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ORIGINAL ARTICLE

C-X-C motif chemokine ligand 1 induced by Hedgehog signaling promotes mouse extrahepatic bile duct repair after acute injury

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Abstract

Background and Aims: In extrahepatic bile duct (EHBD) cholangiopathies, including primary sclerosing cholangitis, a reactive cholangiocyte phenotype is associated with inflammation and epithelial hyperproliferation. The signaling pathways involved in EHBD injury response are poorly understood. In this study, we investigated the role of Hedgehog (HH) signaling and its downstream effectors in controlling biliary proliferation and inflammation after EHBD injury.

Approach and Results: Using mouse bile duct ligation as an acute EHBD injury model, we used inhibitory paradigms to uncover mechanisms promoting the proliferative response. HH signaling was inhibited genetically in *Gli1*$^{-/-}$ mice or by treating wild-type mice with LDE225. The role of neutrophils was tested using chemical (SB225002) and biological (lymphocyte antigen 6 complex locus G6D [Ly6G] antibodies) inhibitors of neutrophil recruitment. The cellular response was defined through morphometric quantification of proliferating cells and CD45+ and Ly6G+ immune cell populations. Key signaling component expression was measured and localized to specific EHBD cellular compartments by *in situ* hybridization, reporter strain analysis, and immunohistochemistry. Epithelial cell proliferation peaked 24 h after EHBD injury, preceded stromal cell proliferation, and was associated with neutrophil influx. Indian HH ligand expression in the biliary epithelium rapidly increased after injury. HH-responding cells and neutrophil chemoattractant C-X-C motif chemokine ligand 1 (CXCL1) expression mapped to EHBD stromal cells. Inhibition of HH signaling blocked CXCL1 induction, diminishing neutrophil

Abbreviations: BDL, bile duct ligation; CK19, cytokeratin 19; CXCL, C-X-C motif chemokine ligand; CXCR2, C-X-C motif chemokine receptor 2; EdU, 5-ethynyl-2′-deoxyuridine; EHBD, extrahepatic bile duct; GBS, Glioma-Associated Oncogene binding site; GLI1, GLI family zinc finger 1; H&E, hematoxylin and eosin; HH, Hedgehog; IHBD, intrahepatic bile duct; IHH, Indian Hedgehog; Ly6G, lymphocyte antigen 6 complex locus G6D; PSC, primary sclerosing cholangitis; Ptch1, Patched1; SHH, Sonic Hedgehog; WT, wild type.

recruitment and the biliary proliferative response to injury. Directly targeting neutrophils by inhibition of the CXCL1/C-X-C motif chemokine receptor 2/Ly6G signaling axis also decreased biliary proliferation.

Conclusions: HH-regulated CXCL1 orchestrates the early inflammatory response and biliary proliferation after EHBD injury through complex cellular crosstalk.

INTRODUCTION

Cholangiopathies are disorders that affect the biliary epithelium in intrahepatic and extrahepatic bile ducts (IHBDs, EHBDs).[1] Biliary atresia and primary sclerosing cholangitis (PSC) are incurable cholangiopathies with poorly understood mechanisms driving pathophysiology that affect large EHBDs. Identification of the pathways affecting EHBD injury and repair will help to uncover key contributors to maladaptive pathological responses. During the initial tissue insult through environmental exposure, microorganism infection, or autoimmune stimulation, cholangiocytes develop a reactive phenotype.[2,3] This phenotype includes up-regulation of cytokines and developmental signaling pathways, including Hedgehog (HH),[4] as well as an inflammatory response.[5–7] The reactive phenotype contributes to tissue restoration. Biliary proliferation is one of the central events during the EHBD response to injury with cholangiocyte hyperproliferation induced to repair EHBD function. After acute insult, the tissue either recovers or proceeds to chronic inflammation, leading to biliary strictures, cholestasis, cirrhosis, and malignant transformation of the biliary epithelium. Most prior studies have focused on liver and IHBD responses to injury; EHBD physiology and pathophysiology are comparatively understudied.

Cytokines are commonly up-regulated in cholangiopathies, including IL-6, IL-8, IL-17, IL-33, C-X-C motif chemokine ligand (CXCL) 8, TNF-α, and TGF-β2.[2,8–11] Our group and others recently demonstrated in mouse models that acute inflammatory challenge with IL-33 stimulates biliary proliferation in EHBDs.[8,9,12] Further, we showed an important role for HH signaling in the IL-33 proliferative response.[8] Thus, transgenic overexpression of HH enhanced the IL-33 proliferative response, and HH inhibition through deletion of the gene encoding the key transcriptional effector GLI family zinc finger 1 (*Gli1*) blunted the response.[8] Notably, the EHBD HH signaling axis involved epithelial-stromal cell crosstalk, where Indian Hedgehog (IHH) ligand expression in EHBD cholangiocytes signaled to stromal cells expressing transcriptional response genes *Gli1*, *Gli2*, and *Gli3* and the HH receptor *Patched1* (*Ptch1*).[8] The identities of the HH-dependent stromal cell signals that provide feedback to promote epithelial cell proliferation are unknown. HH signaling up-regulation was also reported in human PSC and mouse models of chronic cholestatic injury.[13–17]

Inflammatory cells are recruited to sites of tissue injury, where they orchestrate complex tissue responses to resolve or exacerbate the injury. Neutrophils in particular play a major role after tissue injury, serving as first responders of the innate immune system to initiate tissue repair.[18] Although their involvement in liver diseases, including PSC,[11,19] has been well established, specific neutrophil function after EHBD injury is poorly understood. The potent neutrophil chemoattractant CXCL1 signaling through its receptor C-X-C motif chemokine receptor 2 (CXCR2) has been implicated in several EHBD inflammation-induced disorders, including biliary atresia[20] and cholangiocarcinoma,[21] suggesting that the CXCL1-CXCR2-neutrophil axis may play a role in EHBD disease.

To investigate molecular mechanisms of biliary cell responses to injury, bile duct ligation (BDL) in mice is commonly used to emulate typical features of human cholestatic liver diseases including biliary obstruction, inflammation, and bile acid stasis.[22] Interestingly, following 5-day BDL-induced cholestasis, IHH was reported to signal to GLI1-positive mesenchymal cells surrounding large IHBDs[14] and was associated with neutrophil recruitment into the injured liver.[23] This suggests that HH signaling may play a role in neutrophil recruitment into the hepatobiliary system.

In the current work, we investigated the mechanisms promoting EHBD biliary cell proliferation in acute obstructive injury. Here we demonstrate that, in response to short-term bile duct obstruction, there is a major biliary proliferative surge at 24 h, which rapidly subsides. During this proliferative response, IHH ligand is induced, which stimulates GLI1-positive stromal cells to up-regulate expression of the neutrophil chemoattractant CXCL1. In turn, CXCL1 recruits neutrophils into EHBD tissue to promote biliary proliferation in an HH-dependent manner. This work highlights the complex epithelial-stromal-inflammatory cell interactions that occur during the early response to EHBD injury. It also mechanistically unveils a previously unrecognized link between HH signaling and the CXCL1-CXCR2 signaling axis to modulate epithelial cell proliferation, a process potentially relevant to a broad spectrum of organ systems.

MATERIALS AND METHODS

Mouse experiments

All mouse experiments were approved by the University of Michigan Institutional Animal Care and Use Committee. For genetic inhibition of HH signaling, *Gli1*$^{lacZ/lacZ}$ mice (*Gli1*$^{-/-}$) containing a nuclear β-galactosidase construct knocked into exon 2 of the mouse *Gli1* gene were used.[24] HH signaling cells were identified using *Ptch1*$^{lacZ/+}$ reporter mice.[25] LDE225, a small molecule inhibitor of the HH signaling protein Smoothened, was used for pharmacologic inhibition of HH signaling (Chemietek). SB225002 was used as a selective small molecule CXCR2 antagonist (B8200, ApeXBio). For lymphocyte antigen 6 complex locus G6D (Ly6G) neutralization, mice were treated with rat anti-mouse Ly6G (clone 1A8, BE0075-1, BioXcell). Further details on mouse experiments, including BDL procedure and pharmacological and genetic models of HH, CXCR2, and Ly6G inhibition, can be found in the Supporting Materials.

Human samples

Human EHBD tissue from cholangiocarcinoma and adjacent noncancerous tissue was collected at the University of Michigan. Human EHBD tissue affected by PSC with and without dysplasia was procured during liver transplantation at the University of Nagasaki, Japan. Tissues were collected with Institutional Review Board approval at both institutions according to the principles embodied in the Declaration of Helsinki. For this archived sample analysis, the requirement for informed consent was waived by the institutional review committee. Paraffin-embedded human tissue was sectioned at 5 μm for analysis.

Immunostaining and histological staining

Mouse EHBD and liver tissue was isolated 12, 24, or 48 h after operation and fixed in 10% neutral buffered formalin (Thermo Fisher Scientific) at 4°C overnight, followed by processing for immunofluorescence as described.[8] Antibodies for immunofluorescence are listed in Table S1. Hematoxylin and eosin (H&E) staining was done following manufacturer's protocol (Vector Labs). X-gal staining was performed as described.[8]

Cell proliferation

Proliferating cells were labeled with 5-ethynyl-2′-deoxyuridine (EdU; 2.5 mg/kg in phosphate-buffered saline) administered intraperitoneally 2 h before tissue collection and detected using Click-It EdU Alexa Fluor 488 Imaging Kit (Life Technologies). Epithelial cell compartment was marked by cytokeratin 19 (CK19) immunoreactivity. Epithelial and stromal cell compartments were analyzed for the percentage of proliferating EdU$^+$ cells, which were expressed as a proportion of either epithelial cells in the CK19$^+$ cell compartment or DAPI$^+$ cells, respectively. Proliferating EdU$^+$ IHBDs epithelial cells were quantified among CK19$^+$ cells.

Supplementary materials

Additional information on staining, morphometric analysis, *in situ* hybridization, quantitative real time PCR, growth factor gene expression using the PCR array and statistical analysis can be found in the Supporting Materials.

RESULTS

Acute EHBD injury induces a surge in biliary cell proliferation

To examine the dynamic response of mouse EHBD to acute injury, we conducted BDL (Figure S1) and analyzed tissue responses, including proliferation, at 12, 24, and 48 h after injury (Figure 1). H&E imaging showed injury-induced expansion of both epithelial and stromal cell compartments by 24 h after BDL (Figure 1A). We examined proliferating cells in epithelial (CK19$^+$) and stromal cell compartments (Figure 1B–D). EHBD tissue was quiescent in sham controls at all examined timepoints (Figure 1B). In contrast, we observed extensive cellular proliferation in both epithelial and stromal cells after BDL (Figure 1B). Morphometric analysis demonstrated that epithelial cell proliferation peaked at 24 h after BDL at the times analyzed (Figure 1C). Stromal cell proliferation was less profound and developed more slowly, with a significant increase observed 48 h after BDL (Figure 1D). Thus, acute EHBD injury leads to a rapid surge in biliary epithelial cell proliferation and a delayed stromal proliferative response.

Because cholangiopathies also affect the intrahepatic biliary tree, we examined proliferative responses in IHBDs (Figure S2). Unlike EHBDs, there was no increase in cholangiocyte proliferation in small (Figure 2A–C) and large (Figure 2D–F) IHBDs at 24 h after BDL based on H&E, immunofluorescence, and morphometric analyses. There was a modest increase in small (Figure S2A–C) and large (Figure S2D–F) IHBD cholangiocyte proliferation at 48 h after BDL. These data suggest that the early epithelial proliferative surge is specific to EHBDs.

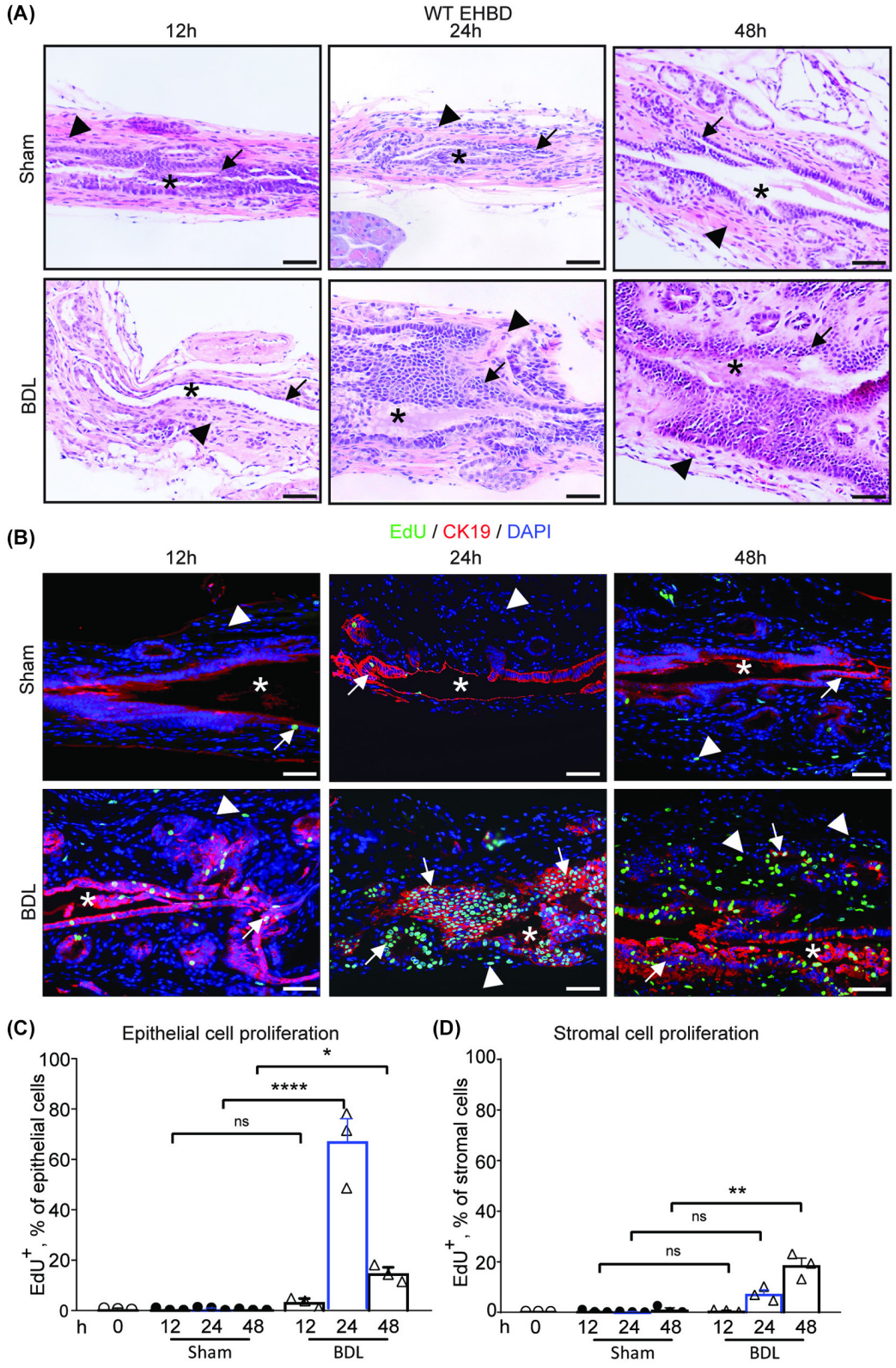

FIGURE 1 EHBD cell proliferation after acute injury. WT mouse EHBD H&E at 12, 24, and 48 h after sham or BDL (A). EdU (green) marked proliferating cells, CK19 (red) marked epithelial cells, and DAPI (blue) marked nuclei (B). Proliferating cells were enumerated, expressed as a percentage of either epithelial cells in the $CK19^+$ cell compartment (C) or $DAPI^+$ cells in the stromal compartment (D) compared with sham. Asterisks mark EHBD lumen; arrows and arrowheads mark epithelial and stromal cells, respectively. The data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. $n = 3$ mice/group. One-way ANOVA. Scale bars, 50 μm

HH signaling is up-regulated in injured EHBDs

The HH signaling pathway is induced in hepatobiliary tissues in response to chronic obstructive cholestatic injury.[5,14,16] We confirmed that HH signaling was induced after acute BDL in EHBDs. Measuring whole EHBD mRNA abundance for HH signaling components at 24 h after BDL showed up-regulation of *Ihh* (Figure 2A) but not Sonic Hedgehog (*Shh*) ligand (Figure 2B), suggesting that IHH is the primary HH ligand in EHBD tissues during both homeostasis and injury. Further, two HH-responsive genes, *Gli1* (Figure 2C) and *Ptch1* (Figure 2D), were both significantly up-regulated.

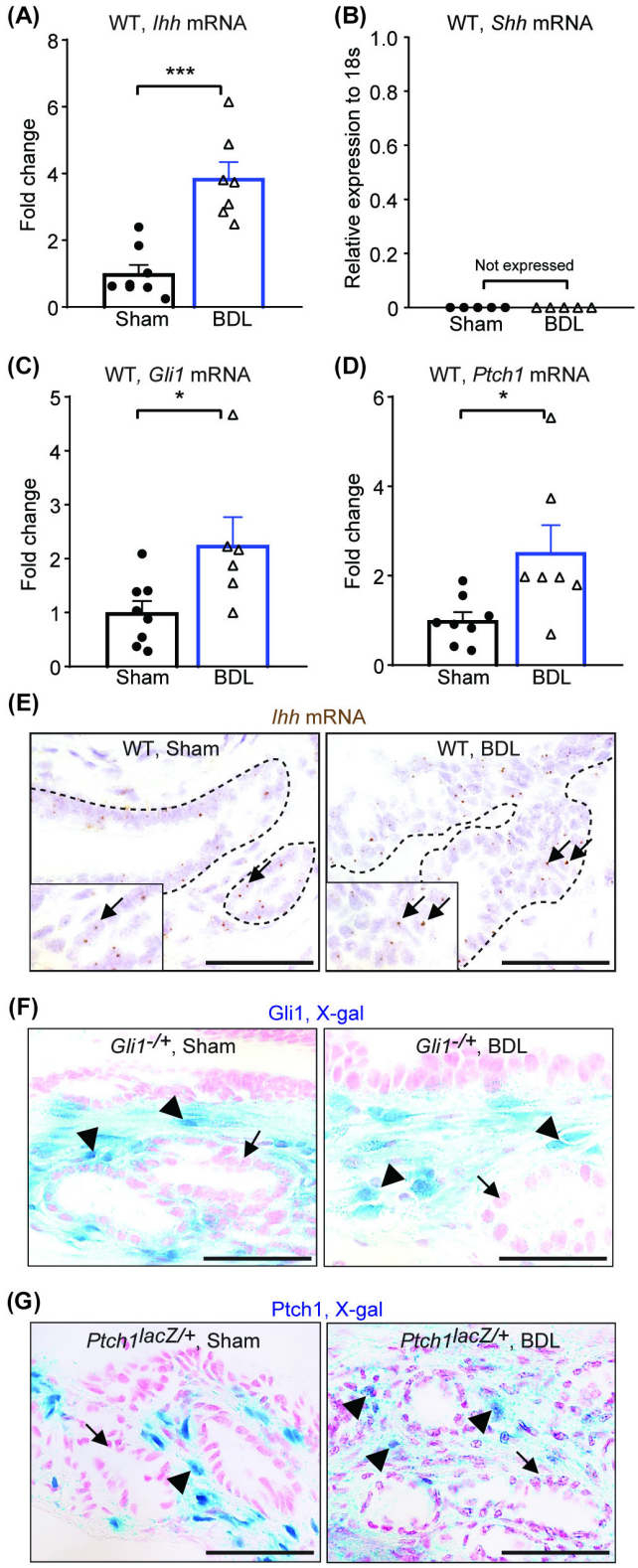

FIGURE 2 EHBD HH ligand and target gene expression after acute injury. EHBDs were analyzed 24 h after sham or BDL. mRNA abundance for *Ihh* (A), *Shh* (B), *Gli1* (C), and *Ptch1* (D) was determined by quantitative Reverse Transcription PCR in whole EHBD from WT mice and expressed as fold-change over sham. *In situ* hybridization for *Ihh* mRNA 24 h after sham or BDL procedures (E) (n = 5 mice/group). X-gal staining in $Gli1^{lacZ/+}$ ($Gli1^{-/+}$; F) and $Ptch1^{lacZ/+}$ mice (G) (n = 2–3 mice/group). Arrows and arrowheads mark epithelial and stromal cells, respectively. Dashed lines demarcate the epithelial compartment. The data are presented as the mean ± SEM. $^*p < 0.05$, $^{***}p < 0.001$. n = 5–8 mice/group (A–D). Unpaired Student t test. Scale bars, 150 μm

To define the EHBD tissue compartments involved in HH signaling during acute obstructive injury, we conducted *in situ* hybridization for *Ihh* mRNA and X-gal staining in $Gli1^{lacZ/+}$ and $Ptch1^{lacZ/+}$ reporter mice. These analyses showed that *Ihh* mRNA was restricted to biliary epithelial cells (Figure 2E), whereas *Gli1* and *Ptch1* were expressed in stromal cells (Figure 2F,G) in sham and BDL mice. These findings suggest that HH signaling occurs from epithelial to stromal cells.

Genetic and pharmacologic HH pathway inhibition decreases epithelial cell proliferation after EHBD injury

To determine the contribution of HH signaling to the proliferative response to acute obstructive EHBD injury, we used genetic (Figure 3A–C) and pharmacologic (Figure 3D–G) inhibitory paradigms. We confirmed the absence of *Gli1* expression in EHBDs from $Gli1^{-/-}$ mice (Figure S3A). As expected, BDL induced *Ihh* mRNA in $Gli1^{-/-}$ mice (Figure S3B), whereas the GLI1 target *Ptch1* remained unchanged (Figure S3C), demonstrating effective HH pathway inhibition in $Gli1^{-/-}$ mice. We also used a pharmacologic approach, inhibiting HH signaling with LDE225, which is used clinically for treatment of basal cell carcinoma.[26] A hair pluck assay confirmed suppression of HH signaling in LDE225-treated mice[27] (Figure S3D). Further, *Gli1* mRNA abundance was decreased in ligated EHBDs from LDE225-treated animals (Figure S3E), demonstrating effective pharmacologic HH signaling inhibition in EHBD tissue.

H&E analysis of EHBD tissue sections showed decreased cellularity in the epithelial cell compartment of $Gli1^{-/-}$ mice after BDL as compared with wild-type (WT) controls (Figure 3A). Morphometric analysis of EdU^+ cells in *Gli1* null mice showed a blunted proliferative

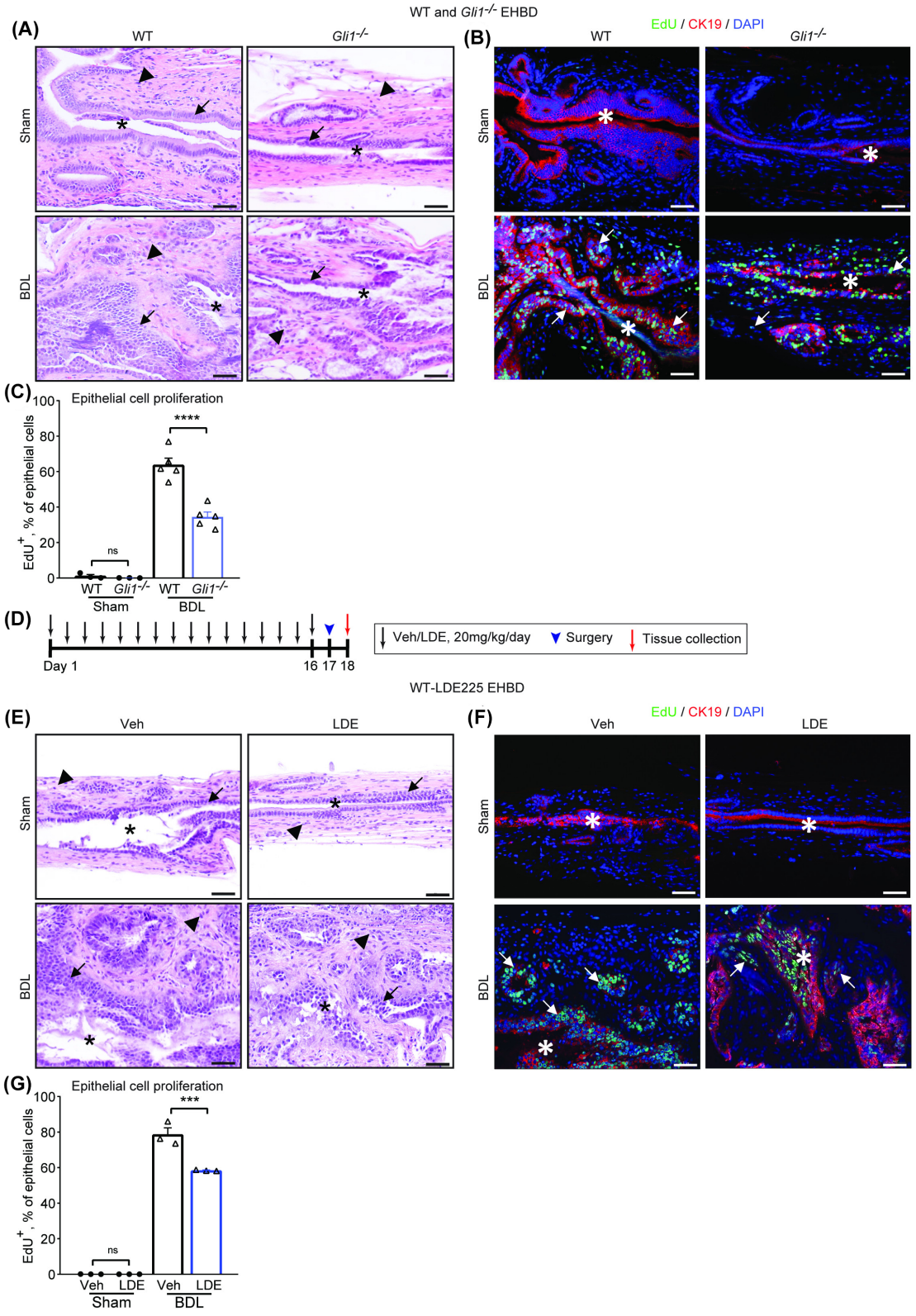

FIGURE 3 HH pathway inhibition reduces EHBD cell proliferation after acute injury. EHBDs of WT and *Gli1*$^{-/-}$ mice were examined 24-h after operation with H&E (A) and immunofluorescence (B) (proliferating cells, EdU, green; epithelial cells, CK19, red). Proliferating cells were enumerated and compared with WT controls (C). Experimental schema of WT mice treated with the Vehicle (Veh) or HH inhibitor LDE225 (LDE) before sham or BDL operation with analysis after 24 h (D). LDE- or Veh-treated mouse EHBDs were examined with H&E (E) and immunofluorescence (F). Morphometric analysis of LDE- or Veh-treated mice (G) where proliferating cells were enumerated and compared with Veh-treated controls. Proliferating cells were expressed as a percentage of all epithelial cells in the CK19^{+} cell compartment. Asterisks mark EHBD lumen; arrows and arrowheads mark epithelial and stromal cells, respectively. The data are presented as the mean ± SEM. ***p < 0.001, ****p < 0.0001. n = 3–5 mice/group. One-way ANOVA. Scale bars, 50 μm

response (Figure 3B,C). Similarly, mice subjected to pharmacologic HH inhibition (Figure 3D) had a blunted response to BDL, based on H&E staining analysis (Figure 3E) and EdU^+ cell enumeration in the $CK19^+$ epithelial cell compartment (Figure 3F,G). Together, these findings suggest that stromal HH signaling through GLI1 promotes the proliferative epithelial response to acute obstructive EHBD injury.

HH induces CXCL1 to recruit neutrophils and promote biliary proliferation in injured EHBDs

To identify potential HH-regulated growth factors that might affect proliferation, we performed an exploratory gene expression analysis of 84 growth factors using the Qiagen RT^2 Profiler PCR array on whole EHBD RNAs isolated at 12, 24, and 48 h after operation. Interestingly, this screen showed an increase in *Cxcl1* mRNA rapidly following EHBD injury (Figure S4A). The array data are available in GEO with accession number GSE182049. CXCL1 is a potent neutrophil chemoattractant relevant to the pathophysiology of inflammation-mediated diseases.[28] Notably, mRNA abundance of *Cxcl12*, encoding a cytokine that is important for the recruitment of T-lymphocytes and monocytes, but not neutrophils,[29] was decreased in acutely injured EHBDs (Figure S4B). We validated BDL-related induction of *Cxcl1* mRNA by quantitative PCR analysis, showing a 38-fold increase in *Cxcl1* mRNA abundance at 12 h after operation (Figure S4C).

We performed immunostaining to determine which tissue compartment expressed CXCL1 24 h after operation. This analysis localized CXCL1 to stromal cells and confirmed a marked increase in protein levels after BDL in WT mice (Figure 4A). Because EHBD stromal cells are HH-responsive, we hypothesized that CXCL1 was induced by HH signaling. To test this hypothesis, we examined EHBDs from *Gli1*$^{-/-}$ (Figure 4A) and LDE225-treated (Figure 4B) mice. This analysis showed that both CXCL1 protein (Figure 4A,B) and mRNA (Figure 4C,D) levels were significantly decreased in injured EHBDs on genetic (Figure 4A,C) and pharmacologic (Figure 4B,D) HH signaling inhibition. These findings demonstrated HH-dependent induction of CXCL1 in acute biliary injury. To investigate potential direct regulation of *Cxcl1* expression, we conducted an *in silico* analysis for the presence of high affinity consensus Glioma-Associated Oncogene binding sites (GBS) GACCACCC[30] using the ApE tool (https://jorgensen.biology.utah.edu/wayned/ape/) in the neighborhood of the mouse *Cxcl1* and human *CXCL1* loci, including 50 kb upstream and 10 kb downstream to cover potential promoter and enhancer regions. In both species, we identified a single GBS 270 bp downstream of *Cxcl1* and 2687 bp downstream of *CXCL1* (Figure S5), suggesting potential direct transcriptional regulation of *Cxcl1* by HH signaling.

CXCL1 induction in EHBDs of patients with cholangiopathies

Histological analysis of a limited number of patient tissues, including PSC without (Figure S6A) and with dysplasia (Figure S6B) and extrahepatic cholangiocarcinoma (Figure S6C), also showed CXCL1 in the bile duct stroma. Increased CXCL1 was observed in 5/10 patients with PSC without dysplasia, 2/2 patients with PSC with dysplasia, and 2/3 patients with extrahepatic cholangiocarcinoma (Figure S6A–C). Due to biliary obstruction, patients with cholangiopathies often have biliary infection treated with antibiotics and bile acid dysregulation treated with ursodeoxycholic acid, a secondary bile acid, and bile acid binders. We examined if these treatments were associated with increased CXCL1 expression in our patient samples. Patients who received antibiotics were more likely to demonstrate CXCL1 immunoreactivity (7/9) than those who did not receive antibiotics (1/6; Table S3). This suggests that CXCL1 expression might be associated with inflammation and neutrophil recruitment during infection in cholangiopathies. We observed no effect of ursodeoxycholic acid and bile acid binders on CXCL1 immunoreactivity (Table S3). To expand this analysis, we examined *CXCL1* mRNA expression in 36 patients with intrahepatic cholangiocarcinoma in the Cancer Genome Atlas database,[31] demonstrating increased expression in comparison to normal controls (Figure S6D). These data suggest that bile ducts from a subset of patients with cholangiopathies exhibit increased stromal *CXCL1* expression.

HH signaling regulates influx of neutrophils into injured EHBDs

We postulated that BDL induces IHH ligand in biliary cells to signal to stromal cells, stimulating secretion of factors recruiting leukocytes into injured EHBDs. Accordingly, we demonstrated by histological staining that, 24 h after BDL, $CD45^+$ leukocytes infiltrated the EHBD stroma (Figure 5A). We tested whether this cellular influx was HH-dependent by analysis of the HH inhibition models. Morphometric analysis demonstrated that the BDL-induced influx of $CD45^+$ cells was effectively suppressed by genetic (Figure 5A,B) and pharmacologic (Figure 5C,D) inhibition of HH signaling.

CXCL1 is a potent neutrophil attractant.[28] To confirm that neutrophils were included among the

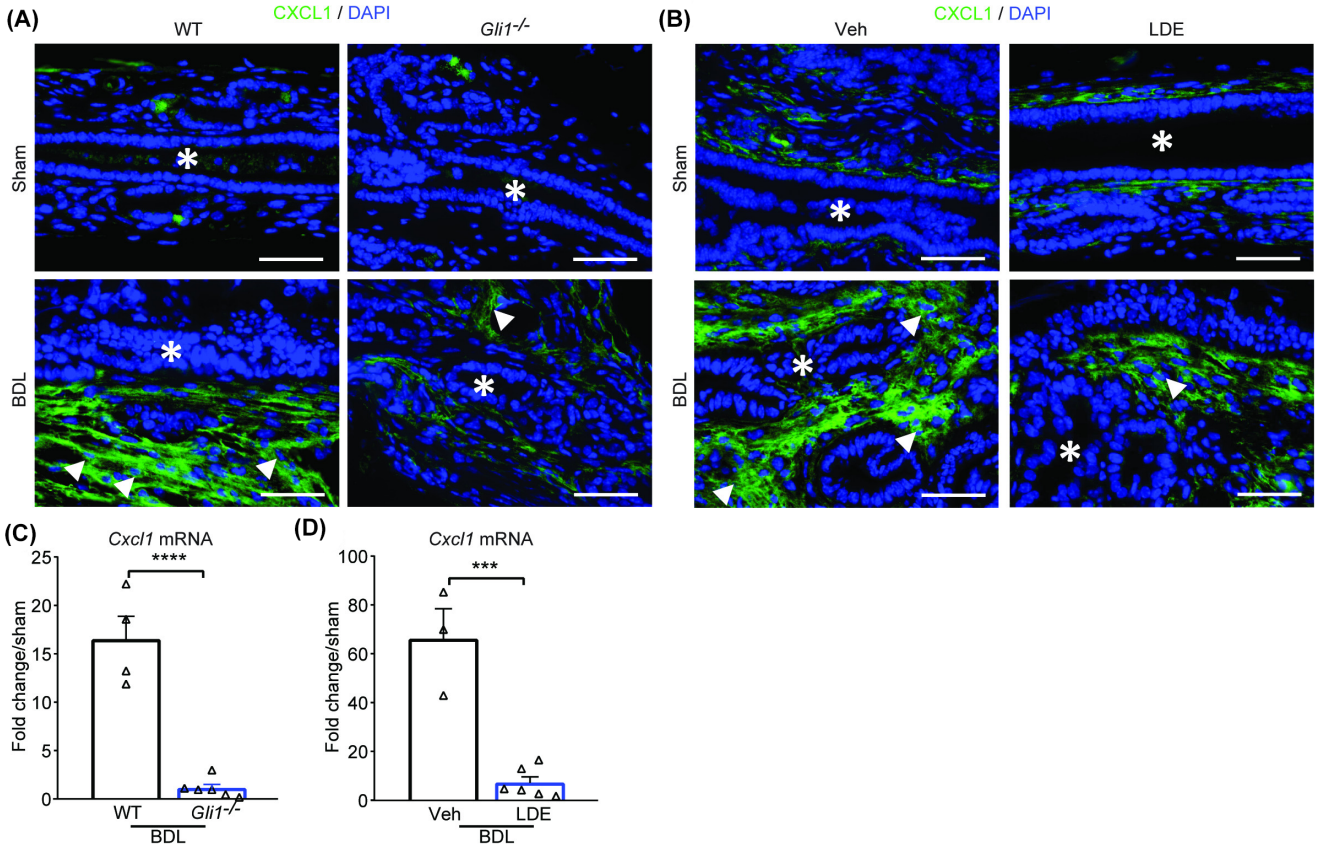

FIGURE 4 HH-dependent CXCL1 expression after acute EHBD injury. EHBDs of WT and *Gli1*$^{-/-}$ mice (A) or Veh- and LDE-treated WT mice (B) were examined for expression of CXCL1 (green) by immunofluorescence 24 h after operation. *Cxcl1* mRNA abundance in whole mouse EHBD tissue was measured by quantitative Reverse Transcription PCR in *Gli1*$^{-/-}$ (C) and LDE (D) mice and compared with respective controls. DAPI (blue) marks nuclei, asterisks mark EHBD lumen, and arrowheads mark CXCL1. The data are presented as the mean ± SEM. ***$p < 0.001$, ****$p < 0.0001$. $n = 3–6$ mice/group. Unpaired Student *t* test. Scale bars, 100 μm

infiltrating CD45$^+$ cells, we examined the expression of the neutrophil marker Ly6G by immunostaining, showing damage-induced ingress of neutrophils (Figure 5E). Comparing the number of CD45$^+$ cells with the number of Ly6G$^+$ cells suggested that neutrophils account for approximately half of the infiltrating leukocytes at 24 h after BDL. To further confirm neutrophil identity, we inspected H&E-stained EHBDs at high power for the characteristic neutrophil nuclear morphology, confirming a significant influx of neutrophils into EHBDs after acute obstructive cholestatic injury (Figure S7A). Together, these findings demonstrate robust recruitment of neutrophils in EHBDs after BDL.

We next tested whether the neutrophil influx was HH signaling-dependent. Analysis of *Gli1*$^{-/-}$ (Figure 5E,F) and LDE225-treated (Figure 5G,H) mice showed a significant reduction in neutrophil influx 24 h after BDL. Finally, we showed that induction of the neutrophil marker and CXCL1 receptor *Cxcr2* mRNA was blunted in HH-inhibited mice (Figure S7B,C). These data support our hypothesis that stromal HH signaling induces expression of the growth factor CXCL1, which recruits neutrophils into the acutely damaged EHBDs.

Blocking recruitment of Ly6G-expressing cells into damaged EHBDs decreases epithelial cell proliferation

To determine whether the HH-GLI1-CXCL1 axis-dependent neutrophil influx is important for biliary cell proliferation, we blocked CXCL1-CXCR2 interaction with the selective CXCR2 antagonist SB225002. Mice were treated with SB225002 or vehicle 1 h before and 6 h after operation, with tissue analysis 24 h after BDL (Figure 6A). CXCR2 inhibition reduced the number of Ly6G$^+$ neutrophils (Figure 6B,C) and, more importantly, suppressed epithelial cell proliferation compared with vehicle-treated controls (Figure 6D,E). These findings suggest that neutrophils play an important role in stimulating the cellular proliferative response to acute EHBD injury.

It was reported that Ly6G expression directly correlates with neutrophil differentiation and maturation

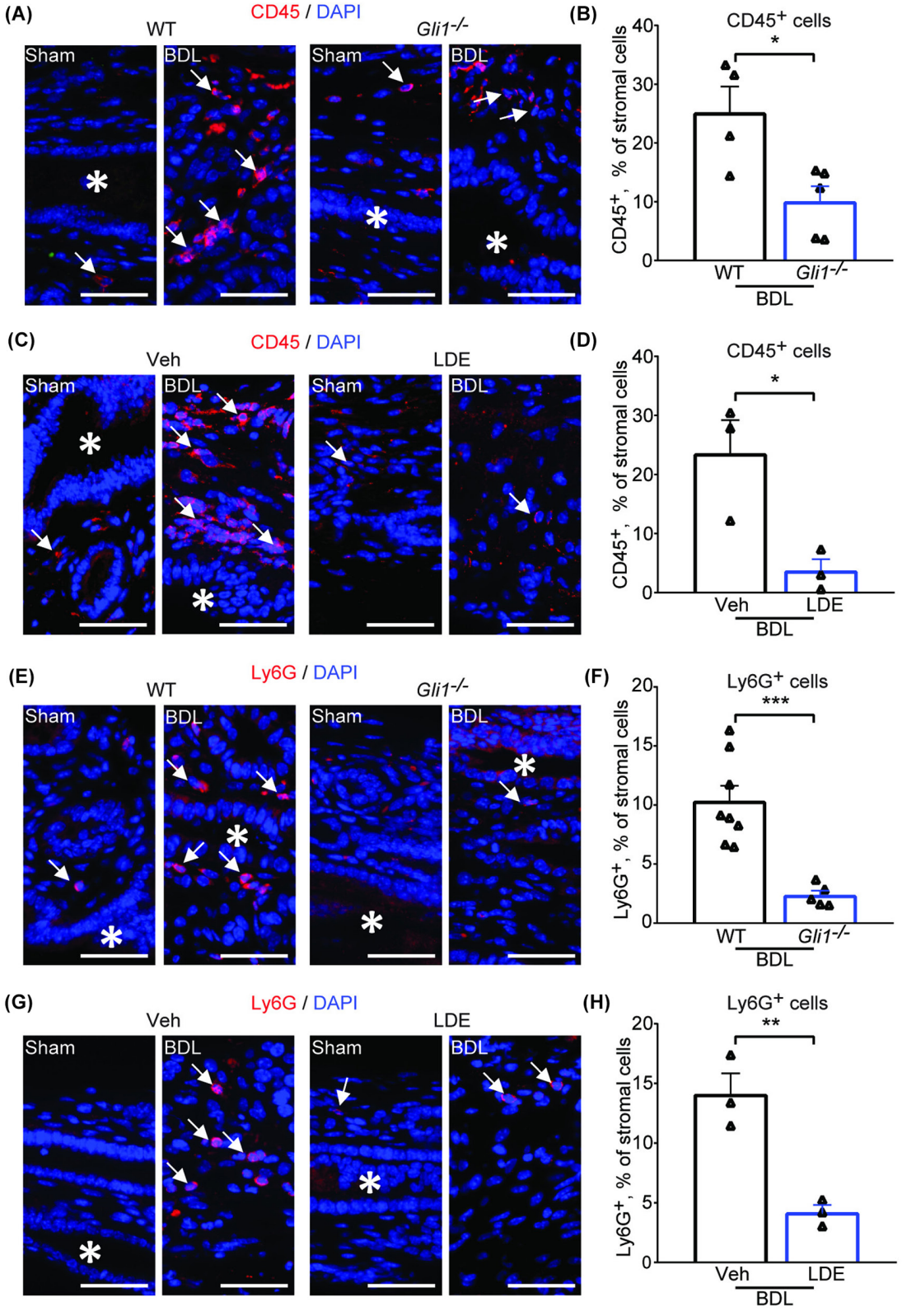

FIGURE 5 Immune cell recruitment into EHBD after acute injury. EHBDs of *Gli1*$^{-/-}$ (A,B) and LDE-treated (C,D) mice were examined for pan-leukocyte marker CD45 (red) by immunofluorescence and compared with WT controls and Veh-treated mice, respectively. Enumerated CD45^{+} cells were expressed as a percentage of DAPI^{+} stromal cells after BDL (B,D). Activated neutrophils were detected by Ly6G immunostaining (red) in *Gli1*$^{-/-}$ (E) and LDE-treated (G) mice, quantified as a subset of DAPI^{+} stromal cells after BDL and compared with respective controls (F,H). Asterisks mark lumen, and arrowheads mark either CD45^{+} or Ly6G^{+} cells. The data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001. n = 3–8 mice/group. Unpaired Student t test. Scale bars, 100 μm

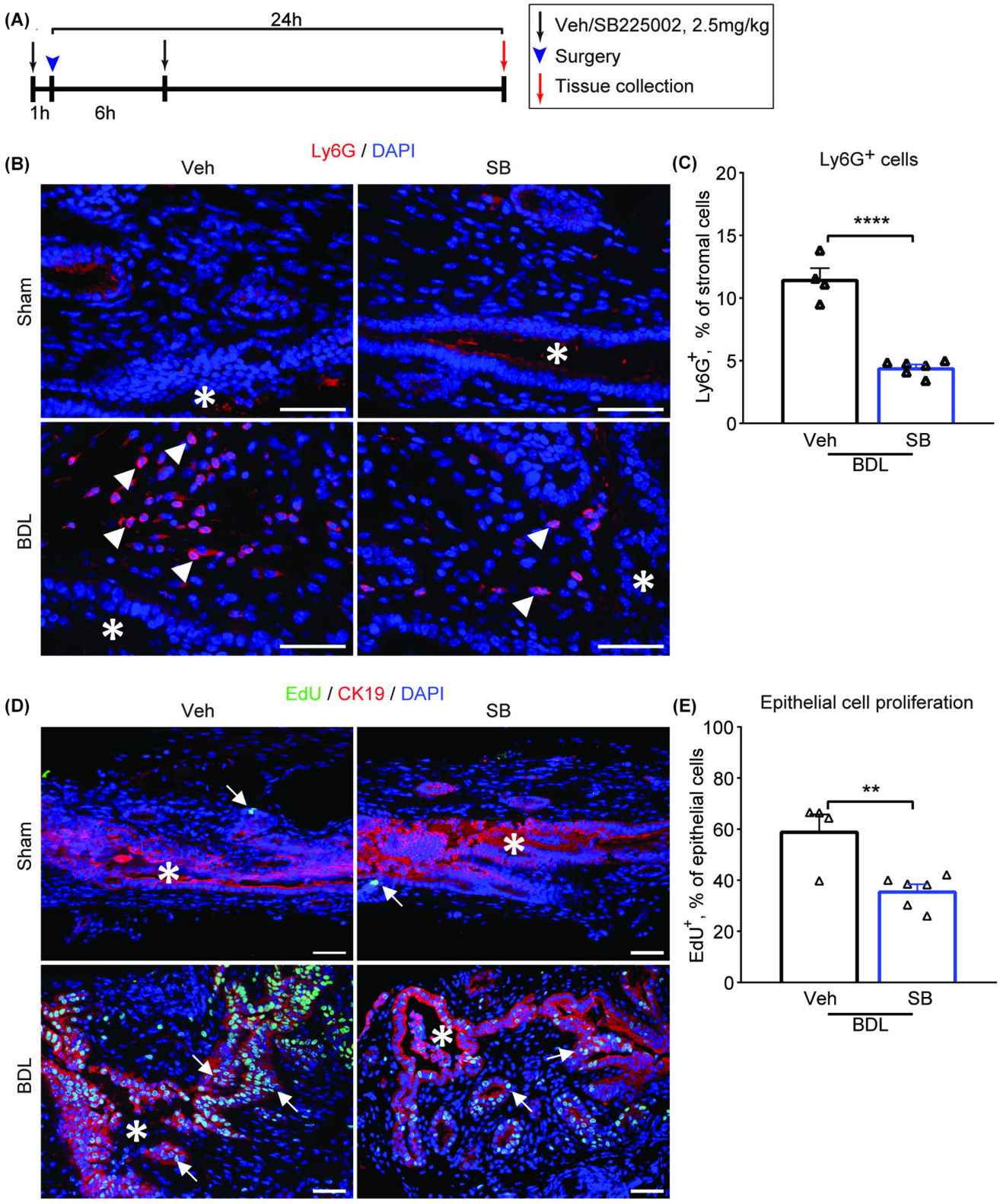

FIGURE 6 CXCR2 inhibition reduces epithelial cell proliferation after acute injury. WT mice were treated with SB225002 or Veh (A), and EHBDs were examined 24 h after BDL (B–E). Neutrophils were marked with Ly6G (red) using immunofluorescence (B), enumerated, expressed as a percentage of all stromal cells, and compared with Veh-treated controls (C). Proliferating epithelial cells were marked with EdU (green) (D), enumerated, expressed as a percentage of all epithelial cells in the $CK19^+$ cell compartment, and compared with Veh-treated controls (E). Asterisks mark EHBD lumen, arrows mark epithelial cells, and arrowheads mark $Ly6G^+$ cells. The data are presented as the mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$. n = 4–6 mice/group. Unpaired Student t test. Scale bars, 100 μm (B) and 50 μm (D)

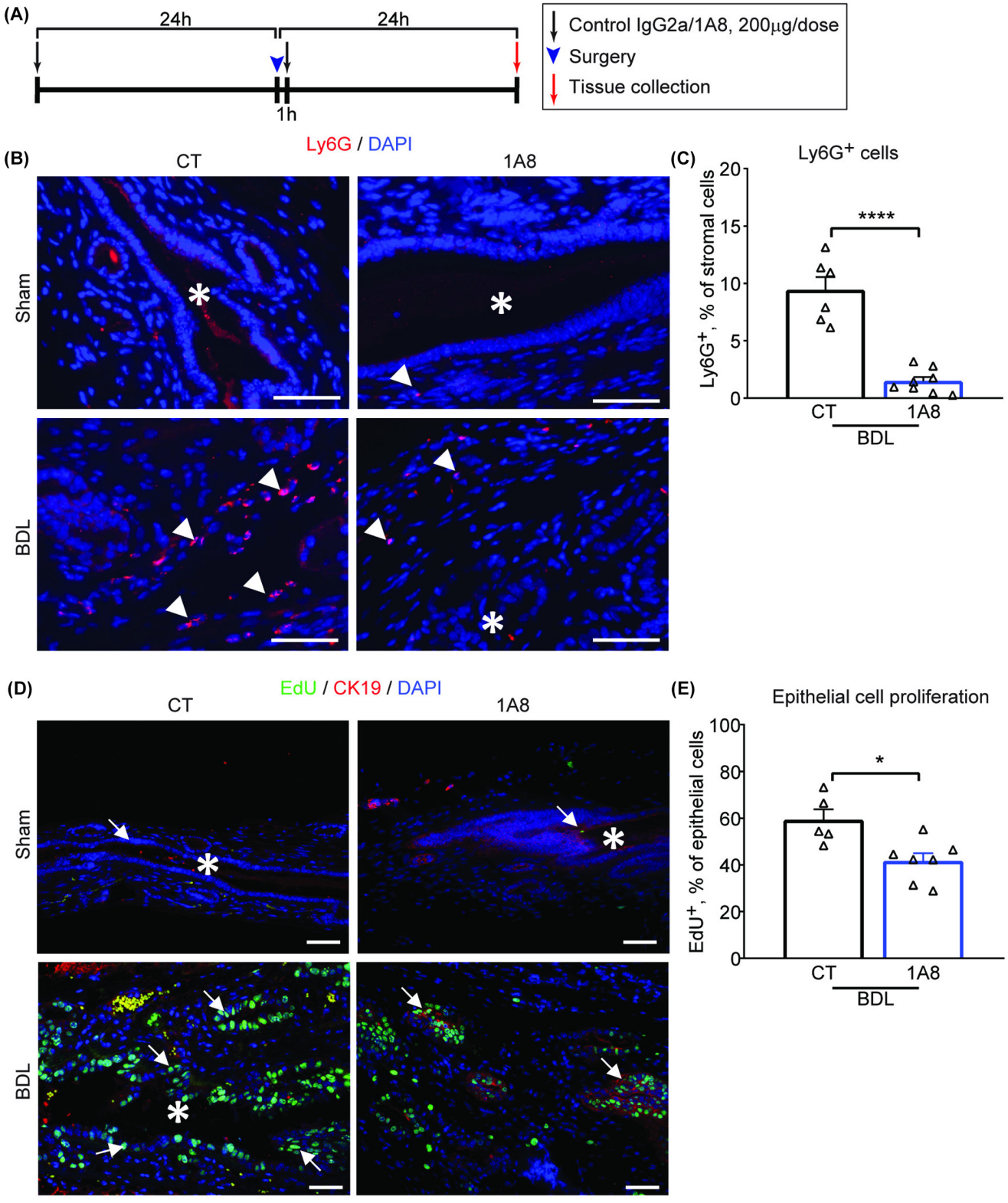

FIGURE 7 Ly6G inhibition reduces epithelial cell proliferation after acute injury. Ly6G$^+$ cells were depleted in WT mice with anti-Ly6G antibody (A), and EHBDs were examined 24 h after BDL (B–E). Neutrophils were marked with Ly6G (red; arrowheads) (B), enumerated, expressed as a percentage of all stromal cells, and compared with control antibody-treated mice (CT) (C). Proliferating epithelial cells were marked with EdU (green) (D), enumerated, expressed as a percentage of all epithelial cells in the CK19$^+$ cell compartment, and compared with control antibody-treated mice (E). DAPI (blue) marks nuclei, asterisks mark EHBD lumen, arrows mark epithelial cells, and arrowheads mark Ly6G$^+$ cells. The data are presented as the mean ± SEM. *$p < 0.05$, ****$p < 0.0001$. n = 5–7 mice/group. Unpaired Student t test. Scale bars, 100 μm (B) and 50 μm (D)

and is required for neutrophil influx into damaged tissues.[32] We blocked Ly6G by treating mice with neutralizing antibody (1A8) before operation (Figure 7A) as an alternative approach to inhibit neutrophil function. As expected, Ly6G neutralization, but not 2A3 isotype control administration, effectively reduced neutrophil tissue infiltration (Figure 7B,C) and blunted the proliferative response to acute EHBD injury (Figure 7D,E). Together, our findings show that Ly6G$^+$ neutrophil influx into injured EHDBs plays a role in promoting biliary proliferation.

DISCUSSION

Key hallmarks of cholangiopathies include the presence of inflammation, epithelial cell hyperproliferation, fibrosis, and biliary obstruction. Regulation of biliary proliferation is central to restoration of EHBD homeostasis after injury. Accordingly, the focus of the present study was to investigate mechanisms regulating biliary hyperproliferation after acute obstructive EHBD injury. Using an obstructive cholestatic EHBD injury model and testing inhibitory paradigms in mice, we demonstrated that IHH ligand up-regulated in injured cholangiocytes promotes biliary proliferation through epithelial to mesenchymal signaling and engagement of the inflammatory CXCL1-CXCR2-neutrophil axis through up-regulation of CXCL1 (Figure 8). Notably, our analysis of human EHBD tissues suggests that this mechanism is conserved across species.

To date, cholangiopathy and cholangiocarcinoma research has primarily focused on IHBDs. However, a better understanding of the fundamental biological differences between IHBDs and EHBDs has recently revealed a significant knowledge gap in EHBD cholangiopathies, preventing the development of effective therapies for these disorders.[17] IHBDs and EHBDs have distinct embryonic origins, progenitor cell compartments, and microenvironment composition.[33] Cholangiocytes in IHBDs are in close proximity to hepatocytes, resident liver immune cells, hepatic stellate cells, and periportal fibroblasts; stromal cells of EHBDs are less well-characterized but appear to be mainly represented by myofibroblasts and blood vessels.

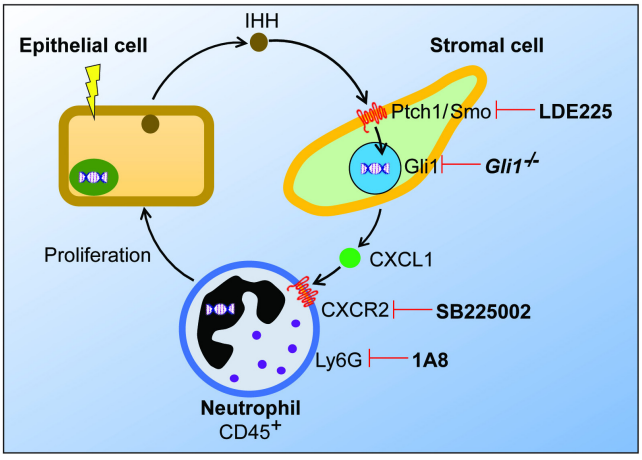

FIGURE 8 Model of proposed HH and CXCL1 signaling interactions in the biliary proliferative response. IHH induced in cholangiocytes after injury signals to GLI1$^+$ stromal cells to induce CXCL1 expression, which recruits CD45$^+$Ly6G$^+$ neutrophils to promote a proliferative response in cholangiocytes

The role of HH signaling in gastrointestinal injuries differs between organs and the type of injury (e.g., acute versus chronic). Thus, HH signaling has been implicated in cholestatic liver disorders in humans (e.g., PSC) and experimental animal models of chronic cholestasis, where after chronic BDL, IHH is overexpressed in both cholangiocytes and myofibroblasts and associated with progenitor cell proliferation and fibrogenesis.[13–17] Interestingly, cholestasis is associated with dysregulation of bile acid metabolism,[17] and bile acid precursors, oxysterols, modulate HH signaling through receptor Smoothened.[34,35] We previously showed that IHH is important for EHBD proliferative responses to acute cytokine IL-33–induced inflammation.[7] In mouse models of chemical colitis, IHH acts as a suppressor of inflammation through inhibition of CXCL12 secretion by fibroblasts.[36] This finding is consistent with decreased *Cxcl12* expression from the growth factor array in our study. In contrast, in the stomach, SHH ligand is secreted by parietal cells and acts directly as a chemoattractant of myeloid-derived suppressor cells during chronic *Helicobacter* infection.[37] Our current study that focused on acute obstructive damage revealed a rapid and highly dynamic epithelial cell proliferative response after BDL that utilizes HH signaling components expressed by epithelial (IHH$^+$, HH-producing) and stromal (GLI1$^+$, HH-responding) cells to facilitate early inflammatory and biliary proliferative responses. This early proliferative response was absent in IHBDs in our acute obstructive injury model, which suggests that mechanisms involved in responses to injury are different between IHBDs and EHBDs.

The role of the CXCL1-CXCR2-neutrophil axis has been previously reported in liver injury, though not necessarily cholangiopathies. It was demonstrated that increased CXCL1 expression in hepatic stellate cells after BDL for 4 weeks results in recruitment of sinusoidal neutrophils interacting with cholangiocytes, causing microthrombi development with portal hypertension.[38] In an alcohol-associated hepatitis study, the CXCL1-neutrophil axis is linked to worsening of cholestasis through inhibition of bicarbonate secretion from cholangiocytes through c-Jun up-regulation.[39] In polycystic liver disease, CXCR2 overexpression is associated with increased cyst cholangiocyte proliferation; however, it was not indicated to be a neutrophil-mediated

effect.[40] CXCL1-CXCR2 receptor signaling is linked to immune-mediated fibroproliferative disorders, such as biliary atresia, and cancer promotion, including cholangiocarcinoma, stomach, colorectal, breast, esophageal, pancreatic, lung, and ovarian cancers.[20,21,41,42] CXCR2 overexpression is associated with increased cell proliferation, migration, and poor patient prognosis in intrahepatic cholangiocarcinoma.[43] CXCR2 inhibition protected mice from liver injury 3 and 14 days after BDL, although this effect was independent from neutrophil accumulation.[44] Our data suggest that HH signaling induces CXCL1 expression in EHBD stromal cells to recruit $Ly6G^+$ neutrophils, which induce biliary proliferation (Figure 8).

CXCL1 chemokine expression is regulated by NF-κB and STAT1 transcription factors in murine hepatocytes during hepatocellular carcinogenesis, malignant and immortalized melanocytes, pancreatic β-cells, and cancer-associated fibroblasts in esophageal squamous cell cancer.[28,45–47] Our discovery of a high affinity consensus GBS in close proximity to the mouse *Cxcl1* and human *CXCL1* genes suggests a potential mechanism of HH induction of *Cxcl1* through transcriptional regulation. Future studies will be required to validate direct GLI-mediated regulation of *Cxcl1* expression.

Neutrophils have dual roles during tissue injury. They can intensify the immune response by affecting epithelial cell integrity and release of cytotoxic components, but they can also mediate inflammation resolution by clearing infection, secreting growth factors enhancing angiogenesis, facilitating debris clearance, and signaling to other immune cells to cease the inflammatory reaction.[6,18,19] Notably, neutrophils were identified as a major cell type of the immune landscape in bile ducts of patients with PSC.[11] In our human sample analysis, CXCL1 expression was increased in bile ducts of patients with cholangiopathies, especially those who received antibiotics for cholangitis, which is consistent with recruitment of inflammatory cells in biliary infection. Our work implies that recruitment of neutrophils, the first responders during acute inflammation in EHBD injury, promotes biliary proliferation, suggesting that neutrophils contribute to tissue repair in this context. Accordingly, HH pathway inhibition not only dampened neutrophil recruitment but significantly blunted EHBD proliferation after injury.

Our study demonstrated that HH signaling promotes an early inflammatory response to EHBD damage. Cholangiopathies, such as primary biliary cholangitis and PSC, are thought to be immune-mediated and occur when an early inflammatory response evolves into a chronic process.[48] The ability to direct immune responses toward EHBD repair is very attractive for clinical management of cholangiopathies, and several therapeutic approaches have been proposed targeting HH[49] or CXCL1-CXCR2[41] pathways. In this study, we determined that HH signaling promotes an early inflammatory response to EHBD injury through CXCL1-mediated neutrophil recruitment. Recognition of this HH-CXCL1-CXCR2-neutrophil axis regulating the inflammatory and epithelial proliferative response to EHBD injury offers potential targets for disease-modifying agents in EHBD cholangiopathies and other immune-mediated disorders.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

Nataliya Razumilava and Nureen H. Mohamad Zaki devised the study design, main conceptual ideas, and project outline. Nataliya Razumilava, Nureen H. Mohamad Zaki, Junya Shiota, Ashley N. Calder, Theresa M. Keeley, and Benjamin L. Allen contributed to experiments. Nataliya Razumilava and Nureen H. Mohamad Zaki processed and analyzed the data with Junya Shiota and Ashley N. Calder's assistance. Kazuhiko Nakao and Junya Shiota procured human samples. Nataliya Razumilava and Nureen H. Mohamad Zaki wrote the manuscript. Linda C. Samuelson and Benjamin L. Allen provided critical feedback on the manuscript and interpretation of results.

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