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Targeting Extracellular Cyclophilins Ameliorates Disease Progression in Experimental Biliary Atresia

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Biliary atresia (BA) is a devastating liver disease of unknown etiology affecting children generally within the first 3 months of life. The disease is manifested by inflammation and subsequent obstruction of the extrahepatic bile ducts, fibrosis and liver failure. The mechanisms responsible for disease pathogenesis are not fully understood, but a number of factors controlled by the SMAD signaling pathway have been implicated. In this study, we investigated the role of a known proinflammatory factor, extracellular cyclophilin A (CypA), in the pathogenesis of biliary atresia using the rhesus rotavirus (RRV) murine model. We used a unique cyclosporine A derivative, MM284, which does not enter cells and therefore inactivates exclusively extracellular cyclophilins, as a potential treatment. We demonstrated that levels of CypA in plasma of RRV-infected mice were increased significantly, and that treatment of mice with MM284 prior to or one day after disease initiation by RRV infection significantly improved the status of mice with experimental BA: weight gain was restored, bilirubinuria was abrogated, liver infiltration by inflammatory cells was reduced and activation of the SMAD pathway and SMAD-controlled fibrosis mediators and tissue inhibitor of metalloproteinases (TIMP)-4 and matrix metalloproteinase (MMP)-7 was alleviated. Furthermore, treatment of human hepatic stellate cells with recombinant cyclophilin recapitulated SMAD2/3 activation, which was also suppressed by MM284 treatment. Our data provide the first evidence that extracellular cyclophilins activate the SMAD pathway and promote inflammation in experimental BA, and suggest that MM284 may be a promising therapeutic agent for treating BA and possibly other intrahepatic chronic disorders.

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INTRODUCTION

Biliary atresia (BA) is a devastating liver disease in children that results in obstruction of the biliary system and, without treatment, death within two years of birth due to hepatic cirrhosis. The incidence of BA is quite rare; it is diagnosed in approximately one in 10,000 children in the first three months of life. The pathophysiology of BA is inflammation and obstruction of the extrahepatic bile ducts that results in fibrosis, subsequent cirrhosis and eventual liver failure. Surgical treatment via portoenterostomy results in only ~60% transplant-free survival 2 years after surgery (1). Since the exact cause of the disease is unknown and medical and surgical treatments remain suboptimal, clarification of the mechanisms involved in initial induction and progression of biliary inflammation and identification of potential targets for therapeutic intervention remain essential for new treatment strategies.

The murine model of BA in neonatal mice infected with rhesus rotavirus (RRV) has been used to study the pathogenesis of the disease (2,3). In our early studies using this model, we demonstrated increased mRNA expression of the matrix metalloproteinases (MMPs) in liver tissue samples of infected mice (4). We had initially suspected that these genes may be upregulated via the transforming growth factor (TGF)β pathway, as we had also seen perturbations in mRNA expression of tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-4

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as well as plasminogen activator inhibitor-1 (PAI-1), which are all regulated by TGFβ. However, several experiments utilizing anti-TGF strategies, comprised mainly of antibody blockade of TGF and its pathway members, failed to demonstrate any effect in this animal model (data not shown). Therefore, we began to explore alternative mechanisms that could be responsible for BA-associated inflammation.

Studies of tumorigenesis have shown that increased levels of MMPs are detected in stromal fibroblasts, endothelial cells and in the tumor cells themselves in response to activation of extracellular matrix metalloproteinase inducer (EMMPRIN) or CD147 (5). CD147 is a type I transmembrane glycoprotein, a member of the immunoglobulin super-family, that is expressed by a wide array of cell types, including epithelial, endothelial and hematopoietic cells (6). CD147 is a signaling receptor for extracellular cyclophilins (Cyp) A and B (7,8), and recent publications have identified the CD147 signaling pathway induced by extracellular cyclophilins as a triggering mechanism regulating MMP expression and inflammation in atherosclerosis, rheumatoid arthritis, allergic and chronic lung disease (9–14). Thus, we hypothesized that CD147 activation by extracellular cyclophilin may play a role in experimental BA as well.

Previous studies showed that the interaction of extracellular CypA with CD147 induces activation of extracellular signal-regulated kinase (ERK) pathway and a downstream increase in the expression of interleukins, MMPs and CD147 itself, resulting in the progression of inflammation (9,15,16). Thus, blockade of extracellular cyclophilin-CD147 interactions may have a potential to reduce inflammation and infiltration of inflammatory cells into infected tissues. In fact, studies of serum inflammatory cytokine profiles demonstrated that treatment of acute systemic vasculitis (Kawasaki disease) with cyclosporine (a known cyclophilin inhibitor) resulted in downregulation of cyclophilin-induced pathways, including CD147-mediated inflammation (17). Therefore, specific targeting of extracellular cyclophilins may be a valid therapeutic strategy to inhibit CD147-dependent pathways. MM284 is a nonimmunosuppressive cell-impermeable cyclosporine derivative that does not penetrate the plasma membrane and, therefore, cannot interact with intracellular cyclophilins and mediate intracellular immunosuppressive activity; thus MM284 targets only the extracellular pool of cyclophilins (12,18). We hypothesized that the liver inflammation associated with BA may be induced or activated by the interaction of extracellular CypA with CD147 located at the plasma membrane of hepatocytes and hepatic stellate cells, and blocking this interaction by MM284 would inhibit this inflammatory response and subsequent liver inflammation and, potentially, fibrosis.

MATERIALS AND METHODS

Murine Model of Biliary Atresia

Pregnant time-dated BALB/c mice (Charles River Labs) were kept with one animal per cage with free access to water and the standard laboratory diet. After spontaneous vaginal delivery, the newborn mice were randomly divided into four groups. During the first 24 h of life, mice in the control group (n = 10) received an intraperitoneal injection of 15% Cremophor EL (Sigma-Aldrich) or saline. The initial set of animals in this group (n = 5) was injected with 15% Cremophor EL, and no effect of the Cremophor EL on mouse weight or bilirubinuria was confirmed by comparing these mice to mice receiving saline alone (n = 5) (data not shown). In the second group (RRV group, n = 23), 1.5×10^6 fluorescence forming units of rhesus rotavirus (RRV) was administered, and 15% Cremophor EL was injected on d 0, 2, 4, 6, 8, 10 and 12. The third group (MM284 + RRV group, n = 20 total) received RRV plus 20 mg/kg of MM284 in 15% Cremophor EL. The fourth group (MM284 group, n = 5) received 20 mg/kg of MM284 in 15% Cremophor EL only. The animal data were collected from three separate animal trials to account for the variability in the RRV model. We used two different schedules for MM284 treatment for group 3. In the first set of experiments (n = 15), animals received the drug on d 0, 2, 4, 6, 8, 10 and 12. This represented initiation of MM284 treatment prior to RRV administration and consequently prior to the onset of disease (pretreatment). In the second set of experiments (n = 5), treatment with MM284 was initiated on d 1 after RRV treatment. The treated mice were kept with their mothers, maintained in normal environment and housed in a room with a standard 12-h dark–light cycle. Subsequent experimental procedures (such as clinical phenotyping, subcutaneous saline injection and organ harvest) were performed as previously described (4). Liver specimens were harvested for protein and RNA isolation, as well as histological and immunohistochemical analysis. All procedures were approved by the Children's National Medical Center Institutional Animal Care and Use Committee (IACUC).

RNA Isolation and Real-time Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Mice were euthanized on d 14 after RRV injection, their livers were harvested, and mRNA expression for inflammatory cytokines and fibrosis mediators was quantified by RT-PCR. Total RNA from the homogenized livers was extracted using TRI reagent (Molecular Research Center) and purified using RNeasy Mini Kits (Qiagen) according to the manufacturer's instructions. The cDNA pools were generated by the RT reaction using standard reagents (Invitrogen) and Oligo-dT primer and subjected to RT-PCR on an ABI PCR machine using SYBR GreenPCR Master mix (BioRad). The mRNA expression of selected cytokines and fibrotic mediators were then quantified relative to the reference housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using techniques previously described (4). All measurements were done in triplicate. The primers are listed in Table 1.

Table 1. Primers.

Primers for proinflammatory cytokines		
Mediator	Forward primer (5′-3′)	Reverse primer (5′-3′)
TNF-α	AGCCCCCAGTCTGTATCCTT	CTCCCTTTGCAGAACTCAGG
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
IL-1β	AGGCCACAGGTATTTTGTCG	GCCCATCCTCTGTGACTCAT
Primers for fibrosis mediators		
Mediator	Forward primer (5′-3′)	Reverse primer (5′-3′)
PAI-1	CCGATCCTTTCTCTTTGTGG	TCAAGGCTCCATCACTTGG
Integrin αVβ6	GAACGCTCTAAGGCCAAGTG	TGCTTCTCCCTGTGCTTGTA
TIMP-1	GACCTATAGTGCTGGCTGTG	TCATCTTCCAGTTTGCAAGG
TIMP-4	TGCCAAATCACCACTTGCTA	ATAGAGCTTCCGTTCCAGCA
MMP-7	CCCTACCTATCAAAGAGACT	CAGCGTGTTCCTCTTTCCAT
MMP-9	CCTGGAACTCACACGACATC	GGAAACTCACACGCCAGAA
GAPDH	GGCATTGCTCTCAATGACAA	CCCTGTTGCTGTAGCCGTAT

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of phosphorylated SMAD2 was determined using the commercially available PathScan P-SMAD2 Sandwich ELISA kit (Cell Signaling Technology). Liver samples were homogenized according to the manufacturer's instructions in lysis buffer using homogenizer FastPrep-24 (MP Biomedicals). Subsequent manipulations were performed as described earlier (19). The concentration of murine CypA in plasma was determined using ELISA kits from MyBioSource following the manufacturer's instructions.

MM284

MM284, a side-chain modification in the 1-position of cyclosporine A (CsA) was prepared according to published procedures (20). For injection, the powder was first dissolved in 100% ethanol (Sigma-Aldrich) and then diluted in sterile 15% solution of Cremophor EL (Sigma-Aldrich) in PBS.

Cyclophilin A

Human recombinant cyclophilin A was an Enzo Life Science product.

Cell Culture

Human hepatic stellate cells (HSC) were a product of ScienCell Research Laboratories. Cells were cultured in the special stellate cell medium supplemented with 1% stellate cell grow supplement, 2% fetal bovine serum and penicillin-streptomycin solution (100 U/mL and 100 μg/mL, respectively). HSC cells were cultured in poly-D-lysine-coated 75-cm^2 culture flasks (Becton Dickinson Labware) at 37°C and 5% CO^2/95% air atmosphere. Cultured HSCs were treated with recombinant CypA (800 ng/mL) with or without MM284 (400 ng/mL) and then incubated for 72 h. The dose of CypA used in this experiment was about 15-fold higher than the level measured in the plasma of BA mice to account for higher concentration of cyclophilin at the sites of inflammation; this concentration is similar to CypA levels in the serum of patients with systemic inflammation observed in sepsis (22).

RESULTS

Presence of CypA in RRV-Treated Mice

To determine whether extracellular cyclophilins are upregulated in the BA model, we measured CypA levels in plasma of RRV-treated mice. As shown in Figure 1, CypA was increased from 17.6 ± 3.9 ng/mL in saline-treated control mice to 59.0 ± 18.0 ng/mL in RRV-treated mice ($p < 0.0001$), suggesting that extracellular CypA may contribute to pathophysiology of disease in this model. Of note, the actual concentration of CypA at the site of inflammation (the place where it is released) is likely considerably higher than the plasma levels. Thus, we proceeded to employ MM284 in the experimental model of BA to assess whether blockade of the extracellular cyclophilins may have a beneficial effect. In initial experiments, we attempted a dose of 10 mg/kg for MM284 administration, but this dose did not have any measurable effect (data not shown). The foregoing data are only for animals treated with the 20-mg/kg dose of the compound.

MM284 Treatment Restores Weight Gain and Prevents Bilirubinuria in BA Mice

In our previous studies we showed that mice with experimental BA displayed significantly less weight gain than the normal controls (4). Our current data confirm that from d 7 to d 14, experimental BA mice demonstrated 30% less weight gain compared with control mice (Figure 2A). After d 12, RRV-infected mice did not increase their weight at all, whereas control animals and RRV-infected mice treated with MM284 continued to gain weight (Figures 2A, B). Interestingly both pretreatment with MM284 before RRV infection (panel A) and treatment with MM284 24 h postinfection

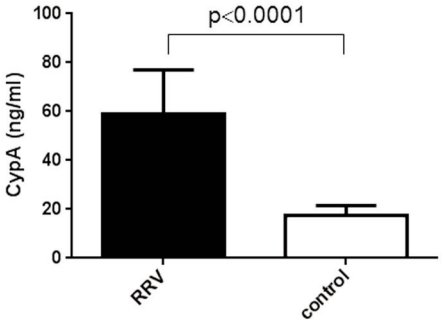

Figure 1. Increased levels of CypA in plasma of mice with biliary atresia. Serum CypA levels were measured 14 d after RRV infection (n = 10) and compared with 10 uninfected (control) mice. Results represent mean ± SD. *P* value was calculated using Student unpaired t-test.

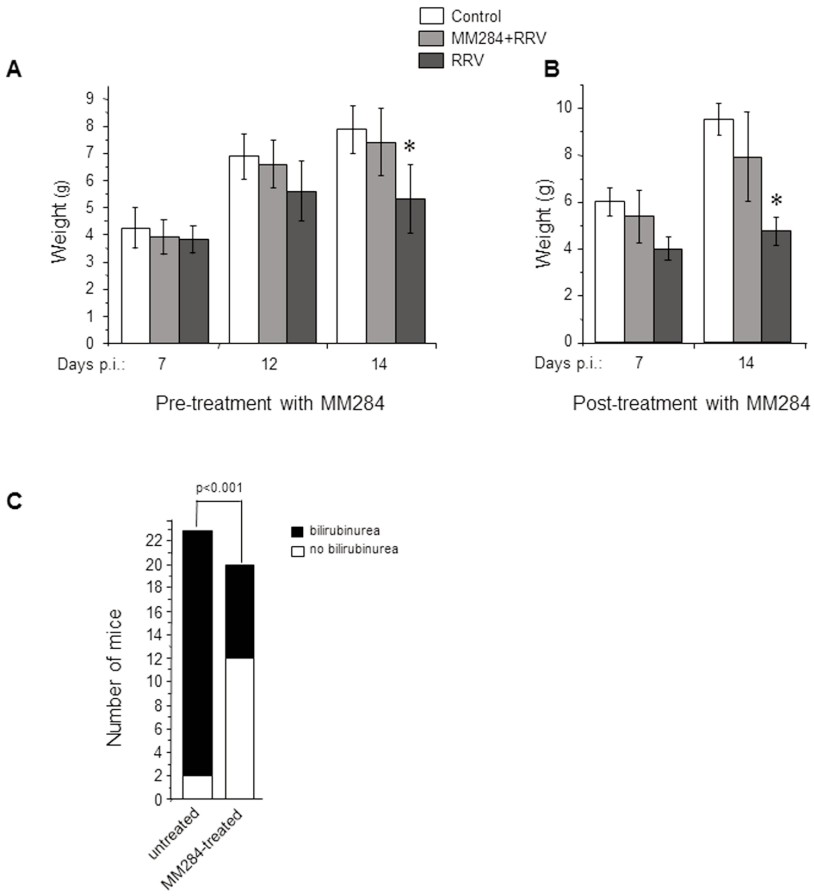

Figure 2. MM284 prevented weight loss in newborn mice with biliary atresia induced by rhesus rotavirus. Newborn BALB/c mice were either pretreated with MM284 on the first day of life and injected with Rhesus Rotavirus (RRV) 24 h posttreatment (A) or infected with RRV and then treated with MM284 on d 1 postinfection (B). Subsequent MM284 injections were performed on d 4, 6, 8, 10 and 12 (A), or on d 2, 3, 4, 6, 8, 10 and 12 (B). Weight was measured daily from d 7 to d 14 when the animals were killed. Results are shown as a mean ± SD for each group. Asterisks represent *p* values <0.05. (C) Bilirubinuria was measured on d 14 in a group of untreated mice (n = 23) and compared with a combined group of mice pretreated or posttreated with MM284 (n = 20). Results were compared using chi-square test and the *p* value is shown above the bars.

(panel B) resulted in similar weight gain trajectories as control mice. There were no significant differences in weight gain between control and RRV-infected MM284-treated groups. When compared with RRV-infected untreated mice, infected mice treated with MM284 showed statistically significant weight increase: 7.4 ± 1.2 g versus 5.3 ± 1.2 g ($p < 0.01$) in the experiment with MM284 pretreatment, and 7.92 ± 1.9 g versus 4.0 ± 0.5 g ($p < 0.001$) when the mice were given MM284 after viral infection.

We have previously shown that bilirubinuria correlates well with biliary obstruction and with histologic BA in the experimental model of BA (4). In the current study, no animals treated with saline or Cremophor EL alone without RRV infection displayed bilirubinuria on a urine dipstick. In animals infected with RRV but not receiving MM284, 21 of the 23 were positive for bilirubinuria on d 14 (Figure 2C). In contrast, only 8 out of 20 MM284-treated mice were positive for bilirubinuria after RRV infection. Both the pretreatment and posttreatment groups had the same proportion of bilirubinuria-positive mice (6/15 and 2/5, respectively, $p = 1.0$ Fischer's Exact test). When combining the pretreatment and posttreatment groups and comparing them to untreated animals, the chances that the difference between MM284-treated and control animals was due to chance alone were insignificant ($p < 0.001$, chi-square test).

MM284 Ameliorates the Inflammatory Response in BA Mice

We initially performed routine hematoxylin and eosin staining, which revealed neutrophil infiltration and bile duct proliferation after RRV infection, but lack of fibrosis (Figure 3A), similar to previously described findings in this model (4,5). Treatment with MM284 prior to RRV infection revealed a reduction of infiltration of neutrophils and inflammatory foci in the liver confirmed by myeloperoxidase staining when compared with the RRV-only group (Figure 3A).

We previously demonstrated a dramatic increase of mRNA expression of certain mediators of inflammation and fibrosis, such as TIMP-1, TIMP-4, PAI-1, MMP-7 and MMP-9 in the mouse model of BA (4). In the present study, we found that treatment with MM284 significantly reduced expression of two of these mediators, TIMP-4 and MMP-7, in the liver homogenates of mice with RRV-induced BA. Pretreatment with MM284 resulted in an over six-fold (6.6 ± 1.1, $p < 0.001$) reduction of TIMP-4 mRNA in the liver relative to untreated animals on d 14 (Figure 3B), and posttreatment with MM284 decreased the TIMP-4 mRNA approximately five-fold (5.4 ± 1.12, $p < 0.01$) (Figure 3C). An even more pronounced effect was observed on MMP-7 mRNA expression: it was reduced about ten-fold on d 14 postinfection in both mice pretreated and posttreated with MM284 (10.7 ± 2.2, $p < 0.001$ and 9.9 ± 0.7, $p < 0.01$ respectively, relative to untreated mice) (Figures 3D, E). However, TIMP-4 and MMP-7 mRNA expression levels in

MM284-treated animals were still higher than those in control animals. Quantitative RT-PCR did not detect significant differences in TIMP-1, PAI-1 and MMP-9 expression, although there was a trend toward decreased expression of these mediators as well (data not shown).

MM284 Reduces Interleukin (IL)-6 mRNA Expression in the Liver of BA Mice

IL-6 is a multifunctional cytokine that is implicated in the pathogenesis of multiple diseases associated with inflammation, including inflammatory liver diseases, and is known to be secreted by macrophages in response to extracellular cyclophilin (24,25). Thus, we next investigated whether MM284 had an effect on IL-6 production in our model. RRV infection increased IL-6 mRNA expression five-fold relative to control mice 14 d after RRV infection (5.4 ± 0.3, $p < 0.05$); pretreatment with MM284 significantly abrogated this elevated expression (1.65 ± 0.5, $p < 0.05$ relative to untreated infected mice) (Figure 3F). In the experiment with MM284 posttreatment, RRV increased IL-6 mRNA expression approximately four-fold relative to the uninfected group (4.44 ± 0.2, $p < 0.01$) and MM284 treatment reduced IL-6 to the level similar to that in the control group (1.0 ± 0.4) (Figure 3G).

MM284 Reduces SMAD2 Activation

Activation (phosphorylation) of SMAD2 leads to increased expression of such fibrotic mediators as PAI-1, TIMP-1, TIMP-4 and MMP-7 (26). As TIMP-4 and MMP-7 mRNA expression was decreased with MM284 administration, we evaluated whether SMAD2 phosphorylation was affected by MM284 as well. Treatment of mice with MM284 prior to RRV infection led to a dramatic decrease of SMAD2 phosphorylation relative to RRV-infected mice without MM284 treatment (Figure 4A), suggesting that the mechanism by which MM284 downregulated inflammatory and fibrotic mediators in our model was at least in part via inhibition of SMAD2 phosphorylation. Surpris-

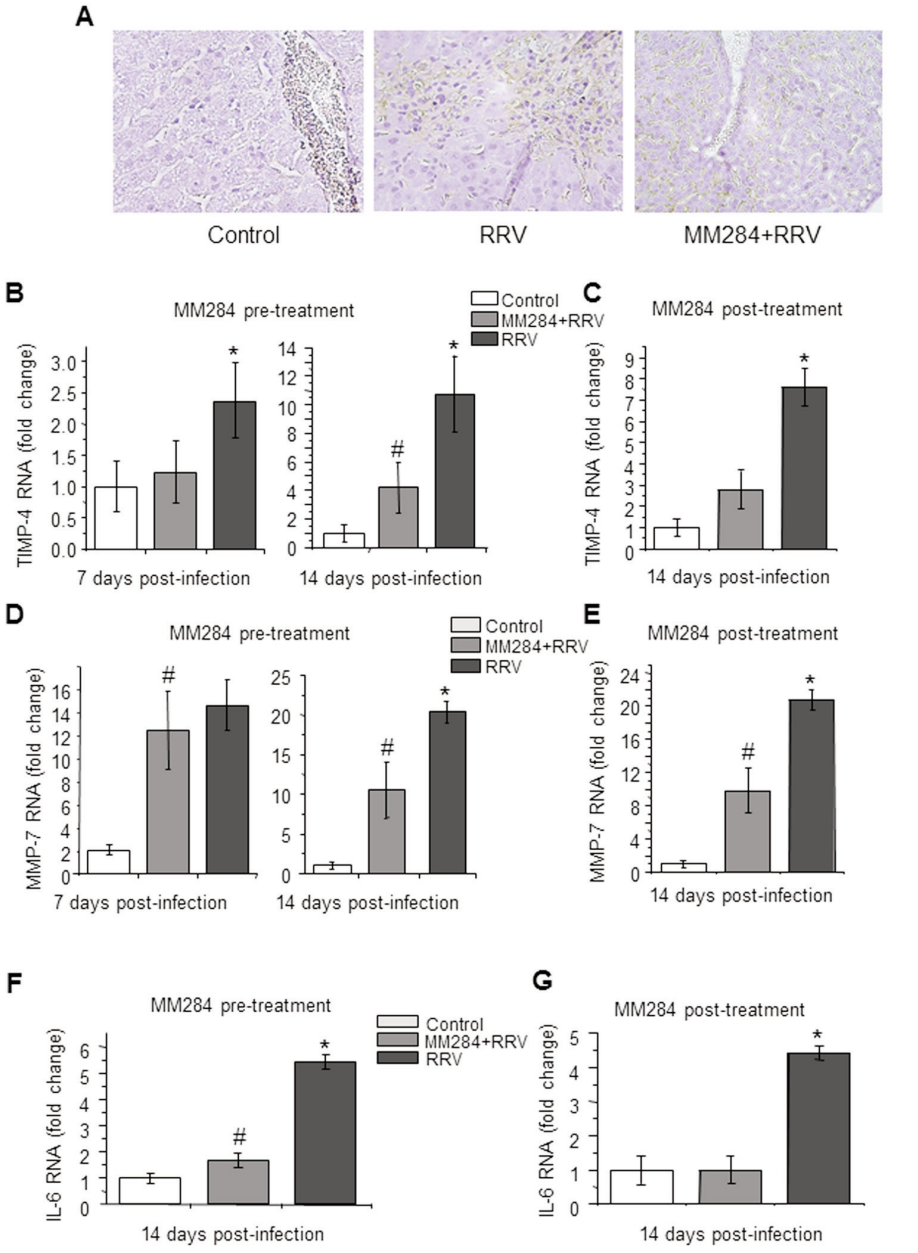

Figure 3. MM284 reduced inflammation in the liver of mice with RRV-induced BA. (A) Myeloperoxidase staining of liver tissue. Representative images are shown for three groups of animals. (B, C) Quantitative RT-PCR analysis of TIMP-4 mRNA in the liver extracts of RRV-infected mice pretreated (B) or posttreated (C) with MM284. Results are shown as mean ± SD of three independent experiments. Asterisks represent p values <0.05 between RRV and RRV + MM284 groups. The pound sign represents p values <0.05 between RRV + MM284 and control groups. (D,E) Quantitative RT-PCR analysis of MMP-7 mRNA in the liver extracts of RRV-infected mice pretreated (D) or posttreated (C) with MM284. Results are shown as mean ± SD of three independent experiments. Asterisks represent p values <0.05 between the RRV and RRV + MM284 groups. The pound sign represents p values <0.05 between the RRV + MM284 and control groups. (F,G) Quantitative RT-PCR results of IL-6 mRNA in the liver extracts of RRV-infected mice pretreated (F) or posttreated with MM284 (G). Asterisks represent p value <0.01 between RRV and RRV + MM284 groups, and the pound sign represents p values <0.05 between RRV + MM284 and control groups.

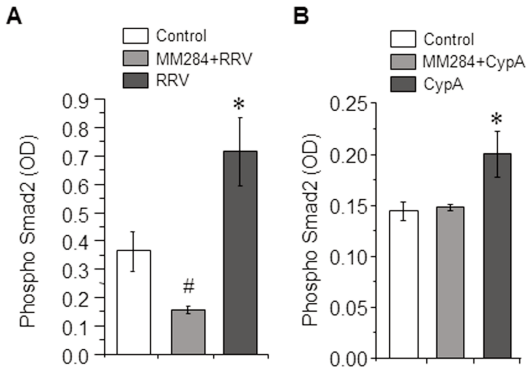

Figure 4. MM284 treatment decreased SMAD2 phosphorylation. (A) The mice were euthanized on d 14 post-RRV infection, and portions of liver were harvested for protein extraction. Total protein lysates were used for PathScan Phospho-SMAD2 sandwich ELISA. Data represent absorbance at λ = 450 nm. Asterisks represent *p* values <0.05 between the RRV treated and control groups; the pound sign represents *p* values <0.05 between control and RRV + MM284 groups. (B) HSCs were treated with 800 ng/mL of Cyclophilin A (CypA) with or without 400 ng/mL of MM284. Cells were harvested at 72 h posttreatment and SMAD2/3 phosphorylation was analyzed in lysates by PathScan Phospho-SMAD2/3 sandwich ELISA. The experiment was performed in triplicate, and the error bars represent the standard deviations. Asterisks represent *p* values <0.01 between CypA treated and both control and CypA + MM284 groups.

ingly, MM284-treated mice demonstrated decreased SMAD2 phosphorylation compared with the control group. This suggests that extracellular cyclophilin may play a physiological role as a regulator of SMAD2 activation, as well as mediating the pathogenesis of experimental BA.

SMAD activation is a previously unreported action of extracellular cyclophilins. To confirm this finding, we analyzed the effect of recombinant human CypA on the inflammatory signaling pathways in human hepatic stellate cells (HSCs), which are known contributors to the pathogenesis of fibrotic liver diseases (27,28). Similar to the findings in the BA mice, lysates from CypA-treated cells demonstrated a 1.5-fold increase in SMAD2/3 phosphorylation relative to untreated cells, and a complete absence of the increased SMAD2/3 phosphorylation when the cells were treated with CypA in combination with MM284 (Figure 4B). Importantly, sensitivity to MM284 indicates that the effect of recombinant CypA was not due to possible endotoxin contamination but was mediated by a cyclosporine-sensitive interaction (29).

DISCUSSION

Increased levels of extracellular cyclophilins (mostly CypA) have been documented in many inflammatory diseases, and their role in disease pathogenesis has been verified in a variety of animal models of human diseases, including rheumatoid arthritis, sepsis, asthma, atherosclerosis and a number of viral infections (33,34). Similarly, we reported a marked inhibitory effect of an extracellularly restricted inhibitor of the peptidyl prolyl isomerase activity of cyclophilins, the highly branched CsA-like molecule MM218, on the inflammatory response in a mouse model of allergic lung inflammation (13). Furthermore, a smaller molecule member of this type of cell-impermeable cyclosporine derivatives, MM284, inhibited the recruitment of leukocytes during inflammation in a mouse model of experimentally induced peritonitis and delayed-type hypersensitivity reaction (21). Thus, we hypothesized that MM284 may provide a benefit in the experimental model of BA comprised of neonatal mice infected with RRV.

In this study we demonstrated that MM284 reduced inflammation and phenotypic signs of disease in the RRV-induced mouse model of BA. Given that interaction between virus-incorporated cyclophilin and CD147 has been implicated in infection by HIV-1 and measles (35,36), we also evaluated a group of mice which were infected with RRV first and then treated with MM284 starting 24 h after RRV infection to exclude the possibility that MM284 prevents RRV infection of the animal directly. The fact that both pretreatment and posttreatment with MM284 had similar effects on disease manifestation (less bilirubinuria, improved weight gain) indicates that the drug inhibited the inflammatory response after RRV infection rather than RRV infection itself. We found that MM284 administration also reduced mRNA expression of a number of proinflammatory mediators downstream from SMAD2 activation, which was also inhibited, suggesting that extracellular cyclophilin plays a role in the inflammation associated with this model of BA. Given that the major signaling receptor for extracellular cyclophilins is CD147 (30), our data suggest that interaction between extracellular cyclophilins and CD147 plays a significant role in inflammation and hepatic pathology in experimental BA. Indeed, several reports have demonstrated activation of the CD147-induced signaling pathways by CypA circulating in extracellular media in a number of inflammatory diseases (31). CypA has been shown to be secreted by different types of cells in response to inflammatory stimuli and oxidative stress (32–36). While the cells responsible for secretion of extracellular cyclophilin in our BA model have not been identified, hepatocytes may be involved as these cells have been shown to secrete CypA in response to infection by another virus, hepatitis B (37). HSCs are another type of cells that could contribute to the etiology of BA, although we did not assess whether HSCs secrete CypA in the BA model used in this study. Further experiments isolating hepatocytes and HSCs from the liver would be required to sort out the relative importance of these two cell types, however, such experiments are technically challenging in neonatal mice.

CD147 is expressed on a wide variety of cells, which potentially can respond to extracellular cyclophilin and contribute to the pathogenesis of inflammatory diseases. Several beneficial effects of MM284 treatment on the experimental BA progression identified in this study allow us to infer possible mechanisms for the effects of extracellular cyclophilins in experimental BA. Most likely, the reduction of neutrophil infiltration in the livers of MM284-treated BA animals due to inhibition of chemotactic activity of extracellular cyclophilins (31) is the key component. Indeed, the inflammatory response in BA, both in the mouse model and in humans, is associated with infiltration by neutrophils, which form inflammatory foci around the areas of bile duct obstruction (21–23). Modulation of neutrophil infiltration by the inhibition of extracellular cyclophilins or their receptor CD147 has been reported in rheumatoid arthritis (38,39), lung inflammation (14) and myocardial ischemia and reperfusion injury (40). The decreased migration of neutrophils and other inflammatory cells to the liver may explain the decreased levels of IL-6 observed in MM284-treated animals in our study. Downregulation of IL-6 by MM284 is consistent with previous reports demonstrating induction of IL-6, IL-8, IL-1β, MCP-1 and tumor necrosis factor (TNF)-α in monocytes by extracellular CypA (24,25). Another cell type likely contributing to disease pathogenesis are HSCs, as our data showed activation of SMAD2 in human HSCs after CypA exposure and inhibition of this effect by MM284 treatment, mimicking the effects observed with whole liver. Activation (phosphorylation) of SMAD2 is a known mediator of increased expression of such markers of fibrosis as PAI-1, TIMP-1, TIMP-4 and MMP-7 (26), and inhibition of the expression of TIMP-4 and MMP-7 by MM284 was observed in our study. Suppression by MM284 of SMAD2/3 activation suggests a novel, previously unrecognized activity of extracellular cyclophilins. Previously, SMAD activation was considered a characteristic feature of the TGFβ signaling pathway (41), raising the possibility that TGF was involved in the pathogenesis of BA. However, we did not observe any improvement in disease status or reduction of SMAD2/3 activation after treatment of mice with anti-TGFβ antibody or TGFβ soluble receptor (data not shown). Our results thus indicate that extracellular cyclophilin may initiate the SMAD-dependent signaling pathway directly. SMAD activation by extracellular cyclophilins may be mediated by activation of ERK, a known effector of cyclophilin-CD147 interaction (42). Crosstalk between ERK and SMAD2/3 pathways has been reported in certain cell types (43) and may well occur in the liver as well. Figure 5 outlines the proposed mechanism by which CypA and MM284 may influence the ongoing inflammation in experimental BA, summarizes the findings of this study and indicates remaining questions that will be addressed in future experiments.

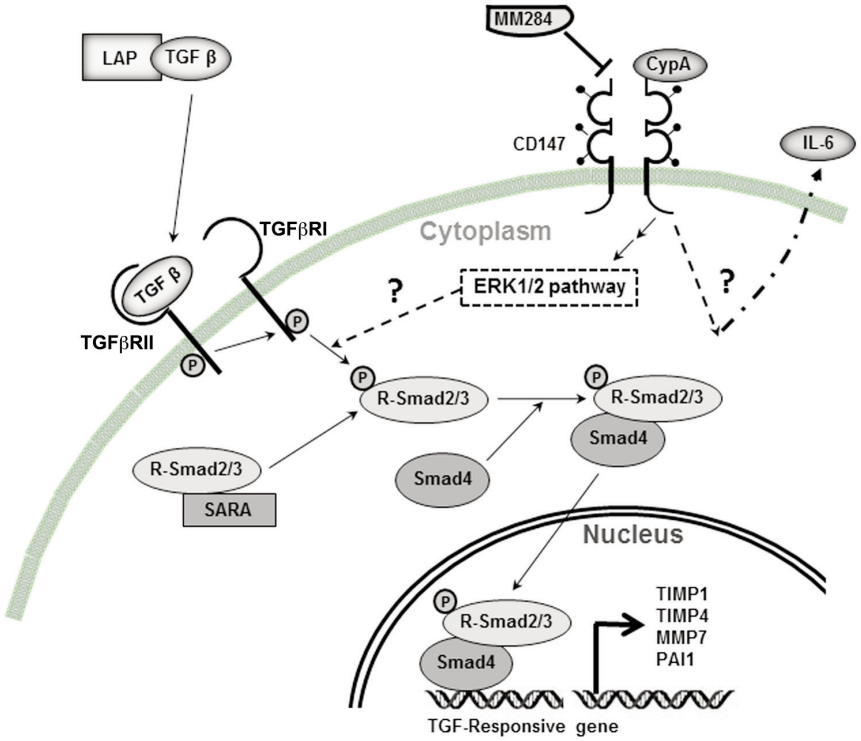

Figure 5. Schematic representation of the effects of extracellular CypA and MM284 in experimental BA. Extracellular CypA produced by resident liver cells attracts inflammatory cells and stimulates them to produce IL-6. It also activates the SMAD pathway resulting in production of TIMP-4, MMP-7 and other mediators. Question marks denote effects with unknown mechanism.

CONCLUSION

In summary, our study adds biliary atresia to the list of inflammatory diseases where extracellular cyclophilins may play a role in exacerbating disease pathology. While it would be interesting to confirm the role of CypA in experimental BA using CypA knockout mice, the specificity of the RRV model to BALB/c mice renders those experiments unfeasible at present, as the knockout mice have a different strain as their background. Our findings suggest that extracellular cyclophilins are important contributors to the liver inflammation associated with experimental BA. Most importantly, results reported here provide a validation for development of cell-impermeable cyclosporine A derivatives as therapeutics for treatment of BA or other inflammatory diseases of the liver where SMAD2 activation may be involved. As this animal model is not well suited to address fibrotic liver disease,

whether or not extracellular cyclophilins in general or CypA in particular may be putative targets for therapy once fibrosis or cirrhosis has been established remains in question.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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