nonparametric statistical tests were used for statistical analysis. ALT, glutamic pyruvic transaminase; AST, glutamic oxaloacetic transaminase; BAX, BCL2-associated X; BCL2, B-cell leukemia/lymphoma 2; BCL6, B-cell lymphoma 6; CCL2, C-C motif chemokine ligand 2; IL, interleukin; I/R, ischemia/reperfusion; TNF-α, tumor necrosis factor alpha.

BCL6 Deficiency Aggravates I/R-induced Liver Damage

Hepatocyte-specific BCL6 knockout ($BCL6^{CKO}$) mice were generated (**Figure S5A, SDC,** http://links.lww.com/TP/D222), and Western blot analysis confirmed the effectiveness of the knockout (**Figure S5B, SDC,** http://links.lww.com/TP/D222). In contrast to $BCL6^{flox/flox}$ male mice, post-I/R $BCL6^{CKO}$ male mice exhibited more extensive damage, with significantly enlarged areas of liver necrosis and elevated serum transaminase levels (Figure 3A–D). BCL6 ablation increased macrophage accumulation and enhanced inflammatory cytokine expression (Figure 3E and F). Consistently, upregulation of the serum concentrations of inflammatory cytokines and the increased p-p65 in the post-I/R $BCL6^{CKO}$ mice livers indicated further activation of NF-κB signaling (**Figure S6A–D, SDC,** http://links.lww.com/TP/D222). As demonstrated by c-Caspase3 immunohistochemistry, apoptotic cell number in the post-I/R $BCL6^{CKO}$ mice livers was increased significantly (Figure 3E). Moreover, the protein level of the BCL2 (an antiapoptotic gene) was reduced significantly, and BCL2-associated X expression (a proapoptotic marker) was increased in the post-I/R $BCL6^{CKO}$ group compared with $BCL6^{flox/flox}$ mice (Figure 3G). These results demonstrated that BCL6 deficiency aggravated I/R-induced cell death, inflammation, and apoptosis. Compared with control female mice, I/R-induced hepatocyte necrosis in $BCL6^{CKO}$ mice was more severe (**Figure S7A–D, SDC,** http://links.lww.com/TP/D222).

BCL6 Modulates NLRP3 Inflammasome Signaling in HIRI

To elucidate the mechanisms behind the protective role of BCL6 against HIRI, we conducted RNA-seq on primary hepatocytes extracted from $BCL6^{CKO}$ and $BCL6^{flox/flox}$ mice under H/R condition. Volcano Plot analysis confirmed that gene expression patterns significantly differed between the $BCL6^{CKO}$ and $BCL6^{flox/flox}$ groups (Figure 4A). Gene Set Enrichment Analysis showed that BCL6 deficiency systemically activated inflammation-related pathways and genes (Figure 4B and C). Figure 4D showed an inflammation-related gene expression heatmap. NLRP3 inflammasome signaling was found to be activated in BCL6-deficient hepatocytes. Consequently, we hypothesized that BCL6 may inhibit the NLRP3 inflammasome pathway, thereby mitigating the adverse effects of HIR.

Western blot results showed that H/R-induced overexpression of NLRP3 inflammasome components (NLRP3, ASC, and pre-Caspase1) and mature IL-1β proteins in primary hepatocytes, and the NLRP3/caspase1/ASC/IL-1β pathway in the inflammasome was further activated when BCL6 was knocked down by the recombinant adenovirus in normal control and H/R-treated groups (**Figure S8A and B, SDC,** http://links.lww.com/TP/D222). According to the in vivo data, inflammasome protein expression was also reduced in the liver tissue of post-I/R Ad BCL6 mice (Figure 4E). Knockout of BCL6, on the other hand, showed the opposite phenotype, upregulating the number of inflammasome proteins in sham control and I/R groups (Figure 4F). Moreover, Pearson correlation analysis showcased that BCL6 and NLRP3 mRNA levels were significantly negatively correlated in human and mouse livers, according to the statistical results from GSE107170 and GSE339549 of the Gene Expression Omnibus data repository (**Figure S8C and D, SDC,** http://links.lww.com/TP/D222). Based on these results, we speculated that BCL6 protects the liver from IRI by inactivating the NLRP3 inflammasome pathway.

BCL6 Inhibits NLRP3 Transcription by Recruiting SIRT1 to Repress the NF-κB/NLRP3 Pathway

Previous studies showed that BCL6 could inhibit the mRNA expression of target genes by binding to their promoter as a repressive transcription factor.[12,18,21] We sought to determine whether BCL6 directly regulates NLRP3 expression. Under the H/R condition, we overexpressed exogenous wild-type (BCL6-wt) and transcriptionally active mutant BCL6 (BCL6-mut), respectively, on the basis of knocking down endogenous BCL6 (Figure 5A). The results showed that transcriptionally active mutant BCL6 could also inhibit NLRP3 promoter activity. RT-PCR and Western blot experiments confirmed that transcriptionally active mutant BCL6 could also inhibit NLRP3 expression (Figure 5B and C), suggesting that there might be other mechanisms for BCL6 to regulate NLRP3. Previous studies showed that BCL6 could play a transcriptional regulatory role through binding proteins.[22-26] The liquid chromatography-mass spectrometry was applied to detect the binding protein of BCL6 in hepatocytes under the H/R condition. The binding protein of BCL6 is shown in **Table S4** (**SDC,** http://links.lww.com/TP/D222). The results showed that NF-κB p65 could bind with BCL6. Multiple studies have shown that NF-κB p65 could promote the NLRP3 pathway activation. To determine whether NF-κB p65 mediates the inhibitory effect of BCL6 on NLRP3 expression, we performed Co-IP experiments to confirm that BCL6 could bind with p65 under H/R condition (Figure 5D). The transcriptionally active mutant BCL6 could also bind with p65 under the H/R condition (**Figure S9A, SDC,** http://links.lww.com/TP/D222). To determine whether BCL6 inhibits NLRP3 through the NF-κB p65 pathway, knockdown of p65 could abolish the promoting effect of BCL6 knockdown on NLRP3 promoter activity, mRNA, and protein levels under the H/R condition (Figure 5E–G). NF-κB p65 can directly bind to the NLRP3 promoter and enhance NLRP3 expression.[27] An NLRP3 promoter-reporter gene plasmid with a mutation in the p65 binding site was constructed. Luciferase assays revealed that BCL6 knockdown did not enhance the activity of the NLRP3 promoter with the p65 mutation under the H/R condition (Figure 5H). These results suggest that BCL6 inhibits NLRP3 transcriptional activity via interaction with p65. Mass spectrometry showed that SIRT1 could bind to BCL6 (**Figure S9B, SDC,** http://links.lww.com/TP/D222). It has been reported that SIRT1 could deacetylate p65 to inhibit NF-κB and its downstream pathway.[9-11] To clarify whether the inhibitory effect of p65-mediated BCL6 on NLRP3 is related to SIRT1, IP results showed that overexpression of BCL6 could promote the binding of SIRT1 and p65 while knocking down BCL6 could weaken the binding of SIRT1 and p65 under H/R condition (**Figure S9C, SDC,** http://links.lww.com/TP/D222; Figure 5I). The dual-luciferase reporter assay

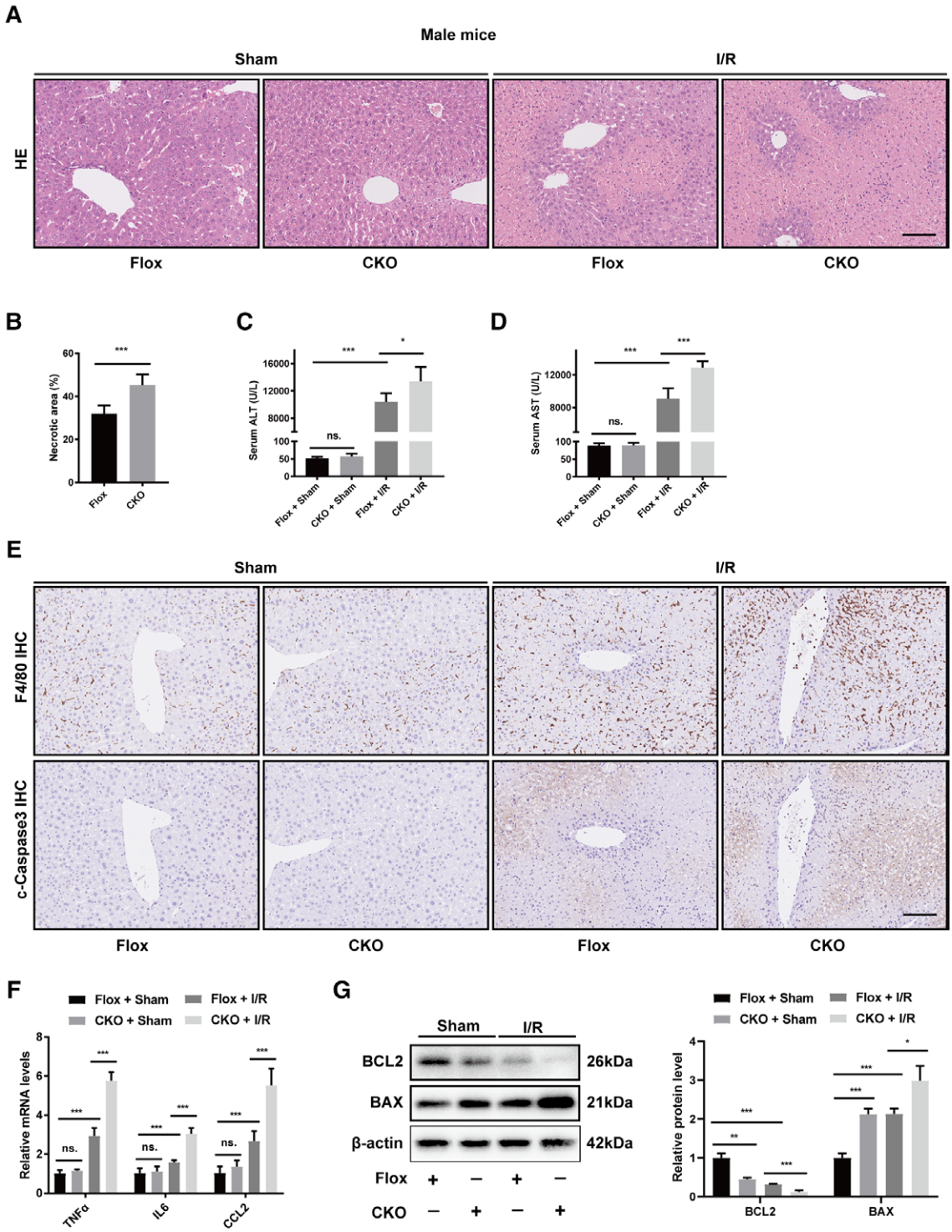

FIGURE 3. BCL6 deficiency aggravates liver damage, inflammatory responses, and apoptosis during hepatic I/R injury. A and B, Representative histological H&E-stained images and statistics showing necrotic areas in liver tissue of BCL6$^{flox/flox}$ and BCL6^{CKO} male mice at 12 h after hepatic I/R surgery (n = 6 per group). Scale bar, 100 μm. C and D, Serum ALT/AST activities in BCL6$^{flox/flox}$ and BCL6^{CKO} male mice at 12 h after hepatic I/R surgery (n = 6 per group). E, Representative immunohistochemical staining of F4/80 and c-Caspase3 in the liver of BCL6$^{flox/flox}$ and BCL6^{CKO} male mice at 12 h after hepatic I/R surgery (n = 6 per group). Scale bar, 100 μm. F, The mRNA levels of inflammatory factors TNF-α, IL-6, and CCL2 in the liver of BCL6$^{flox/flox}$ and BCL6^{CKO} male mice at 12 h after hepatic I/R surgery (n = 6 per group). G, The protein levels of BCL2 and BAX in the liver of BCL6$^{flox/flox}$ and BCL6^{CKO} male mice at 12 h after hepatic I/R surgery. β-actin served as the loading control (n = 3 per group). Statistical significance is indicated as $^{*}P < 0.05$, $^{***}P < 0.001$, and ns = not significant. Student *t* test, ANOVA, or Kruskal-Wallis nonparametric statistical tests were used for statistical analysis. ALT, glutamic pyruvic transaminase; AST, glutamic oxaloacetic transaminase; BAX, BCL2-associated X; BCL2, B-cell leukemia/lymphoma 2; BCL6, B-cell lymphoma 6; CCL2, C-C motif chemokine ligand 2; IL, interleukin; I/R, ischemia/reperfusion; TNF-α, tumor necrosis factor alpha.

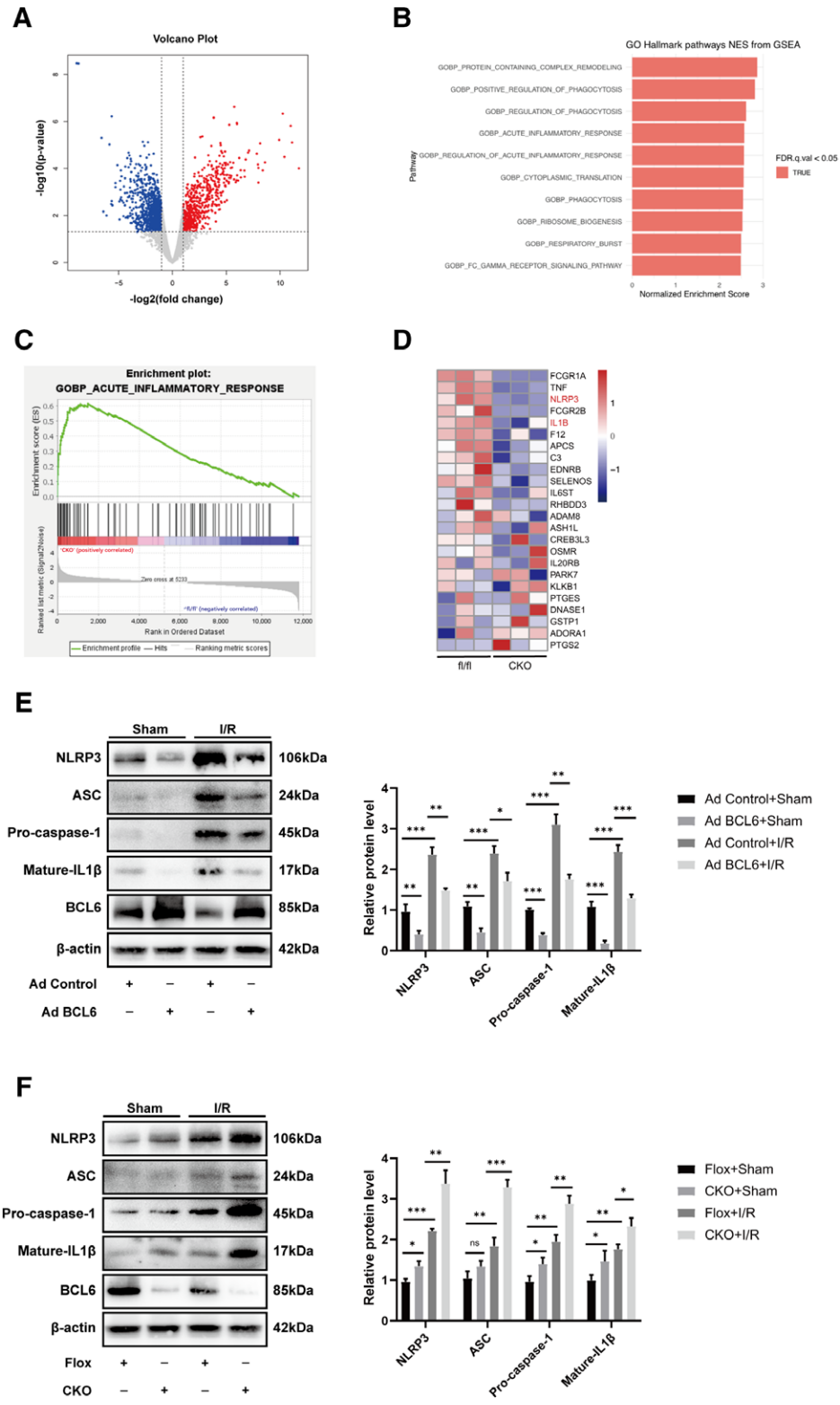

FIGURE 4. BCL6 modulates NLRP3 inflammasome signaling both in vitro and in vivo. A, Volcano Plot analysis showing global mRNA distribution profiles of primary hepatocytes extracted from BCL6^{CKO} and BCL6$^{flox/flox}$ mice under H/R condition. B, KEGG pathway enrichment analysis of the major biological pathways contributing to BCL6 function based on leading-edge subsets of GSEA-enriched inflammation-related pathways. The top 10 most significantly enriched pathways are shown. C, GSEA of inflammation-related gene co-expression networks. D, Heatmap of the inflammation-related gene. E, The expressions of NLRP3, ASC, pro-IL-1β, mature IL-1β, and BCL6 in the Ad control group and Ad BCL6 group with or without I/R injury were shown by Western blot. β-actin served as the loading control (n = 3 per group). F, The expressions of NLRP3, ASC, pro-IL-1β, mature IL-1β, and BCL6 in the Flox group and CKO group with or without I/R injury were shown by Western blot. β-actin served as the loading control (n = 3 per group). ASC, apoptosis-associated speck-like protein containing a CARD; BCL6, B-cell lymphoma 6; GSEA, Gene Set Enrichment Analysis; H/R, hypoxia/reoxygenation; IL, interleukin; I/R, ischemia/reperfusion; KEGG, Kyoto Encyclopedia of Genes and Genomes; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3.

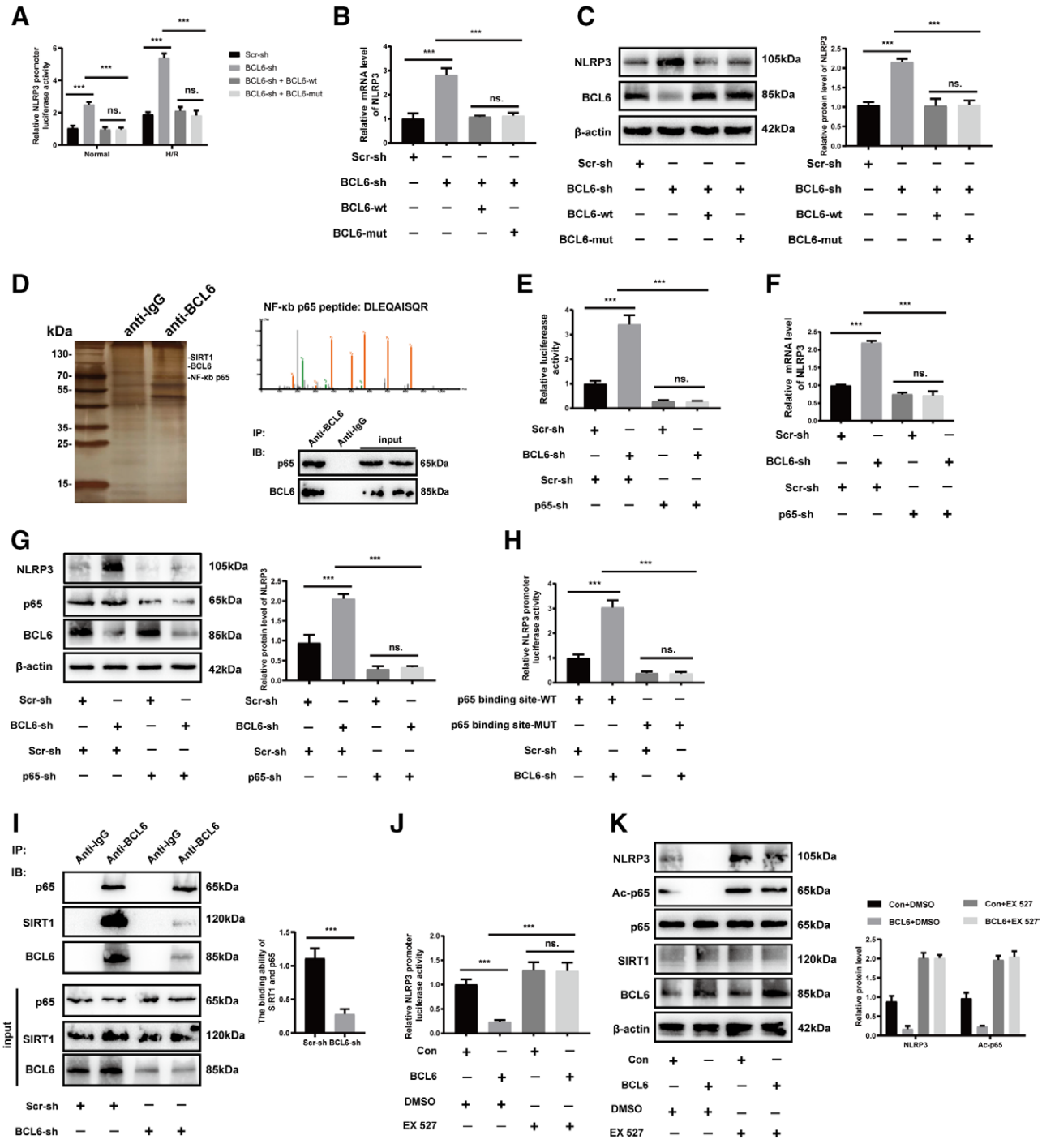

FIGURE 5. BCL6 inhibits NLRP3 transcription by recruiting SIRT1 to repress the NF-κB/NLRP3 pathway. A, Effects of wild-type and transcriptionally active mutant BCL6 overexpression on luciferase activities in mice primary hepatocytes with endogenous BCL6 knockdown under H/R condition. B, Effects of wild-type and transcriptionally active mutant BCL6 overexpression on NLRP3 mRNA in mice primary hepatocytes with endogenous BCL6 knockdown under H/R condition. C, Effects of wild-type and transcriptionally active mutant BCL6 overexpression on NLRP3 protein in mice primary hepatocytes with endogenous BCL6 knockdown under H/R condition. β-actin served as the loading control (n = 3 per group). D, Silver staining of BCL6-interacting protein under H/R condition, NF-κB p65 mass spectrometry peptide, IP results of BCL6 and NF-κB p65 under H/R condition. E, Effects of BCL6 knockdown on NLRP3 promoter luciferase activities in mice primary hepatocytes with NF-κB p65 knockdown under H/R condition. F, Effects of BCL6 knockdown on NLRP3 mRNA in mice primary hepatocytes with NF-κB p65 knockdown under H/R condition. G, Effects of BCL6 knockdown on NLRP3 protein in mice primary hepatocytes with NF-κB p65 knockdown under H/R condition. β-actin served as the loading control (n = 3 per group). H, Effects of BCL6 knockdown on NLRP3 wild-type and NF-κB-binding site mutant luciferase activities in mice primary hepatocytes under H/R condition. I, Effects of BCL6 knockdown on SIRT1 and p65 binding capacity under H/R condition. J, Effects of SIRT1 inhibitor (EX 527) on the NLRP3 promoter luciferase activities in mice primary hepatocytes with BCL6 overexpress under H/R condition. K, Effects of SIRT1 inhibitor (EX 527) on the protein level of NLRP3, Ac-p65 in mice primary hepatocytes with BCL6 overexpress under H/R condition. β-actin served as the loading control (n = 3 per group). Statistical significance is indicated as ***$P < 0.001$, and ns = not significant. Student *t* test, ANOVA, or Kruskal-Wallis nonparametric statistical tests were used for statistical analysis. BCL6, B-cell lymphoma 6; H/R, hypoxia/reoxygenation; IP, immunoprecipitation; NF-κB, nuclear factor kappa-B; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3; SIRT1, sirtuin 1.

showed that the SIRT1 inhibitor (EX 527) reversed the inhibition of NLRP3 promoter activity by BCL6 under the H/R condition (Figure 5J). Western blot confirmed that BCL6 overexpression could inhibit p65 lys310 acetylation and NLRP3 expression, and SIRT1 inhibitor (EX 527) could relieve these effects of BCL6 under the H/R condition (Figure 5K). In conclusion, our findings indicate that BCL6 reduces the acetylation level of p65 at lysine 310 by recruiting SIRT1, thereby inhibiting the NF-κB/NLRP3 pathway.

SIRT1 Abolished the Exacerbating Effect of BCL6 Deficiency on HIR

In this study, we evaluated whether excessive inflammation and damage in post-I/R BCL6^{CKO} male and female mice when SIRT1 was overexpressed. These mice were administered adenovirus via the tail vein 48 h before I/R (**Figure S10A, SDC,** http://links.lww.com/TP/D222). HIR models were established for all 4 groups. Hematoxylin and eosin staining and liver function analysis revealed that SIRT1 overexpression has a significant protective effect on HIRI exacerbated by BCL6 deficiency (Figure 6A–D). More importantly, the increased liver macrophage infiltration, the number of apoptotic cells, and the elevated proinflammatory cytokines mRNA levels were inhibited by SIRT1 overexpression in BCL6^{CKO} mouse livers underwent HIR treatment (Figure 6E; **Figure S10B–E, SDC,** http://links.lww.com/TP/D222).

Moreover, Western blot showed that SIRT1 overexpression blocked the activity of p65 lys310 acetylation and NLRP3 inflammasome signaling in BCL6^{CKO} mice after reperfusion insult (Figure 6F). These results indicate that SIRT1 is essential in exacerbating the impacts of BCL6 deficiency on HIRI. Moreover, we administered the SIRT1 inhibitor (EX 527) to BCL6 overexpression male and female mice and found that the SIRT1 inhibitor could abolish the protective influence of BCL6 overexpression on I/R mice (Figure 7A–E). Altogether, the BCL6 impact on HIR was SIRT1 dependent, and SIRT1 mediated the inhibitory effect of BCL6 on the p65-NLRP3 pathway.

TCF7 Modulated BCL6 Transcription in HIR

To elucidate the mechanism of BCL6 expression decline in HIR, Biotin-dUTP was used to label the BCL6 promoter. Then, we incubated the biotin-labeled promoter DNA and hepatocytes nuclear protein and then used streptomycin beads to pull-down proteins for possible binding under the H/R condition. A series of proteins were found by LC-MS, as shown in **Table S5** (**SDC,** http://links.lww.com/TP/D222), among which TCF7 had been reported to promote BCL6 expression[28] (Figure 8A). DNA pull-down and Western blot experiments were used to verify that TCF7 could indeed bind to the promoter under the H/R condition (Figure 8B). We found that H/R stimulation could downregulate TCF7, and TCF7 overexpression could relieve the H/R inhibitory impact on BCL6 (Figure 8C and D). With the help of the database JAPAR (http://jaspar.genereg.net/), one possible binding site (–1624 to –1614 bp) was predicted for TCF7 in BCL6 promoter, followed by the construction of reporter genes with the binding site mutated (Figure 8E). Herein, the influence of TCF7 on BCL6 promoter activity in primary mouse hepatocytes was investigated, revealing that TCF7 did not activate the BCL6-mut promoter activity (Figure 8F). Chromatin immunoprecipitation assay also confirmed that TCF7 can bind to the BCL6 promoter under the H/R condition (Figure 8G). Collectively, TCF7 mediated the H/R inhibitory effect on BCL6.

DISCUSSION

This study found that BCL6 expression decreased significantly in HIR, and overexpression of BCL6 attenuated HIRI by inhibiting NLRP3 inflammasome formation in vivo and in vitro. Mechanistically, BCL6 recruited SIRT1 to deacetylate NF-κB p65 310 lysine, thereby inhibiting the NF-κB/NLRP3 pathway. Moreover, TCF7 was found to mediate the transcription regulation of BCL6 upon H/R challenge.

The I/R process mediated by complex interactions between hepatocytes and other cells through complex signaling pathways can lead to severe aseptic inflammatory responses and liver damage.[6,10,29-31] Hepatocytes are considered to be the main cell type that promotes liver IRI, activating inflammatory cells by secreting proinflammatory mediators, thereby causing uncontrolled aseptic inflammatory response.[31] Although the initial stage of HIRI is relatively mild, it induces a series of subsequent events, such as the release of proinflammatory mediators, recruitment and activation of white blood cells, and severe liver injury.[1]

The NF-κB signaling activation is crucial in aseptic inflammatory responses induced by I/R and remains an extensively researched inflammatory pathway.[32-34] Besides the classical activation pathway, NF-κB activity can also be regulated by posttranscriptional modifications of proteins (including deacetylation). For example, Yeung et al[35] showed that SIRT1 can physically bind with NF-κB p65 and inhibit transcription through deacetylation of p65 at lysine 310. Our results for the first time showed that overexpression of BCL6 could promote the binding of SIRT1 and p65, whereas knocking down BCL6 could weaken the binding of SIRT1 and p65.

The NF-κB/NLRP3 inflammasome pathway is a key regulator of inflammation and liver injury,[36-38] particularly in hepatocytes, which are the primary targets of IRI. Although liver immune activation in IRI involves hepatocyte-derived DAMPs activating innate immune cells,[39-41] our study specifically investigates the role of BCL6 in hepatocytes because NLRP3 activation in these cells is a critical driver of cell death, a major consequence of IRI. NF-κB activation in hepatocytes has dual roles, functioning both as a cytoprotective mechanism to prevent inflammatory cell death and as a proinflammatory driver of injury.[42] Our findings suggest that BCL6 selectively modulates NF-κB activity to inhibit NLRP3 transcription, thereby balancing these dual roles to protect against liver IRI. Zhu et al[43] confirmed that NLRP3 is involved in HIRI, besides showing that NLRP3 silencing by short hairpin RNA can reduce the expression of proinflammatory cytokines and protect the liver from IRI. Our results showed that BCL6 protects against HIRI by inactivating the NLRP3 inflammasome pathway, which was consistent with results of Zhu et al.

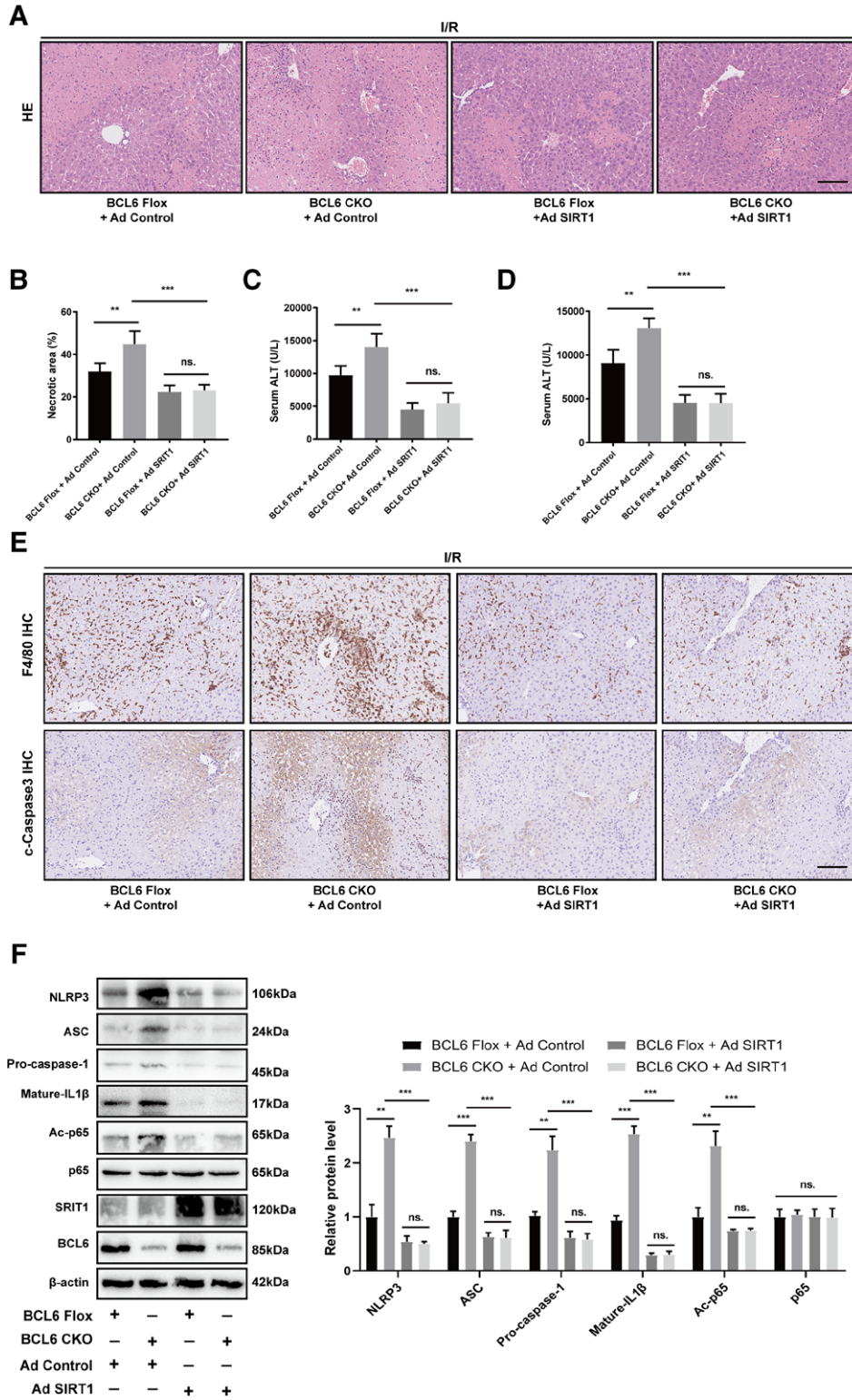

FIGURE 6. SIRT1 abolished the exacerbating effect of BCL6 deficiency on hepatic I/R. A and B, Representative histological H&E-stained images and statistics showing necrotic areas in liver tissue of $BCL6^{flox/flox}$ + Ad control, $BCL6^{CKO}$ + Ad control, $BCL6^{flox/flox}$ + Ad SIRT1, and $BCL6^{CKO}$ + Ad SIRT1 mice at 12 h after hepatic I/R surgery (n = 6 per group). Scale bar, 100 μm. C and D, Serum ALT/AST activities in $BCL6^{flox/flox}$ + Ad control, $BCL6^{CKO}$ + Ad control, $BCL6^{flox/flox}$ + Ad SIRT1, and $BCL6^{CKO}$ + Ad SIRT1 mice at 12 h after hepatic I/R surgery (n = 6 per group). E, Representative immunohistochemical staining of F4/80 and c-Caspase3 in the liver of $BCL6^{flox/flox}$ + Ad control, $BCL6^{CKO}$ + Ad control, $BCL6^{flox/flox}$ + Ad SIRT1, and $BCL6^{CKO}$ + Ad SIRT1 mice at 12 h after hepatic I/R surgery (n = 6 per group). Scale bar, 100 μm. F, The protein levels of NLRP3, ASC, pro-Caspase1, mature-IL-1β, Ac-p65, p65, SIRT1, and BCL6 in the liver of $BCL6^{flox/flox}$ + Ad control, $BCL6^{CKO}$ + Ad control, $BCL6^{flox/flox}$ + Ad SIRT1, and $BCL6^{CKO}$ + Ad SIRT1 mice at 12 h after hepatic I/R surgery. β-actin served as the loading control (n = 3 per group). Statistical significance is indicated as $^{**}P < 0.01$, $^{***}P < 0.001$, and ns = not significant. Student *t* test, ANOVA, or Kruskal-Wallis nonparametric statistical tests were used for statistical analysis. ALT, glutamic pyruvic transaminase; ASC, apoptosis-associated speck-like protein containing a CARD; AST, glutamic oxaloacetic transaminase; BCL6, B-cell lymphoma 6; H&E, hematoxylin and eosin; I/R, ischemia/reperfusion; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3; SIRT1, sirtuin 1.

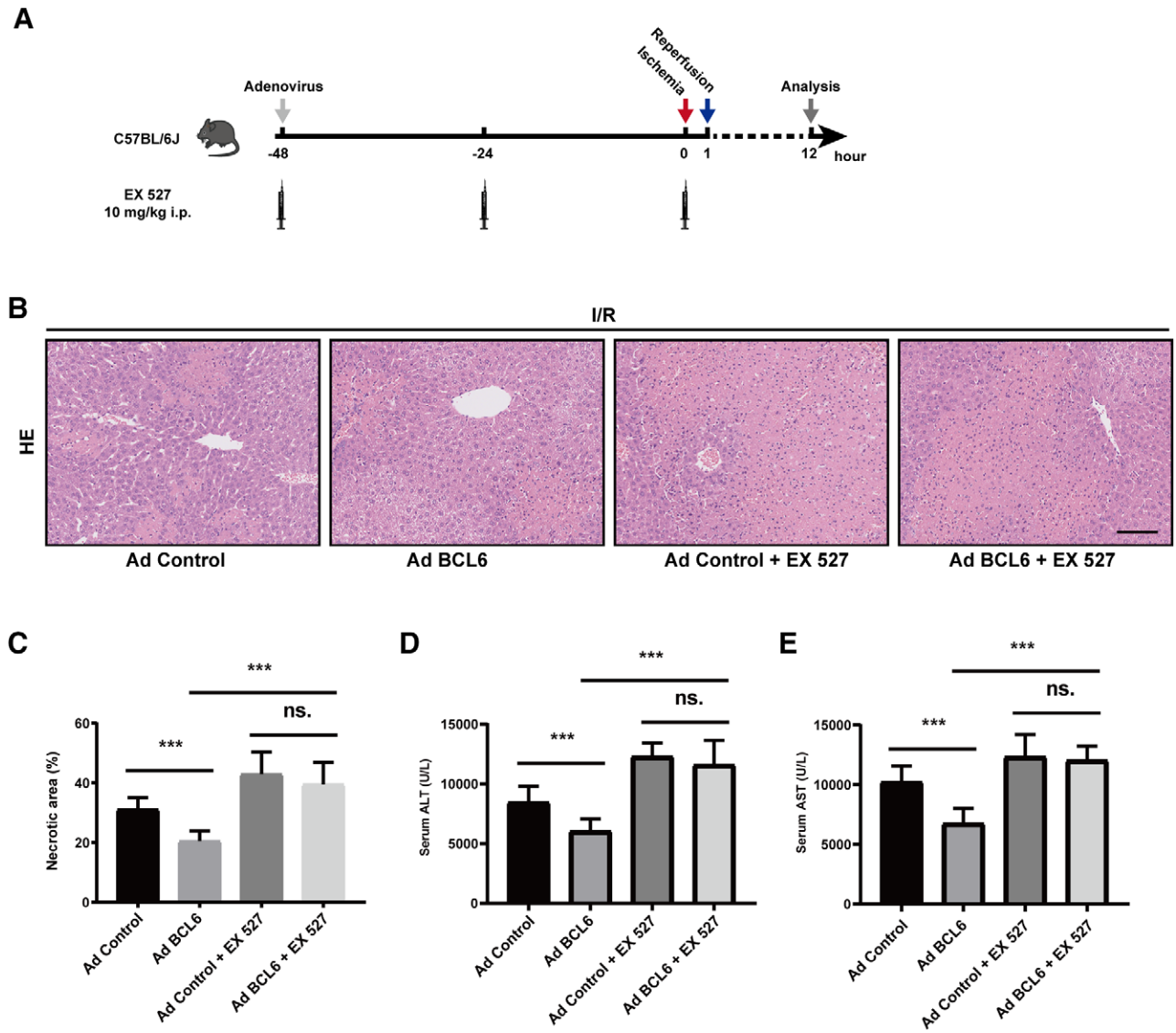

FIGURE 7. SIRT1 inhibitor EX527 abolished the protective influence of BCL6 overexpression on I/R mice. A, Schematic representation of adenovirus, EX 527 injection time point, and C57BL/6J mice hepatic I/R model. B and C, Representative histological H&E-stained images and statistics showing necrotic areas in liver tissue of Ad control, Ad BCL6, Ad control + EX 527, and Ad BCL6 + EX 527 male mice at 12 h after hepatic I/R surgery (n = 6 per group). Scale bar, 100 μm. D and E, Serum ALT/AST activities in Ad control, Ad BCL6, Ad control + EX 527, and Ad BCL6 + EX 527 male mice at 12 h after hepatic I/R surgery (n = 6 per group). Statistical significance is indicated as $^{***}P < 0.001$ and ns = not significant. Student *t* test, ANOVA, or Kruskal-Wallis nonparametric statistical tests were used for statistical analysis. ALT, glutamic pyruvic transaminase; AST, glutamic oxaloacetic transaminase; BCL6, B-cell lymphoma 6; H&E, hematoxylin and eosin; I/R, ischemia/reperfusion; SIRT1, sirtuin 1.

Our previous research shows that BCL6 alleviates nonalcoholic fatty liver disease in mice.[16] This study aimed to ascertain the BCL6 involvement in HIR, which has not been studied before. Herein, we found that BCL6 was downregulated 12 h post-IRI. Hepatic BCL6 alleviated inflammation and apoptosis throughout IRI. BCL6 deficiency aggravates I/R-induced liver damage. The protective effect of BCL6 is independent of sex. RNA-seq results indicated that BCL6 could modulate NLRP3 inflammasome signaling in HIRI. Chen et al[44] elucidated that BCL6 attenuates renal inflammation by negatively governing NLRP3 transcription. Previous studies showed that BCL6 could play a transcriptional regulatory role through binding proteins. Our Co-IP experiments confirmed that BCL6 could bind with p65. Mass spectrometry results showed that SIRT1 could also bind to BCL6. We found that BCL6 deacetylated NF-κB p65 lys310 by recruiting SIRT1, thereby inhibiting the NF-κB/NLRP3 pathway, which aligned with the results of Chen et al.

Biel et al[45] showcased that SIRT1 was significantly downregulated post-I/R in both human and mouse livers, and SIRT1 loss causes hepatocyte death after I/R. Nakamura et al[41] showed that high SIRT1 levels can improve hepatocellular function while reducing proinflammatory cytokine levels, thereby improving survival in human liver transplant patients. Our results showed that overexpression of SIRT1 blocked the detrimental effects of BCL6 depletion on liver IRI. Moreover, EX 527, a SIRT1 inhibitor, vanished the protection effect of BCL6 overexpression. Our results were consistent with those of Biel et al and Nakamura et al. This suggests that the influence of BCL6 on HIR was SIRT1 dependent, and SIRT1 mediated

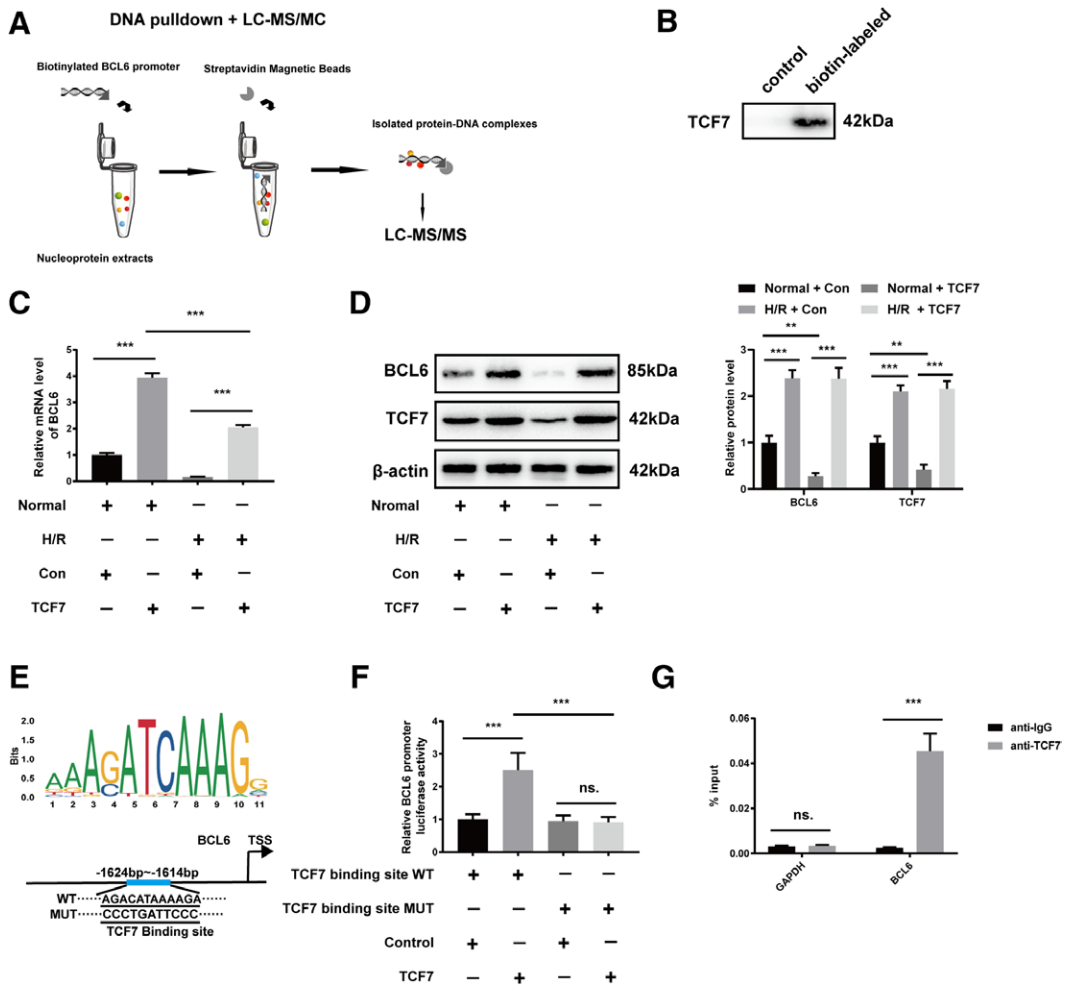

FIGURE 8. TCF7 modulated BCL6 transcription in hepatic I/R. A, Schematic diagram of DNA pull-down and LC/MS under H/R condition. B, Western blot detection of biotin-labeled BCL6 promoter pull-down TCF7 under H/R condition. C, Effects of TCF7 overexpress on mRNA level of BCL6 in mice primary hepatocytes under normal or H/R condition. D, Effects of TCF7 overexpress on the protein level of BCL6 in mice primary hepatocytes under normal or H/R conditions. β-actin served as the loading control (n = 3 per group). E, Common DNA motif sequences of TCF7, putative TCF7 responsive elements in the BCL6 promoter, and sequences of responsive elements used for constructing the MUT vector in the subsequent dual-luciferase reporter gene experiment. F, Effects of TCF7 overexpress on BCL6 wild-type and TCF7-binding site mutant luciferase activities in mice primary hepatocytes under H/R condition (n = 6 per group). G, Quantitative ChIP was performed in mice primary hepatocytes using antibodies for TCF7 or IgG control to enrich for possible TCF7-binding sites in the BCL6 or GAPDH loci under H/R condition. The y-axis represents fold enrichment of binding vs input, as compared with IgG control. Statistical significance is indicated as $^{***}P < 0.001$ and ns = not significant. Student *t* test, ANOVA, or Kruskal-Wallis nonparametric statistical tests were used for statistical analysis. BCL6, B-cell lymphoma 6; ChIP, chromatin immunoprecipitation; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; LC/MS, liquid chromatograph/mass spectrometer; MUT, mutant; TCF7, transcription factor 7.

the inhibitory effect of BCL6 on the p65-NLRP3 pathway. Furthermore, TCF7 was found to mediate the transcription regulation of BCL6 upon H/R challenge. Xu et al[46] found that at the beginning of the follicular regulatory T-cell differentiation program, TCF1 (encoded by TCF7) could bind to the Bcl6 promoter and induce Bcl6 expression, which is consistent with our findings. Summing up the above, our data demonstrate that BCL6 is a regulator in NF-κB/NLRP3-mediated HIRI.

Although our study primarily focuses on the role of BCL6 and SIRT1 in hepatocytes, it is important to acknowledge that these factors may also exert protective effects on liver IRI through mechanisms involving nonparenchymal cells, such as Kupffer cells or endothelial cells. In vivo adenoviral transfection of BCL6 or SIRT1 was not cell type-specific; their cytoprotective effect could be hepatocyte independent. Future studies using cell type-specific approaches are needed to clarify the contributions of BCL6 and SIRT1 in different liver cell populations.

The liver can be affected positively or negatively by inflammation, depending on various factors. Limited inflammatory responses, which are mild in intensity and expected to resolve, have demonstrated consistent hepatoprotective effects by aiding tissue repair and facilitating the restoration of homeostasis. In contrast, an excessive amount of inflammation, which is out of proportion in intensity and long-lasting, can cause a significant reduction in hepatocytes and worsen the seriousness of different liver conditions, including irreversible IRI-caused liver damage.[47] However, new insights into the activation of inflammatory responses in HIRI may stimulate the development of new interventions.[48] Blocking specific components or steps of the inflammatory cascade may disrupt

the self-amplifying cycle of cell death, leukocyte activation, and reactive oxygen species production. This approach could lead to the development of novel therapies that offer a promising alternative to conventional treatments for HIRIs.[5,49] Translating our findings on BCL6-mediated protection into clinical therapy could involve developing strategies to enhance BCL6 expression or activity specifically in the liver during IRI. Potential therapeutic approaches could include gene therapy to increase BCL6 expression, small molecules that activate BCL6, or drugs that mimic its protective effects. Additionally, given the role of BCL6 in inhibiting HBV replication,[16] therapies could also be designed to target BCL6 in cases where HBV-related liver injury is a concern. Future studies will focus on refining these strategies, evaluating their safety and efficacy in preclinical models, and determining the most effective way to translate these findings into treatments for liver injury in clinical settings. Overall, we suggest that BCL6 is crucial in the HIRI and may act as a potential therapeutic target.

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