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Interleukin-20 exacerbates acute hepatitis and bacterial infection by downregulating IκBζ target genes in hepatocytes

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Abstract

Background & Aims: Interleukin-20 (IL-20) and IL-22 belong to the IL-10 family. IL-10 is a well-documented anti-inflammatory cytokine while IL-22 is well-known for its epithelial protection and anti-bacterial function, showing great therapeutic potential for organ damage; but the function of IL-20 remains largely unknown.

Methods: *IL-20* knockout ($Il20^{-/-}$) mice and wild-type littermates were generated and injected with Concanavalin A (ConA) and *Klebsiella pneumoniae* (*K.P.*) to induce acute hepatitis and bacterial infection, respectively.

Results: $Il20^{-/-}$ mice were resistant to acute hepatitis with selective elevation of the hepatoprotective cytokine IL-6 levels without affecting most other cytokines. Such selective inhibition of IL-6 by IL-20 was due to IL-20 targeting-hepatocytes that produce high levels of IL-6 but a limited number of other cytokines. Mechanistically, IL-20 upregulated NAD(P)H: quinone oxidoreductase 1 (NQO1) expression and subsequently promoted the protein degradation of transcription factor IκBζ, resulting in selective downregulation of the IκBζ-dependent gene *Il6* as well several other IκBζ-dependent genes including lipocalin-2 (*Lcn2*). Given an important role of IL-6 and LCN2 in limiting bacterial infection, we examined the effect of IL-20 on bacterial infection and found $Il20^{-/-}$ mice were resistant to *K.P.* infection accompanied with an elevation of

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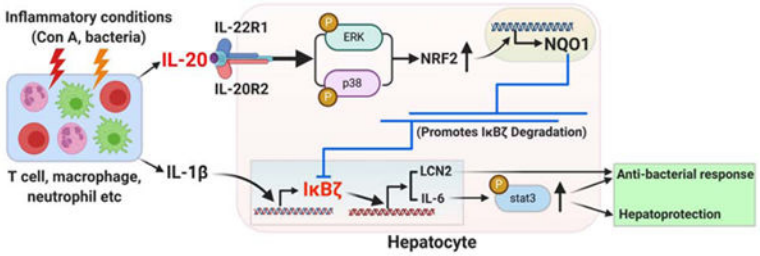
hepatic IκBζ-dependent antibacterial genes. Moreover, IL-20 upregulated hepatic NQO1 by activating ERK/p38MAPK/NRF2 signaling pathways via the binding of IL-22R1/IL-20R2. Finally, hepatic *IL1B, IL20*, and IκBζ target genes are elevated and correlated each other in patients with acute alcoholic hepatitis.

Conclusions: IL-20 selectively inhibits hepatic IL-6 production rather than exerts an IL-10 like broad anti-inflammatory properties and has opposing functions compared to IL-22 by aggravating acute hepatitis and bacterial infection. Thus, anti-IL-20 therapy may have benefits to control acute hepatitis and bacterial infection.

Lay Summary:

Immune cell-derived IL-20 induces NQO1 expression in hepatocytes by activating ERK/NRF2 and p38/NRF2 signaling pathway via the binding of IL-22R1/IL-20R2. The elevated NQO1 promotes IκBζ degradation in hepatocytes and subsequently downregulates IκBζ-target hepatoprotective and anti-bacterial genes (e.g. *Il6* and *Lcn2*), thereby accelerating acute hepatitis and bacterial infection. Collectively, IL-20 exacerbates acute hepatitis and bacterial infection by downregulating IκBζ-target genes in hepatocytes.

Graphical Abstract



Keywords

IL-10; IL-22; NQO1; liver; *Klebsiella pneumoniae*

Introduction

IL-20 subfamily of cytokines, which belong to IL-10 family, is comprised of IL-19, IL-20, IL-22, IL-24, and IL-26.[1] IL-20 subfamily cytokines signal through heterodimeric receptors comprising various combinations of several shared receptor subunits, namely, IL-20R1, IL-20R2, IL-10R2, and IL-22R1.[1] Among them, IL-22 is the best characterized cytokine that plays a crucial role in ameliorating epithelial cell injury and bacterial infection via the activation of STAT3 in epithelial cells, showing great therapeutic potential for various diseases.[2,3] In contrast to well-characterized IL-22, the functions of other IL-20 family cytokines are less clear. For example, although it was discovered almost 20 years ago and the data from IL-20 transgenic mice suggest IL-20 plays a role in controlling epidermal function and psoriasis,[4] the functions of IL-20 in other organs remain poorly understood and the use of IL-20 deficient ($Il20^{-/-}$) mice to explore its function has not been reported. Most data on IL-20 functions were obtained from using antibodies against IL-20, IL-20R1, or IL-20R2, or using mice deficient in *Il20r1* or *Il20r2*. Previous studies reported that treatment

with monoclonal antibodies against IL-20 or IL-20R1 attenuated liver injury induced by carbon tetrachloride (CCl_4).[5] The functions of IL-20R signaling have been investigated via the blockade of IL-20R1 or IL-20R2 using antibodies or using knockout mice.[6–8] For instance, knockout of *Il20r2* or treatment with anti-IL-20R2 antibodies attenuated *Staphylococcus aureus* infection, suggesting that IL-20R2 signaling promotes bacterial infection.[9] However, the data from the blockade of IL-20R1 or IL-20R2 cannot distinguish the function of IL-20 from other cytokines (such as IL-19, IL-24, and IL-26) that also signal via these receptors.

To precisely define the functions of IL-20 *in vivo*, we generated $Il20^{-/-}$ mice and used these mice to characterize IL-20 functions in several models of liver injury and bacterial infection. The most interesting finding we observed was that $Il20^{-/-}$ mice had much higher serum IL-6 levels (~3000 pg/ml) than WT mice (~1000 pg/ml) in Concanavalin A (ConA)-induced T-cell hepatitis model; while most other cytokines were barely affected. These data suggest that IL-20 selectively inhibits IL-6 production but does not have an IL-10 like broad anti-inflammatory property although IL-20 belongs to IL-10 family, which puzzled us because IL-20 activates STAT3 in a similar manner to IL-10. Our further studies revealed that IL-20 directly targets hepatocytes and subsequently upregulates the expression of NAD(P)H: quinone oxidoreductase 1 (NQO1) via the activation of ERK/ NRF2 and p38/NRF2 signaling pathways. Upregulated NQO1 induces hepatic transcription factor IκBζ ubiquitination, degradation, and subsequent downregulation of IκBζ-dependent genes including IL-6 that protects against liver injury and bacterial infection. In addition to IL-6, several other IκBζ-dependent genes in hepatocytes are also suppressed by IL-20, and some of them also play an important role in regulating acute hepatitis and bacterial infection.

Material and Methods:

Animal experiments

Eight- to ten-week-old male mice were used in all experiments. IL-20 deficient mice ($Il20^{-/-}$) (B6N.129S5-*Il20*^{tm1Lex}/Mmucd) were generated by deleting the IL-20 gene exon1 and described in Supplemental Materials and Fig. S1. $Il20^{-/-}$ mice and littermate wild-type (WT) controls were used.

For ConA-induced acute hepatitis model, mice were injected intravenously with ConA (12 mg/kg). For the bacterial infection model, mice were injected intraperitoneally with 3000 CFU *Klebsiella pneumonia* (*K.P*) strain 43816 (ATCC, Manassas, VA). All mouse studies were approved by the NIAAA Animal Care and Use Committee.

Human liver samples

Liver tissue samples from two cohorts of healthy control (HC), patients with severe alcoholic hepatitis (SAH) or cirrhosis are described in Supporting Materials and Supporting Table S1 and S2. These samples were obtained from the Liver Tissue Cell Distribution System (Minneapolis, Minnesota, the NIH #HHSN276201200017C), John Hopkins Hospital (R24AA025017, Clinical resources for alcoholic hepatitis investigators), and Indiana

University and Roudebush Veterans Administration Medical Center (VAMC). The study was approved by the IRB at the Indiana/Purdue University and Roudebush VAMC Research.

The following methods are described in Supplemental Materials:

*Human samples; In viv*o treatment with adenovirus *Nfkbiz* shRNA, *Nqo1* shRNA and control shRNA; *In vitro treatment with cycloheximide (CHX) and MG132 treatment*; and *Ubiquitination assay.*

Other common methods are described in Supplemental Materials

Statistical analysis—Data are expressed as the means ± SD for all *in vivo* experiments, means ± SEM for all *in vitro* experiments, and were analyzed using GraphPad Prism software (v. 8.0a; GraphPad Software, La Jolla, CA). To compare values obtained from three or more groups, a one-way ANOVA was used, followed by the Tukey post-hoc test. The two-tailed Student t test was performed to compare values obtained from two groups. *P* values of <0.05 were considered significant.

Results:

IL-20 selectively inhibits the expression of the hepatoprotective cytokine IL-6 in hepatocytes in acute hepatitis

Previous studies reported that IL-20 antibody treatment ameliorates CCl_4-induced liver fibrosis;[5] however, we found that although serum IL-20 levels were elevated after CCl_4 injection, there was no difference in the extent of liver fibrosis after CCl_4 injection between *Il20*$^{-/-}$ and littermate controls (Fig. S2A-D). Next, we used another model of ConA-induced acute T-cell hepatitis and found that in this model, serum IL-20 levels as well as IL-20 mRNA levels in the liver and spleen were highly elevated (Fig. 1A-B). *Il20*$^{-/-}$ mice were resistant to ConA-induced hepatitis as evidenced by lower serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), and the degree of liver necrosis compared with WT mice (Fig. 1C-D). Furthermore, *Il20*$^{-/-}$ mice had higher serum levels of the hepatoprotective cytokine IL-6 and IL-22 at the 6-hour (6h) time point post ConA injection compared to WT mice, while no differences in other cytokines were observed (IFN-γ, TNF-α, IL-4, and IL-12p70) (Fig. 1E). In agreement with elevated serum IL-6, hepatic *Il6* mRNA levels were also higher in *Il20*$^{-/-}$ mice compared to WT mice at an earlier time point (3h) post ConA injection, but other cytokine mRNA levels were comparable between WT and *Il20*$^{-/-}$ mice (Fig. 1F). Moreover, activation of STAT3, the downstream signal of IL-6, in the liver was greater in *Il20*$^{-/-}$ mice than in WT mice (Fig. 1G, Fig. S3A). Although serum IFN-γ levels were comparable between *Il20*$^{-/-}$ and WT mice, activation of its downstream signal STAT1 was attenuated in *Il20*$^{-/-}$ mice (Fig. 1G, Fig. S3A), which is probably due to stronger IL-6-induced STAT3 activation that is known to downregulate IFN-γ/STAT1 signaling.[10] Finally, hepatic expression of STAT3 downstream proteins cyclin D1 and BCL-X_L were higher while the expression of pro-apoptosis proteins BIM and cleaved caspase 3 were lower in ConA-treated *Il20*$^{-/-}$ mice compared to WT mice (Fig. 1H, Fig. S3B).

To understand how IL-20 regulates *Il6* gene expression in the liver, we first examined the expression of IL-20 receptors. As shown in Fig. S4A-B, *Il20r1* and *Il20r2* mRNAs were detected in different organs and cell types including immune cells from WT mice, while *Il22r1* mRNA was detected in various organs and hepatocytes but not in immune cells. On the other hand, ConA injection upregulated the shared receptor *Il20r2* mRNA expression in the liver and spleen, and upregulated *Il22r1* in the liver without affecting *Il20r1* (Fig. 2A). Protein levels of these three receptors in the liver were examined by western blotting (Fig. 2B) and immunohistochemistry staining (Fig. 2C), showing greater elevation of IL-20R2 after ConA injection.

Hepatocytes have been reported to produce IL-6 but the extent to which hepatocytes contribute to IL-6 production *in vivo* remains unknown.[11] To clarify this question, we performed immunofluorescence and immunohistochemistry staining of hepatic IL-6 protein and found IL-6 protein expression was markedly elevated in hepatocytes after ConA administration, and such expression was enhanced in $Il20^{-/-}$ mice compared with WT mice (Fig. 2D-E, Fig. S4C). To conclusively determine whether hepatocytes produce IL-6 and are an important source of serum IL-6, we generated hepatocyte-specific *Il6* deficient mice ($Il6^{\text{Hep}-/-}$) and found that serum IL-6 levels were reduced by ~50% in $Il6^{\text{Hep}-/-}$ mice compared to WT mice post Con A administration (Fig. 2F), suggesting that hepatocytes are one of the major sources for the production of serum IL-6 in ConA-mediated acute hepatitis.

IL-20 inhibits hepatic expression of IκBζ and its target genes including *Il6* in acute hepatitis

Next, we examined the molecular mechanisms underlying IL-20 regulation of hepatic IL-6 expression by examining an inducible nuclear IκB protein IκBζ, which is rapidly induced by IL-1β and plays a critical role in controlling IL-6 production.[12] As illustrated in Fig. 3A-B, in WT mice, hepatic expression of IκBζ protein and *Nfkbiz* mRNA (*Nfkbiz* encodes IκBζ protein) was strongly induced at 1h and rapidly declined 3h post ConA injection, while $Il20^{-/-}$ mice had greater hepatic upregulation of IκBζ protein but comparable *Nfkbiz* mRNA expression compared with WT mice (Fig. 3A-B), suggesting that IL-20 reduces IκBζ protein stability without affecting the mRNA expression.

In addition to *Il6*, several other genes are also controlled by IκBζ, including *Lcn2*, *Ccl2*, *Ccl20*, *Csf3*, *Il23a*, *S100a8*, and *S100a9*.[13–16] Most of these IκBζ-dependent genes were upregulated post ConA injection in the liver with greater elevation in $Il20^{-/-}$ mice compared with WT mice (Fig. 3C). Interestingly, the induction of hepatic IκBζ is not completely in accordance with the mRNA levels of its target genes along the different timepoints in Fig. 3C, which was probably because these genes are also regulated by other factors or expressed in a cell-specific manner. For example, Ccl20 gene expression is controlled by NF-κB, SP1, and AP1 in addition to IκBζ.[17] *S100a8* and *S100a9* genes are mainly expressed in neutrophils [18] but very low in hepatocytes.

To better understand the function of hepatic IκBζ, we silenced hepatic IκBζ with Ad-sh*Nfkbiz* (Fig. 3D), which reduced serum IL-6 levels but elevated serum ALT levels after ConA administration (Fig. 3E). Ad-sh*Nfkbiz* treatment also reduced hepatic expression of

IκBζ-dependent genes except *Ccl2* and *Ccl20* (Fig. 3F) but did not affect IκBζ-independent cytokine genes including *Ifng*, *Tnfa*, and IL-22 (Fig. S5).

IL-20 promotes bacterial infection by attenuating hepatic IκBζ-dependent antibacterial genes (*Il6* and *Lcn2)*

The above data suggest that IL-20 inhibits hepatic expression of IL-6 and other IκBζ target genes such as *Lcn2*, which are known to play a key role in controlling bacterial infection,[19] and thus we hypothesized IL-20 may affect bacterial infection. To test this hypothesis, we used *Klebsiella pneumoniae* (*K.P.*) infection model and found that serum IL-20 levels were significantly elevated post *K.P.* injection (Fig. 4A). *Il20*$^{-/-}$ mice had lower mortality and lower levels of circulating bacterial load than WT mice (Fig. 4B-C). Furthermore, hepatic expression of several IκBζ-dependent genes except for *Il23a* and *Ccl20* was much higher in *Il20*$^{-/-}$ mice than that in WT mice 3h post infection (Fig. 4D, left panel). Moreover, we examined several other IκBζ-independent antibacterial genes that are not controlled by IκBζ. [20] Many of them in the liver were upregulated in both WT and *Il20*$^{-/-}$ mice post bacterial infection, but there were no significant differences in such elevation between WT and *Il20*$^{-/-}$ mice (Fig. 4D, right panel). In addition, serum IL-6 and LCN2 levels were higher in *Il20*$^{-/-}$ mice compared with WT mice after *K.P.* infection, while serum IL-22 levels were comparable between these two groups (Fig. 4E). Finally, hepatic mRNA expressions of three *Il20r*s were upregulated 3h post infection (Fig. 4F). Hepatic upregulation of IL-20R1, IL-20R2 and IL-22R1 proteins was further confirmed by immunohistochemistry analyses (Fig. 4G).

IL-20 promotes IκBζ degradation in hepatocytes via the induction of NAD(P)H: quinone oxidoreductase 1 (NQO1)

To understand the mechanism by which IL-20 affects IκBζ protein expression, we performed *in vitro* cell culture experiments. As shown in Fig. 5A-B, treatment with IL-1β, which is known to induce IκBζ expression,[12] upregulated the expression of IκBζ protein and *Nfkbiz* mRNA in mouse hepatocyte AML12 cells. Interestingly, pre-treatment with IL-20 markedly reduced IL1β-induction of IκBζ protein expression without inhibiting *Nfkbiz* mRNA. Treatment of IL-20 alone did not affect either IκBζ protein or mRNA expression. Meanwhile, IL-1β treatment upregulated IκBζ-dependent genes, many of which were downregulated after pre-treatment with IL-20 (Fig. 5B).

Next, we evaluated whether IL-20 affects IκBζ protein stability by using *de novo* protein synthesis inhibitor cycloheximide (CHX). As illustrated in Fig. 5C, upon blockade of *de novo* protein synthesis after CHX treatment, IL-1β-induced IκBζ protein was rapidly degraded, such degradation was faster after pre-treatment with IL-20. Furthermore, treatment with MG132, a proteasome inhibitor that reduces the degradation of ubiquitin-conjugated proteins, equally prevented IL-1β-induced IκBζ degradation with or without treatment of IL-20 in hepatocytes. IκB degradation is known to depend on ubiquitin-mediated proteasomal degradation.[21] Thus, we wondered whether IL-20 affects IκBζ protein stability by regulating IκBζ ubiquitination. Indeed, our data revealed that IL-20 markedly enhanced IL-1β-induced IκBζ polyubiquitination in hepatocytes in the absence or

presence of MG132, with the stronger polyubiquitination signal in the MG132 group (Fig. 5D).

To understand how IL-20 promotes IκBζ degradation, we examined the role of NQO1, which is one of the two major quinone reductases that regulates protein stability by either promoting or attenuating the degradation of target proteins.[22,23] IL-20 treatment upregulated NQO1 expression in mouse AML12 hepatocytes and primary mouse hepatocytes (Fig. 6A). Knockdown of *Nqo1* in AML12 cells markedly enhanced IL-1β induction of IκBζ protein expression (Fig. 6B) and its dependent genes without affecting *Nfkbiz* mRNA levels (Fig. S6A-B). Furthermore, knockdown of *Nqo1* delayed IκBζ protein degradation without inhibiting its protein synthesis in cultured hepatocytes (Fig. S6C).

Hepatic expression of NQO1 mRNA and protein was also elevated in WT mice after ConA injection or *K.P* infection, while such elevation was diminished in *Il20*$^{-/-}$ mice (Fig. 6C-D). Knockdown of hepatic *Nqo1* expression with Ad-sh*Nqo1* (as demonstrated by immunofluorescence staining in Fig. 6E) upregulated hepatic IκBζ expression in mice compared to those treated with Ad-*Gfp* (Fig. 6F). Furthermore, compared with Ad-*Gfp*-treated mice, Ad-sh*Nqo1*-treated mice had lower serum ALT but higher serum IL-6 (Fig. 6G) and higher mRNA levels of IκBζ-dependent genes in the liver (Fig. 6H). In addition, IκBζ-independent genes including *Ifng* and *Tnfa* were not affected by *Nqo1* knockdown (Fig. S7).

IL-20 induces hepatic NQO1 expression by activating ERK/NRF2 and p38/NRF2 signaling pathways via the binding of IL-22R1/IL-20R2

To explore which IL-20 receptor complex is involved in IL-20 induction of hepatic NQO1 expression, we silenced different IL-20 receptor subunits and found knockdown of *Il20r2* and *Il22r1* but not *Il20r1* blocked IL-20 induction of NQO1 in hepatocytes (Fig. 7A), suggesting that IL-20 induces NQO1 via the binding of the IL-22R1/IL-20R2 not IL-20R1/IL-20R2 complex.

Next, we examined which signaling pathways are involved in IL-20 induction of NQO1 in hepatocytes. As illustrated in Fig. 7B and Fig. S8, IL-20 treatment activated STAT3 (p-STAT3) in primary mouse hepatocytes, which was much weaker than IL-22 treatment. Interestingly, IL-22 induced weaker STAT3 activation than IL-6 in primary hepatocytes (Fig. S9). Furthermore, IL-20 also weakly activated extracellular signal-related kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38) in hepatocytes. Moreover, IL-20 treatment markedly downregulated KEAP1 protein expression but upregulated NRF2 protein expression (Fig. 7B-C, Fig. S8). Immunofluorescence staining demonstrated that IL-20 treatment induced nuclear translocation of NRF2 in hepatocytes (Fig. 7C). In addition, IL-20 treatment did not activate aryl hydrocarbon receptor (AhR) (Fig. S10), another inducer of NQO1 expression.[24] Finally, pretreatment with ERK1/2 or p38 inhibitor but not JNK inhibitor blocked IL-20 induction of NQO1 in hepatocytes (Fig. 7D), suggesting that IL-20 induction of NQO1 is dependent on ERK/NRF2 and p38/NRF2 signaling pathways.

Hepatic mRNA levels of *IL1B*, *IL20,* and IκBζ-target genes are elevated and correlated in patients with SAH

The above data suggest that IL-1β upregulates IκBζ-target genes in hepatocytes, which is blocked by IL-20 via the induction of NQO1. To further explore whether this regulatory pathway is clinically relevant in the presence of severe hepatitis. We first measured hepatic expression of *IL1B* and IκBζ-target genes and determined their correlation in human samples from healthy controls (HC), SAH and alcohol/HCV cirrhosis. As demonstrated in Fig. 8A, *IL1B* and IκBζtarget gene levels including *IL6* and *CCL2* were remarkably upregulated in ASH and cirrhotic patients. Several other IκBζ-target gene levels were elevated in SAH patients but not in cirrhotic patients. Moreover, hepatic *IL1B* mRNA levels positively correlated with these IκBζtarget gene levels (Fig. 8B).

Furthermore, we also measured IL-20 and *NQO1* mRNA levels and found that compared to HC, IL-20 levels were significantly higher in SAH patients but not in cirrhotic patients, while hepatic *NQO1* mRNA levels trended higher in SAH but it did not reach to statistical difference (Fig. 8C). Interestingly, hepatic IL-20 levels also correlated with *NQO1* (Fig. 8C) and IκBζ-target gene levels (Fig. S11A). Finally, serum IL-20 levels were not elevated in SAH (Fig. S11B).

Discussion:

The first striking finding from the current study was that serum and hepatic IL-6 levels were highly upregulated in acute hepatitis from *Il20*$^{-/-}$ mice compared to WT mice while most other cytokines were comparable. Such IL-6 elevation is accompanied by the upregulation of hepatic STAT3 activation, a well-documented hepatoprotective signal,[10] which likely contributes to the resistance of *Il20*$^{-/-}$ mice to acute hepatitis. By using immunofluorescence staining and *Il6*$^{\text{Hep}-/-}$ mice, we provided conclusive evidence that hepatocytes are one of the major contributors for IL-6 production in acute hepatitis and such production is selectively attenuated by IL-20. Our mechanistic study suggests a model in which inflammatory mediators such as IL-1β upregulates hepatic IκBζ and its dependent genes, which is blocked by IL-20 (Figure 8D).

Our *in vivo* and *in vitro* data suggest that IL-20 attenuates the expression of several IκBζ -target genes in hepatocytes including *Il6*, *Lcn2*, *Ccl2*, *Ccl20*, *Csf3*, *S100a8*, and *S100a9*. Many of them are known to play an important role in ameliorating acute hepatitis and bacterial infection. For example, IL-6 is a well-documented hepatoprotective cytokine and plays a key role in controlling bacterial infection.[10,19] Hepatocytes are also the major source of the production of LCN2, which protects against ConA-induced hepatitis and bacterial infection.[19,25] CCL2/MCP-1 is a hepatoprotective chemokine in acute hepatitis[26] and plays a crucial role in the resolution and repair processes of acute bacterial infection.[27] S100A8 and S100A9 are well known to have antibacterial potential[28] and promote neutrophil chemotaxis, migration, and accumulation.[29] Collectively, IL-20 inhibition of these IκBζ-target genes discussed above likely contributes to IL-20 promotion of acute hepatitis and bacterial infection. In addition, IκBζ repression by IL-20 may also explain some previously reported IL-20 functions, such as IL-20 inhibition of neutrophil migration and function[30] and IL-20R2 signaling promotion of cutaneous infection with *staphylococcus aureus*.[9]

IκBζ is a member of the nuclear IκB family of proteins that serves as a key transcriptional factor via the association with NF-κB. IκBζ is undetectable in unstimulated cells but treatment with TLR ligands or IL-1β robustly induces IκBζ expression in macrophages.[12,31] However, the role of IκBζ in hepatocytes remains unknown. In the current study, we demonstrated that knockdown of *Nfkbiz* expression in hepatocytes exacerbated ConA-induced hepatitis via the downregulation of several IκBζ-target genes including *Il6*, *Lcn2*, *Csf3*, and *Il23a*, indicating that hepatic IκBζ plays an important role in controlling acute hepatitis. Moreover, our data revealed that IL-20 attenuated IκBζ protein expression without altering *Nfkbiz* mRNA expression *in vivo* and *in vitro*, and that IL-20 treatment markedly enhanced IκBζ ubiquitination and promoted IκBζ degradation in hepatocytes, which is mediated via the induction of NQO1.

NQO1 is expressed ubiquitously and was originally identified as the enzyme that catalyzes the reduction and detoxification of quinones and their derivatives.[32] In addition to its antioxidant properties, NQO1 also functions to either promote or inhibit protein stabilization, such as inhibition of the proteasomal degradation of tumor suppressor p53 and hypoxia-inducible factor-1α[33,34] but promotion of the ubiquitin-dependent IκBζ degradation in macrophages.[23] Our data here revealed that silencing of *Nqo1* upregulated IκBζ protein expression and IκBζ-dependent gene expression without affecting *Nfkbiz* mRNA expression in hepatocytes, suggesting that NQO1 promotes IκBζ degradation in hepatocytes.

The next obvious question is what signal activated by IL-20 induces NQO1 in hepatocytes. IL-20 exerts its function via the binding of IL-22R1/IL-20R2 or IL-20R1/IL-20R2. By using siRNA inhibition of receptor expression, we demonstrated that the IL-22R1/IL-20R2 but not IL-20R1/IL-20R2 complex is involved in IL-20 induction of NQO1 expression in hepatocytes, as deletion of *Il22r1* or *Il20r2* but not *Il20r1* blunted such induction. Interestingly, IL-20 induces weak hepatoprotective STAT3 activation in hepatocytes, which may be why IL-20 does not protect against hepatocyte damage. Hepatocytes express high level of IL-22R1 but relatively low levels of IL-20R1 and IL-20R2; thus, we speculate that high levels of IL-22R1 paired with low levels of IL-20R2 are sufficient to promote IL-20 induction of NQO1 expression but not strong STAT3 activation in hepatocytes. Furthermore, we found that IL-20 treatment leads to KEAP1 degradation and NRF2 nuclear translocation in hepatocytes, which depends on ERK and p38 MAPK activation. Because activation of Keap1/NRF2 signaling is an important mechanism for the induction of NQO1,[35] IL-20 activation of the ERK- and p38 MAPK-dependent Keap1/NRF2 signaling pathway likely contributes to IL-20 induction of NQO1 in hepatocytes. Further studies are required to identify how IL-20 activates ERK and p38 MAPK in hepatocytes.

In conclusion, IL-20 exacerbates acute hepatitis and bacterial infection via the reduction of IκBζ and its target genes in hepatocytes. Because of the broad expression of IL-20R1 and IL-20R2 in different cell types, it will be interesting to explore whether IL-20 exacerbates acute hepatitis and bacterial infection by also targeting IκBζ in other cell types outside of hepatocytes, including immune cells. Finally, our model described in Figure 8D is clinically relevant because both IL-β and IL-20 are elevated in many types of liver diseases [11,36] such as SAH as shown in our study. Although both IL-β and IL-20 correlated with hepatic levels

of IκBζ- dependent genes in SAH, we believe that IL-1β induces hepatic expression of IκBζ and its dependent genes, while elevated IL-20 blocks such induction and subsequently exacerbates liver injury and bacterial infection. Therefore, anti-IL-20 may prove to be a novel and potential therapeutic target for treatment of acute hepatitis and bacterial infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment:

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Abbreviations:

Ad	Adenovirus
AhR	aryl hydrocarbon receptor
ALC	alcoholic liver cirrhosis
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CASP3	caspase-3
Ccl2	chemokine (C-C motif) ligand 2
Ccl20	chemokine (C-C motif) ligand 20
CCl_4	carbon tetrachloride
CFU	colony-forming unit
ConA	Concanavalin A
Csf3	colony stimulating factor 3
HC	healthy control
IκBζ	Inhibitor of kappa B zeta
IL-20	interleukin-20
K.P.	*Klebsiella pneumoniae*
LCN2	lipocalin 2
Nfkbiz	NF-kappa-B inhibitor zeta

NQO1	NAD(P)H: quinone oxidoreductase 1
SAH	severe alcoholic hepatitis
siRNA	small interfering RNA
STAT1	transducer and activator of transcription 1
STAT3	transducer and activator of transcription 3
WT	wild-type

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Highlights:

- IL-20 exacerbates acute hepatitis and bacterial infection.
- IL-20 selectively inhibits IL-6 and LCN2 expression in hepatocytes by promoting IκBζ protein degradation.
- IL-20 induces NQO1 expression in hepatocytes, therefore promoting IκBζ degradation.

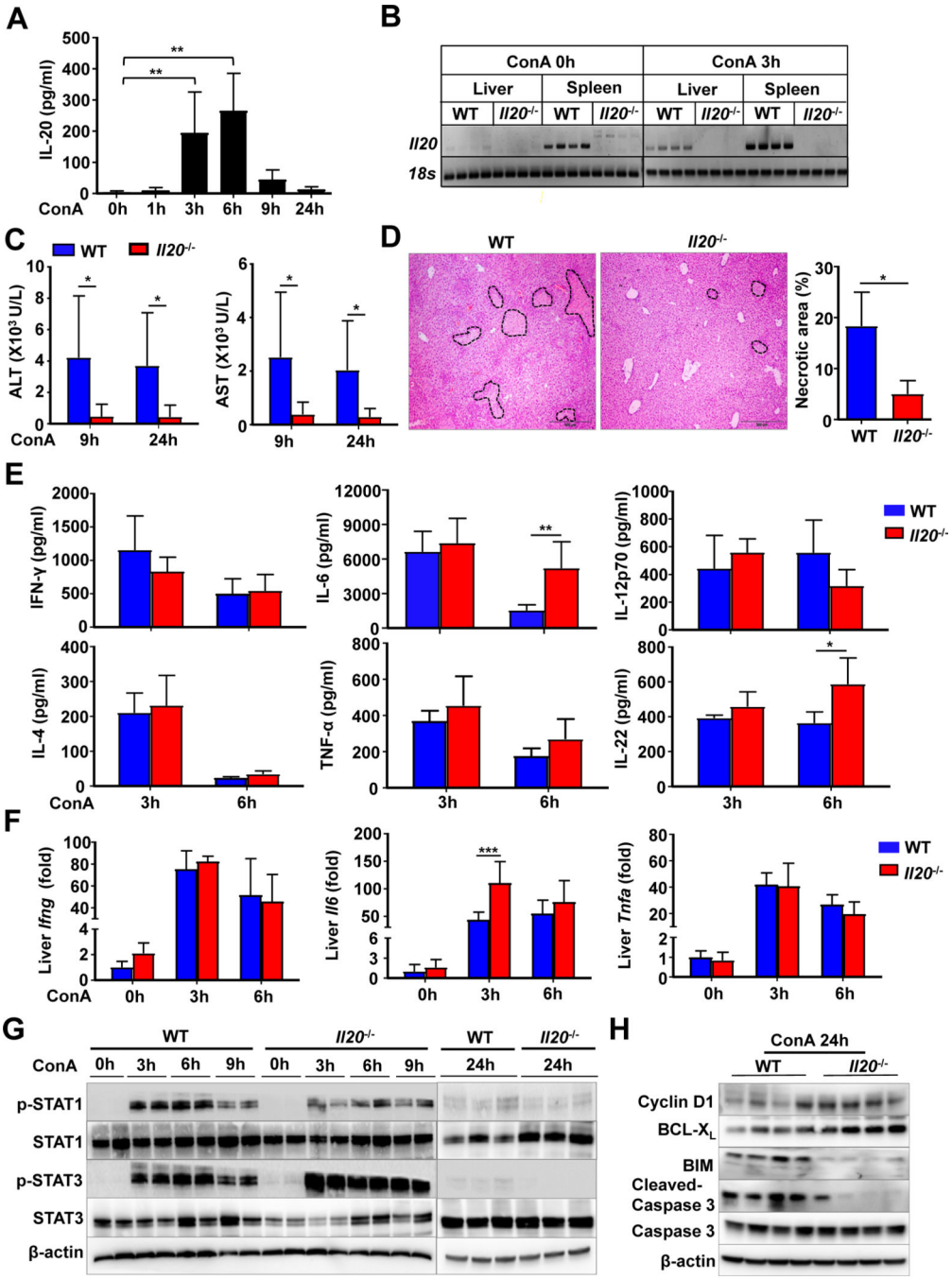

Figure 1. *Il20*$^{-/-}$ mice are resistant to ConA-mediated acute hepatitis by elevating hepatoprotective cytokine IL-6 in the liver.

(A) C57BL/6N mice (n=6) were intravenously injected with ConA (12 mg/kg). Serum IL-20 levels were measured. (B-H) WT (n=8) and *Il20*$^{-/-}$ mice (n=10) were intravenously injected with ConA. (B) RT-qPCR of IL-20 mRNA levels. (C) Serum ALT and AST levels. (D) Representative images of H&E staining are shown, the percentage of necrotic area per field was quantified. (E, F) Serum cytokine levels and hepatic cytokine mRNA levels. (G, H) Western blot analyses of IL-6 and IFN-γ downstream signaling pathways, and pro- and anti-

apoptotic protein levels in the liver post ConA injection. Values represent means ± SD. *P< 0.05, **P< 0.01, ***P< 0.001. ConA: Concanavalin A.

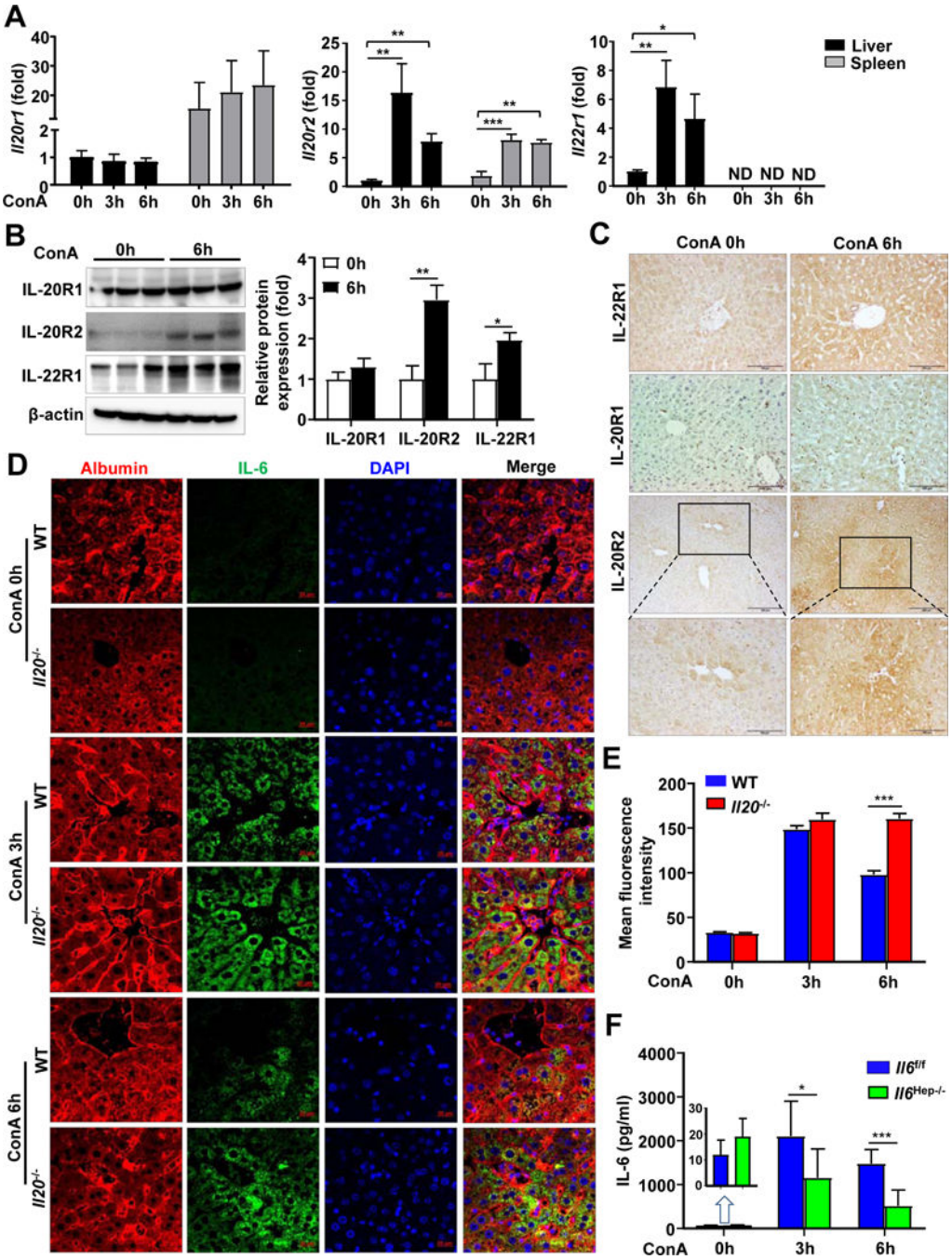

Figure 2. *Il20*$^{-/-}$ mice have greater IL-6 protein expression in hepatocytes than WT mice in acute hepatitis.

(A-C) C57BL/6N mice (n=4–6) were intravenously injected with ConA (12 mg/kg). (A) RT-qPCR analyses of liver mRNA levels. (B) Western analyses and quantification of IL-20 receptors. (C) Representative images of immunohistochemistry staining of IL-20 receptors. (D, E) WT (n=4–8) and *Il20*$^{-/-}$ mice (n=4–8) were intravenously injected with ConA. (D) Representative images of immunofluorescence staining of IL-6. (E) Mean IL-6 fluorescence intensity was quantified. (F) *Il6*$^{\text{Hep}-/-}$ (n=8) and littermate *Il6*$^{\text{f/f}}$ control mice (n=7) were

intravenously injected with ConA (12 mg/kg). Serum IL-6 levels were measured. Values represent means ± SD. *P< 0.05, **P< 0.01, ***P< 0.001. ConA: Concanavalin A.

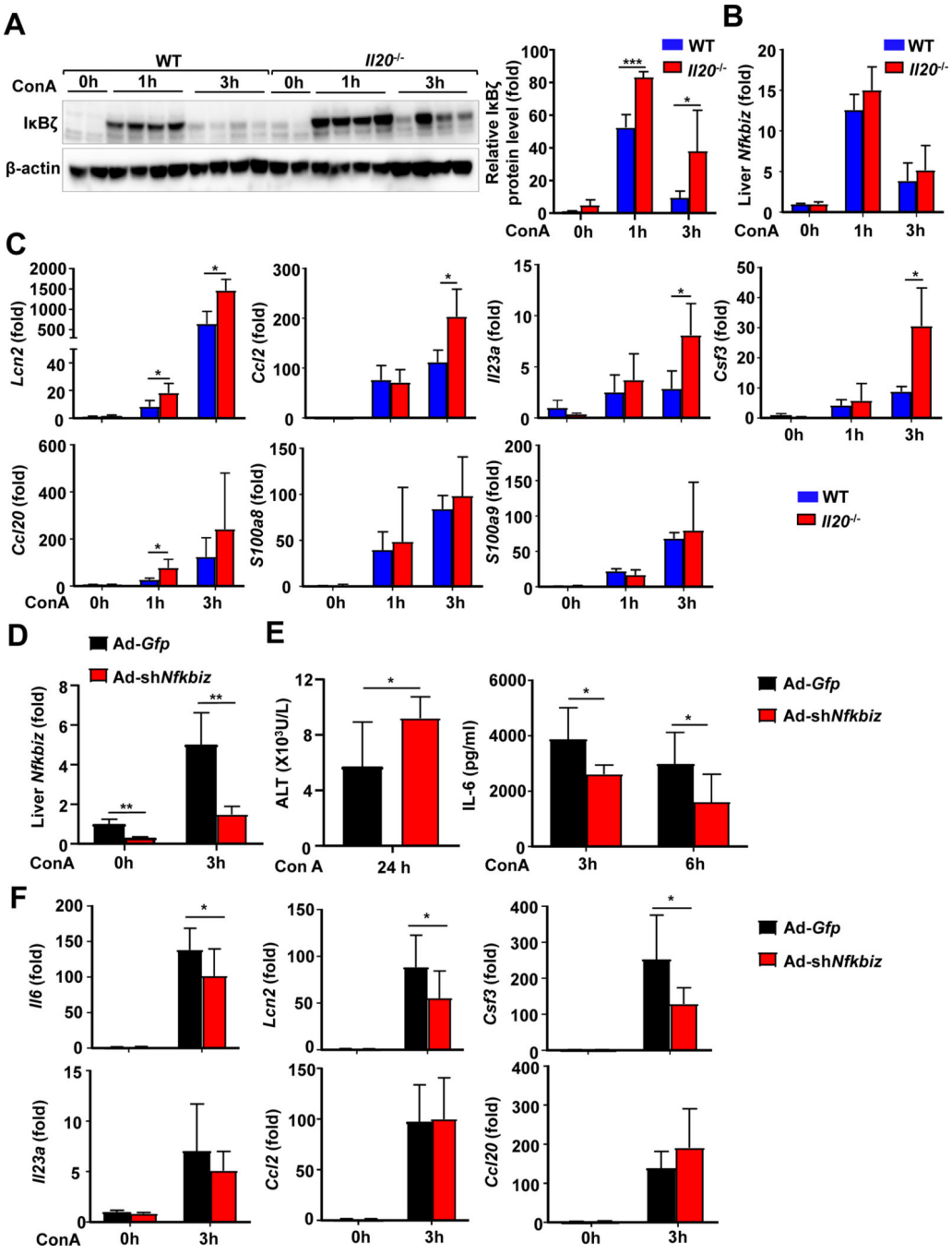

Figure 3. *Il20* deletion selectively enhances the expression of IκBζ-dependent genes including *Il6* and *Lcn2* in hepatocytes in acute hepatitis.

I (A-C) WT (n=4–8) and *Il20*$^{-/-}$ mice (n=4–8) were injected with ConA (12 mg/kg). (A) Western blot analyses of liver IκBζ expression. (B, C) RT-qPCR analyses of liver *Nfkbiz* mRNA and IκBζ-target genes. (D-F) C57BL/6N mice were injected with Ad-*Gfp* (n=3 in control group, n=5 in ConA-treated group) and Ad-sh*Nfkbiz* (n=3 in control group, n=6 in ConA-treated group) for 7 days, and followed by ConA injection. (D) RT-qPCR analyses of *Nfkbiz* mRNA. (E) Serum ALT and IL-6 levels were determined. (F) RT-qPCR analyses of

liver IκBζ-target genes. Values represent means ± SD. *P< 0.05, **P< 0.01, ***P< 0.001. ConA: Concanavalin A

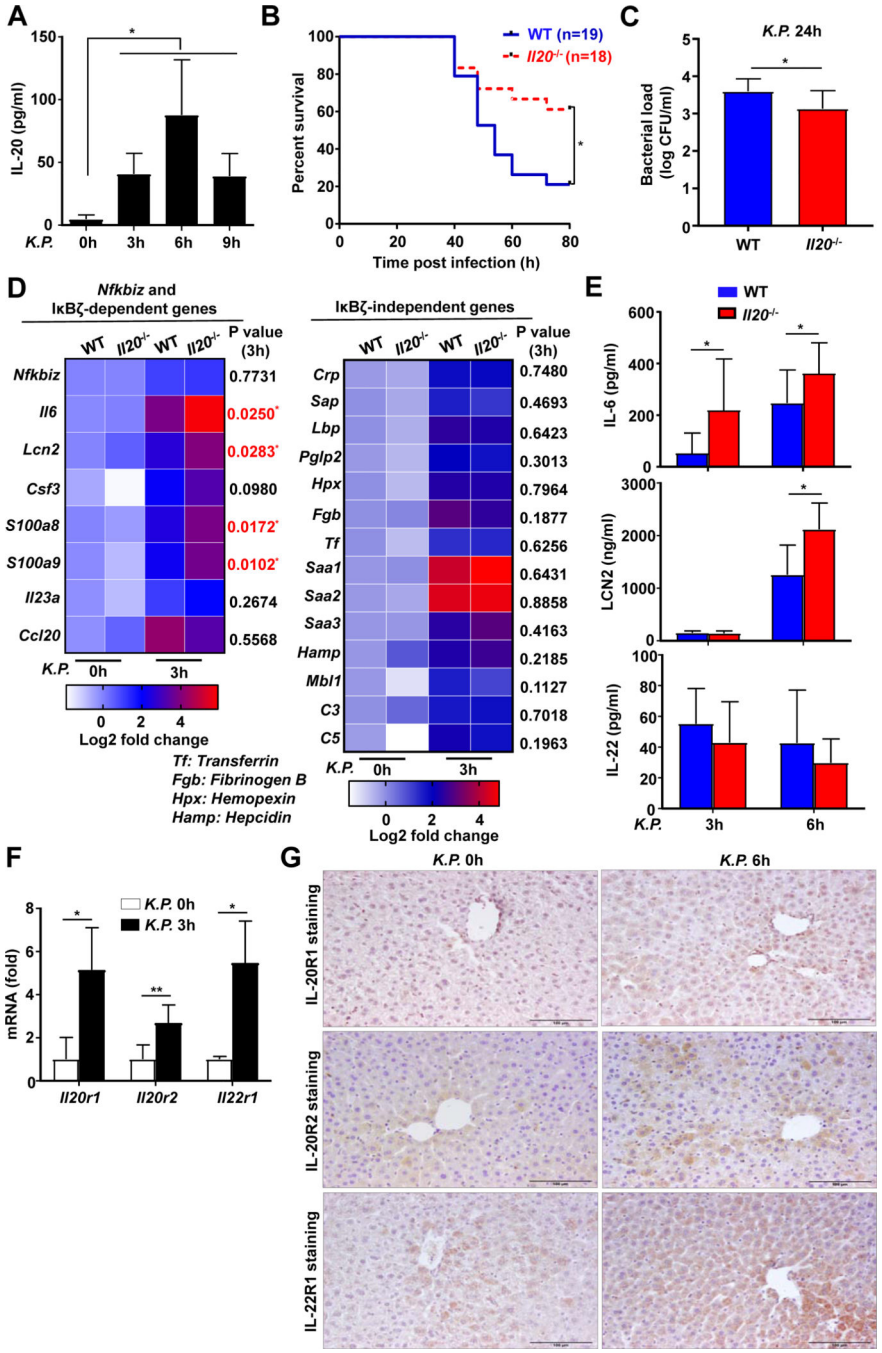

Figure 4. IL-20 deletion ameliorates bacterial infection by elevating hepatic expression of IκBζ-target antibacterial genes (eg. *Il6* and *Lcn2*).

(A) C57BL/6N mice (n=6) were infected with *K.P.* (3000 CFU). Serum IL-20 levels were measured. (B, C) WT (n=19) and *Il20*$^{-/-}$ mice (n=18) were infected with *K.P.*. (B) Survival rates were analyzed. **P*< 0.05. (C) Blood bacterial load was measured. (D, E) WT (n=4–7) and *Il20*$^{-/-}$ mice (n=4–8) were infected with *K.P.* (D) RT-qPCR analyses of hepatic expression of IκBζ-target genes and other antibacterial genes. P values (*Il20*$^{-/-}$ *versus* WT mice at 3-h time point) are indicated. (E) Serum IL-6, LCN2 and IL-22 were measured. (F, G) RT-qPCR analyses of hepatic expression of *Il20r* mRNAs and representative images of

liver IL-20 receptor staining in *K.P.*-infected mice. Values represent means ± SD. **P*< 0.05.
K.P.: Klebsiella pneumoniae

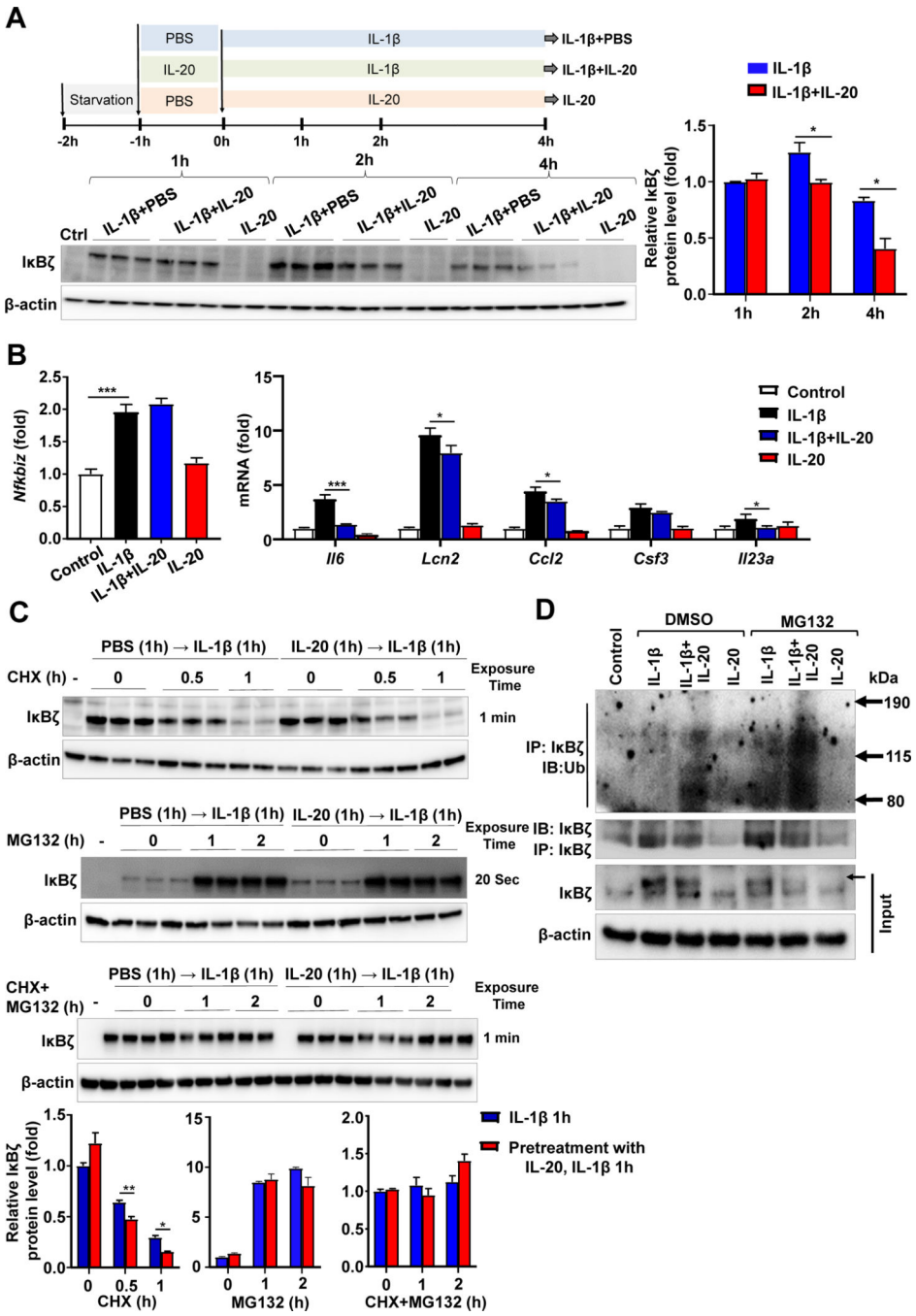

Figure 5. IL-20 downregulates the expression of IκBζ protein and its target genes in hepatocytes by promoting IκBζ degradation.

(A, B) Schematic treatment timeline of mouse AML12 hepatocytes. (A) Western blot analysis and quantification of IκBζ expression. (B) RT-qPCR analyses of *Nfkbiz* and IκBζ-target gene mRNAs at the 3h time point. (C) Serum-starved AML12 cells were treated with IL-20, IL-1β, and/or various inhibitors as indicated. Western blot analysis and quantification of IκBζ expression. (D) Serum-starved AML12 cells were pretreated with IL-20 for 1h, and then stimulated with IL-1β in the presence or absence of MG132 for 2 h. Ubiquitination assay for IκBζ was detected with anti-ubiquitin. Values represent means ± SEM from three

to four independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001. *K.P.: Klebsiella pneumoniae.* IL-20 (50 ng/ml); IL-1β (20 ng/ml); CHX (100 μg/ml); MG132 (20 μM)

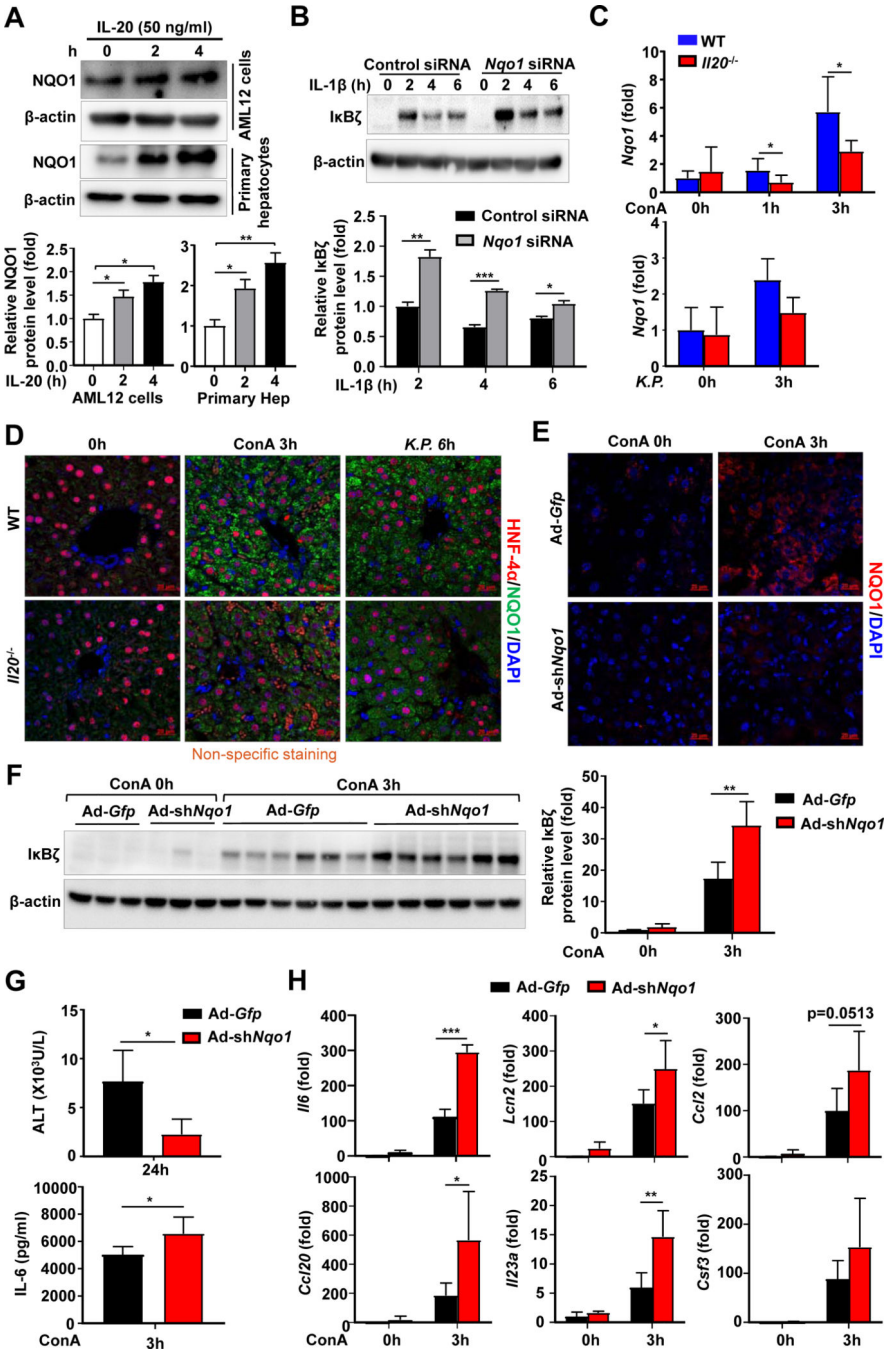

Figure 6. IL-20 promotes IκBζ degradation in hepatocytes via the induction of NQO1.

(A) Serum-starved AML12 cells or primary hepatocytes were treated with IL-20 (50 ng/ml) for the indicated time points. Western blot analysis and quantification of NQO1 expression. (B) AML12 cells were transfected with control siRNA or *Nqo1* siRNA for 24 h, and then treated with IL-1β (20 ng/ml) for the indicated time points. Western blot analysis and quantification of IκBζ expression. (C, D) WT and *Il20*$^{-/-}$ mice were injected with ConA (12 mg/kg) or *K.P.* (3000 CFU) for the indicated time points. Liver tissues were subjected to the measurement of *Nqo1* mRNA levels (panel C). Representative images of NQO1 (green),

hepatocyte marker HNF-4α (red), and nuclei (blue) are shown in panel D. (E-H) C57BL/6N mice were intravenously injected with Ad-*Gfp* (n=3 in control group, n=6 in ConA-treated group) and Ad-sh*Nqo1* (n=3 in control group, n=6 in ConA-treated group) for 7 days, and then followed by ConA injection. (E) Representative images of NQO1 (red) and nuclei (blue). (F) Western blot analysis and quantification of IκBζ expression. (G) Serum ALT and IL-6 levels. (H) RT-qPCR analyses of hepatic expression of IκBζ-dependent genes. Values represent means ± SEM from three to four independent *in vitro* experiments or means ± SD from *in vivo* experiments. *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$.

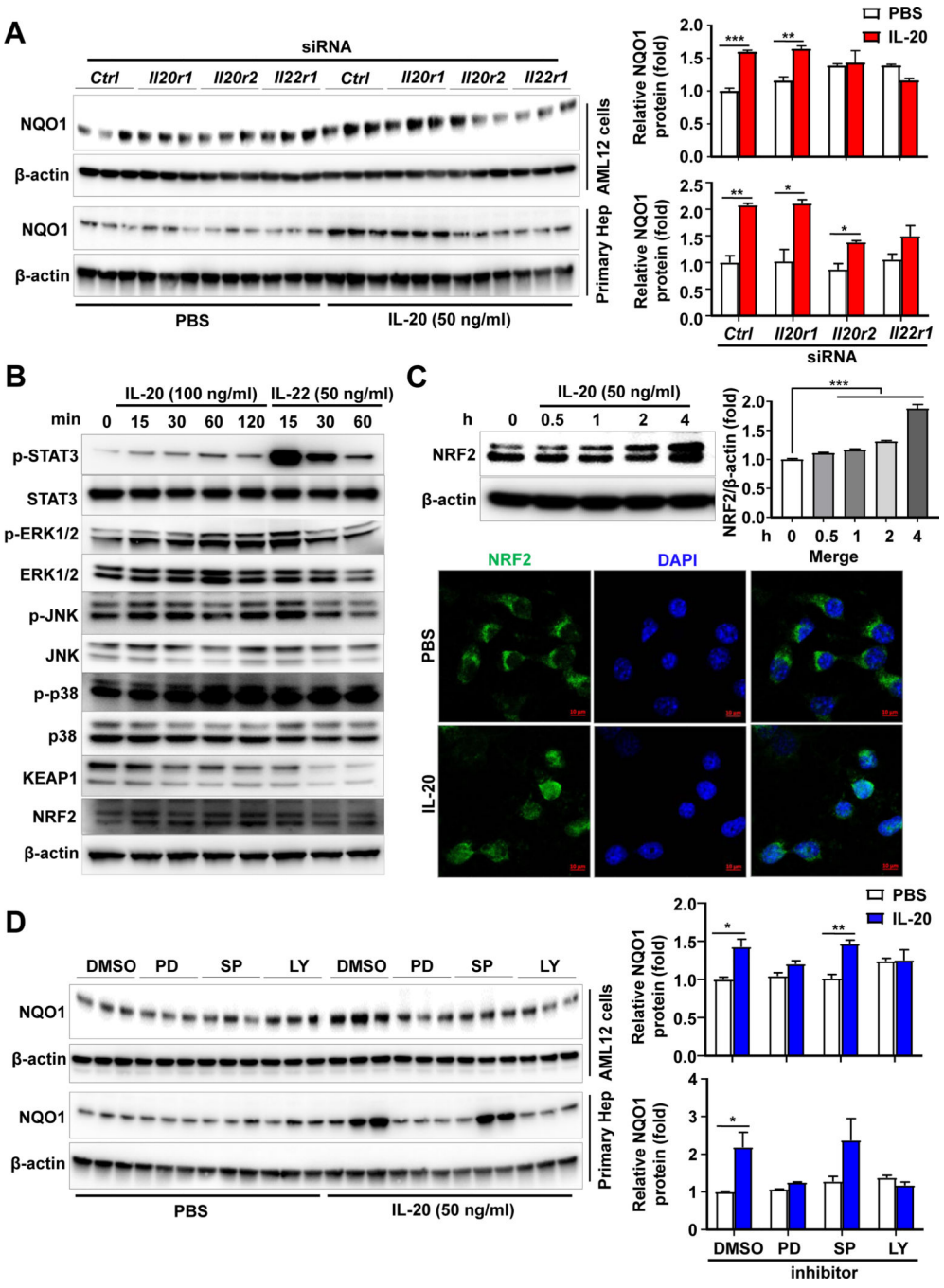

Figure 7. IL-20 induces NQO1 expression in hepatocytes by activating ERK/NRF2 and p38/NRF2 signaling via the binding of IL-22R1/IL-20R2.

(A) Silencing of different IL-20 receptors in AML12 cells or primary hepatocytes was followed by serum-starvation for 1h, and treatment with IL-20 (50 ng/ml) for 4h. Western blot analysis and quantification of NQO1 expression. (B) Serum-starved primary hepatocytes were treated with IL-20 (100 ng/ml) or IL-22 (50 ng/ml) for the indicated time points. Western blot analyses of various proteins. (C) Serum-starved AML12 cells were treated with IL-20 (50 ng/ml) for the indicated time points. Western blot analysis and quantification of NRF2 expression (top panel). NRF2 nuclear translocation was analyzed by

NRF2 immunofluorescence staining (lower panel). Representative images of NRF2 (green) and nuclei (blue) are shown. (D) Serum-starved AML12 cells or primary hepatocytes were pretreated with ERK1/2 inhibitor (PD98059, 50 μM), JNK inhibitor (SP600125, 50 μM), or p38 MAPK inhibitor (LY2228820, 2 μM) for 1h, and then stimulated with IL-20 (50 ng/ml) for 4h. Western blot analysis and quantification of NQO1 expression. Values represent means ± SEM from three to four independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001.

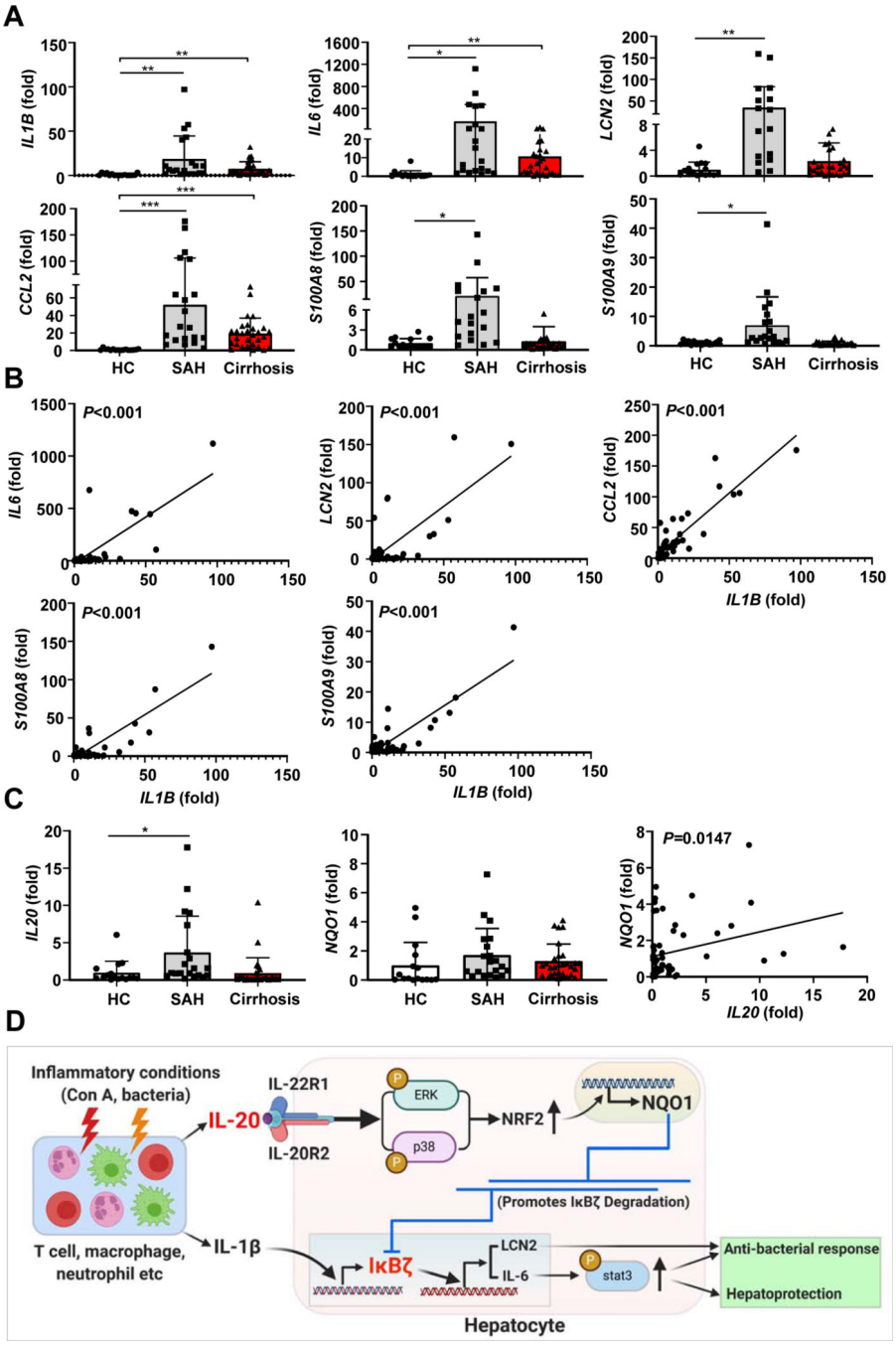

Figure 8. Hepatic expression and correlation of *IL1B*, *IL20*, and IκBζ-target genes in SAH patients.

Human liver samples from healthy controls (HC, n=16), severe alcoholic hepatitis (SAH, n=20), and alcohol/HCV cirrhosis (Cirrhosis, n=28) were used. (A) RT-qPCR analyses. (B) A positive correlation of *IL1B* with IκBζ-target genes in human liver samples. (C) RT-qPCR analyses of **IL-20** and *NQO1* mRNA levels and a positive correlation of IL-20 with *NQO1* in human liver samples. Values in panels A, C represent means ± SD. **P*< 0.05, ***P*< 0.01, ****P*< 0.001. **(D) The schematic model depicting IL-20 exacerbates acute hepatitis and**

bacterial infection by downregulating IκBζ-target genes in hepatocytes. Under inflammatory conditions, inflammatory cells release various cytokines including IL-1β and IL-20. IL-1β induces IκBζ expression in hepatocytes and subsequently elevates its target genes including *Il6* and *Lcn2*, which exert hepatoprotective function and limit bacterial infection. Meanwhile, IL-20 induces hepatic NQO1 expression by activating ERK/NRF2 and p38/NRF2 signaling pathway via the binding of IL-22R1/IL-20R2. The elevated NQO1 accelerates liver injury and bacterial infection by promoting IκBζ degradation and subsequent downregulation of IκBζ-target gene expression in hepatocytes.