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High Mobility Group Box 1 Release by Cholangiocytes Governs Biliary Atresia Pathogenesis and Correlates With Increases in Afflicted Infants

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BACKGROUND AND AIMS: Biliary atresia (BA) is a devastating cholangiopathy of infancy. Upon diagnosis, surgical reconstruction by Kasai hepatoportoenterostomy (HPE) restores biliary drainage in a subset of patients, but most patients develop fibrosis and progress to end-stage liver disease requiring liver transplantation for survival. In the murine model of BA, rhesus rotavirus (RRV) infection of newborn pups results in a cholangiopathy paralleling that of human BA. High-mobility group box 1 (HMGB1) is an important member of the danger-associated molecular patterns capable of mediating inflammation during infection-associated responses. In this study, we investigated the role of HMGB1 in BA pathogenesis.

APPROACH AND RESULTS: In cholangiocytes, RRV induced the expression and release of HMGB1 through the p38 mitogen-activated protein kinase signaling pathway, and inhibition of p38 blocked HMGB1 release. Treatment of cholangiocytes with ethyl pyruvate suppressed the release of HMGB1. Administration of glycyrrhizin *in vivo* decreased symptoms and increased survival in the murine model of BA. HMGB1 levels were measured in serum obtained from infants with BA enrolled in the PROBE and START studies conducted by the Childhood Liver Disease Research Network. High HMGB1 levels were found in a subset of patients at the time of HPE. These patients had higher bilirubin levels 3 months post-HPE and a lower survival of their native liver at 2 years.

CONCLUSIONS: These results suggest that HMGB1 plays a role in virus induced BA pathogenesis and could be a target for therapeutic interventions in a subset of patients with BA and high HMGB1. (Hepatology 2021;74:864-878).

Biliary atresia (BA) is a disease of infancy in which a devastating fibroinflammatory cholangiopathy occurs, leading to obstructive jaundice. BA leads to end-stage liver disease (ESLD).[1-4] In the United States, the incidence of BA is 1 in 15,000 births,[5] and it is the most common indication for pediatric liver transplantation.[6,7] In an effort to restore bile flow, a Kasai hepatoportoenterostomy (HPE) is typically performed soon after diagnosis.[8] However, even if an HPE is performed and cholestasis resolves, bile duct proliferation and fibrosis may progress,[7] resulting in the development of portal hypertension and the complications of ESLD.[9] It is estimated that 60% of the patients who overcome perinatal cholestasis will still need a liver transplantation before the age

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BA, biliary atresia; BAhiH, patients with BA and high HMGB1; BAhiH-S, patients with BA and high HMGB1 treated with steroids; BAloH, patients with BA and low HMGB1; CD, cluster of differentiation; ChiLDReN, Childhood Liver Disease Research Network; EP, ethyl pyruvate; EUK-8, Eukarion-8; GRV, goat rotavirus; HMGB1, high-mobility group box 1; HPE, hepatoportoenterostomy; IHC, intrahepatic cholestasis; MAPK, mitogen-activated protein kinase; MAPKAPK2, MAPK-activated protein kinase 2; mCL, murine cholangiocyte; MFI, mean fluorescence intensity; MOI, multiplicity of infection; NK, natural killer; p-, phosphorylated; ROS, reactive oxygen species; RRV, Rhesus rotavirus; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; UV, ultraviolet; VP4, viral protein 4.

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of 20 years.[10] Although the management of patients with BA has improved, their long-term survival still relies on invasive interventions, highlighting the necessity of targeted therapies to prevent liver injury.

The etiology of BA remains uncertain. A perinatal infection is a potential cause as viruses including rotavirus, reovirus, human papillomavirus, Epstein-Barr virus, and cytomegalovirus have been found in the liver explants of children afflicted with BA.[4,5] Additional evidence supporting a viral etiology is the murine model of BA where perinatal exposure to rhesus rotavirus (RRV) triggers biliary obstruction in a fashion that parallels human BA.[11]

Rotavirus infection is the most common cause of diarrheal outbreaks worldwide and is associated with significant morbidity and mortality. Because rotavirus has been found in the blood of infected infants, it is possible that perinatal rotavirus exposure could result in cholangiocyte infection.[12,13] We have shown that human cholangiocytes are susceptible to rotavirus infection in a strain-specific pattern that is analogous to the murine model of BA.[14]

High-mobility group box 1 (HMGB1), a member of the alarmin family, is a nuclear chromatin protein; however, when released extracellularly, it alerts the immune system to tissue damage and triggers an immediate response.[15] Extracellular HMGB1 has been shown to be a key signaling molecule involved in many pathological conditions including cancer,[16] ischemia/reperfusion injury,[17] cardiovascular disease, and inflammatory lung diseases.[18] HMGB1, in response to exogenous and endogenous stimuli (cytokines, lipopolysaccharide [LPS]), hypoxia, and infection,[15,19-21] can either be released passively by necrotic or damaged cells[22] or be actively secreted by various cell types including monocytes, macrophages, natural killer (NK) cells, dendritic cells, and hepatocytes. Upon release, HMGB1 mediates innate and adaptive immune responses.[23,24] Ethyl pyruvate (EP), a simple lipophilic pyruvate ester, inhibits the release of HMGB1.[25] Glycyrrhizin, another natural anti-inflammatory agent, is a pharmacological inhibitor that blocks HMGB1-induced inflammatory responses. Glycyrrhizin has been used to treat patients with chronic hepatitis, and one study showed that glycyrrhizin reduces liver disease in a mouse model of hepatitis B by interfering with HMGB1-induced recruitment of inflammatory cells in the liver.[26]

The molecular mechanisms underlying RRV-induced BA remain unclear; however, experimental evidence suggests that the hepatic inflammatory response

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plays a fundamental role in disease pathogenesis. In the liver, the major targets of RRV infection are biliary epithelial cells (cholangiocytes), which respond to infection by producing a variety of proinflammatory mediators involved in liver immune/inflammatory responses.[27] The mechanisms by which cholangiocytes trigger inflammatory responses have not been clearly defined. In this study, we focused on determining the mechanisms of active release of HMGB1 by RRV-infected cholangiocytes. We used an *in vitro* model of RRV infection of cholangiocytes to define the basis by which HMGB1 is secreted and how it contributes to biliary obstruction in the murine model of BA. We observed a dose-dependent release of HMGB1 upon RRV infection, which can be blocked by EP. Mice treated with glycyrrhizin were protected from murine BA. We also investigated the involvement of signal transducer and activator of transcription 1 (STAT1) and the p38 axis, which played a role in HMGB1 release. We analyzed preexisting proteomic data obtained from infants afflicted with BA and found that HMGB1 protein levels were higher in a cohort of patients that correlated with persistent jaundice and decreased native liver survival. Our results implicate HMGB1 in BA pathogenesis.

Materials and Methods

CELLS AND VIRUSES

MA104, monkey kidney cells (BioWhittaker, Walkersville, MD); a murine cholangiocyte (mCL) cell line generously provided by the laboratory of James Boyer (Yale Liver Care Center, Hartford, CT); and H2.35, a hepatocyte cell line, purchased from the ATCC (Manassas, VA) were cultured as described.[28] Five culture-adapted rotavirus strains were grown in MA104 cells and used in this study: RRV, goat rotavirus (GRV; Dr. Osamu Nakagomi, Nagasaki University), Ro1845 (Dr. Yasutaka Hoshino, National Institute of Allergy and Infectious Diseases, National Institutes of Health), two reassortant strains ($Ro1845^{RRV(VP4)}$ and $RRV^{Ro1845(VP4)}$) generated in our lab,[11,29] and RRV that had been purified by cesium chloride centrifugation and inactivated by psoralen with long-wave ultraviolet (UV) light.[30] The human cholangiocytes (H69), provided by Dr. Douglas Jefferson (New England Medical Center, Tufts University, Boston, MA), were cultured as described.[14]

INFECTION OF CELLS AND INHIBITORS

RRV infection of cell lines was performed in 24-well culture plates containing cells seeded at 4×10^5/well and incubated at 37°C for 3 days. Confluent plates were washed twice with media and infected with RRV at varying multiplicities of infection (MOIs). An MOI of 1 indicates that the amount of infectious virus is equal to the number of cells in the culture condition. Incubation was performed at 37°C on a plate rocker for 1 hour. The cultures were washed and incubated with serum-free DMEM at 37°C for varying amounts of time (1-24 hours). For treatment studies, cultures were overlaid with serum-free DMEM plus either 2.5 μg of EP (Fisher), 10 μg of fludarabine (Sigma), 20 μM of SB203580 (Invitrogen), or DMSO (Sigma) for 5 hours. Media was then removed and replaced by fresh serum-free DMEM and incubated for an additional 18 hours.

ELISA FOR DETECTION OF HMGB1, ASPARTATE AMINOTRANSFERASE, AND ALANINE AMINOTRANSFERASE

Quantitative assessment of HMGB1, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels was done using an enzyme immunoassay kit (Aviva Systems Biology, San Diego, CA; Abcam, Cambridge, MA). Serum samples were diluted 1:100 for HMGB1, 1:600 for AST, and 1:10 for ALT, followed by assays performed according to the manufacturer's instructions. The optical density was determined using a microplate reader (Spectramax Plus; Molecular Devices, Sunnyvale, CA) set to 450 nm, and HMGB1 concentrations were calculated by a computer program (Softmax Pro Version 2.2.1; Molecular Devices).

QUANTIFICATION OF REACTIVE OXYGEN SPECIES

Eight-well chamber slides were used to quantify the amount of reactive oxygen species (ROS) produced in cholangiocytes following rotavirus infection. Confluent cell monolayers in chamber slides were washed twice and infected with RRV and Ro1845 at an MOI of 10 for 1 hour at 37°C. Cells were then

washed twice, overlaid with serum-free media containing 100 μM 2′,7′-dichlorofluorescein diacetate (Invitrogen; a fluorogenic dye that measures hydroxyl, peroxyl, and other ROS activity within the cell), and incubated at 37°C for 2 hours. Following incubation, the cells were washed once, overlaid with phosphate-buffered saline, and visualized by fluorescent microscopy (Nikon Ti Eclipse, Japan) at 495 nm. Images were quantified by mean fluorescence intensity (MFI) using Nikon Elements software. Inhibition studies included the addition of 2 mM Eukarion-8 (EUK-8), a salen manganese complex (Sigma) in overlay media.

HUMAN STUDY SAMPLES

The details of human subjects, serum samples, and clinical and histological data were previously reported[31] and relate to patients enrolled in the two studies of the Childhood Liver Disease Research Network (ChiLDReN): the PROBE (Clinical Trials.gov identifier NCT00061828) and START trials. The source of the patient samples is described in Supporting Fig. S1. Sera were collected at the time of diagnosis (initial evaluation prior to or at the time of Kasai portoenterostomy) from 124 subjects with BA who did not receive corticosteroids, 51 subjects with BA who received corticosteroids, 70 subjects with neonatal intrahepatic cholestasis (IHC) serving as disease controls, and 9 subjects at 2-3 years of age (organ donors without history of prior liver disease) serving as normal controls. The serum samples of subjects with BA, those with IHC, and normal controls were analyzed by the SOMAscan assay (SomaLogic, Inc., Boulder, CO) as described.[31]

See the Supporting Information for further details on materials and methods.

STATISTICAL ANALYSIS

Assessment of the development of symptoms and mortality rates following inoculation with each rotavirus strain was based on experimental groups of at least 20 pups each. The findings were presented as percentages of pups expressing at least two disease symptoms and percent survival. Analysis of noncontinuous variables was performed using chi-squared and Fisher exact testing. Each subset of tissues used for histology and viral assays was derived from at least six pups. These continuous variables were analyzed for variance, with *post hoc* testing where appropriate, and expressed as means ± standard error of the mean. The *in vitro* data were analyzed with an unpaired *t* test between groups. Assessment of log-transformed 3-month bilirubin by experimental group was performed using linear regression. Comparison of 2-year native liver survival across experimental groups was performed using log-binomial regression modeling. $P < 0.05$ was considered significant.

Results

HMGB1 IS INCREASED IN THE SERUM OF MICE EXHIBITING SYMPTOMS OF BA

All animal research was performed in accordance with regulations and protocols approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital Medical Center (protocol number IACUC2019-0063), which adheres to the National Institutes of Health OLAW regulation (Animal Assurance number A3108) and the Animal Welfare Act (certification number 31-8-001).

It has been shown that HMGB1 levels were elevated in serum samples of infants with BA and positively correlated with gamma-glutamyl transferase levels.[32] In the murine model of BA, serum samples collected at different time points following RRV infection were analyzed. We found that the concentration of HMGB1 was increased by 2.5-fold to 3.5-fold at 5, 7, and 12 days post-RRV inoculation. In contrast, HMGB1 levels in serum collected from Ro1845 (a human strain which does not induce BA)–inoculated mice remained stable and similar to those of saline-injected control mice (Fig. 1A).

RRV INFECTION INDUCED HMGB1 SECRETION IN CHOLANGIOCYTES

We have shown that RRV infects cholangiocytes.[11,28] To determine if cholangiocytes are the source of HMGB1 in the BA model, mCL and murine H2.35 (hepatocyte) cells were infected with RRV at an MOI of 10 and 100.[28] Supernatants were harvested at 16 hours postinfection and analyzed by western blotting for HMGB1. We found that RRV induced a substantial increase in HMGB1 release

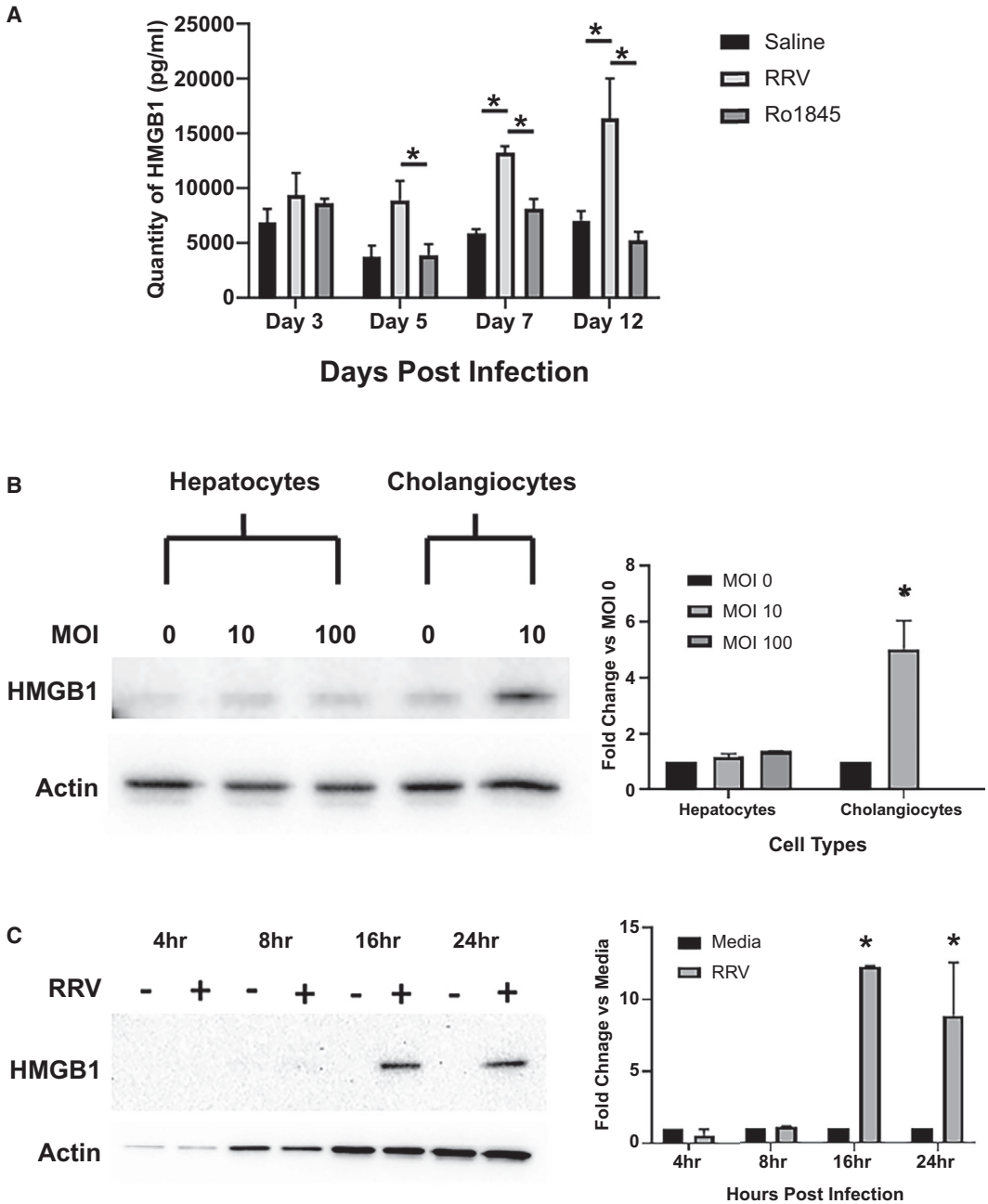

FIG. 1. Quantification of serum levels of HMGB1 *in vivo* and temporal release from cholangiocytes *in vitro*. Serum harvested from RRV-injected, Ro1845-injected, and saline-injected mice at 3, 5, 7, and 12 days postinfection demonstrates significantly higher levels of HMGB1 in RRV-injected mice starting at day 7 when compared to both saline-injected and Ro1845-injected pups (A). Cholangiocytes infected with RRV at an MOI of 10 resulted in a significant increase in HMGB1 release into the supernatant at 24 hours postinfection; conversely, H2.35 infection demonstrated no increase of HMGB1 even at a 10-fold higher MOI (B). Following RRV infection of cholangiocytes, HMGB1 release was detected in the supernatant at 16 hours and continued until 24 hours (C) (*$P < 0.05$).

from cholangiocytes. In contrast, there was no increase in H2.35 cells even at an MOI of 100 (Fig. 1B). To establish the time course for HMGB1 release, cholangiocytes were infected with RRV at an MOI of 10 for 4, 8, 16, and 24 hours. Analysis of the supernatants revealed a time-dependent release when compared with uninfected cells beginning at 16 hours (Fig. 1C). Densitometric analysis of the western blot showed

that RRV infection after 16 and 24 hours increased the HMGB1 release by 12-fold and 8-fold, respectively. HMGB1 release *in vitro* can be a function of active release but also of cell death. To determine which process was occurring in RRV-infected cholangiocytes, cell viability was determined using trypan blue exclusion at 24 hours after infection. We found no difference in cell count between RRV-infected and uninfected cells (Supporting Fig. S2), suggesting that HMGB1 release from cholangiocytes was a function of RRV infection and not a result of cell death.

Replication-Competent RRV is Required for HMGB1 Release from Cholangiocytes

To determine whether viral infectivity is required for HMGB1 release from cholangiocytes, we analyzed HMGB1 protein levels in cells incubated with UV-inactivated virus. Cholangiocytes were infected with RRV and inactivated RRV, and supernatants were harvested at 16 hours postinfection. We found that UV inactivation of RRV did not induce the release of HMGB1 from cholangiocytes when compared with cells infected with live RRV (Fig. 2A). These results suggested that the active replication of virus was required to trigger HMGB1 release from cholangiocytes.

HMGB1 RELEASE FROM CHOLANGIOCYTES IS ROTAVIRUS STRAIN–SPECIFIC AND RRV VIRAL PROTEIN 4–DEPENDENT

We have shown in the murine model that induction of BA is RRV viral protein 4 (VP4)–dependent and that the rotavirus strains which have a significant homology with VP4 are able to induce BA as

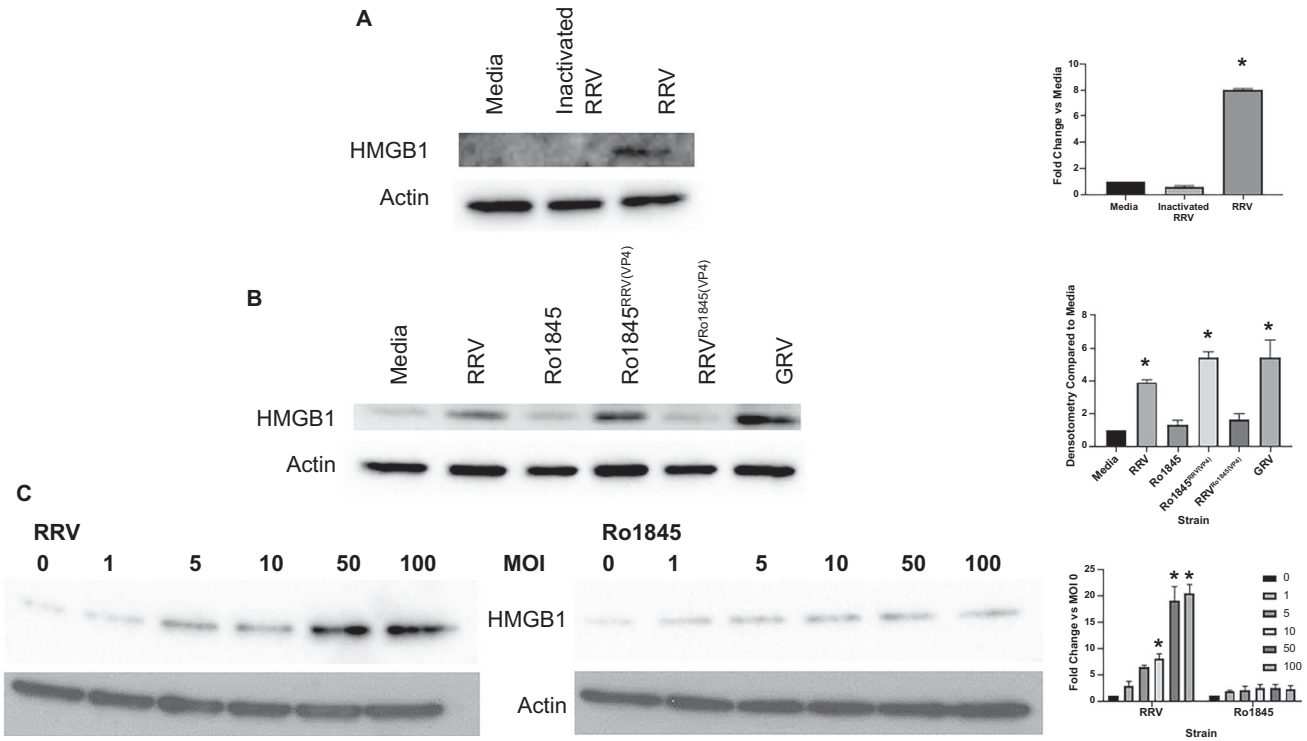

FIG. 2. HMGB1 release from cholangiocytes is replication-dependent, strain-dependent, and dose-dependent. Infection with inactivated RRV resulted in no HMGB1 release from cholangiocytes at 16 hours postinfection when compared to infectious RRV (A). Cholangiocytes infected with multiple strains of rotavirus demonstrated HMGB1 release from RRV and GRV, while no release was observed from Ro1845. Additionally, HMGB1 release is facilitated by the rotavirus gene VP4 as $Ro1845^{RRV(VP4)}$ is capable of inducing HMGB1 secretion, while the incorporation of Ro1845's VP4 to RRV ($RRV^{Ro1845(VP4)}$) results in loss of HMGB1 release (B). Cholangiocytes infected with increasing MOIs of RRV lead to a dose-dependent increase in HMGB1 release; conversely, no increase was witnessed with increasing MOIs of Ro1845 (C) (*$P < 0.05$).

well.[29] We next assessed if RRV VP4 was required for HMGB1 release from cholangiocytes. To test this, cholangiocytes were infected with RRV, Ro1845, GRV (induces BA), and reassortants ($Ro1845^{RRV(VP4)}$ and $RRV^{Ro1845(VP4)}$). The supernatants collected from BA model–inducing strains, RRV and GRV, had significantly higher levels of HMGB1 as detected by western blot (4-fold and 5-fold changes, respectively) over Ro1845 and control ($P < 0.05$). We next sought to determine whether this increase is mediated by RRV's VP4 gene. Significantly increased levels of HMGB1 were noted in RRV and $Ro1845^{RRV(VP4)}$ (Ro1845 reassortant in which the VP4 gene is from RRV) but not in Ro1845 or saline controls ($P < 0.05$). In the RRV reassortant $RRV^{Ro1845(VP4)}$ (where RRV VP4 is replaced by Ro1845 VP4), the release of HMGB1 was abolished (Fig. 2B), suggesting that RRV VP4 is required for HMGB1 release. To determine if Ro1845's inability to induce the release of HMGB1 was simply a function of viral titer, cholangiocytes were infected with increasing MOIs of both RRV and Ro1845. A significant dose-dependent response was observed following infection with RRV, whereas infection with Ro1845 resulted in no significant increase in HMGB1 release even at higher MOIs (Fig. 2C).

EP INHIBITED HMGB1 RELEASE FROM CHOLANGIOCYTES

To determine the effect of EP on HMGB1 release, cholangiocytes were infected for 16 hours with or without EP, and HMGB1 release was measured in the supernatants. EP treatment significantly inhibited RRV-induced HMGB1 secretion (Fig. 3A). There was no significant difference in the virus titer between cells treated with or without EP, suggesting that EP itself has no inhibitory effect on virus replication (Fig. 3B).

HMGB1 RELEASE FROM CHOLANGIOCYTES IS p38/STAT1-DEPENDENT

Mitogen-activated protein kinases (MAPKs) are involved in regulating the expression of various cytokines and chemokines. We sought to identify if MAPKs are involved in HMGB1 release in cholangiocytes. Following infection with RRV, Ro1845, and

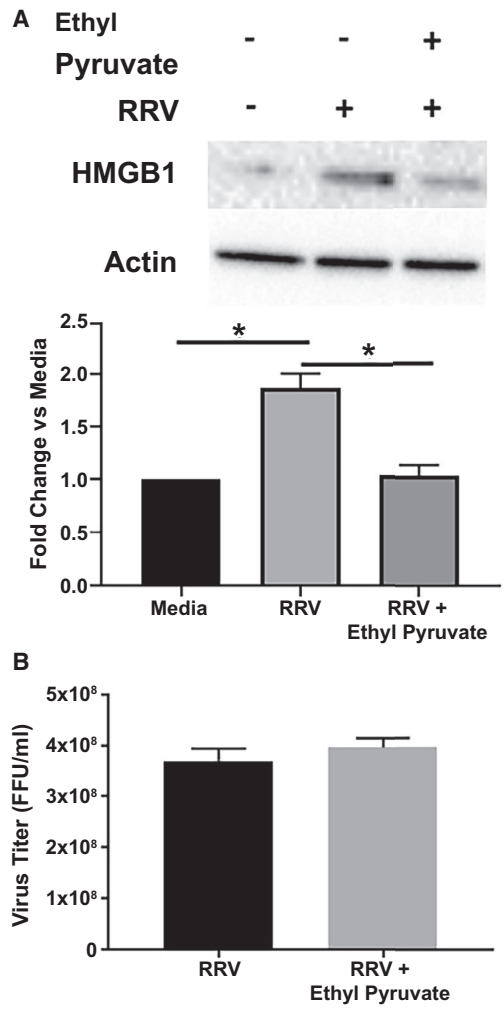

FIG. 3. Effect of EP on HMGB1 release. Cholangiocytes treated with EP showed significantly reduced HMGB1 levels following infection (A). EP had no effect on viral titers (B) (*$P < 0.05$). Abbreviation: FFU, focus-forming unit.

$Ro1845^{RRV(VP4)}$ in cholangiocytes, we determined the activation of p38 at 8 hours. The levels of phospho-p38 (p-p38) and its downstream target p-STAT1 were low in cholangiocytes before treatment and increased at 8 hours after RRV and $Ro1845^{RRV(VP4)}$ infection ($P < 0.01$), paralleling the expression pattern of HMGB1 (Fig. 4A). In contrast, no increase in p-p38 and p-STAT1 was observed after infection with Ro1845 corresponding with low HMGB1 secretion. To determine if p-p38 is involved in HMGB1 release, a specific p38 inhibitor, SB203580, was used to treat the cells following RRV infection. This treatment resulted in a decrease in p38-regulated

MAPK-activated protein kinase 2 (MAPKAPK2) phosphorylation, consequently resulting in reduced p-STAT1 levels and HMGB1 release (Fig. 4B), suggesting that p38 plays a role in HMGB1 secretion in cholangiocytes. To ascertain if the phosphorylation of STAT1 is required for HMGB1 release, cholangiocytes were treated with fludarabine, an inhibitor of STAT1, which resulted in a significant decrease in HMGB1 release (Fig. 4C). Additionally, when we assessed the effect of EP on intracellular signaling, we observed a similar significant decrease in p-STAT1 following treatment (Supporting Fig. S3).

RRV INFECTION INCREASES ROS LEVELS IN CHOLANGIOCYTES

To identify the upstream activator of p38, we evaluated ROS levels within cholangiocytes following rotavirus infection. Fluorescence microscopy detected a significant increase in ROS after 2 hours of infection with RRV compared to Ro1845 or media control (Fig. 5A). When the cholangiocytes were treated with EUK-8, a synthetic catalytic ROS scavenger, the levels of ROS were significantly reduced (Fig. 5A), while no significant effect on virus titer was observed (Fig. 5B). This inhibition of ROS subsequently led to a reduction in both the phosphorylation of p38 and the release of HMGB1 (Fig. 5C).

INTRAPERITONEAL ADMINISTRATION OF GLYCYRRHIZIN ATTENUATED RRV-INDUCED BA IN MICE

Based on the above results, we hypothesized that inhibiting extracellular HMGB1 would decrease inflammation/severity and increase survival in the murine model of BA. Mice were injected with RRV followed by daily IP injections of glycyrrhizin or saline beginning 1 day postinoculation, then every other day for 7 days. Mice were monitored daily for the symptoms of BA (acholic stool, bilirubinuria,

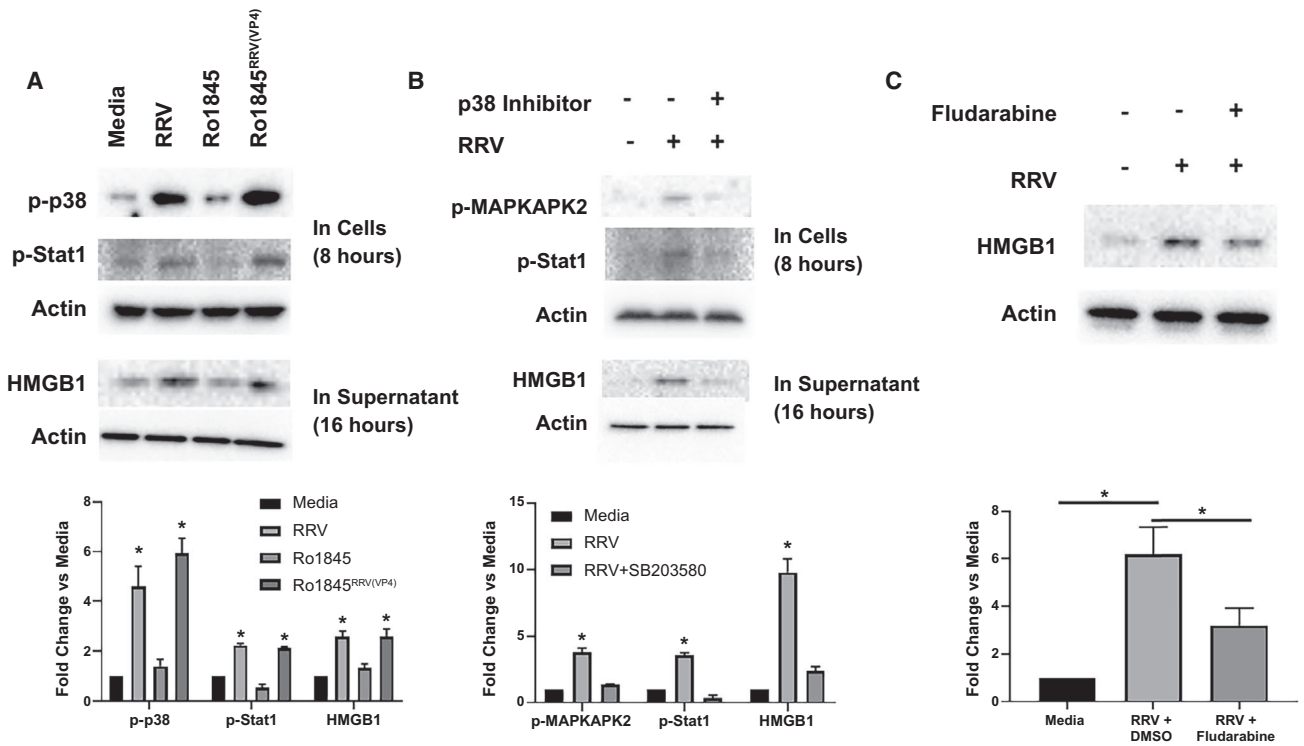

FIG. 4. HMGB1 release is p38-dependent and STAT1-dependent. Protein isolated from cholangiocytes 8 hours postinfection revealed a significant increase in the phosphorylation of both p38 and STAT1 in a similar pattern by those virus strains which were capable of inducing HMGB1 release (A). Cholangiocytes treated with SB203580, a p38 inhibitor, resulted in reduced levels of p-MAPKAPK2 (control) and p-STAT1 along with reduced HMGB1 release (B). A significant decrease in HMGB1 release was witnessed following cholangiocyte treatment with fludarabine, a STAT1 inhibitor (C) (*$P < 0.05$).

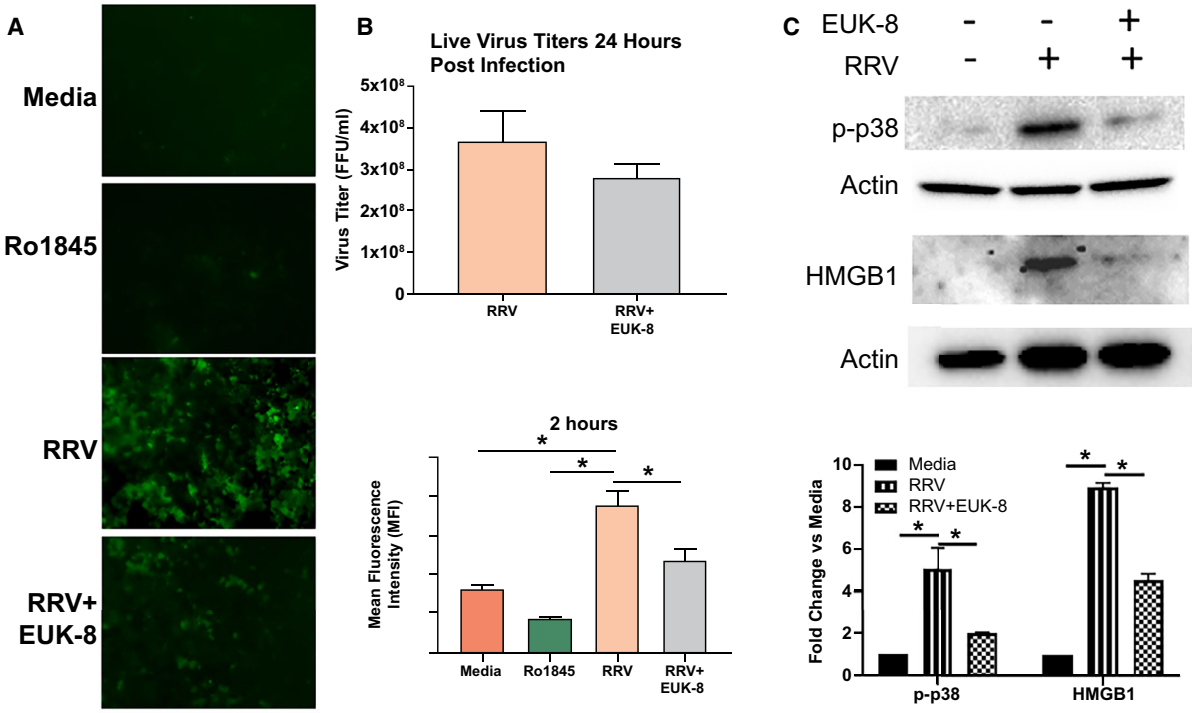

FIG. 5. HMGB1 release is ROS-dependent. At 2 hours postinfection of cholangiocytes with RRV, a significant increase in ROS was measured by MFI. This increase was not observed by infection with Ro1845. Treatment with a ROS inhibitor, EUK-8, significantly reduced the levels of ROS within the cells (A), without affecting the virus titer (B). Additionally, treatment with EUK-8 was able to reduce the phosphorylation levels of p38 at 8 hours and HMGB1 release from the cells at 16 hours (C) (*$P < 0.05$). Abbreviation: FFU, focus-forming unit.

jaundice, and weight loss) from 5 days postinoculation until 21 days. Mice injected with glycyrrhizin following RRV infection developed significantly fewer symptoms when compared with those injected with RRV/saline (73% versus 95%, respectively; $P < 0.05$) (Fig. 6A). Survival was similarly improved when pups were given glycyrrhizin, with only 36% mortality when compared with 95% with RRV/saline (Fig. 6B). We analyzed the virus titer in all groups of mice and found no significant difference in virus titers, suggesting that glycyrrhizin has no direct antiviral activity (Fig. 6C). To evaluate the ameliorative effect of glycyrrhizin treatment on the liver pathology induced by RRV infection, ALT and AST were measured in the sera at 7 and 10 days postinfection. The AST (Fig. 6D) and ALT (Fig. 6E) levels were both significantly reduced in glycyrrhizin-treated mice when compared with RRV/saline mice (993.7 ± 47.7 versus 3290.3 ± 729.4 and 43.4 ± 3.9 versus 78.3 ± 1.2, $P < 0.05$, respectively). Histological analysis of the extrahepatic bile ducts at 10 days postinfection revealed a complete obstruction of the RRV-injected pups, while those treated with glycyrrhizin remained patent (Fig. 6F). Similarly, hematoxylin and eosin–stained liver sections demonstrated less infiltration of inflammatory cells in mice that were treated with glycyrrhizin (Fig. 7A). These findings were confirmed through immunohistochemical staining, demonstrating a significant decrease in both macrophage and NK-cell populations in the glycyrrhizin-treated pups while having no effect on neutrophils (Fig. 7B-D). Flow cytometry was used to verify and further characterize the mononuclear populations between treated and untreated mice. Complementary to the immunohistochemistry, macrophages and NK cells were significantly reduced in the glycyrrhizin-treated mice (8.5 ± 0.4% versus 6.3 ± 0.2% and 12.0 ± 0.9% versus 8.9 ± 0.8%, respectively) along with cluster of differentiation 4 (CD4) and CD8 cell populations (11.3 ± 0.9% versus 6.3 ± 0.8% and 8.5 ± 0.4% versus 5.7 ± 0.7%, respectively) (Supporting Fig. S4).

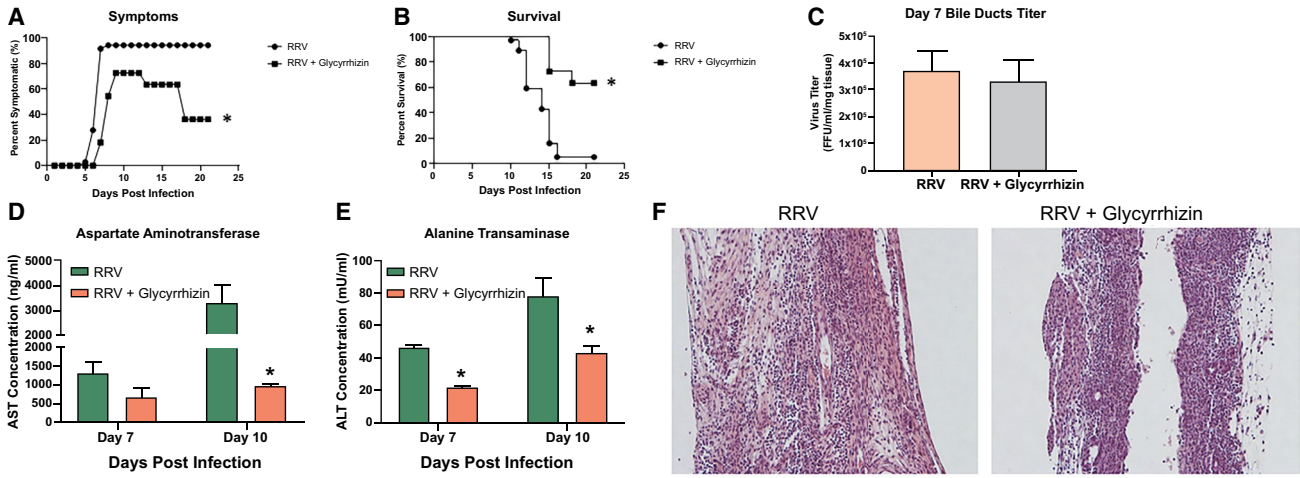

FIG. 6. Glycyrrhizin treatment attenuates a murine model of BA. Newborn mice were infected with RRV (7.5×10^5 focus-forming units/pup) followed by injections of glycyrrhizin or saline on days of life 1, 3, 5, and 7. Symptoms were monitored for 21 days postinjection. There was a significant reduction in symptoms following glycyrrhizin treatment (A). A significant decrease in mortality was also observed in glycyrrhizin-treated pups versus saline (36.4% versus 87.5% respectively; $P < 0.05$) (B). Glycyrrhizin treatment had no effect on viral titer (C) but did decrease both AST and ALT levels (D and E, respectively). Histological evaluation of the extrahepatic bile duct performed by hematoxylin and eosin 10 days postinfection with RRV demonstrated a complete obstruction, while, in contrast, the bile ducts from pups infected with RRV and treated with glycyrrhizin remained patent (×10 magnification) (F) (*$P < 0.05$). Abbreviation: FFU, focus-forming unit.

RRV INFECTION OF HUMAN CHOLANGIOCYTES RESULTS IN HMGB1 RELEASE

To evaluate the relevance of these results to the human disease, we examined the effect of RRV infection on HMGB1 release from human cholangiocytes. We have shown that human cholangiocytes had a similar pattern in terms of chemokine/cytokine gene expression and signaling pathway activation following RRV infection.[14] The HMGB1 secreted from RRV-infected human cholangiocytes mirrored those results as demonstrated in mouse cholangiocytes (Fig. 8A).

HMGB1 mRNA IS UP-REGULATED IN HUMAN PATIENTS WITH BA

To identify the mRNA expression levels of HMGB1 in human BA liver samples, we assessed the publicly available microarray data sets GSE46960 for patients with BA from the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) database. Patients were categorized into three groups: normal control, non-BA disease control, and BA. The available microarray data sets were reanalyzed using an online database, GEO2R. We observed a significant increase in levels of mRNA expression of HMGB1 in patients with BA compared to the non-BA and control groups ($P < 0.05$) (Supporting Fig. S5A).

ELEVATED SERUM HMGB1 IN HUMAN BA CORRELATES WITH HIGH BILIRUBIN AND DECREASED NATIVE LIVER SURVIVAL

To confirm whether the mRNA expression of HMGB1 is translated to protein, we analyzed data from a patient-based SOMAscan assay performed on samples obtained from patients with BA[31] and from patients with BA enrolled in the ChiLDReN-sponsored PROBE and START trials. When these two data sets were combined, we found that, although HMGB1 levels appeared higher in patients with BA and patients with IHC compared to healthy controls, this did not achieve statistical significance (Supporting Fig. S5B). Further analysis revealed that, when we used a cutoff value

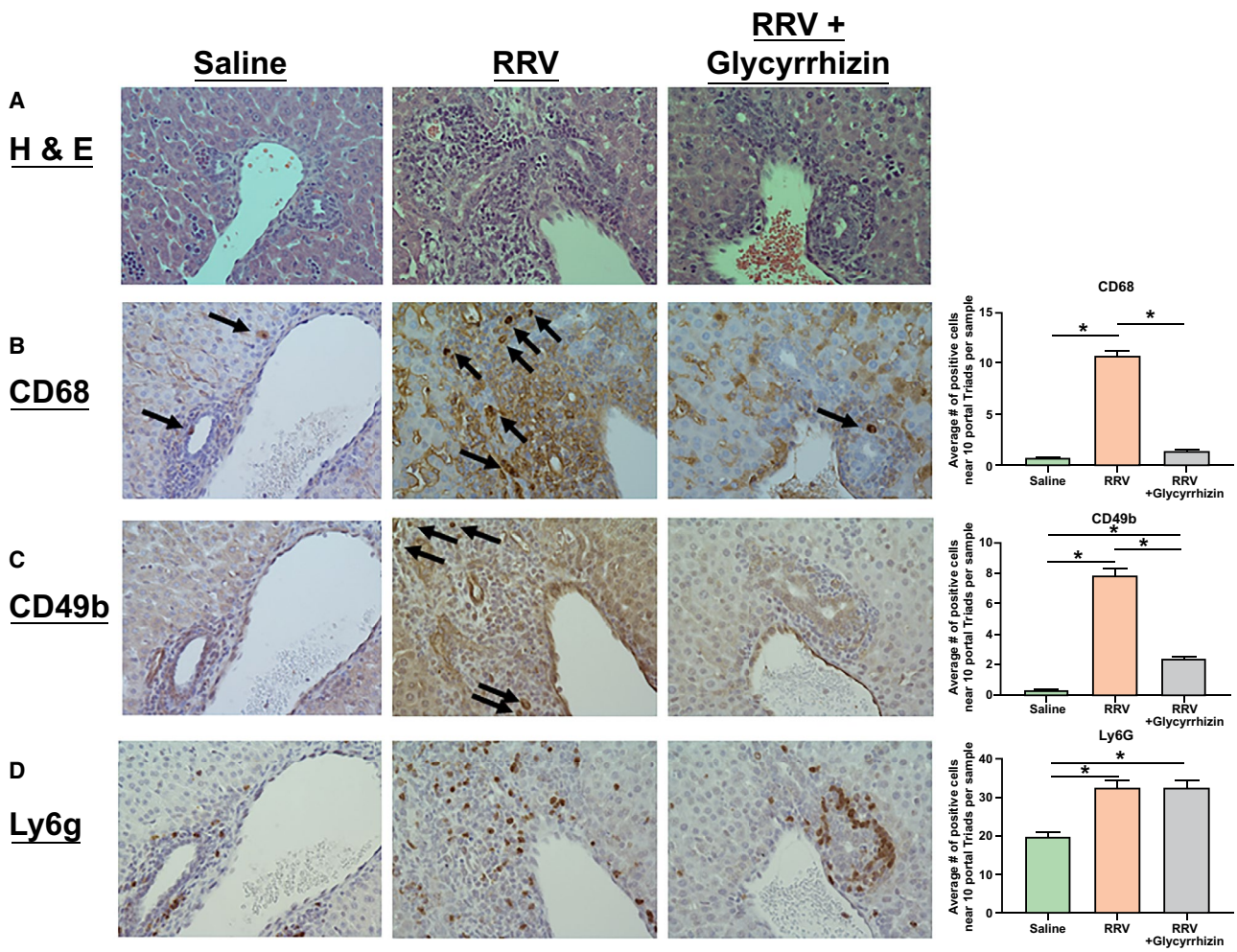

FIG. 7. Liver immune cell profile following glycyrrhizin treatment. Histological evaluation performed by hematoxylin and eosin illustrated a reduced inflammatory response in the liver after glycyrrhizin treatment (A). Immunohistochemistry was used to quantify specific immune cells, which demonstrated a significant reduction in macrophages stained with CD68 (B) and NK cells stained with CD49b (C) in the glycyrrhizin-treated pups; conversely, there was no difference in neutrophils stained with Ly6G (D) (*$P < 0.05$). Abbreviation: Ly6G, lymphocyte antigen 6 complex locus G.

of mean ± 1 standard deviation of healthy control patients, we identified a subpopulation of subjects with BA (29.8% of the cohort) which had high levels of HMGB1 (BAhiH group) with the remaining patients cohorted as BA low HMGB1 (BAloH group) (Fig. 8B). At 3 months post-HPE, the BAhiH group had higher bilirubin levels than the BAloH group, which approached significance (Fig. 8C). High level of total bilirubin 3 months after HPE is often an indicator of failed HPE, and at 2 year follow-up, only 33% of patients in the BAhiH group had survived with their native liver versus 54% of patients in the BAloH group ($P < 0.035$) (Fig. 8D).

When we analyzed the serum from the cohort of patients who were enrolled in the START trial and received corticosteroids, we again found a cohort of patients with high HMGB1 levels (BAhiH-S, BA with high HMGB1 treated with steroids) (23.5%) (Supporting Fig. S6A). By adding this subgroup to the original analysis, we found that bilirubin levels at 3 months post-HPE appeared to normalize and that survival with native liver in the BAhiH-S group was increased to 67%, of which both approached significance (Supporting Fig. S6B,C). This increase in liver survival within the BAhiH-S cohort contradicts expected outcomes as their ages when the HPE

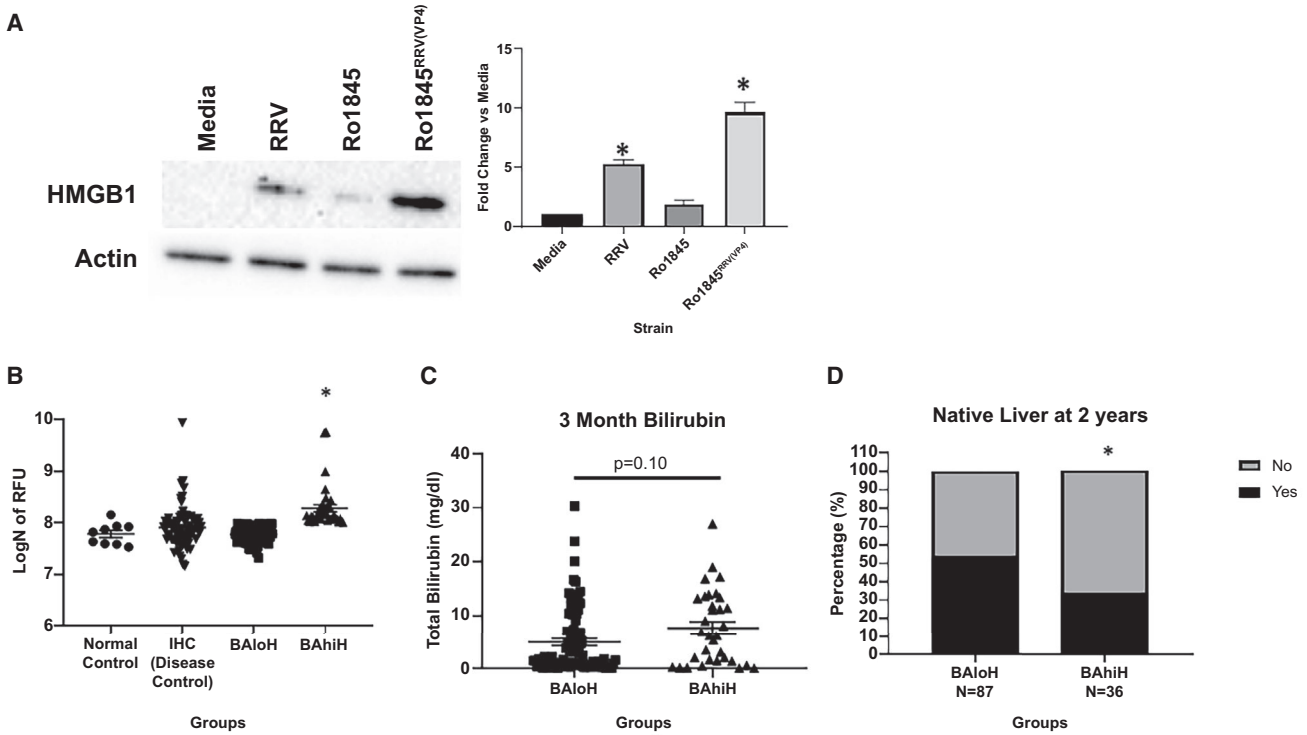

FIG. 8. HMGB1 in human cholangiocytes and patients with BA. Infection with RRV and Ro1845$^{RRV(VP4)}$ triggered the release of HMGB1 from human cholangiocytes (H69); conversely, Ro1845 failed to induce HMGB1 secretions, a pattern similar to that seen in the mCLs (A). A mean ± 1 standard deviation based on the logN of reference fluorescence units in healthy controls was used to identify a subpopulation of subjects with BA expressing higher levels of HMGB1 (BAhiH) versus the remaining patients (BAloH) (B). Comparison analysis of total bilirubin at 3 months post-HPE showed higher levels in the BAhiH group versus the BAloH group, which approached significance (C). At 2 years post-HPE a significantly lower percentage of patients initially in the BAhiH group retained their native liver compared to those in the BAloH group (D) (*$P < 0.05$). Abbreviation: RFU, relative fluorescence units.

procedures were performed were significantly higher, which usually leads to poor liver survival (Supporting Fig. S6D). These results suggest that HMGB1 could be used as a biomarker for a subgroup of patients with BA who may be responsive to anti-inflammatory interventions.

Discussion

BA, the most common cause of neonatal cholestasis, results from an inflammatory and fibrosing obstruction of intrahepatic and extrahepatic bile ducts progressing to liver fibrosis and requiring liver transplantation for survival. The pathogenesis of the disease remains unclear; therefore, it remains a devastating disease with no proven medical therapy.

HMGB1 was originally discovered as a 25-kDa DNA-binding protein typically located in the nucleus of cells, but upon cell activation and/or cell death, it undergoes significant posttranslational modifications, such as acetylation, phosphorylation, and methylation, resulting in translocation to the cytoplasm and subsequent release into the extracellular space.[33,34] It acts as a potent proinflammatory cytokine involved in delayed endotoxin lethality and systemic inflammatory response.[33] It has been demonstrated that HMGB1 is a cytokine that can mediate inflammation and a potential therapeutic target in various experimental models. HMGB1 initiates cellular responses by interacting with several cell surface receptors, such as the receptor for advanced glycation end products, toll-like receptor 2 (TLR2), and TLR4.[35,36] It can interact with TLR4 and generate inflammatory responses that are similar to those initiated by LPS.

After release, HMGB1 acts as a damage-associated molecular pattern functioning like a

proinflammatory cytokine, contributing to various types of tissue injury. Recently, HMGB1 was shown to play an important role in the pathogenesis of acute and chronic liver disease. HMGB1 binding to hepatic stellate cells induces a fibrogenic response.[37] The role of HMGB1 in liver fibrosis was confirmed by a study that showed that the neutralization of HMGB1 ameliorated liver fibrosis, whereas injection of HMGB1 promoted liver fibrosis.[37] Furthermore, it was shown that hepatocytes and myeloid cells are the major sources of HMGB1 and that HMGB1 ablation from both cell types reduced fibrosis.[37] Of note, high levels of HMGB1 were previously detected in the sera of patients with hepatitis B and hepatic fibrosis, suggesting that HMGB1 is an important and reliable noninvasive biomarker of liver fibrosis.[38]

We observed increased levels of HMGB1 in the sera of mice with BA, mirroring the previously reported detection of high levels of HMGB1 in the sera of patients with BA.[32] BA is considered an inflammatory hepatic disease; and in the experimental model, cholangiocytes are preferentially the target for infection by RRV, and how they respond is central to disease initiation. Thus, the HMGB1 released by these cholangiocytes may act as a major regulatory cytokine in this process by recruiting neutrophils, macrophages, dendritic cells, lymphocytes, and hepatic stellate cells to the portal areas. Later activation of macrophages and hepatic stellate cells, which are also a good source of HMGB1, might contribute more HMGB1 to the inflammatory environment.

Our findings indicated that RRV induced HMGB1 secretion, but the molecular basis of its nuclear translocation and the mechanism of its release to the extracellular milieu remain to be determined. Although HMGB1 can be passively released after cell death or necrosis, our results demonstrated that active RRV infection in cholangiocytes leads to translocation of HMGB1 to the extracellular space, independent of cell death. HMGB1 release into the extracellular milieu is a prerequisite to exert its inflammatory effects. In this study, we found an increase in ROS levels following RRV infection (mainly mediated by RRV's VP4), leading to signal transduction through the p38 MAPK pathways as a critical step in HMGB1 release. We observed that p-p38 MAPK increased significantly 8 hours after RRV infection, suggesting that RRV activates the p38 MAPK pathway, with the time point coinciding with HMGB1 expression and increased release. We also observed STAT1 phosphorylation 16 hours after infection. Therefore, STAT1 might be involved in transcription of the HMGB1 gene (Supporting Fig. S7).

Inflammatory responses in BA are complex and mutually reinforcing. In the mouse model, the primary response to RRV infection leads to the onset of the systemic inflammatory response involving activation of CD8 T cells, NK cells, and macrophages, which release proinflammatory cytokines. Of these, interferon gamma (IFNγ), TNFα, and IL6 are the potent cytokines that are rapidly produced by activated immune cells, with direct proinflammatory properties. Serum IFNγ, TNFα, and IL6 have been shown to reach toxic levels in mice and human patients with BA.[5,39,40] HMGB1 detection in human patients indicates that release of HMGB1 may aggravate the inflammatory response. HMGB1 has been implicated as an important mediator in the pathogenesis of various viral infections such as influenza virus H5N1, hepatitis C virus, West Nile virus, dengue virus, and HIV-1.[19,41-43]

EP is a pharmacological inhibitor of HMGB1 secretion.[44,45] Therefore, treatment with EP has been shown to improve survival and alleviate organ dysfunction in a wide variety of preclinical models.[25] We observed that EP inhibited the p-STAT1 signaling pathway and later inhibited HMGB1 release and expression induced by RRV infection. Glycyrrhizin, a natural anti-inflammatory agent/steroid, is used as a pharmacological inhibitor in blocking HMGB1 cytokine activities by binding directly to both HMG boxes in HMGB1. Glycyrrhizin has been shown to interfere with HMGB1-induced recruitment of inflammatory cells in the liver.[26] In our study, mice injected with glycyrrhizin along with RRV developed significantly fewer symptoms of obstruction compared to those injected with RRV and saline, and survival was also significantly improved when pups were given glycyrrhizin. It is likely that HMGB1 exerts its chemotactic function toward mononuclear cells extracellularly, and treatment with glycyrrhizin may hinder this process by inhibiting inflammatory cell accumulation. When we quantified the inflammatory cells around the portal tract, we found a significant decrease in the number of macrophages, NK cells, and T cells (with no change in neutrophils) in glycyrrhizin-treated mice following RRV infection, suggesting the importance of these cells in disease pathogenesis.

Currently, there is no defined effective medical therapy available that alters the course of BA in afflicted infants. Administration of corticosteroids has been studied in several different constructs, with institutional case series and a meta-analysis reporting a potential benefit[46]; however, several prospective trials including the ChiLDReN-sponsored START randomized study[47] showed that among infants with BA who have undergone HPE, high doses of steroids postoperatively did not improve bile drainage at 6 months, and the use of steroids was associated with an earlier onset of serious adverse events. In this study, we analyzed HMGB1 levels in serum obtained at the time of portoenterostomy from patients enrolled in the PROBE and START studies and identified a population of subjects with BA who had high levels of HMGB1. This cohort had a less favorable response to portoenterostomy with higher 3-month bilirubin levels and, even more importantly, significantly lower native liver survival at 2 years. When we analyzed serum obtained from patients enrolled in START who received corticosteroids, we again found a cohort who had high serum HMGB1 levels, but in this group there appears to be an improved response to the Kasai with decreased bilirubin levels and increased native liver survival; however, these data were from an available set of samples which was statistically underpowered to detect meaningful group differences. These results suggest that serum levels of HMGB1 at the time of HPE could be used as a biomarker of an inflammatory phenotype, allowing for the identification of patients who are responsive to anti-inflammatory treatments. i.e., corticosteroids or even glycyrrhizin. Although exciting, the results must be interpreted cautiously as this was a *post hoc* analysis from a study focused on a different treatment goal. Future research will require a properly designed and adequately powered study focused on the predictive value of HMGB1.

In summary, we have demonstrated the potential importance of HMGB1 in BA disease pathogenesis. Further studies focusing on HMGB1 are required, including a prospective clinical trial, to fully validate these interesting findings.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31745/suppinfo.