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Perforin and granzymes work in synergy to mediate cholangiocyte injury in experimental biliary atresia

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Abstract

Background and Aims—Biliary atresia represents obstructive cholangiopathy in infants progressing rapidly to cirrhosis and end-stage liver disease. Activated NK cells expressing Nkg2d have been linked to bile duct injury and obstruction by establishing contact with cholangiocytes. To define the mechanisms used by cytotoxic cells, we investigated the role of perforin and granzymes in a neonatal mouse model of rotavirus (RRV)-induced biliary atresia.

Methods—We used complementary cell lysis assays, flow cytometric analyses, quantitative PCRs and in vivo systems to determine the mechanisms of bile duct epithelial injury and the control of the tissue phenotype in experimental biliary atresia.

Results—RRV-infected hepatic NK and CD8 T cells increased the expression of perforin and injured cholangiocytes in short-term culture in a perforin-dependent fashion. However, the loss of perforin in vivo delayed but did not prevent the obstruction of bile ducts. Based on the increased expression of granzymes by perforin-deficient cytotoxic cells in long-term cytolytic assays, we found that the inhibition of granzymes by nafamostat mesilate (FUT-175) blocked cholangiocyte lysis. Administration of FUT-175 to perforin-deficient mice after RRV infection decreased the development of jaundice, minimized epithelial injury, and improved long-term survival. However, the inhibition of granzymes alone in wild-type mice was not sufficient to prevent the atresia phenotype in newborn mice. In infants with biliary atresia, hepatic *Granzymes A and B* mRNA, but not *Perforin*, increased at the time of portoenterostomy.

Conclusions—Perforin and granzymes have complementary roles mediating epithelial injury by NK and CD8 T cells. The prevention of experimental biliary atresia can only be achieved by inhibiting both granules.

INTRODUCTION

Biliary atresia is a progressive liver disease of infancy resulting from an inflammatory and fibrosing obstruction of extrahepatic bile ducts. Although early diagnosis and surgical

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Conflict of interest:

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intervention by Kasai portoenterostomy may restore continuity of bile flow from the liver to duodenum, the majority of patients die from end-stage biliary cirrhosis in early childhood. Studies using livers and/or biliary remnants from infants with biliary atresia reported an enhanced proinflammatory footprint and an infiltration by CD4⁺/CD8⁺ T lymphocytes, natural killer (NK) and dendritic cells [1-8]. In mechanistic experiments using a neonatal mouse model of *Rhesus* rotavirus (RRV)-induced biliary atresia, disruption of the adaptive immune response by loss of Ifn γ or CD8 T cells reduced bile duct obstruction and improved the cholestasis phenotype [9, 10]. Recently, we ascribed a key function for NK cells in the initiation of epithelial injury by engaging and lyzing cholangiocytes through the Nkg2d receptor [11]. The effector mechanisms used by NK cells to target cholangiocytes, however, remain largely unknown.

Innate immune lymphocytes are critical for early host defenses against viral infections and exert cytotoxic effects against virus-infected cells primarily by granule exocytosis [12]. This potent cytolytic process is housed within the cytoplasmic granules rich in perforin, granzymes and other effector molecules. In addition, binding of stimulatory receptors like Nkg2d on cytotoxic cells by ligands of target cells activates a cascade of intracellular signaling events resulting in the secretion of Ifn γ and Tnf α , and in the polarization and exocytosis of cytolytic granules [13, 14]. Chief among these granules are perforin and granzymes that work in concert to clear virus-infected cells [15]. Based on the central role of NK and CD8 T cell signaling in cholangiocyte injury and on the increased expression of perforin and granzymes in livers of patients with biliary atresia [10, 11], we hypothesized that the perforin-granzyme system is required for epithelial injury of bile ducts. Testing this hypothesis using complementary in vitro and animal approaches, we found that the individual loss of perforin or inhibition of granzymes had minimal impact on the development of bile duct injury after RRV. However, the simultaneous loss/inhibition of both granules prevented cholangiocyte lysis and bile duct obstruction, and improved the phenotype of experimental biliary atresia.

MATERIALS AND METHODS

Experimental model of biliary atresia

BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and Balb/c *Perforin* knockout (PKO) mice were a kind gift from Dr. John T. Harty (University of Iowa, Iowa City, IA). Newborn PKO and WT mice were injected intraperitoneally with 1.5×10^6 fluorescence-forming units (ffu) of RRV in 20 μ l volume within 24 hours of birth to induce experimental biliary atresia as described previously [9]. In granzyme blocking studies, the protease inhibitor nafamostat mesilate (FUT-175, Enzo Life Sciences, Inc., Farmingdale, NY) was administered intraperitoneally at a dose of 15 μ g/g body weight in 20 μ l 1X phosphate buffered saline (PBS) soon after birth followed by RRV infection 24 hours later; control mice received 20 μ l of PBS [9-11]. Thereafter, FUT-175 was administered daily until 14 days of life. Groups of mice were sacrificed between 3–14 days and the extent of duct injury was determined [16]. The Institutional Animal Care and Use Committee (IACUC) of the Cincinnati Children's Research Foundation approved all the animal experiments and protocols.

Human livers

Liver RNA was isolated from 1–3 month old infants at the time of diagnosis of biliary atresia. Control biopsies were obtained from livers of deceased donors aged 2–3.5 years being used for transplantation; age-matched donors from healthy subjects were not pursued due to ethical considerations. These subjects were described in a previous publication [3, 11]. Written informed consent was obtained from the patients' guardians.

Liver function tests

Serum total bilirubin (Total Bilirubin Reagent Set; Pointe Scientific, Inc. Canton, MI) and alanine transaminase (DiscretPak ALT Reagent Kit; Catamount, Inc. Oxford, CT) were quantified according to the manufacturer's instructions.

Flow cytometric analysis and cytotoxicity assays

Mononuclear cells were isolated from livers of neonatal mice and subjected to antibody-staining for flow cytometric analysis and cytotoxicity assays for NK and CD8 T cells as described previously [11]. Description of the assays and a list of antibodies and reagents are provided as Supplementary Materials and Methods.

Protein and gene expression

Supernatants from co-culture experiments were used to quantify granzyme B, IL-1 α , IL-10, IL-12p40, IL-15 and IL-17 by ELISA or protein Milliplex™. Gene expression was quantified by real-time PCR using specific primers and a Stratagene Mx3005P thermocycler (Agilent Technologies, Inc., Santa Clara, CA), as described previously [9]. Description of the assays for protein and gene expression is provided as Supplementary Materials and Methods.

Statistical analysis

The numbers of mice or tissues used in each experiment are presented in the text or figure legends. All in vitro experiments were performed in 6 wells for each condition. Values are expressed as mean $\pm$ standard deviation (S.D.) and statistical significance was determined by unpaired *t* test with or without Welch's correction with significance set at *P* < 0.05. Survival curves were created by Kaplan-Meier analysis using the GraphPad Prism version 5.00 Software (GraphPad Software, San Diego, CA).

RESULTS

Increased perforin expression and requirement for cell lysis

To test the hypothesis that the perforin-granzyme system is required for epithelial injury of bile ducts, we quantified the expression of *Perforin* mRNA in extrahepatic bile ducts and of its protein in cytotoxic cells after RRV challenge. Inoculation of RRV into newborn mice resulted in poor growth, jaundice, and mortality in >95% of mice by 14 days of age, as reported previously [9]. At the mRNA level, *Perforin* progressively increased above saline controls from the onset of epithelial injury (3 days) through the time of duct obstruction (7 days; Figure 1A). Using FACS, the expression of perforin in NK, CD8 and CD4 T cells increased after RRV (Figure 1B; Supplementary Figure 1). To explore whether this increased expression represents a mechanism of cytotoxicity, we co-cultured NK and CD8 T cells from WT or PKO mice with murine cholangiocytes (mCL) at different ratios in a cytolytic assay. As expected for WT mice, mCL lysis occurred when they were co-cultured with RRV-primed but not naïve cells after 5 hours of co-culture; however, in PKO mice, the peak lysis by RRV-primed NK and CD8 T cells was much lower than WT cells at 1:10 and 1:25 ratios to target mCL cells (Figure 1C, D). This decreased cell lysis and the increased expression of perforin after RRV further strengthened the possibility that perforin is required by these cells to injure the duct epithelium in experimental biliary atresia.

Epithelial injury and duct obstruction in PKO mice

To directly examine this possibility, we injected RRV into WT and PKO newborn mice and monitored the development of cholestasis, biliary injury and duct obstruction. As expected, WT mice receiving RRV failed to gain weight, developed jaundice and died between days 8-14 (Figure 2A). To our surprise, while newborn PKO mice injected with saline grew well

and remained asymptomatic, exposure to RRV produced cholestasis (jaundice, acholic stools, bilirubinuria) and decreased survival similarly to WT mice, with the exception that mortality was significantly delayed to days 12-20 after challenge (Figure 2A; $P<0.001$). Microscopically, inflammation and epithelial integrity were similar between the two genotypes at 3 days after RRV, appeared less intense in PKO mice at 5 days, and severe with complete occlusion of the duct lumen at 7 days (Figure 2B, Supplementary Figure 2A). Biochemically, ALT and total bilirubin levels increased in WT and PKO mice after RRV infection, with levels in PKO mice trending to a lower peak (Supplementary Figure 2B, C). The abundance of RRV was similar in extrahepatic bile ducts of both genotypes at 7 days (WT = $1.2\pm0.7\times10^6$ ffu vs PKO = $0.7\pm0.5\times10^6$ ffu; $P=0.36$).

Based on the milder tissue injury at 5 days and delayed mortality in PKO mice, we performed new co-culture experiments to determine whether prolonged incubation of RRV-primed cytotoxic cells from PKO mice produced increased lysis of mCL cells. Indeed, after 24 hours of co-culture, RRV-primed NK and CD8 T cells from PKO and WT mice induced similar levels of mCL lysis (Figure 2C, D). Collectively, the in vivo and cell culture data suggested that in the absence of perforin, mice use alternative mechanisms that ultimately produce cholangiocyte lysis and the biliary atresia phenotype.

Granzymes mediate cholangiocyte lysis by PKO-NK cells

To investigate whether the alternative mechanisms involve functionally related granules, we quantified the levels of granzyme B in supernatants of the co-culture cytolytic assay described above; we also quantified the cytokines Il-1 α , Il-10, Il-12p40, Il-15 and Il-17 to explore whether other molecules were potentially involved in perforin-independent lysis of mCL. Granzyme B was very low in control wells and increased several fold after 24 hours of incubation (Figure 3A), while the levels of other cytokines did not reach statistical significance (Supplementary Table 2). The expression of granzyme B depended on direct contact between NK cells and mCL, as evidenced by the baseline levels when a transwell membrane was interposed between the two cell types (Figure 3A). Further, to determine whether PKO-NK cells used granzyme-mediated signals to injure cholangiocytes, we repeated the lysis assay with the addition of granzyme B antibodies or the synthetic pan-granzyme inhibitor FUT-175. We found that the inhibitor significantly suppressed cholangiocyte lysis to a greater degree than antibodies to granzyme B at 5 and 24 hours of incubation (Figure 3B). These data identified granzymes as important mediators of the delayed cytolytic properties of NK cells on cholangiocytes, perhaps compensating for the absence of perforin.

Granzyme inhibition prevents duct injury and obstruction in PKO mice

To investigate whether granzymes may constitute important effectors of bile duct injury, we quantified the percent of NK and CD8 T cells expressing granzyme B from livers of PKO mice injected with RRV or saline. At 7 days after RRV, $64.5\pm3.5\%$ of PKO-NK and $41.4\pm8.2\%$ of CD8 T cells expressed granzyme B, compared to $21.8\pm0.5\%$ and $6.2\pm1.1\%$ respectively in age-matched saline controls ($P<0.001$; Figure 3C). At the mRNA level, the expression for *GzmA* and *GzmB* in PKO-livers increased after RRV about 3 fold above saline controls in early phases of duct injury (day 3), and 6 fold at the time of duct obstruction (day 7, Figure 3D). Based on these data, we injected FUT-175 (15 μ g/g body weight) or PBS intraperitoneally soon after birth of PKO mice, followed by injection of RRV (or saline as controls) 24 hours later; similar doses of FUT-175 or PBS were administered daily until 14 days of life. Injections were well tolerated as demonstrated by normal appearance and growth beyond 2 weeks in saline-injected mice receiving either FUT-175 or PBS. Administration of FUT-175 to PKO mice did not prevent the poor weight gain observed in the first 2-3 weeks after RRV infection (Figure 4A), but the growth

improved subsequently, jaundice cleared, and mice survived beyond 21 days in 77% of PKO mice (Figure 4A). Microscopically, extrahepatic bile ducts of PKO mice treated with FUT-175 showed mild epithelial injury and submucosal lymphocytic infiltration, and no obstruction of the duct mucosa, in contrast to the severe histological changes in PKO mice that did not receive FUT-175 (Figure 4B). Combined, the in vitro and in vivo data demonstrate that the added inhibition of granzymes to the state of perforin deficiency reduced cholangiocyte injury by the cytotoxic cells in vitro and improved the biliary phenotype in vivo. It remained to be determined whether the granzyme inhibition by FUT-175 decreases NK cell killing and epithelial injury independently of perforin.

FUT-175 decreases cholangiocyte lysis but does not prevent biliary injury in WT mice

To investigate whether inhibition of granzymes by FUT-175 can decrease NK-induced mCL lysis in the presence of perforin, we performed cytolytic assays using RRV-primed and naïve NK cells from WT mice and mCL cells in the presence or absence of FUT-175. mCL lysis after 5 hours of incubation was prevented by FUT-175 (<5%), but prolongation of the assay to 24 hours showed some increase in mCL lysis but kept it at about half of the lysis of untreated cells (Supplementary Figure 3A). Consistent with this partial effect, administration of FUT-175 to WT mice showed a mild improvement in the incidence of jaundice, but did not prevent growth failure or the overall mortality induced by RRV (85% treated FUT-175 versus 100% treated with PBS; $P>0.05$; Supplementary Figure 3B) or the luminal obstruction of extrahepatic bile ducts (Supplementary Figure 3C). Thus, inhibition of granzyme alone is not sufficient to prevent the biliary phenotype induced by RRV, and underscores the existence of compensatory mechanisms to overcome the functional deficit of individual granules.

Granzymes are increased in livers of infants with biliary atresia

To further explore the potential involvement of perforin and granzymes in the pathogenesis of biliary atresia in humans, we quantified the hepatic mRNA expression for *PERFORIN*, *GRANZYME A (GZMA)* and *GRANZYME B (GZMB)* in 1-3 month old infants and control livers. While mRNA expression for *PERFORIN* did not differ between the two groups (Supplementary Figure 4A), *GZMA* and *GZMB* mRNA significantly increased in subjects with biliary atresia above controls (Supplementary Figure 4B, C). These findings support an enrichment of the hepatic environment with granzymes at the time of diagnosis.

DISCUSSION

Seeking to understand the molecular circuits used by NK and CD8 T cells to injure the bile duct epithelium in experimental biliary atresia, we found that both perforin and granzymes are required for the rapid cytolytic properties against cholangiocytes in short-term co-culture. If one of them is lost, the immediate cytotoxic cell-induced lysis is impaired. In long-term culture, however, the loss of perforin or inhibition of granzymes does not substantially suppress cholangiocyte injury within 24 hours of co-culture. A similar finding was reproduced in vivo, in which the genetic loss of perforin or pharmacologic inhibition of granzymes did not prevent the final outcome of bile duct injury, persistent jaundice, and the high mortality rate. However, the simultaneous loss/inactivation of both molecules effectively blocked cytotoxic cell-induced cholangiocyte injury in prolonged culture, suggesting that at least one of the exocytic granules must be present for effector cell function. The apparent lack of alternative cytolytic mechanisms to compensate for the deficiency of perforin/granzyme was reproduced in vivo, with a reduction in bile duct injury, prevention of luminal obstruction, and lower mortality of neonatal mice infected with RRV.

The ability of NK and CD8 T cells to injure cholangiocytes with either perforin or granzyme alone suggests that each granule can exert cytotoxicity independently, or play a secondary role in cell injury, perhaps in association with other proinflammatory factors that these cells produce within or outside of the immunological synapse. This is supported by previous studies reporting the ability of granzymes to enter target cells by endocytosis independently of perforin [17]; however, sub-lytic amounts of perforin are still required to induce rapid apoptosis of target cells. In perforin-deficient states, granzymes have been implicated in extracellular inflammation, leukocyte extravasation, and delayed cell death, as well as cleavage of extracellular matrix proteins, vitronectin, fibronectin and laminin resulting in detachment and death of susceptible target cells [18, 19]. A novel role for granzyme uptake independent of perforin in cells expressing Hsp70 is of particular relevance based on the proposed link of Hsp70 with several cholangiopathies [20]. Additionally, cytolytic cells can also use cytokine- and Fas/FasL-dependent pathways as effector mechanisms of cell death [21, 22]. One of these pathways is likely to involve $\text{Ifn}\gamma$ and Tnfa , which have been shown to work in synergy to display co-stimulatory activity and trigger apoptosis in the experimental model of biliary atresia [16]. Although our experimental approach did not investigate the possibility that granzymes or perforin work in concert with these cytokines, the prevention of cholangiocyte lysis and suppression of bile duct injury when the pan-granzyme inhibitor FUT-175 was superimposed to perforin deficiency supports a synergistic role for both types of granules as effector mechanisms used by NK cells to produce the phenotype of experimental biliary atresia.

Differently than perforin-deficient mice, pharmacologic inhibition of granzymes in WT mice did not modify the NK cell-dependent killing of cholangiocytes or the bile duct injury induced by RRV. This is consistent with a role for perforin independent of granzymes. In support of this concept, perforin has been shown to regulate the rejection of tumors in an Nkg2d-mediated, perforin-dependent process, with Nkg2d acting as a dominant cytotoxicity receptor [23]. Despite the effector function for perforin and granzyme independently of each other, our data clearly show that the loss/inhibition of both renders NK cells unable to injure cholangiocytes in short- or long-term co-culture. The lack of effective compensatory mechanisms is supported by the suppression of biliary injury in PKO mice treated with FUT-175. Thus, although individually not required, the functional synergy between perforin and granzymes plays key a role in NK cell targeting of the duct epithelium. .

In addition to NK cells, perforin and granzymes are also expressed by CD8 T lymphocytes and function as effectors of cholangiocyte lysis. Both NK and CD8 cells have been shown to regulate pathogenesis of bile duct injury in neonatal mice [10, 11]. In this work, we focused on investigating the role of perforin and granzymes in NK and CD8 T cell cytotoxicity because they use granule exocytosis as a chief mechanism of lysis in susceptible target cells, with a primary event involving the release of pore-forming granules rich in perforin [24]. In keeping with this possibility, the numbers of perforin-positive NK and CD8 T cells and expression of perforin and granzymes increased significantly in the livers of RRV-challenged mice up to the time of bile duct obstruction. However, the levels of intracellular granzyme content and degree of cholangiocyte cytotoxicity executed by CD8 T cells were lower than the activity of NK cells. The degree of improvement in the biliary phenotype produced by the loss of perforin and granzyme inhibition by FUT-175 is less than the improvement observed in mice depleted of NK cells, as supported by the mild epithelial injury observed in early stages following RRV infection. Consistent with this injury, newborn mice also had infiltration of inflammatory cells in the subepithelial compartment. Despite these changes, the dual loss of functional perforin and granzymes prevented the obstruction of extrahepatic bile ducts and fostered long-term survival in most mice in a fashion similar to mice depleted of NK or CD8 cells [10, 11]. In humans, we found an increased expression for granzymes but not for perforin at diagnosis of biliary atresia. The

differences between the experimental model and patients with BA may relate to variable stages of liver injury at the time of diagnosis, as supported by previous studies demonstrating that the degree of inflammation may vary, even when patients are matched by age [25]. It is also possible that there may be differences in the neonatal immune system and in the bile duct response to injuries between human and rodents. Further, the RRV-model of experimental biliary atresia does not display prominent fibrosis, a key feature of the liver disease in humans. Therefore, any potential translation of the findings to the human disease requires careful validation of biological principles and additional pre-clinical studies.

Our findings broaden the repertoire of effector molecules used by the neonatal immune system to produce the phenotype of experimental biliary atresia. Perforin and granzymes work cooperatively to induce cholangiocyte lysis by NK cells, and do so requiring direct contact between the two cells. Other effector molecules include the Th1 soluble ligands *Ifn γ* and *Il-15* [8, 9], and possibly *Tnfa* [16], as well as the Th2 cytokine *Il-13* [3]. Notably, previous studies reported an increase in the mRNA expression of these cytokines, including mRNA for *GRANZYMES A and B*, in the livers of infants at the time of diagnosis of biliary atresia [3, 8, 9, 11]. While each one of these molecules can be the focus of future clinical trials [26], our data show that therapeutic strategies to block the perforin-granzyme system must target both molecules simultaneously to be effective in reducing biliary injury and blocking progression of liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

PKO	perforin knockout
ffu	fluorescence-forming units
RRV	<i>Rhesus</i> rotavirus type A
WT	wild type

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Fig. 1. Perforin expression and cholangiocyte lysis

(A) mRNA expression for *Perforin* in extrahepatic bile ducts from WT mice at 3, 5 and 7 days after saline or RRV challenge as a ratio to mouse *Gapdh*; N=3 per time-point; *= $P<0.001$. (B) Median Fluorescence Intensity (MFI) histograms showing perforin expression by NK and CD8 T cells after saline or RRV challenge. N=3 per time-point; Mean $\pm$ SD MFI values are shown. Lower panels show percent cholangiocyte lysis after incubation with NK (C) or CD8 T cells (D) from RRV- WT (grey bars), PKO (black bars) and saline-injected mice (open bars). Hepatic NK and CD8 T cells were obtained from a pool of 6–8 livers; *= $P<0.01$, **= $P<0.003$, ns: not significant.

Fig. 2. Impact of perforin deficiency on outcome and cholangiocyte lysis after RRV infection (A) RRV infection resulted in poor growth and onset of jaundice, acholic stools and bilirubinuria in WT and PKO mice, but mortality was delayed in PKO mice (Kaplan-Meier survival analysis, N=9-33 per group in each experiment). In panel (B), sections of extrahepatic bile ducts from RRV-challenged WT (top panel) and PKO (middle panel) mice show severe cholangitis and luminal obstruction at 5-7 days. Lower panel shows normal anatomy in age-matched control PKO mice. Arrows: subepithelial inflammation; arrowheads: intact duct epithelium; asterisks: lumen. Percent cholangiocyte lysis after incubation with NK (C) and CD8 T cells (D) from RRV-WT (grey bars), PKO (black bars) and saline-injected mice (open bars). Hepatic NK and CD8 T cells were obtained from a pool of 6-8 livers; *= $P < 0.001$.

Fig. 3. Contribution of NK cell granzymes to cholangiocyte lysis

(A) Granzyme-B levels in supernatants following co-culture of PKO NK cells and cholangiocytes (mCL). Arrows indicate non-detectable levels in saline group (blue) or in the RRV group (green) with a transwell between NK and mCL. N=6-8 mice per group; $^*P<0.0001$. (B) Percent lysis after co-culture of PKO NK cells with mCL in the presence or absence of 10 μ g/mL granzyme-B antibodies or 15mg/mL FUT-175. Hepatic NK cells were obtained from a pool of 6-8 livers; $^*P<0.006$. (C) Scatter plots showing granzyme-B expression by NK and CD8 T cells from PKO mice 7 days after RRV infection. Numbers represent percent NK or CD8 T cells expressing granzyme-B; N=3 mice per time-point. (D) mRNA expression of *Granzyme A* and *B* in PKO mice expressed as a ratio to mouse *Gapdh*. N=3 replicates per time-point; $^*P<0.003$.

Fig. 4. Prevention of experimental biliary atresia by FUT-175 treatment of perforin-deficient mice

(A) Daily injections of FUT-175 improved long-term weight, jaundice, acholic stools, bilirubinuria and long-term survival of Perforin knockout (PKO) mice challenged with RRV. N=9-29 mice per group; $^{*}P<0.001$; Kaplan-Meier survival analysis shows increased survival after FUT-175 treatment ($P=0.013$). (B) Longitudinal sections of extrahepatic bile ducts from PKO mice showing normal histology after saline (top panel), typical duct inflammation and obstruction after RRV (middle panel), and prevention of duct obstruction in mice receiving FUT-175 (lower panel). Arrows point to inflammation, arrowheads to the duct epithelium, and asterisks to the lumen.