Interleukin-32 production associated with biliary innate immunity and proinflammatory cytokines contributes to the pathogenesis of cholangitis in biliary atresia

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Interleukin-32 production associated with biliary innate immunity and proinflammatory cytokines contributes to the pathogenesis of cholangitis in biliary atresia

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ABSTRACT

Aims: Biliary atresia (BA) is thought to be associated with infections by viruses such as Reoviridae and is characterized histologically by fibrosclerosing cholangitis with proinflammatory cytokine-mediated inflammation. IL-32 affects the continuous inflammation by increasing the production of proinflammatory cytokines. In this study, the role of IL-32 in the cholangitis of BA was examined. **Methods**: Immunohistochemistry for IL-32 and caspase 1 was performed using 21 samples of extrahepatic bile ducts resected from BA patients. Moreover, using cultured human biliary epithelial cells (BECs), the expression of IL-32 and its induction on stimulation with a Toll-like receptor (TLR) 3 ligand (poly(I:C)) and proinflammatory cytokines was examined. **Results**: BECs composing extrahepatic bile ducts showing cholangitis expressed IL-32 in BA, but not in controls. Caspase 1 was constantly expressed on BECs of both BA and control subjects. Furthermore, poly(I:C) and proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α) strongly induced IL-32 expression in cultured BECs, accompanying the constant expression of TLR3 and caspase 1. **Conclusions**: Our results imply that the expression of IL-32 in BECs was found in the damaged bile ducts of BA and induced by biliary innate immunity via TLR3 and proinflammatory cytokines. These findings suggest that IL-32 is initially involved in the pathogenic mechanisms of cholangitis in BA and also plays an important role in the amplification and continuance of periductal inflammatory reactions. It is therefore tempting to speculate that inhibitors of IL-32 could be useful for attenuating cholangitis in BA.

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INTRODUCTION

The obliterative lesion of biliary atresia (BA) is characterized by a progressive sclerosing cholangitis accompanying severe inflammation, fibrosis, and epithelial injuries and this characteristic feature is known as fibrosclerosing cholangitis. Little is known about the etiology and pathogenesis of BA, but infections by viruses such as Reoviridae (reovirus and rotavirus) having a double-stranded RNA (dsRNA) have been implicated, though conflicting results also have been reported [1-8]. Our recent study has demonstrated that biliary epithelial cells (BECs) possess an innate immune system consisting of Toll-like receptors (TLR), especially TLR3 which is an innate immune-recognition receptor recognizing dsRNA including dsRNA viruses as pathogen-associated molecular patterns (PAMPs) [9, 10]. Furthermore, the biliary innate immune response to artificial dsRNA was also shown to be associated with the induction of biliary apoptosis via the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and, differing from the innate immune response to TLR4 ligand (LPS), lack of subsequent tolerance to dsRNA using cultured human biliary epithelial cells [9-11].

Interleukin (IL)-32 is a recently described cytokine produced by T lymphocytes, natural killer (NK) cells, monocytes and some epithelial cells [12, 13]. Primarily, IL-32 was discovered in the synovial fluid of patients with rheumatoid arthritis and first reported as a transcript in IL-2 activated NK and T cells [14, 15]. There are six isoforms (α, β, γ, δ, ε, and ξ) caused by alternative mRNA splicing, resulting in proteins with a molecular weight ranging from 14.9 to 26.7 kD. IL-32α is the most abundant transcript. IL-32 exhibits several properties typical of proinflammatory cytokines [16]. For example, it stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1α, TNF-α, IL-6, IL-8 and VEGF through the activation of nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinases (MAPKs) [15, 17, 18]. In contrast, the production of IL-32 is induced or enhanced by the presence of proinflammatory cytokines including IL-1β, IFN-γ, and TNF-α via the activation of caspase 1 [17, 19, 20]. IL-32 has been implicated in

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inflammatory disorders such as rheumatoid arthritis, inflammatory bowel diseases, chronic obstructive pulmonary diseases, atopic dermatitis, and allergic rhinitis [14, 19-22].

Although human hepatocytes and hepatoma cells express IL-32 in HCV-associated chronic hepatitis and the expression is regulated by proinflammatory stimuli [23], the pathophysiological role of IL-32 in innate immune-related biliary diseases including BA remains unclear. We therefore investigated the IL-32 expression in the inflamed bile ducts of BA patients and the effect of innate immune stimulation by ligands of TLR3 and cytokines on IL-32 expression in cultured human BECs. Our results provide evidence that biliary epithelial cells are sufficient sources of IL-32 for the biliary inflammation at sites of BA and IL-32 may therefore play a role in the pathophysiology of BA.

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MATERIALS AND METHODS

Patients and tissue preparations

A total of 21 patients with BA (surgical specimens; average age 1.7 months; range of age 0.7-12 months; male/female=9/12) and age-matched control patients consisting of one neonatal hepatitis (giant cell hepatitis; wedge biopsy; 3 months; male) and 6 non-hepatobiliary diseases (congenital heart anomalies; autopsied specimens; average age 2.5 months; male/female=3/3) were examined. Resected common bile ducts and wedge liver biopsy specimens obtained at Kasai procedure from the patients with BA were used. These specimens had been fixed in 10% neutral-buffered formalin and embedded in paraffin; 4μm-thick sections were prepared for histologic observation and immunohistochemistry.

Immunohistochemistry and immunocytochemistry

For the immunocytochemistry using cultured BECs, formalin-fixed, paraffin-embedded sections of cell blocks were prepared according to the protocol reported by Mayall, *et al* [24]. The deparaffinized and rehydrated sections were heated in 10mM citrate buffer for 20min in a microwave oven. Following the blocking of endogenous peroxidase, these sections were incubated at

4ºC overnight with antibody against the C-terminus of IL-32 (rabbit polyclonal IgG, 1μg/ml, Lifespan, Seattle, WA, USA), TLR3 (rabbit polyclonal IgG, 1μg/ml, Santa Cruz, Santa Cruz, CA, USA), and caspase 1 (rabbit monoclonal IgG, diluted 1:1,000, Abcam, Tokyo, Japan) and then at room temperature for 1h with anti-rabbit immunoglobulins conjugated to a peroxidase-labeled dextran polymer (Simple staining kit, Nichirei, Tokyo, Japan). After a benzidine reaction, sections were lightly counterstained with hematoxylin. As a negative control, normal rabbit IgG was used as the primary antibody: no staining was obtained.

For the semi-quantitative evaluation of the immunohistochemistry, intrahepatic bile ducts and extrahepatic common bile ducts were chosen in each section for assessment and IL-32 immunoreactivity in these bile ducts was semiquantitatively graded as follows: score 0, absence of expression; score 1, low constitutive expression; score 2, intermediate expression; score 3, high expression.

In addition, simultaneous detection of IL-32 and cytokeratin (CK)19 was done using double immunohistochemical staining. After IL-32 immunostaining, CK19 antibody (mouse monoclonal IgG1kappa, 0.45μg/ml, Dako Japan, Tokyo, Japan) was applied overnight at 4ºC, followed by immunoglobulins conjugated to alkaline phosphatase labeled-dextran polymer (Nichirei). Color development of IL-32 and CK19 was achieved with diaminobenzidine (brown) and Vector blue (Vector Lab, Burlingame, CA, USA.), respectively.

Cultured human BECs and stimulation with PAMPs and proinflammatory cytokines

A cultured cell line of human intrahepatic BECs was established from the explant liver of a 24-year-old male with BA who had already received Kasai procedure during the newborn period, and cultured as previously reported [25] The cultured BECs were incubated with a culture medium composed of D-MEM/F-12 (Invitrogen, Tokyo, Japan), 5% newborn calf serum (Invitrogen), 0.18mM adenine (Sigma, Saint Louis, MO, USA), hydrocortisone (0.4μg/ml), cholera toxin (10ng/ml), tri-iodo-thyronine (1.3μg/l), ITS+ (Becton Dickinson, Franklin Lakes, NJ, USA), 25mM sodium bicarbonate (Sigma), 1% antibiotics antimycotic, human epidermal growth factor(20ng/ml)

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(Invitrogen), and human hepatocyte growth factor(10ng/ml) (Invitrogen). The cells were grown as monolayers in a humidified incubator with 5% CO_2 at 37°C. More than 95% of the cells were confirmed to be biliary epithelial cells by the expression of a biliary-type cytokeratin (CK19). The cultured BECs were used between passages 4 and 9. Informed consent for human research was obtained from the patient prior to surgery. This study was approved by the Kanazawa University Ethics Committee. Moreover, as control cultured cells, a commercially available cell line derived from human hepatocellular carcinoma, HepG2, was obtained from Health Science Research Resources Bank (Osaka, Japan).

These cultured cells were stimulated with a TLR3 ligand, polyinosinic-polycytidylic acid (poly(I:C), a synthetic analogue of viral dsRNA, 25μg/ml, Invivogen, San Diego, CA, USA) and recombinant cytokines (IL-1β, IFN-γ, TNF-α, TGF-β1, and IL-10, l,000U/ml, PeproTech, London, and IL-32, 1,000U/ml, R&D system, Minneapolis, MN, USA) for 3 hrs (molecular analysis) and 48 hrs (protein analysis by immunocytochemistry and Western blotting analysis).

Isolation of RNA, reverse transcription-polymerase chain reaction (RT-PCR), and real-time PCR

For the evaluation of mRNA of IL-32, caspase 1, TLR3, IL-1β, and IL-6, in cultured BECs, isolation of RNA from BECs and reverse transcription were performed using the RNeasy Total RNA System (Qiagen, Hilden, Germany) and ReverTra Ace (Toyobo, Osaka, Japan). First, to examine the presence of target molecules and the validity of the newly designed primers, conventional PCR was performed. Specific primers for IL-32, caspase 1, TLR3, and glyceraldehyde 3 phosphate dehydrogenase (GAPDH, positive control) were designed: IL-32 forward, 5’-AGCTGGAGGACGACTTCAAA-3’, and reverse, 5’-TTGAGGATTGGGGTTCAGAG-3’ (predicted size, 258bp); TLR3 forward, 5’-CCATTCCAGCCTCTTCGTAA-3’, and reverse, 5’-GGATGTTGGTATGGGTCTCG-3’ (predicted size, 505bp); caspase 1 forward, 5’-CCACAATGGGCTCTGTTTTT-3’, and reverse, 5’-CATCTGGCTGCTCAAATGAA-3’ (predicted size, 117); IL-1β, forward, 5'-CCAGGGACAGGATATGGAGCA-3' and reverse, 5'-TTCAACACGCAGGACAGGTACAG-3' (predicted size, 129bp); IL-6, forward,

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5'-AGTGAGGAACAAGCCAGAGC-3' and reverse, 5'-AAGCTGCGCAGAATGAGAT-3' (predicted size, 189bp); GAPDH, forward, 5'-GGCCTCCAAGGAGTAAGACC-3', and reverse, 5'-AGGGGTCTACATGGCAACTG-3' (predicted size, 147bp). The reaction profile consisted of initial denaturation at 94ºC for 3min followed by 25-40 cycles with 30sec of denaturation at 94ºC, 30sec of annealing of primers at 55ºC, and a 60sec extension at 72ºC. Next, to carry out relative quantification, real-time quantitative PCR was performed according to a standard protocol using the Brilliant II SYBR Green QPCR Reagents and Mx300P QPCR system (Stratagene Japan, Tokyo, Japan). Relative gene expression was calculated using the comparative cycle threshold method and adjusted based on the expression of house-keeping gene (GAPDH). Results were obtained from three independent experiments and shown as relative mRNA expression compared with the level without any treatments. Negative controls were obtained by replacing the reverse transcriptase or cDNA samples with RNase and DNase free water.

Western blotting

Cell lysates of poly(I:C)-stimulated or unstimulated cultured cell lines (10μg protein/lane) and the culture medium were subjected to SDS-PAGE. Recombinant IL32 protein (0.1μg, R&D) was used as a positive control. Separated proteins were transferred to a nitrocellulose membrane; the membrane was blocked in 5% bovine serum albumin, and then probed for 1h with a primary antibody against human IL-32 (0.1μg/ml). After a wash, the membrane was incubated for 1h with a Simple Staining Kit, and visualized with the benzidine reaction. The density of bands was quantitatively evaluated by using NIH images.

Statistical analysis

Data were analyzed using the paired *t*-test or Welch's *t*-test; $p<0.05$ was considered statistically significant.

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RESULTS

Expression of IL-32, caspase 1, and TLR3 in extrahepatic bile ducts of BA

Immunohistochemistry revealed the expression of IL-32 in BECs, infiltrating inflammatory cells, and endothelial cells at various intensities. In particular, damaged common bile ducts showing cholangitis in BA strongly expressed IL-32, accompanying many IL-32-positive inflammatory cells and vessels (Fig.1A, 1B, 1C). As shown in Fig.1F, the double immunohistochemistry highlighted that CK19-postive bile ducts clearly expressed IL-32. However, non-damaged biliary epithelium found at the margin of resected common bile ducts did not express IL-32 (Fig.1G and 1H). In wedge liver biopsies, hepatocytes were also positive for IL-32 in addition to small bile ducts (interlobular bile ducts), but the intensity was lower than that in damaged common bile ducts (Fig.1I and 1J). Moreover, congestive bile in intrahepatic bile ducts was also strongly positive for IL-32 (Fig.1J). In contrast, BECs in common bile ducts and intrahepatic bile ducts of age-matched controls, only weakly expressed or lacked IL-32 (Fig2A and 2D). The semi-quantitative analysis for immunoreaction confirmed the expression of IL-32 in damaged common bile ducts of BA was significantly upregulated, compared with those in non-damaged/normal bile ducts of BA and age-matched controls (Fig.3). Caspase 1 and TLR3 were constantly expressed in BECs of extrahepatic bile ducts, in both the BA and control patients (Fig.2B and 2C).

Induction of IL-32 expression by PAMPs and cytokines in cultured BECs

To examine the presence of target molecules and the validity of the newly designed primers, RT-PCR at 40 cycles was performed and an amplification of all molecules could be detected as a single band from cultured BECs at the expected size. Moreover, the BECs constantly expressed the mRNA of TLR3 and caspase 1, which is necessary for the recognition of poly(I:C) and the production of functional IL-32 protein, respectively. The real-time PCR analysis revealed that TLR3 ligand, poly(I:C) and proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α), but not regulatory cytokines (TGF-β1 and IL-10), enhanced the mRNA expression of IL-32, with the increases being

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statistically significant (Fig.4A). In contrast, the stimulation with IL-32 did not significantly upregulated the expression of BEC-producing cytokines (IL-1β, IL-6, and IL-32), TLR3, and caspase 1 in cultured BECs (Fig.4B). Although the control cell line, HepG2, also expressed IL-32 mRNA, the upregulation of IL-32 was not significant by the stimulation with poly(I:C) or IL-32 (Fig.4C).

Detection of intracytoplasmic and secreted IL-32protein

To investigate the secretion of the IL-32 protein, Western blotting was performed using the cell lysate and culture medium of BECs. IL-32 was detected in the medium as well as lysate from the poly(I:C)-stimulated BECs (Fig.5A). Semi-quantitative analysis using NIH image analysis revealed that the density of bands was upregulated in cell lysate and culture medium by the stimulation with poly(I:C) (Fig.5A). Moreover, immunocytochemistry also demonstrated that IL-32 protein was strongly expressed in poly(I:C)-stimulated BECs, compared with non-stimulated BECs (Fig.5B).

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DISCUSSION

BA is initially characterized by periductal inflammation and fibrosis, and the obstruction of common bile ducts, known as fibrosclerosing cholangitis. Recruitment of inflammatory cells results in the release of other proinflammatory cytokines and chemokines, sustaining the cholangitis associated with the biliary innate immune response and promoting the chronic cholangitis associated with the subsequent acquired immune response in a later phase [26]. IL-32 is a recently described cytokine that is a strong inducer of pro-inflammatory cytokines and whose expression is markedly increased in several inflammatory disorders including RA and IBD and correlated with the severity of these diseases [14, 19]. In the present study, human BECs were demonstrated to be the local

source of IL-32. Immunohistochemical analysis showed a cytoplasmic distribution of IL-32 in BECs of the damaged common bile ducts in the cases of BA, though BECs of common bile ducts in age-matched controls were negative or only weakly positive for IL-32, suggesting that IL-32 is closely associated with the histogenesis of periductal inflammation in BA. However, the event of IL-32 production in BECs is not be specific for only BA. In facts, we confirmed the expression of IL-32 in bile ducts of adult biliary diseases such as primary biliary cirrhosis, but its intensity was lower than those in the damaged common bile ducts of BA. Therefore, we speculated that the induction of IL-32 by unique factors such as viral infections in BA was stronger than those in other biliary diseases. Inflammasomes are multi-protein cytoplasmic complexes that mediate the activation of inflammatory caspase-1. For example, caspase-1 cleaves pro-IL-1β to the active form IL-1β. In this manner, caspase-1 controls the maturation of some of the pro-inflammatory cytokines and IL-32 also depended on the activation of caspase 1 [17, 20]. Therefore, the presence of caspase 1 is necessary for the functional expression of IL-32 in BECs. In the present study, BECs constantly expressed caspase 1 *in vitro* and *in vivo*, suggesting the expression of a functional IL-32 in BECs.

Recent studies have focused on the role of innate immunity associated with Reoviridae (reovirus and rotavirus) in the pathogenesis of BA. Reoviridae having a dsRNA genome, in particular, are characterized by epithelial tropism [1, 3, 4, 9, 10, 27, 28]. The initial sensing of innate immunity is mediated by the recognition of PAMPs through TLRs. IL-32 also appears to play an important role in host defense against invading micro-organisms [23, 29, 30]. That is, IL-32 is described as a pro-inflammatory cytokine that enhances host immunity against various microbial pathogens. The present study revealed that stimulation with poly(I:C), a mimic of Reoviridae, enhanced the expression of IL-32 in cultured BECs, suggesting that the biliary innate immune response directly induces the production of IL-32 in BECs. A control cell line used in this study, HepG2, also expressed IL-32 mRNA, but the upregulation of IL-32 was not significant by the stimulation with poly(I:C). It has already been reported that IL-32 expression is induced in peripheral blood mononuclear cells and monocytes by *Mycobacterium tuberculosis* [31], but to our knowledge this is the first description concerning the production of IL-32 in epithelial cells such as

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BECs via an innate immune response.

IL-1β, IFN-γ, and TNF-α were reported to be inducers of IL-32 expression [16, 19]. However, the regulatory mechanism of these pro-inflammatory cytokines remains unclear. In this study, we found that all these pro-inflammatory cytokines are potent stimulators of IL-32 expression in cultured BECs. In contrast, the aforementioned results suggest that the secretion of IL-32 could stimulate periductal inflammatory and/or immune cells to secrete proinflammatory cytokines and contributes to the deterioration of periductal inflammation. Because these inflammatory cytokines and an innate immunity play important roles in the immune-mediated histogenesis of BA, the inflammatory responses and innate immune response in the affected bile ducts of BA patients may be amplified by constant IL-32-induced secretion of proinflammatory cytokines from BECs and periductal inflammatory cells, suggesting that IL-32 plays a central role in the inflammatory responses involved in the pathogenesis of BA. However, IL-32 itself could not upregulate the expression of inflammatory cytokines (IL-1β, IL-6, and IL-32), TLR3, and caspase 1 in cultured BECs, suggesting that IL-32 produced by BECs was unlikely involved in direct reciprocal signaling resulting in upregulation of inflammatory cytokines and of susceptibility to virus in BECs.

In this study, we demonstrated that stimulation with poly(I:C) induced the transcription of IL-32 mRNA in BECs and also confirmed the presence of the protein in the culture medium as well as cell lysate. Moreover, immunohistochemistry also revealed that a condensed bile in intrahepatic small bile ducts was positive for IL-32. These findings suggest the secretion of IL-32 from IL-32-expressing BECs. Therefore, IL-32 is speculated to be extracellularly secreted in periductal tissue fluids and into bile in BA. As mentioned above, the secreted IL-32 induces the production of proinflammatory cytokines in inflammatory and/or immune cells, resulting in a marked amplification of inflammatory cytokine milieu, and these responses may contribute to the aggravation of BA. Moreover, it was recently suggested that IL-32 acts as a cytoplasmic protein: IL-32 was expressed at high levels in human epidermal keratinocytes after stimulation with IFN-γ and TNF-α, but was not secreted by keratinocytes [21]. Moreover, it was also shown that the upregulation of cytoplasmic IL-32 expression induces apoptosis [21, 32]. In IBD, the apoptosis of

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damaged colonic cells by accumulated intracellular IL-32 can be considered a host defense mechanism against invading microorganisms, by which damaged epithelial cells are eliminated efficiently along with invading microorganisms and further invasions of microorganisms can be blocked [19, 33]. In BA, our previous study found that biliary apoptosis was enhanced in the damaged common bile ducts and closely associated with bile duct loss in BA, which was caused by the production of an apoptosis-inducer, TRAIL, in BECs via the biliary innate immune response to a TLR3 ligand, poly(I:C) [10]. However, this TRAIL-mediated biliary apoptosis is only partially involved in the poly(I:C)-induced mechanism and other possible mechanisms also could exist [10]. Therefore, the IL-32-mediated mechanism is also likely in the poly(I:C)-induced biliary apoptosis and might be associated with the enhanced biliary apoptosis in the damaged common bile ducts of BA.

In conclusion, we have demonstrated that IL-32 expression is enhanced in the damaged common bile ducts of BA patients. Expression of IL-32 in BECs was induced by the innate immune response to dsRNA (poly(I:C)) and proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α). This study identified IL-32 as an important inflammatory cytokine involved in the cholangitis of BA. So far, anti-IL32 treatment has been studied in a few diseases such as rheumatoid arthritis [34, 35]. The regulation of IL-32 expression may form the basis of a new strategy for the treatment of BA.

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FIGURE LEGENDS

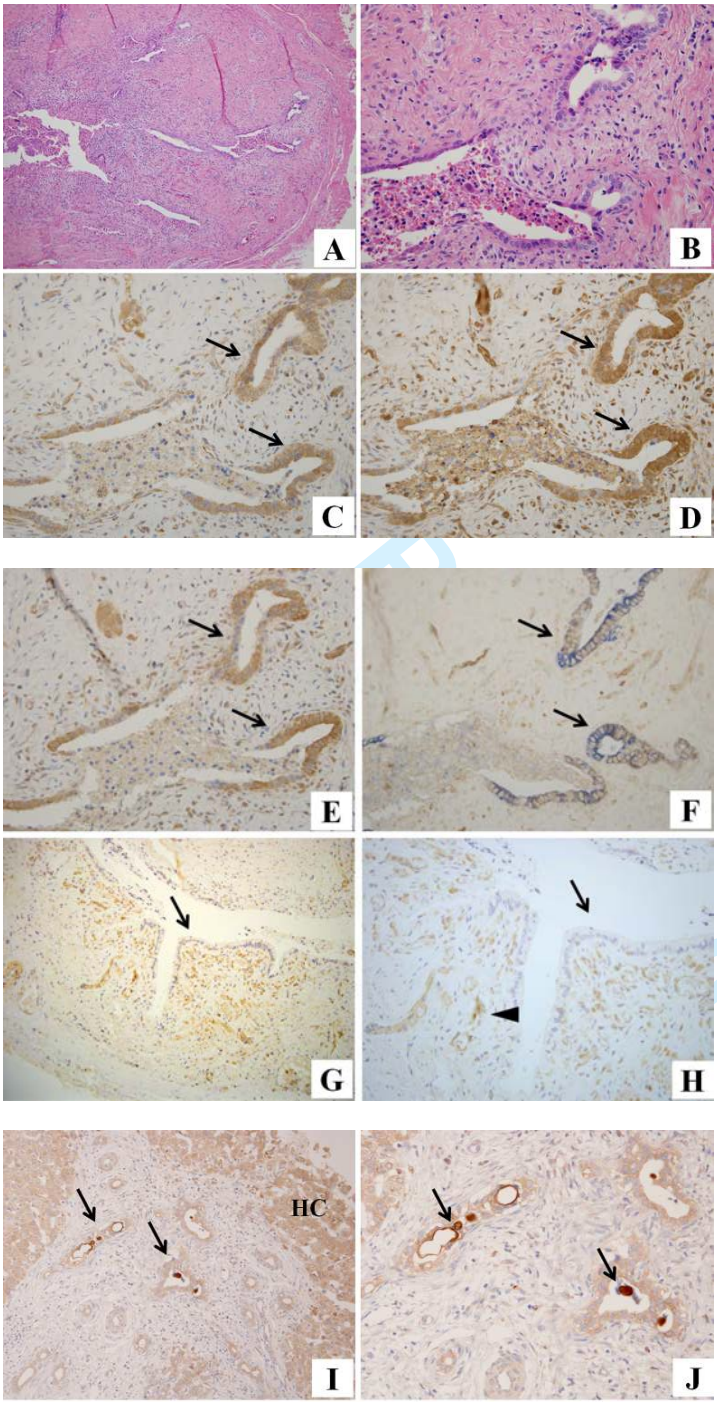

Fig.1 Histology and immunohistochemistry for IL-32, TLR3, and caspase 1 in biliary atresia (BA). A and B: Transverse sections of biliary remnants. Damaged extrahepatic bile ducts inconsistently line by desquamated columnar epithelium and surrounding fibroplasia with an

inflammatory cell infiltrate. B is a higher magnification of A. H&E staining. Original magnification, A, x100 and B, x400. C, D, and E: Immunohistochemistry for IL-32 (C), TLR3 (D), and caspase 1 (E). The strong expression of IL-32, TLR3, and caspase 1 was found in biliary epithelial cells (arrows) of damage bile ducts. Original magnification, x400. F: Double immunohistochemistry for CK19 and IL-32 highlighted the CK19-postive bile ducts (blue) clearly expressed IL-32 (brown) (arrows). Original magnification, x400. G and H: Immunohistochemistry for IL-32. Undamaged extrahepatic bile duct located at the resected margin in BA. IL-32-positive neovascular structures (arrowhead) were found, but undamaged biliary epithelium lacked IL-32 expression (arrows). H is higher magnification of G. Original magnification, G, x200 and H, x400. I and J: Immunohistochemistry for IL-32 using wedge liver specimens of BA. Interlobular bile ducts (arrows in I) and hepatocytes (HC in I) expressed IL-32. Moreover, condensed bile in dilated bile ducts was also strongly positive for IL-32 (arrows in J). J is a higher magnification of I. Original magnification, E, x200 and F, x400.

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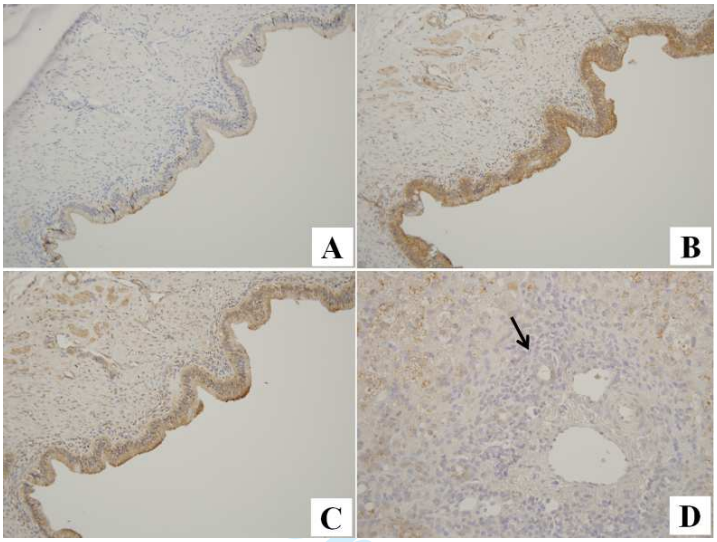

Fig.2 Immunohistochemistry for IL-32 (A and D), TLR3 (B), and caspase 1 (C) in age-matched controls. A, B, and C: Biliary epithelial cells in common bile ducts of non-hepatobiliary diseases (congenital heart anomalies) expressed TLR3 (B) and caspase 1 (C), but lack or faintly expressed IL-32 (A). was faint or negative. Original magnification, x200. D: Interlobular bile duct in neonatal hepatitis was negative for IL-32 (arrow). Original magnification, x400.

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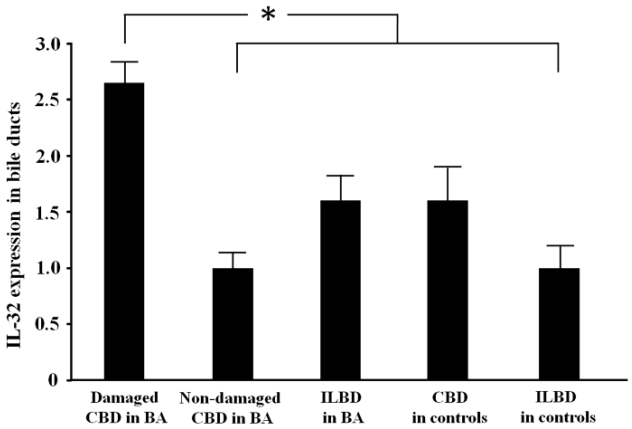

Fig.3

Fig.3 Semi-quantitative analysis of immunohistochemistry for IL-32. The expression of IL-32 in damaged common bile ducts (CBD) of biliary atresia (BA) was significantly upregulated, compared with those of non-damaged CBD and interlobular bile ducts (ILBD) in BA, and of CBD and ILBD in age-matched controls. *<0.05.

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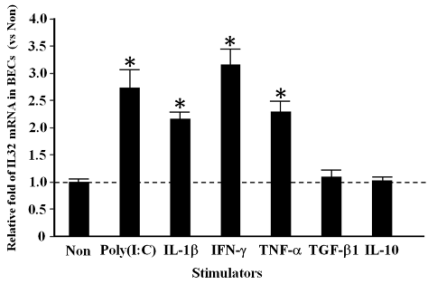

Fig.4A

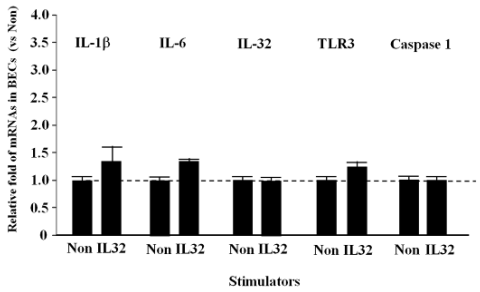

Fig.4B

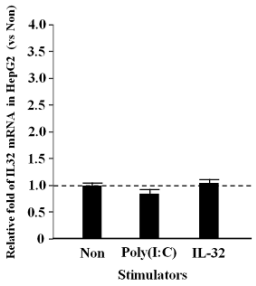

Fig.4C

Fig.4 A: Induction of IL-32 expression by TLR3 ligand (poly I:C) and cytokines in cultured biliary epithelial cells (BECs). Quantitative analysis using real-time PCR revealed that a TLR3 ligand, poly(I:C), and proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α), but not regulatory cytokines (TGF-β1 and IL-10), significantly upregulated the mRNA expression of IL-32. B: Detection of BEC-producing cytokines (IL-1β, IL-6, and IL-32), TLR3, and caspase 1 in cultured BECs. The stimulation with IL-32 did not significantly upregulated the

expression of any cytokines, TLR3, or caspase 1. C: Detection of IL-32 in a control cell line, HepG2. Induction of IL-32 expression was not found by the stimulation with poly(I:C) or IL-32. Results were obtained from three independent experiments and shown as relative mRNA expression compared with the level without any treatments (Non). Bars indicate the mean±S.E.M. *<0.05.

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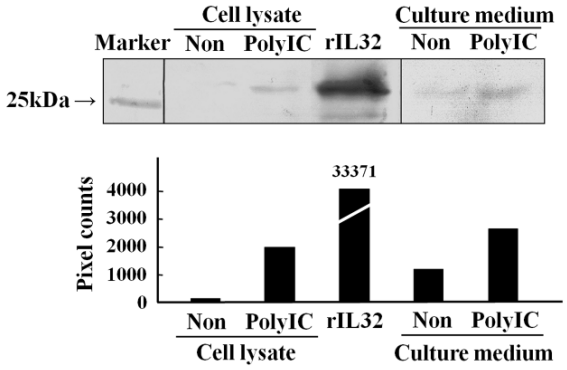

Fig.5A

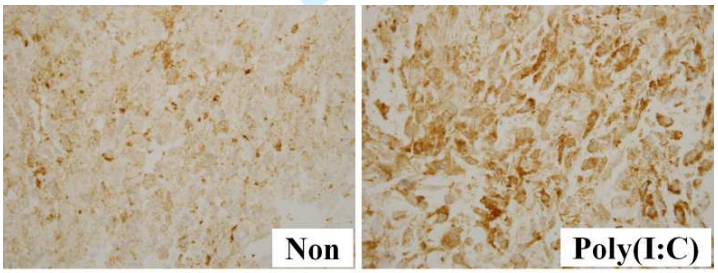

Fig.5B

Fig.5 Detection of intracytoplasmic and secreted IL-32 protein in cultured biliary epithelial cells (BECs). A: Western blotting revealed that the culture medium as well as cell lysate of poly(I:C)-treated cultured cells contained IL-32 protein, but the level was faint in untreated cells (Non). As a positive control, recombinant IL-32 (rIL32, 0.1μg) was used. Semi-quantitative analysis using NIH image analysis confirmed that the density of bands was upregulated in cell lysate and culture medium by the stimulation with poly(I:C). B: Immunocytochemistry also demonstrated that IL-32 was strongly expressed in the poly(I:C)-stimulated BECs, compared with the unstimulated BECs (Non). Original magnification, x400.

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Interleukin-32 production associated with biliary innate immunity and proinflammatory cytokines contributes to the pathogenesis of cholangitis in biliary atresia

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Contribution of the authors: Dr. Okamura* and Dr. Harada* contributed equally in this study, and Dr. Nakanuma and Dr. Nio were mainly involved in the concept of this study and preparation of the manuscript.

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ABSTRACT

Aims: Biliary atresia (BA) is thought to be associated with infections by viruses such as Reoviridae and is characterized histologically by fibrosclerosing cholangitis with proinflammatory cytokine-mediated inflammation. IL-32 affects the continuous inflammation by increasing the production of proinflammatory cytokines. In this study, the role of IL-32 in the cholangitis of BA was examined. **Methods**: Immunohistochemistry for IL-32 and caspase 1 was performed using 21 samples of extrahepatic bile ducts resected from BA patients. Moreover, using cultured human biliary epithelial cells (BECs), the expression of IL-32 and its induction on stimulation with a Toll-like receptor (TLR) 3 ligand (poly(I:C)) and proinflammatory cytokines was examined. **Results**: BECs composing extrahepatic bile ducts showing cholangitis expressed IL-32 in BA, but not in controls. Caspase 1 was constantly expressed on BECs of both BA and control subjects. Furthermore, poly(I:C) and proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α) strongly induced IL-32 expression in cultured BECs, accompanying the constant expression of TLR3 and caspase 1. **Conclusions**: Our results imply that the expression of IL-32 in BECs was found in the damaged bile ducts of BA and induced by biliary innate immunity via TLR3 and proinflammatory cytokines. These findings suggest that IL-32 is initially involved in the pathogenic mechanisms of cholangitis in BA and also plays an important role in the amplification and continuance of periductal inflammatory reactions. It is therefore tempting to speculate that inhibitors of IL-32 could be useful for attenuating cholangitis in BA.

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INTRODUCTION

The obliterative lesion of biliary atresia (BA) is characterized by a progressive sclerosing cholangitis accompanying severe inflammation, fibrosis, and epithelial injuries and this characteristic feature is known as fibrosclerosing cholangitis. Little is known about the etiology and pathogenesis of BA, but infections by viruses such as Reoviridae (reovirus and rotavirus) having a double-stranded RNA (dsRNA) have been implicated, though conflicting results also have been reported [1-8]. Our recent study has demonstrated that biliary epithelial cells (BECs) possess an innate immune system consisting of Toll-like receptors (TLR), especially TLR3 which is an innate immune-recognition receptor recognizing dsRNA including dsRNA viruses as pathogen-associated molecular patterns (PAMPs) [9, 10]. Furthermore, the biliary innate immune response to artificial dsRNA was also shown to be associated with the induction of biliary apoptosis via the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and, differing from the innate immune response to TLR4 ligand (LPS), lack of subsequent tolerance to dsRNA using cultured human biliary epithelial cells [9-11].

Interleukin (IL)-32 is a recently described cytokine produced by T lymphocytes, natural killer (NK) cells, monocytes and some epithelial cells [12, 13]. Primarily, IL-32 was discovered in the synovial fluid of patients with rheumatoid arthritis and first reported as a transcript in IL-2 activated NK and T cells [14, 15]. There are six isoforms (α, β, γ, δ, ε, and ξ) caused by alternative mRNA splicing, resulting in proteins with a molecular weight ranging from 14.9 to 26.7 kD. IL-32α is the most abundant transcript. IL-32 exhibits several properties typical of proinflammatory cytokines [16]. For example, it stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1α, TNF-α, IL-6, IL-8 and VEGF through the activation of nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinases (MAPKs) [15, 17, 18]. In contrast, the production of IL-32 is induced or enhanced by the presence of proinflammatory cytokines including IL-1β, IFN-γ, and TNF-α via the activation of caspase 1 [17, 19, 20]. IL-32 has been implicated in

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inflammatory disorders such as rheumatoid arthritis, inflammatory bowel diseases, chronic obstructive pulmonary diseases, atopic dermatitis, and allergic rhinitis [14, 19-22].

Although human hepatocytes and hepatoma cells express IL-32 in HCV-associated chronic hepatitis and the expression is regulated by proinflammatory stimuli [23], the pathophysiological role of IL-32 in innate immune-related biliary diseases including BA remains unclear. We therefore investigated the IL-32 expression in the inflamed bile ducts of BA patients and the effect of innate immune stimulation by ligands of TLR3 and cytokines on IL-32 expression in cultured human BECs. Our results provide evidence that biliary epithelial cells are sufficient sources of IL-32 for the biliary inflammation at sites of BA and IL-32 may therefore play a role in the pathophysiology of BA.

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MATERIALS AND METHODS

Patients and tissue preparations

A total of 21 patients with BA (surgical specimens; average age 1.7 months; range of age 0.7-12 months; male/female=9/12) and age-matched control patients consisting of one neonatal hepatitis (giant cell hepatitis; wedge biopsy; 3 months; male) and 6 non-hepatobiliary diseases (congenital heart anomalies; autopsied specimens; average age 2.5 months; male/female=3/3) were examined. Resected common bile ducts and wedge liver biopsy specimens obtained at Kasai procedure from the patients with BA were used. These specimens had been fixed in 10% neutral-buffered formalin and embedded in paraffin; 4μm-thick sections were prepared for histologic observation and immunohistochemistry.

Immunohistochemistry and immunocytochemistry

For the immunocytochemistry using cultured BECs, formalin-fixed, paraffin-embedded sections of cell blocks were prepared according to the protocol reported by Mayall, *et al* [24]. The deparaffinized and rehydrated sections were heated in 10mM citrate buffer for 20min in a microwave oven. Following the blocking of endogenous peroxidase, these sections were incubated at

4ºC overnight with antibody against the C-terminus of IL-32 (rabbit polyclonal IgG, 1μg/ml, Lifespan, Seattle, WA, USA), TLR3 (rabbit polyclonal IgG, 1μg/ml, Santa Cruz, Santa Cruz, CA, USA), and caspase 1 (rabbit monoclonal IgG, diluted 1:1,000, Abcam, Tokyo, Japan) and then at room temperature for 1h with anti-rabbit immunoglobulins conjugated to a peroxidase-labeled dextran polymer (Simple staining kit, Nichirei, Tokyo, Japan). After a benzidine reaction, sections were lightly counterstained with hematoxylin. As a negative control, normal rabbit IgG was used as the primary antibody: no staining was obtained.

For the semi-quantitative evaluation of the immunohistochemistry, intrahepatic bile ducts and extrahepatic common bile ducts were chosen in each section for assessment and IL-32 immunoreactivity in these bile ducts was semiquantitatively graded as follows: score 0, absence of expression; score 1, low constitutive expression; score 2, intermediate expression; score 3, high expression.

In addition, simultaneous detection of IL-32 and cytokeratin (CK)19 was done using double immunohistochemical staining. After IL-32 immunostaining, CK19 antibody (mouse monoclonal IgG1kappa, 0.45μg/ml, Dako Japan, Tokyo, Japan) was applied overnight at 4ºC, followed by immunoglobulins conjugated to alkaline phosphatase labeled-dextran polymer (Nichirei). Color development of IL-32 and CK19 was achieved with diaminobenzidine (brown) and Vector blue (Vector Lab, Burlingame, CA, USA.), respectively.

Cultured human BECs and stimulation with PAMPs and proinflammatory cytokines

A cultured cell line of human intrahepatic BECs was established from the explant liver of a 24-year-old male with BA who had already received Kasai procedure during the newborn period, and cultured as previously reported [25] The cultured BECs were incubated with a culture medium composed of D-MEM/F-12 (Invitrogen, Tokyo, Japan), 5% newborn calf serum (Invitrogen), 0.18mM adenine (Sigma, Saint Louis, MO, USA), hydrocortisone (0.4μg/ml), cholera toxin (10ng/ml), tri-iodo-thyronine (1.3μg/l), ITS+ (Becton Dickinson, Franklin Lakes, NJ, USA), 25mM sodium bicarbonate (Sigma), 1% antibiotics antimycotic, human epidermal growth factor(20ng/ml)

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(Invitrogen), and human hepatocyte growth factor(10ng/ml) (Invitrogen). The cells were grown as monolayers in a humidified incubator with 5% CO_2 at 37°C. More than 95% of the cells were confirmed to be biliary epithelial cells by the expression of a biliary-type cytokeratin (CK19). The cultured BECs were used between passages 4 and 9. Informed consent for human research was obtained from the patient prior to surgery. This study was approved by the Kanazawa University Ethics Committee. Moreover, as control cultured cells, a commercially available cell line derived from human hepatocellular carcinoma, HepG2, was obtained from Health Science Research Resources Bank (Osaka, Japan).

These cultured cells were stimulated with a TLR3 ligand, polyinosinic-polycytidylic acid (poly(I:C), a synthetic analogue of viral dsRNA, 25μg/ml, Invivogen, San Diego, CA, USA) and recombinant cytokines (IL-1β, IFN-γ, TNF-α, TGF-β1, and IL-10, l,000U/ml, PeproTech, London, and IL-32, 1,000U/ml, R&D system, Minneapolis, MN, USA) for 3 hrs (molecular analysis) and 48 hrs (protein analysis by immunocytochemistry and Western blotting analysis).

Isolation of RNA, reverse transcription-polymerase chain reaction (RT-PCR), and real-time PCR

For the evaluation of mRNA of IL-32, caspase 1, TLR3, IL-1β, and IL-6, in cultured BECs, isolation of RNA from BECs and reverse transcription were performed using the RNeasy Total RNA System (Qiagen, Hilden, Germany) and ReverTra Ace (Toyobo, Osaka, Japan). First, to examine the presence of target molecules and the validity of the newly designed primers, conventional PCR was performed. Specific primers for IL-32, caspase 1, TLR3, and glyceraldehyde 3 phosphate dehydrogenase (GAPDH, positive control) were designed: IL-32 forward, 5’-AGCTGGAGGACGACTTCAAA-3’, and reverse, 5’-TTGAGGATTGGGGTTCAGAG-3’ (predicted size, 258bp); TLR3 forward, 5’-CCATTCCAGCCTCTTCGTAA-3’, and reverse, 5’-GGATGTTGGTATGGGTCTCG-3’ (predicted size, 505bp); caspase 1 forward, 5’-CCACAATGGGCTCTGTTTTT-3’, and reverse, 5’-CATCTGGCTGCTCAAATGAA-3’ (predicted size, 117); IL-1β, forward, 5'-CCAGGGACAGGATATGGAGCA-3' and reverse, 5'-TTCAACACGCAGGACAGGTACAG-3' (predicted size, 129bp); IL-6, forward,

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5'-AGTGAGGAACAAGCCAGAGC-3' and reverse, 5'-AAGCTGCGCAGAATGAGAT-3' (predicted size, 189bp); GAPDH, forward, 5'-GGCCTCCAAGGAGTAAGACC-3', and reverse, 5'-AGGGGTCTACATGGCAACTG-3' (predicted size, 147bp). The reaction profile consisted of initial denaturation at 94ºC for 3min followed by 25-40 cycles with 30sec of denaturation at 94ºC, 30sec of annealing of primers at 55ºC, and a 60sec extension at 72ºC. Next, to carry out relative quantification, real-time quantitative PCR was performed according to a standard protocol using the Brilliant II SYBR Green QPCR Reagents and Mx300P QPCR system (Stratagene Japan, Tokyo, Japan). Relative gene expression was calculated using the comparative cycle threshold method and adjusted based on the expression of house-keeping gene (GAPDH). Results were obtained from three independent experiments and shown as relative mRNA expression compared with the level without any treatments. Negative controls were obtained by replacing the reverse transcriptase or cDNA samples with RNase and DNase free water.

Western blotting

Cell lysates of poly(I:C)-stimulated or unstimulated cultured cell lines (10μg protein/lane) and the culture medium were subjected to SDS-PAGE. Recombinant IL32 protein (0.1μg, R&D) was used as a positive control. Separated proteins were transferred to a nitrocellulose membrane; the membrane was blocked in 5% bovine serum albumin, and then probed for 1h with a primary antibody against human IL-32 (0.1μg/ml). After a wash, the membrane was incubated for 1h with a Simple Staining Kit, and visualized with the benzidine reaction. The density of bands was quantitatively evaluated by using NIH images.

Statistical analysis

Data were analyzed using the paired *t*-test or Welch's *t*-test; $p<0.05$ was considered statistically significant.

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RESULTS

Expression of IL-32, caspase 1, and TLR3 in extrahepatic bile ducts of BA

Immunohistochemistry revealed the expression of IL-32 in BECs, infiltrating inflammatory cells, and endothelial cells at various intensities. In particular, damaged common bile ducts showing cholangitis in BA strongly expressed IL-32, accompanying many IL-32-positive inflammatory cells and vessels (Fig.1A, 1B, 1C). As shown in Fig.1F, the double immunohistochemistry highlighted that CK19-postive bile ducts clearly expressed IL-32. However, non-damaged biliary epithelium found at the margin of resected common bile ducts did not express IL-32 (Fig.1G and 1H). In wedge liver biopsies, hepatocytes were also positive for IL-32 in addition to small bile ducts (interlobular bile ducts), but the intensity was lower than that in damaged common bile ducts (Fig.1I and 1J). Moreover, congestive bile in intrahepatic bile ducts was also strongly positive for IL-32 (Fig.1J). In contrast, BECs in common bile ducts and intrahepatic bile ducts of age-matched controls, only weakly expressed or lacked IL-32 (Fig2A and 2D). The semi-quantitative analysis for immunoreaction confirmed the expression of IL-32 in damaged common bile ducts of BA was significantly upregulated, compared with those in non-damaged/normal bile ducts of BA and age-matched controls (Fig.3). Caspase 1 and TLR3 were constantly expressed in BECs of extrahepatic bile ducts, in both the BA and control patients (Fig.2B and 2C).

Induction of IL-32 expression by PAMPs and cytokines in cultured BECs

To examine the presence of target molecules and the validity of the newly designed primers, RT-PCR at 40 cycles was performed and an amplification of all molecules could be detected as a single band from cultured BECs at the expected size. Moreover, the BECs constantly expressed the mRNA of TLR3 and caspase 1, which is necessary for the recognition of poly(I:C) and the production of functional IL-32 protein, respectively. The real-time PCR analysis revealed that TLR3 ligand, poly(I:C) and proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α), but not regulatory cytokines (TGF-β1 and IL-10), enhanced the mRNA expression of IL-32, with the increases being

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statistically significant (Fig.4A). In contrast, the stimulation with IL-32 did not significantly upregulated the expression of BEC-producing cytokines (IL-1β, IL-6, and IL-32), TLR3, and caspase 1 in cultured BECs (Fig.4B). Although the control cell line, HepG2, also expressed IL-32 mRNA, the upregulation of IL-32 was not significant by the stimulation with poly(I:C) or IL-32 (Fig.4C).

Detection of intracytoplasmic and secreted IL-32protein

To investigate the secretion of the IL-32 protein, Western blotting was performed using the cell lysate and culture medium of BECs. IL-32 was detected in the medium as well as lysate from the poly(I:C)-stimulated BECs (Fig.5A). Semi-quantitative analysis using NIH image analysis revealed that the density of bands was upregulated in cell lysate and culture medium by the stimulation with poly(I:C) (Fig.5A). Moreover, immunocytochemistry also demonstrated that IL-32 protein was strongly expressed in poly(I:C)-stimulated BECs, compared with non-stimulated BECs (Fig.5B).

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DISCUSSION

BA is initially characterized by periductal inflammation and fibrosis, and the obstruction of common bile ducts, known as fibrosclerosing cholangitis. Recruitment of inflammatory cells results in the release of other proinflammatory cytokines and chemokines, sustaining the cholangitis associated with the biliary innate immune response and promoting the chronic cholangitis associated with the subsequent acquired immune response in a later phase [26]. IL-32 is a recently described cytokine that is a strong inducer of pro-inflammatory cytokines and whose expression is markedly increased in several inflammatory disorders including RA and IBD and correlated with the severity of these diseases [14, 19]. In the present study, human BECs were demonstrated to be the local

source of IL-32. Immunohistochemical analysis showed a cytoplasmic distribution of IL-32 in BECs of the damaged common bile ducts in the cases of BA, though BECs of common bile ducts in age-matched controls were negative or only weakly positive for IL-32, suggesting that IL-32 is closely associated with the histogenesis of periductal inflammation in BA. However, the event of IL-32 production in BECs is not be specific for only BA. In facts, we confirmed the expression of IL-32 in bile ducts of adult biliary diseases such as primary biliary cirrhosis, but its intensity was lower than those in the damaged common bile ducts of BA. Therefore, we speculated that the induction of IL-32 by unique factors such as viral infections in BA was stronger than those in other biliary diseases. Inflammasomes are multi-protein cytoplasmic complexes that mediate the activation of inflammatory caspase-1. For example, caspase-1 cleaves pro-IL-1β to the active form IL-1β. In this manner, caspase-1 controls the maturation of some of the pro-inflammatory cytokines and IL-32 also depended on the activation of caspase 1 [17, 20]. Therefore, the presence of caspase 1 is necessary for the functional expression of IL-32 in BECs. In the present study, BECs constantly expressed caspase 1 *in vitro* and *in vivo*, suggesting the expression of a functional IL-32 in BECs.

Recent studies have focused on the role of innate immunity associated with Reoviridae (reovirus and rotavirus) in the pathogenesis of BA. Reoviridae having a dsRNA genome, in particular, are characterized by epithelial tropism [1, 3, 4, 9, 10, 27, 28]. The initial sensing of innate immunity is mediated by the recognition of PAMPs through TLRs. IL-32 also appears to play an important role in host defense against invading micro-organisms [23, 29, 30]. That is, IL-32 is described as a pro-inflammatory cytokine that enhances host immunity against various microbial pathogens. The present study revealed that stimulation with poly(I:C), a mimic of Reoviridae, enhanced the expression of IL-32 in cultured BECs, suggesting that the biliary innate immune response directly induces the production of IL-32 in BECs. A control cell line used in this study, HepG2, also expressed IL-32 mRNA, but the upregulation of IL-32 was not significant by the stimulation with poly(I:C). It has already been reported that IL-32 expression is induced in peripheral blood mononuclear cells and monocytes by *Mycobacterium tuberculosis* [31], but to our knowledge this is the first description concerning the production of IL-32 in epithelial cells such as

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BECs via an innate immune response.

IL-1β, IFN-γ, and TNF-α were reported to be inducers of IL-32 expression [16, 19]. However, the regulatory mechanism of these pro-inflammatory cytokines remains unclear. In this study, we found that all these pro-inflammatory cytokines are potent stimulators of IL-32 expression in cultured BECs. In contrast, the aforementioned results suggest that the secretion of IL-32 could stimulate periductal inflammatory and/or immune cells to secrete proinflammatory cytokines and contributes to the deterioration of periductal inflammation. Because these inflammatory cytokines and an innate immunity play important roles in the immune-mediated histogenesis of BA, the inflammatory responses and innate immune response in the affected bile ducts of BA patients may be amplified by constant IL-32-induced secretion of proinflammatory cytokines from BECs and periductal inflammatory cells, suggesting that IL-32 plays a central role in the inflammatory responses involved in the pathogenesis of BA. However, IL-32 itself could not upregulate the expression of inflammatory cytokines (IL-1β, IL-6, and IL-32), TLR3, and caspase 1 in cultured BECs, suggesting that IL-32 produced by BECs was unlikely involved in direct reciprocal signaling resulting in upregulation of inflammatory cytokines and of susceptibility to virus in BECs.

In this study, we demonstrated that stimulation with poly(I:C) induced the transcription of IL-32 mRNA in BECs and also confirmed the presence of the protein in the culture medium as well as cell lysate. Moreover, immunohistochemistry also revealed that a condensed bile in intrahepatic small bile ducts was positive for IL-32. These findings suggest the secretion of IL-32 from IL-32-expressing BECs. Therefore, IL-32 is speculated to be extracellularly secreted in periductal tissue fluids and into bile in BA. As mentioned above, the secreted IL-32 induces the production of proinflammatory cytokines in inflammatory and/or immune cells, resulting in a marked amplification of inflammatory cytokine milieu, and these responses may contribute to the aggravation of BA. Moreover, it was recently suggested that IL-32 acts as a cytoplasmic protein: IL-32 was expressed at high levels in human epidermal keratinocytes after stimulation with IFN-γ and TNF-α, but was not secreted by keratinocytes [21]. Moreover, it was also shown that the upregulation of cytoplasmic IL-32 expression induces apoptosis [21, 32]. In IBD, the apoptosis of

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damaged colonic cells by accumulated intracellular IL-32 can be considered a host defense mechanism against invading microorganisms, by which damaged epithelial cells are eliminated efficiently along with invading microorganisms and further invasions of microorganisms can be blocked [19, 33]. In BA, our previous study found that biliary apoptosis was enhanced in the damaged common bile ducts and closely associated with bile duct loss in BA, which was caused by the production of an apoptosis-inducer, TRAIL, in BECs via the biliary innate immune response to a TLR3 ligand, poly(I:C) [10]. However, this TRAIL-mediated biliary apoptosis is only partially involved in the poly(I:C)-induced mechanism and other possible mechanisms also could exist [10]. Therefore, the IL-32-mediated mechanism is also likely in the poly(I:C)-induced biliary apoptosis and might be associated with the enhanced biliary apoptosis in the damaged common bile ducts of BA.

In conclusion, we have demonstrated that IL-32 expression is enhanced in the damaged common bile ducts of BA patients. Expression of IL-32 in BECs was induced by the innate immune response to dsRNA (poly(I:C)) and proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α). This study identified IL-32 as an important inflammatory cytokine involved in the cholangitis of BA. So far, anti-IL32 treatment has been studied in a few diseases such as rheumatoid arthritis [34, 35]. The regulation of IL-32 expression may form the basis of a new strategy for the treatment of BA.

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FIGURE LEGENDS

Fig.1 Histology and immunohistochemistry for IL-32, TLR3, and caspase 1 in biliary atresia (BA). A and B: Transverse sections of biliary remnants. Damaged extrahepatic bile ducts inconsistently line by desquamated columnar epithelium and surrounding fibroplasia with an inflammatory cell infiltrate. B is a higher magnification of A. H&E staining. Original magnification, A, x100 and B, x400. C, D, and E: Immunohistochemistry for IL-32 (C), TLR3 (D), and caspase 1 (E). The strong expression of IL-32, TLR3, and caspase 1 was found in biliary epithelial cells (arrows) of damage bile ducts. Original magnification, x400. F: Double immunohistochemistry for CK19 and IL-32 highlighted the CK19-postive bile ducts (blue) clearly expressed IL-32 (brown) (arrows). Original magnification, x400. G and H: Immunohistochemistry for IL-32. Undamaged extrahepatic bile duct located at the resected margin in BA. IL-32-positive neovascular structures (arrowhead) were found, but undamaged biliary epithelium lacked IL-32 expression (arrows). H is higher magnification of G. Original magnification, G, x200 and H, x400. I and J: Immunohistochemistry for IL-32 using wedge liver specimens of BA. Interlobular bile ducts (arrows in I) and hepatocytes (HC in I) expressed IL-32. Moreover, condensed bile in dilated bile ducts was also strongly positive for IL-32 (arrows in J). J is a higher magnification of I. Original magnification, E, x200 and F, x400.

Fig.2 Immunohistochemistry for IL-32 (A and D), TLR3 (B), and caspase 1 (C) in age-matched controls. A, B, and C: Biliary epithelial cells in common bile ducts of non-hepatobiliary diseases (congenital heart anomalies) expressed TLR3 (B) and caspase 1 (C), but lack or faintly expressed IL-32 (A). was faint or negative. Original magnification, x200. D: Interlobular bile duct in neonatal hepatitis was negative for IL-32 (arrow). Original magnification, x400.

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Fig.3 Semi-quantitative analysis of immunohistochemistry for IL-32. The expression of IL-32 in damaged common bile ducts (CBD) of biliary atresia (BA) was significantly upregulated, compared with those of non-damaged CBD and interlobular bile ducts (ILBD) in BA, and of CBD and ILBD in age-matched controls. *<0.05.

Fig.4 A: Induction of IL-32 expression by TLR3 ligand (poly I:C) and cytokines in cultured biliary epithelial cells (BECs). Quantitative analysis using real-time PCR revealed that a TLR3 ligand, poly(I:C), and proinflammatory cytokines (IL-1β, IFN-γ, and TNF-α), but not regulatory cytokines (TGF-β1 and IL-10), significantly upregulated the mRNA expression of IL-32. B: Detection of BEC-producing cytokines (IL-1β, IL-6, and IL-32), TLR3, and caspase 1 in cultured BECs. The stimulation with IL-32 did not significantly upregulated the expression of any cytokines, TLR3, or caspase 1. C: Detection of IL-32 in a control cell line, HepG2. Induction of IL-32 expression was not found by the stimulation with poly(I:C) or IL-32. Results were obtained from three independent experiments and shown as relative mRNA expression compared with the level without any treatments (Non). Bars indicate the mean±S.E.M. *<0.05.

Fig.5 Detection of intracytoplasmic and secreted IL-32 protein in cultured biliary epithelial cells (BECs). A: Western blotting revealed that the culture medium as well as cell lysate of poly(I:C)-treated cultured cells contained IL-32 protein, but the level was faint in untreated cells (Non). As a positive control, recombinant IL-32 (rIL32, 0.1μg) was used. Semi-quantitative analysis using NIH image analysis confirmed that the density of bands was upregulated in cell lysate and culture medium by the stimulation with poly(I:C). B: Immunocytochemistry also demonstrated that IL-32 was strongly expressed in the poly(I:C)-stimulated BECs, compared with the unstimulated BECs (Non). Original magnification, x400.

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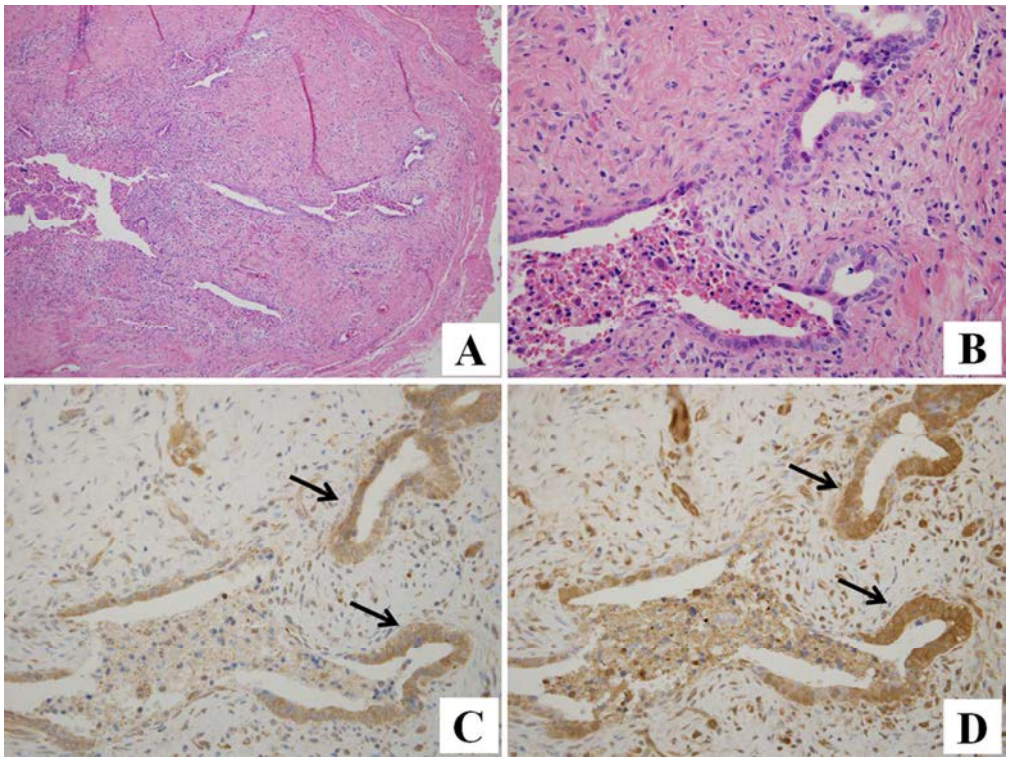

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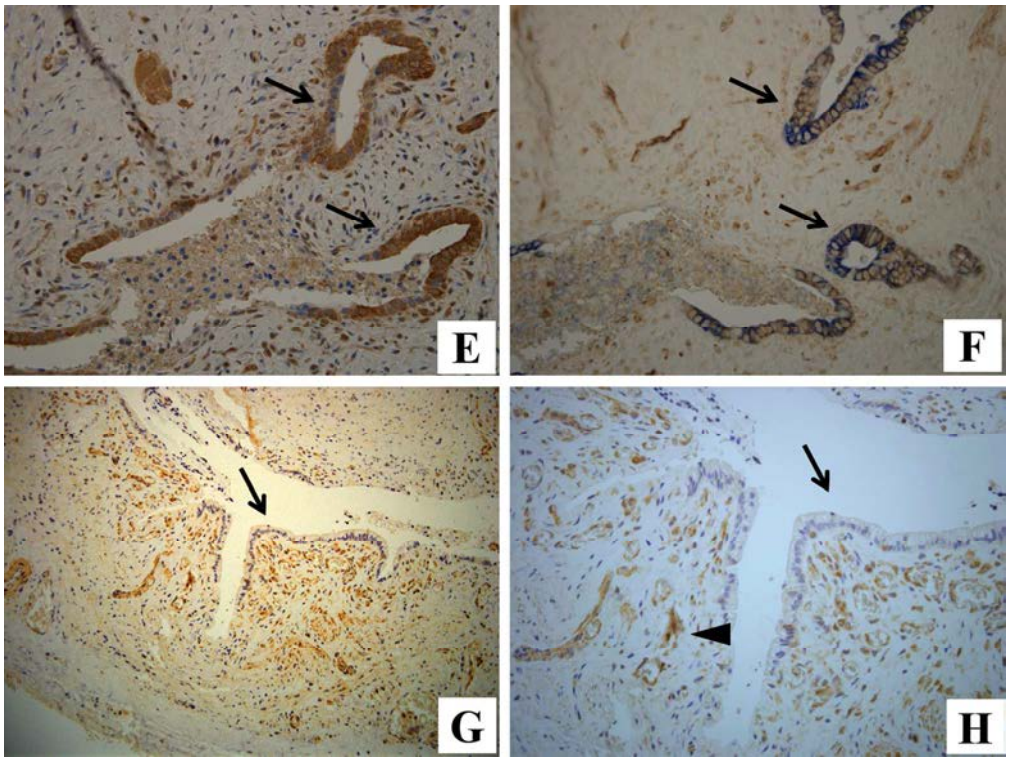

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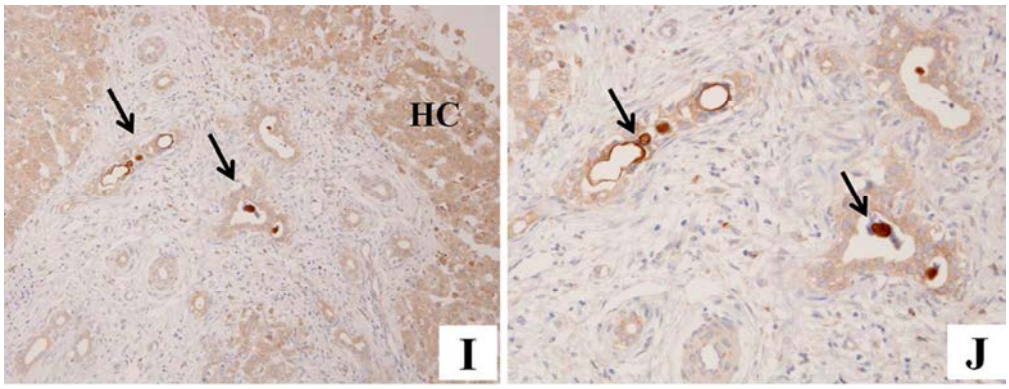

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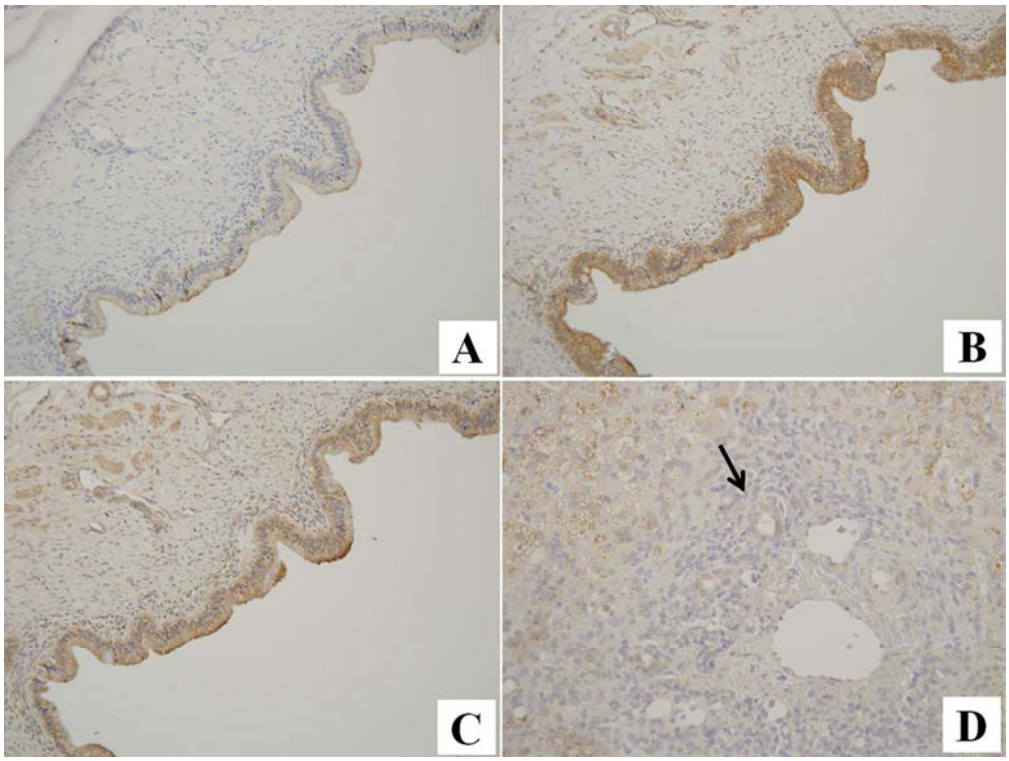

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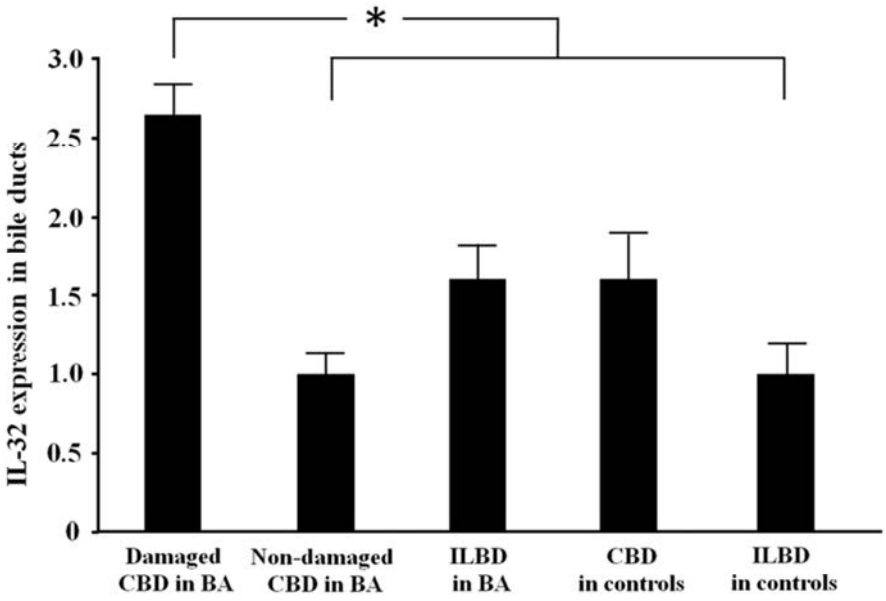

Fig.3

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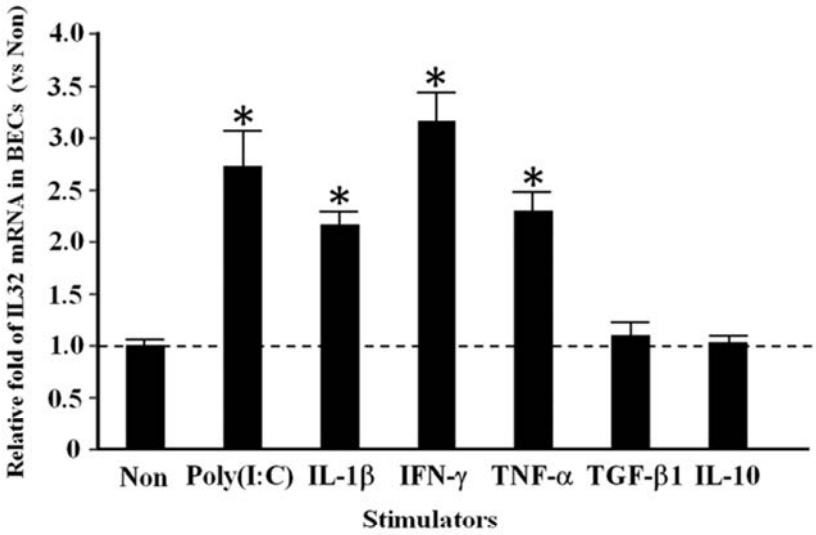

Fig.4A

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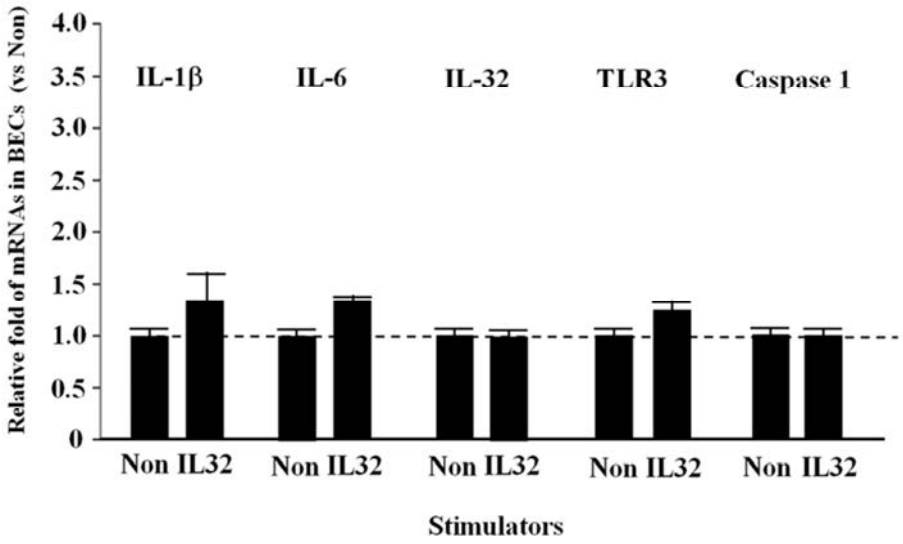

Fig.4B

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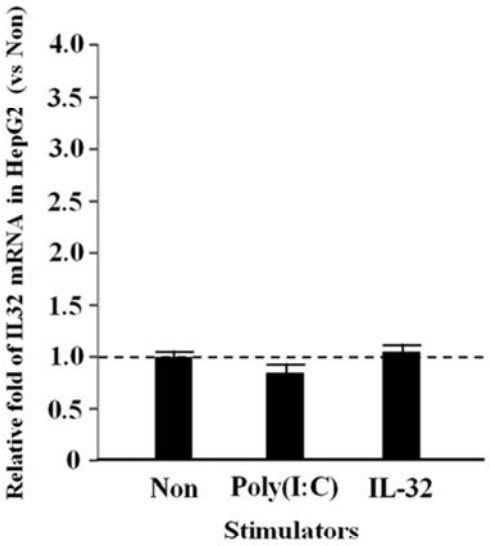

Fig.4C

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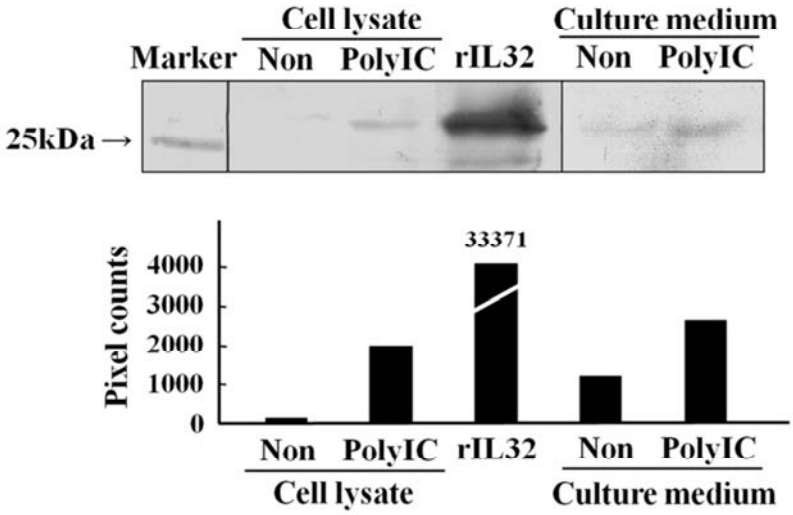

Fig.5A

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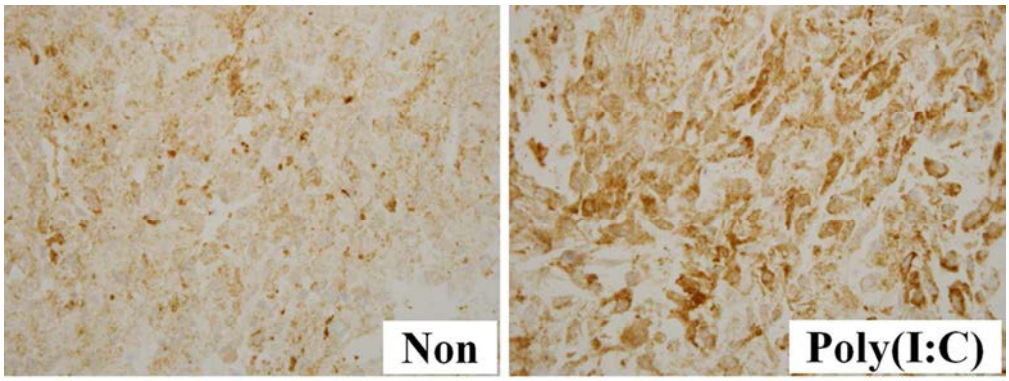

Fig.5B

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