CD8+ T lymphocyte response against extrahepatic biliary epithelium is activated by epitopes within NSP4 in experimental biliary atresia

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Zheng S, Zhang H, Zhang X, Peng F, Chen X, Yang J, Brigstock D, Feng J. CD8+ T lymphocyte response against extrahepatic biliary epithelium is activated by epitopes within NSP4 in experimental biliary atresia. Am J Physiol Gastrointest Liver Physiol 307: G233–G240, 2014. First published May 29, 2014; doi:10.1152/ajpgi.00099.2014.—Interferon (IFN)-γ-driven and CD8+ T cell-dependent inflammatory injury to extrahepatic biliary epithelium (EHBE) is likely to be involved in the development of biliary atresia (BA). We previously showed that viral protein NSP4 is the pathogenic immunogen that causes biliary injury in BA. In this study, NSP4 or four synthetic NSP4 (NSP4157–170, NSP4144–152, NSP493–110, NSP424–32) identified by computer analysis as candidate CD8+ T cell epitopes were injected into neonatal mice. The pathogenic NSP4 epitopes were confirmed by studying extrahepatic bile duct injury, IFN-γ release and CD8+ T cell response against EHBE. The results revealed, at 7 days postinjection, inoculation of glutathione S-transferase (GST)-NSP4 caused EHBE injury and BA in neonatal mice. At 7 or 14 days postinjection, inoculation of GST-NSP4, NSP4144–152, or NSP4157–170 increased IFN-γ release by CD8+ T cells, elevated the population of hepatic memory CD8+ T cells, and augmented cytotoxicity of CD8+ T cells to rhesus rotavirus (RRV)-infected or naive EHBE cells. Furthermore, depletion of CD8+ T cells in mice abrogated the elevation of GST-NSP4-induced serum IFN-γ. Lastly, parenteral immunization of mouse dams with GST-NSP4, NSP4144–152, or NSP4157–170 decreased the incidence of RRV-induced BA in their offspring. Overall, this study reports the CD8+ T cell response against EHBE is activated by epitopes within rotavirus NSP4 in experimental BA. Neonatal passive immunization by maternal vaccination against NSP4144–152 or NSP4157–170 is effective in protecting neonates from developing RRV-related BA.

BILIARY ATRESIA (BA), the most common cause of chronic hepatic disease in children, is characterized as a progressive destruction of extrahepatic bile ducts that leads to impaired bile flow during the first few months of life (25). The prevalence of this disease is ~1 in 5,000–8,000 children (11), and it is the cause of >50% of all pediatric liver transplants (1). Although the etiology of BA is not well understood, viral infection or chronic inflammatory or autoimmune-mediated bile duct injury may be involved, because the pathogenesis of BA entails a progressive inflammatory injury of bile ducts (17, 24, 25).

Multiple lines of evidence suggest that viral agents are associated with BA in humans, although no definite conclusion has been made (9, 13, 27). A murine model of experimental BA, established by peritoneal injection of neonatal BALB/c mice with rhesus rotavirus (RRV), leads to progressive inflammation and obstruction of the extrahepatic bile duct (21). The ability of RRV to target murine cholangiocytes and induce a tissue-specific inflammatory injury indicates that infectious agents may target neonatal cholangiocytes and trigger an undesired inflammatory response that results in occlusion of extrahepatic bile ducts (7, 13). Because the RRV-induced BA model in mice (mrvBA) is highly reproducible and commonly used, it is anticipated that additional investigations with this model will help to establish central mechanisms of biliary epithelial injury.

The RRV genome consists of 11 segments of dsRNA encoding 12 proteins, six of which are nonstructural proteins (NSP1-NSP6) and the other six of which are structural proteins (VP1-VP4, VP6, and VP7) (2, 20). We previously reported that silencing of NSP4 inhibited rotavirus replication and decreased incidence of mrvBA (10). The results suggested that NSP4 may act as a pathogenic immunogen in mrvBA. However, to date, no studies regarding the mechanism by which NSP4 triggers a host inflammatory response have been reported. Recent studies have shown that inactivation of interferon (IFN)-γ or targeted depletion of CD8+ T cells suppressed duct injury, prevented luminal obstruction, and restored bile flow in mice models of experimental BA, revealing key roles for IFN-γ and CD8+ T cells in the development of BA (23, 24).

These findings suggest that after RRV insult to the biliary tree, NSP4 may act as an important immunogen that elicits IFN-γ production and/or CD8+ T cell-mediated inflammation directed at bile duct epithelium. To prove this hypothesis, we injected mice with synthetic peptides corresponding to predicted CD8+ T cell epitopes within the NSP4 protein. Candidate epitopes were screened by interrogating biliary epithelial injury, IFN-γ production, and cytotoxicity of CD8+ T cells against cholangiocytes. We also explored the potential application of passive maternal vaccination against pathogenic epitopes for prevention of RRV-related BA in neonatal mice.

METHODS

Animals. Ten- to twelve-week-old BALB/C, C57BL/6, and C57BL/10 mice were provided by the Experimental Animal Center of Tongji Medical College (Wuhan, People’s Republic of China). These specific pathogen free (SPF) animals were housed routinely in cages, under light and dark schedules (12:12-h) and had free access to both

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food and water. Animals were housed at a 1:2 male-to-female mating ratio, and newborn mice were used for experiments. All of the studies were carried out in accordance with the Communities Council Directive for care of laboratory animals in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility following approval of study design (Permit Number 2009-AR0288) by the Institutional Animal Care and Use of the Committee at Tongji Medical College.

**Virus preparation.** RRV strain mmu18006 was obtained from Dr. Greg M. Tiao (Pediatric Surgery Division and Liver Care Center, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH) (4). RRV was propagated in MA104 rhesus monkey kidney epithelial cells (provided by Dr. Yuanhong Wang, Center of Disease Control, Wuhan, People’s Republic of China) in DMEM (GIBCO, Grand Island, NY) with trypsin (3 mg/ml, Sigma, St. Louis, MO), 1% penicillin, and 1% streptomycin. When cytopathic effect reached 80%, RRV was harvested and digested by Smal and Xhol restriction enzymes and the digested products were separated and detected by gel electrophoresis. Lane M, DNA marker; lane 1, positive control DNA (496 bp); lane 2, NSP4 gene fragment (525 bp). B: after inserting the NSP4 gene fragment into pGEX-4T-1 vector, the recombinant plasmid was double digested by Smal and Xhol restriction enzymes and the digested products were separated and detected by gel electrophoresis. Lane M, DNA marker; lane 1, undigested pGEX-4T-1 vector; lane 2, digested products of pGEX-4T-1 containing NSP4 gene fragment. C: Western blot of GST-NSP4 fusion protein. Beta-actin was used as internal control.

**Fig. 1. Cloning and expression of GST-NSP4 fusion protein.** A: representative gel images of reverse transcription polymerase chain reaction (RT-PCR). NSP4 gene fragment was obtained by RT-PCR amplification and analyzed by gel electrophoresis. Lane M, DNA marker; lane 1, positive control DNA (496 bp); lane 2, NSP4 gene fragment (525 bp). B: after inserting the NSP4 gene fragment into pGEX-4T-1 vector, the recombinant plasmid was double digested by Smal and Xhol restriction enzymes and the digested products were separated and detected by gel electrophoresis. Lane M, DNA marker; lane 1, undigested pGEX-4T-1 vector; lane 2, digested products of pGEX-4T-1 containing NSP4 gene fragment. C: Western blot of GST-NSP4 fusion protein. Beta-actin was used as internal control.

**Isolation of hepatic CD8+ T cells and flow cytometric analysis.** Mice were killed at 7 or 14 dpi. Following liver perfusion with PBS through the portal vein, mononuclear cells were isolated by density gradient centrifugation with Lymphoprep (Axis-Shield, Oslo, Norway). CD8+ T cells were collected by immunomagnetic positive sorting with an anti-CD8a antibody (Bergisch Gladbach). Isolated CD8+ T lymphocytes were further identified by FACS aria flow cytometry (BD Bioscience, San Jose, CA) using PE-labeled rat anti-mouse CD3 (eBioscience) and Fluorescein isothiocyanate (FITC)-labeled rat anti-mouse CD8a. To determine the releasing of IFN-γ by CD8+ T cells, cells were double stained with PE-labeled rat anti-mouse IFN-γ and FITC-labeled CD8a antibodies. To evaluate the proportion of memory CD8+ T lymphocytes in the sample, cells were double stained with PE-labeled rat anti-mouse CD44 and FITC-labeled CD8a antibodies. Results were analyzed with CellQuest software (BD Biosciences).

**Enzyme-linked immuno-spot assay.** Enzyme-linked immuno-spot (ELISPOT) assay was performed using a Quick Spot mouse IFN-γ precoated ELISPOT kit according to the manufacturer’s instructions (Dakewe Biotech, Shenzhen, People’s Republic of China). Briefly, 96-well plates were coated with 10 μg/ml of monoclonal antibody against IFN-γ. As a positive control, some wells received 10 μg/ml phytohemagglutin A (PHA). Unstimulated cells were used as negative controls. The number of IFN-γ-secreting CD8+ T cells was determined in each well in triplicate. After 20 h, plates were stained for 1 h with a solution containing 0.1% crystal violet in 20% methanol. Plates were then washed twice with 2% bovine serum albumin and dried at room temperature. The IFN-γ spots were counted using a 20× objective. The number of spots per well was used to determine the number of IFN-γ-secreting CD8+ T cells.

**Fig. 2. Inoculation of NSP4 enhances the injury of extrahepatic bile ducts in neonatal mice.** (A) Mice received intraperitoneal injection of PBS containing 20 μg rabbit IgG (control group), glutathione S-transferase (GST)-NSP4, NSP424–32, NSP493–110, NSP4144–152, or NSP457–170. rhesus rotavirus (RRV), or GST within the first 12 h after birth. At 7 or 14 days postinjection (dpi), mice were killed. Extrapleural bile ducts were harvested for histologic evaluation. Histologic injury was graded blindly using a standardized injury scoring system according to Petersen et al. (10, 21) as follows: grade 0, no obstruction, stenosis, necrotic epithelia, or inflammatory cells infiltration; grade 1, mild stenosis and several inflammatory cells; grade 2, stenosis or obstruction caused by necrotic cells or inflammatory cells in bile duct lumens; grade 3, complete lumen obstruction. Grade 2 and 3 were considered as BA (21).

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control, and no antigen or cells were added to the background control wells. Cells (300,000/well) suspended in RPMI1640 culture medium (Invitrogen, Carlsbad, CA) were added to wells containing 10 μl/well GST-NSP4, NSP4144–152, or NSP4157–170. After incubation for 24 h, plates were washed, 100 μl (1 μg/ml) of biotinylated antibody was added, and plates were incubated for 1 h. Plates were then washed, and samples were incubated with streptavidin-horseradish peroxidase for another 1 h. After treatment with AEC-substrate solution (3-amino-9-ethylcarbazole) for 25 min, the spots were enumerated with an ELISPOT analyzer (Cellular Technology, Cleveland, OH.).

ELISA for IFN-γ. Peripheral blood of mice was obtained by eye-bleeding under ether anesthesia. After centrifugation, serum was collected and its IFN-γ content was determined using a mouse IFN-γ ELISA kit according to the manufacturer’s instructions (R&D Systems).

Cytotoxicity assay. The cytotoxicity of CD8+ T lymphocytes against extrahepatic biliary epithelial (EHBE) cells were examined using a CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer’s instructions (Promega, Madison, WI). EHBE cells, isolated and cultured as previously described (6), were used as target cells by seeding them into 6-well plate and adding 500 μl of RRV (titer = 2 × 10^6 pfu/ml). The hepatic CD8+ T cells isolated from the mice of experimental groups served as effector cells. Effector cells (12,500 cells/well) were cocultured with target cells (2,500 cells/well) in a 96-well plate. Absorbance data were collected using a 96-well plate reader set to 490 nm.

Cytotoxicity (%) was computed by the formula: (experimental release – spontaneous release of effector cells – spontaneous release of target cells)/maximum release of effector cells × maximum release of target cells) × 100%.

Depletion of CD8+ T lymphocytes. CD8+ T lymphocytes depletion was performed as previously described (24). To deplete CD8+ T lymphocytes, mice also received daily intraperitoneal injections of 80 μg of anti-mouse CD8a functional grade purified antibody (eBioscience). Prior to the antibody injection, within the first 12 h after birth, BALB/c mice were injected intraperitoneal with GST-NSP4. This resulted in >99% loss of CD8+ cells as determined by flow cytometry.

Immunization of pregnant mice. For maternal immunization experiments, RRV-free female BALB/C mice, 10–12 wk of age, were housed in SPF conditions. The day of vaginal plug appearance was considered to be the day 0 of pregnancy. One week after pregnancy, mice were immunized by intraperitoneal injection of 40 μg GST-NSP4, NSP4144–152, or NSP4157–170, each of which had been pre-mixed with 10 μg monophosphoryl lipid A adjuvant (MPL, Glaxo-SmithKline Biologicals, Rixensart, Belgium) to maximize immunological efficacy. Control mice received MPL alone.

RRV infection on neonatal mice. Within the first 6 h after birth, neonates were inoculated intraperitoneally with 10^3 pfu live RRV or saline (controls) as described previously (10). After RRV infection, all pups were monitored for 7 days. At the experimental endpoint, all of the pups were killed, and their bile ducts were evaluated histologically.

Fig. 3. Inoculations of RRV, GST-NSP4, NSP4144–152, or NSP4157–170 elevate serum levels of interferon (IFN)-γ, and augment IFN-γ release by hepatic T lymphocytes or CD8+ T cells at 7 or 14 dpi. A: enzyme-linked immuno-spot (ELISpot) assay for IFN-γ release by hepatic T lymphocytes. X-axis: at 7 or 14 dpi, hepatic T lymphocytes were isolated from the mice inoculated with RRV, GST-NSP4, NSP4144–152, or NSP4157–170. Y-axis: percentage of IFN-γ-positive hepatic CD8+ T cells. B: quantification of IFN-γ release by hepatic T lymphocytes. X-axis: at 7 or 14 dpi, hepatic CD8+ T cells were isolated from the mice inoculated with RRV, GST-NSP4, NSP4144–152, or NSP4157–170. Y-axis: percent of IFN-γ-positive CD8+ T cells as determined by flow cytometry. C: flow cytometric analysis of IFN-γ-positive CD8+ T cells. D: concentration of IFN-γ in peripheral blood serum was examined by ELISA. X-axis: at 7 or 14 dpi, sera were collected from the mice inoculated with RRV, GST-NSP4, NSP4144–152, or NSP4157–170. Y-axis: serum concentration of IFN-γ. *P < 0.05.
Statistical analysis. Analysis of variance (ANOVA) was used to compare mean values. Fisher’s exact test was used to compare the incidence of BA. All data were analyzed with SPSS 11.0 (SPSS, Chicago, IL). *P < 0.05 was considered statistically significant.

RESULTS

Cloning and expression of GST-NSP4 fusion protein. NSP4 was amplified using specific primers by RT-PCR of RNA from culture medium of MA104 cells pretreated with RRV (14). The amplified product was of the predicted size (525 bp; Fig. 1A) and sequence (data not shown) and subsequently inserted into pGEX-4T-1 vector for production in E. coli as a GST-NSP4 fusion protein. The recombinant plasmid (5494 bp in length) underwent double restriction enzyme digestion, yielding two predicted fragments (4,969 and 525 bp) as expected (Fig. 1B). The presence of NSP4 protein was confirmed by Western blotting analysis (Fig. 1C).

Inoculation of NSP4 enhances the injury of extrahepatic bile ducts in neonatal mice. To determine the roles of NSP4 or NSP4 synthetic peptides in the development of experimental BA, mice received abdominal injection of PBS containing rabbit IgG (control), GST-NSP4, NSP424–32, NSP493–110, NSP4144–152, NSP4157–170, RRV, or GST within 12 h of birth (n = 8–13 for each group). At 7 postinjection days (dpi), severities of extrahepatic bile duct injuries were graded blindly using a standardized histologic injury scoring system in which grades 2–3 were considered as BA. Of the 10 neonatal mice injected with RRV, 7 mice (70%) had grade 2 or 3 injuries, and 3 mice had grade 1 injuries (30%). Of the 13 neonatal mice injected with GST-NSP4, 6 mice (46.2%) had grade 2 injuries, and 7 mice had grade 1 injuries (53.8%). However, in the mice injected with NSP424–32, NSP493–110, NSP4144–152, NSP4157–170, or GST, no extrahepatic biliary epithelium injury (grade 0) was observed.

Fig. 4. Depletion of CD8+ T cells abrogates NSP4 or RRV-induced increase of IFN-γ. CD8+ T cells were depleted by intraperitoneal injections of anti-mouse CD8a antibody. A: IFN-γ ELISPOT assays for peptide-reactive CD8+ T cells responses. Depletion of CD8+ T cells abrogated NSP4- or RRV-induced release of IFN-γ by hepatic CD8+ T cells at 7 or 14 dpi. B: serum level of IFN-γ was studied by ELISA. Depletion of CD8+ T cells abrogated the NSP4 or RRV-induced increase in IFN-γ in serum at 7 or 14 dpi. *P < 0.05.

Fig. 5. Inoculation of GST-NSP4, NSP4144–152 or NSP4157–170 elevates the population of memory CD8+ T cells in liver. Neonatal mice were injected with GST-NSP4, NSP4144–152, or NSP4157–170 within 12 h of birth. At 7 or 14 dpi, hepatic T lymphocytes were isolated. A: flow cytometric analysis of hepatic memory CD8+ T cells (CD8+/CD44+ double positive cells). B: quantification of memory CD8+ T cells. Inoculation of GST-NSP4, NSP4144–152 or NSP4157–170 significantly elevated the number of memory CD8+ T lymphocytes in the liver at 7 or 14 dpi. Y-axis: percentage of CD8+/CD44+ double positive cells. *P < 0.05.
inoculation of GST-NSP4, NSP4144–152, or NSP4157–170 elevate the IFN-γ production in mice. Despite the lack of tissue injury by the NSP4 peptides, we next investigated their ability to affect production of IFN-γ after in vivo administration in neonatal mice. At 7 or 14 dpi, inoculation of GST-NSP4, NSP4144–152, or NSP4157–170 or RRV significantly increased IFN-γ release by hepatic T lymphocytes compared with controls (all P < 0.05) (Fig. 3A) but the effect of GST-NSP4 was significantly greater than any of the peptides (all P < 0.05) (Fig. 3A). In contrast, injection of NSP424–32 did not alter IFN-γ release at either 7 or 14 dpi (all P > 0.05) (Fig. 3A). Similar results were obtained by analysis of serum IFN-γ levels (Fig. 3B).

Additionally, at 7 or 14 dpi, inoculation of GST-NSP4, NSP4144–152, or NSP4157–170, or RRV significantly increased the population of IFN-γ-positive CD8+ T cells compared with controls (all P < 0.05; Fig. 3, C and D), and, again, GST-NSP4 was significantly more effective than either of the peptides (all P < 0.05; Fig. 3, C and D). NSP4144–152 or NSP4157–170 had no effect on the population of IFN-γ positive CD8+ T cells (all P > 0.05; Fig. 3, C and D). Depletion of CD8+ T lymphocytes abrogates the elevation of IFN-γ induced by NSP4 or RRV. In an alternative approach, we first depleted >99% of the CD8+ T lymphocytes by antibody neutralization before isolation of the residual <1% CD8+ T lymphocytes at either 7 or 14 dpi. NSP4 or RRV-induced IFN-γ release by this small residual CD8+ T cell population was significantly decreased compared with control animals (P < 0.05; Fig. 4A). Serum levels of IFN-γ in CD8+ T cell-depleted mice at 7 or 14 dpi were ~50% of those in control mice (all P < 0.05; Fig. 4B), demonstrating that CD8+ T lymphocytes contributed at least one-half of the elevated IFN-γ levels in response to NSP4 or RRV.

Inoculation with GST-NSP4, NSP4144–152, or NSP4157–170 elevates the number of memory CD8+ T lymphocytes. The number of memory CD8+ (CD8/CD44 double positive) T lymphocytes was quantified using dual-stain flow-cytometric analysis of mononuclear cells isolated from livers at different time points after synthetic peptides injection. At 7 or 14 dpi, inoculation of GST-NSP4, NSP4144–152, or NSP4157–170 significantly increased the number of memory CD8+ hepatic T lymphocytes more than controls (Fig. 5).

**GST-NSP4-, NSP4144–152-, or NSP4157–170-primed hepatic CD8+ T cells are toxic to extrahepatic biliary epithelial cells.** To determine whether GST-NSP4, NSP4144–152, or NSP4157–170 could activate hepatic CD8+ T cells and damage biliary epithelium, we injected GST-NSP4 or synthetic peptides into neonatal mice, isolated hepatic CD8+ T lymphocytes at 7 or 14 dpi, and tested their respective cytolytic activities for EHBE cells using an in vitro cytotoxicity assay. As shown in Fig. 6, GST-NSP4-, NSP4144–152-, or NSP4157–170-primed CD8+ T cells were more toxic to EHBE cells than naive CD8+ T cells (all P < 0.05), whereas no significant cytotoxicity of NSP424–32- or NSP4144–152-primed CD8+ T cells against EHBE cells was noted (all P > 0.05). GST-NSP4-, NSP4144–152-, or NSP4157–170-, but not NSP424–32- or NSP4144–152-, primed CD8+ T cells were more toxic to RRV-infected EHBE cells than control naive CD8+ T cells at 7 or 14 dpi (all P < 0.05). Notably, cytotoxicity of GST-NSP4-primed CD8+ T cells against RRV-infected EHBE cells was more severe than those against noninfected EHBE cells at 7 or 14 dpi (P = 0.01 at 7 dpi; P < 0.001 at 14 dpi).

**Fig. 6.** Cytotoxicity of hepatic CD8+ T cells against extrahepatic biliary epithelial (EHBE) cells cultured in vitro. Neonatal mice were injected with GST-NSP4 or one of the four synthetic NSP4 peptides within 12 h of birth. At 7 or 14 dpi, CD8+ T cells were isolated from the liver, and cytotoxicity of hepatic CD8+ T cells against EHBE cells was assessed. At 7 or 14 dpi, GST-NSP4-, NSP4144–152-, or NSP4157–170-primed CD8+ T cells were more toxic to EHBE cells than naive CD8+ T cells. However, cytotoxicity of NSP424–32- or NSP4144–152-primed CD8+ T cells against EHBE cells was not increased compared with control naive CD8+ T cells. Similar results were also seen in NSP4- or NSP4 peptide-primed CD8+ T cells to RRV-infected EHBE cells. After 7 or 14 dpi, cytotoxicity of GST-NSP4-primed CD8+ T cells against RRV-infected EHBE cells was higher than that against RRV-uninfected EHBE cells. *P < 0.05.
NSP4144–152 or NSP4157–170 significantly decreased the incidence of BA and improved weight gain in neonatal mice. One week after pregnancy, female BALB/c mice were immunized with GST-NSP4, NSP4144–152, or NSP4157–170. Neonatal mice were infected with RRV to induce biliary atresia (BA) and then killed 7 days later. Histologic injury of extrahepatic bile ducts was graded blindly using a standardized histologic injury scoring system in which grades 2 and 3 are defined as BA. A: representative histological images of the extrahepatic bile duct. B: summary of distribution of biliary injury grading. Seven of the 13 neonatal mice born to the control nonimmunized mothers developed BA, which had a more frequent BA incidence compared with the neonates born to the mothers immunized against GST-NSP4 (2/13), NSP4144–152 (1/10), or NSP4157–170 (1/9). Additionally, the weights of RRV-infected neonates at 7 or 14 days after birth was significantly greater in mice born to mothers immunized with GST-NSP4, NSP4144–152, or NSP4157–170 compared with those from nonimmunized control mothers immunized at 7 or 14 days after RRV infection. *P < 0.05.

**DISCUSSION**

Currently, the cause and pathogenic mechanisms of BA remain largely unknown (13). Human biopsy sampling studies indicate that cellular immunity likely plays a major role in BA. Additionally, analysis of the T-cell receptor variable region of the b-chain within BA liver and extrahepatic bile duct remnants revealed that the CD4+ and CD8+ T cells were oligoclonal, suggesting that T cell expansion occurs in response to specific antigen(s) such as viral proteins or autoantigen in biliary epithelium (15, 18).

With the RRV murine model of BA (mrvBA), previous studies found infiltration of macrophage, CD4+ and CD8+ T cells, and increased expression of IFN-γ-induced chemokines were associated with the development of biliary epithelium injury (5, 16, 19). An elegant study conducted by Shivakumar et al. (23) demonstrated that a RRV challenge triggers a hepatobiliary inflammation by IFN-γ-producing CD4+ and CD8+ lymphocytes. Importantly, the genetic loss of IFN-γ remarkably suppressed the tissue-specific targeting of T lymphocytes and completely prevented the inflammatory and bile duct obstruction (23). Furthermore, depletion of CD4+ or CD8+ cells did not modify expression of IFN-γ by lymphocytes in response to RRV. However, depletion of CD8+ cells but not CD4+ cells remarkably suppressed bile duct injury, prevented luminal obstruction, and restored bile flow (24). These observations suggest that IFN-γ-driven CD8+ T cell-dependent cellular immunity plays an important role in developing mrvBA.

Previously, we showed that silencing of rotavirus NSP4 mRNAs in biliary epithelia by siRNA transfection decreased...
the levels of VP7 or VP4, reduced viral particles, and decreased incidence of mrVBA (10). In the present study, we showed that intraperitoneal injection of purified GST-NSP4 fusion protein caused extrahepatic bile duct injury in neonatal mice, with similar pathological features found in RRV-infected mice. These studies suggest that NSP4 is a pathogenic immunogen that initiates an inflammatory response against biliary epithelium. Because we speculated that CD8+ T cells are the effector cells in the NSP4-induced inflammatory response, we reasoned that identification of CD8+ T cells epitopes in NSP4 would be useful for analysis of the T-cell response to RRV infection and potential immunization strategies. To this end, we found that mice injected with NSP4157–170 or NSP4144–152 had more memory CD8+ T cells compared with those injected with NSP424–32 or NSP493–110. Furthermore, NSP4157–170 or NSP4144–152-primed CD8+ T cells were more toxic to RRV-infected or naive EHBE cells. The ability of NSP4157–170 or NSP4144–152-primed CD8+ T cells to recognize healthy duct epithelium supports a role for autoimmune responses in the pathogenesis of experimental BA. At present the inability of NSP4157–170 or NSP4144–152 to cause BA in neonatal mice is perplexing, because this outcome was inconsistent with in vitro findings. However, it is possible that development of the atresia phenotype requires the synergy of other effector cells (such as natural killer cells) or that CD8+ T cells need multiple epitopes to maximize damage to biliary epithelium and obstruct the duct lumen. Moreover, the use of algorithms for epitope prediction suffers from a significant rate of false-negative results and epitope ranking lists that often are not validated by practical experience. This study does not exclude the possible existence of some other dominant epitopes with low predictive scores in NSP4.

In this study, IFN-γ was chosen as the detection cytokine based on previous studies showing its significant role in inflammation and bile duct obstruction (23, 24). We found the mice injected with NSP4157–170 or NSP4144–152 had higher serum levels of IFN-γ at 7 and 14 dpi. Furthermore, NSP4157–170 or NSP4144–152-primed hepatic T lymphocytes released more IFN-γ. After deleting >99% of CD8+ T cells by antibody neutralizing, there was a 50% reduction in serum IFN-γ levels at 7 or 14 days after NSP4 injection, suggesting that approximately one-half of the NSP4-induced IFN-γ was produced by CD8+ T cells.

At present, the prognosis of BA is still dismal (8). A solution to improve the poor outcome would be to prevent BA before it begins. Maternal immunization during pregnancy is an effective and safe means of eliciting or increasing maternal antibody to infectious diseases (12, 26). In a study performed by Bondoc et al. (4), prenatal intraperitoneal injection in female mice of pure RRV, a live pentavalent rotavirus vaccine containing 5 human-bovine reassortant rotaviruses, prevented development of BA in their offspring. Similar results were also reported by Petersen et al. (22) who showed that prenatal intraperitoneal administration of live RRV protected offspring from developing BA, but the detection of RRV in the liver or brain of fetuses or neonates raised an important safety concern about live RRV vaccines. Peptide-based vaccines for RRV, which offer advantages of safety and a high specificity in eliciting immune responses, have thus been proposed as a better choice in immunization strategies (3). Indeed, the work presented in this report has shown that such an approach is feasible in that mice born to mothers vaccinated against GST-NSP4, NSP4144–152, or NSP4157–170 rarely manifested the sequelae of biliary obstruction and gained weight faster than RRV-infected mice. Our findings thus provide an important proof of principle that immunization of female mice against GST-NSP4, NSP4144–152, or NSP4157–170 is a safe and effective strategy to protect their offspring from developing RRV-related BA.

In summary, our present study demonstrates that amino acid residues 144–152 and 157–170 of NSP4 are epitopes that are recognized by CD8+ T cells and induce CD8+ T cell-mediated inflammatory responses against BA. This study also shows that a CD8+ T cell-mediated autoimmune response represents one of the pathogenic mechanisms in experimental BA. More importantly, maternal immunization against NSP4144–152 or NSP4157–170 can protect the offspring from developing RRV-related BA. Our findings provide important evidence that supports the further investigation of NSP4 epitope-based vaccines in the protection against development of BA in experimental models and that may have broad implications for improved understanding of the underlying pathogenic mechanisms and potential therapeutic targets in human BA.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


