Accelerated and Progressive and Lethal Liver Fibrosis in Mice That Lack Interleukin (IL)-10, IL-12p40, and IL-13Rα2

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BACKGROUND & AIMS: Progressive fibrosis contributes to the morbidity of several chronic diseases; it typically develops slowly, so the mechanisms that control its progression and resolution have been difficult to model. The proteins interleukin (IL)-10, IL-12p40, and IL-13Rα2 regulate hepatic fibrosis following infection with the helminth parasite *Schistosoma mansoni*. We examined whether these mediators interact to slow the progression of hepatic fibrosis in mice with schistosomiasis. **METHODS:** *IL-10*$^{-/-}$, *IL-12/23(p40)*$^{-/-}$, and *IL-13Rα2*$^{-/-}$ mice were crossed to generate triple knockout (TKO) mice. We studied these mice to determine whether the simultaneous deletion of these 3 negative regulators of the immune response accelerated mortality from liver fibrosis following infection with *S mansoni.* **RESULTS:** Induction of inflammation by *S mansoni*, liver fibrosis, and mortality increased greatly in TKO mice compared with wild-type mice; 100% of the TKO mice died by 10 weeks after infection. Morbidity and mortality were associated with the development of portal hypertension, hepatosplenomegaly, gastrointestinal bleeding, ascites, thrombocytopenia, esophageal and gastric varices, anemia, and increased levels of liver enzymes, all features of advanced liver disease. IL-10, IL-12p40, and IL-13Rα2 reduced the production and activity of the profibrotic cytokine IL-13. A neutralizing antibody against IL-13 reduced the morbidity and mortality of the TKO mice following *S mansoni* infection. **CONCLUSIONS: IL-10, IL-12p40, and IL-13Rα2 act cooperatively to suppress liver fibrosis in mice following infection with *S mansoni*. This model rapidly reproduces many of the complications observed in patients with advanced cirrhosis, so it might be used to evaluate the efficacy of antifibrotic reagents being developed for schistosomiasis or other fibrotic diseases associated with a T-helper 2 cell-mediated immune response.**

Keywords: Th2 Response; Mouse Model; Immune Regulation; T-Cell Response.

Liver fibrosis occurs in a variety of clinical settings and is typically associated with chronic diseases, including alcoholism, persistent or untreated infectious diseases (including viral hepatitis and schistosomiasis), and autoimmune hepatitis. Many cases of liver fibrosis in Western societies are linked with nonalcoholic fatty liver disease and associated with the rise in obesity and type 2 diabetes mellitus.[1] Although the initiating stimuli vary, inflammation and fatty changes in the liver lead to hepatic cell damage and death. This in turn generates an immunologic and tissue repair response. In chronic disease, the tissue repair response results in excess collagen deposition and compromised liver function.[2–4]

Immunoregulatory responses governing liver fibrosis have been modeled in mice infected with the parasitic helminth *Schistosoma mansoni*.[5] The parasite eggs become trapped in hepatic portal venules and induce granulomatous inflammation characterized by CD4$^+$ T-helper (Th) 2 cells, producing interleukin (IL)-4, IL-5, and IL-13.[6] The hepatic granulomas in turn elicit a tissue remodeling response, including the induction of matrix metalloproteinases, tissue inhibitors of metalloproteinases, and collagens.[7] In untreated human schistosomiasis, the repetitious cycle of tissue damage, inflammation, and fibrosis can lead to severe life-threatening complications such as portal hypertension, variceal bleeding, and ultimately death, usually after several years of infection.[8]

In addition to schistosome-induced liver fibrosis, other murine models have been developed to help identify factors that regulate liver fibrosis. These include carbon tetrachloride exposure, alcohol-induced fibrosis, and bile duct ligation.[9] Although these models have provided many important insights on the initiation of fibrosis, they often fail to replicate the complications associated with chronic hepatic fibrosis, which include the development of portal hypertension, formation of esophageal varices, ascites, and anemia.[10] Development of these comorbid conditions requires repeated injury to the liver and typically develops over a sustained period. Indeed, other than the chronic schistosome infection model, there are relatively few experimental models of fibrosis that generate the pathological sequelae associated with chronic and progressive disease. Because the primary goal of our research on liver fibrosis is to prevent or slow the development of these life-threatening sequelae,[11] the murine model of schistosomiasis provides a useful system to

Abbreviations used in this paper: IFN, interferon; IL, interleukin; KO, knockout; Th, T-helper; TKO, triple knockout; WT, wild-type.

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evaluate the efficacy of antifibrotic therapies in a well-defined experimental model of chronic liver fibrosis.[12]

Several studies have shown that the Th2-associated cytokine IL-13 serves as the principal driver of fibrosis following *S mansoni* infection.[13–15] For example, mice deficient in IL-13 have reduced fibrosis compared with wild-type (WT) mice despite similar worm burdens and granulomatous inflammation. Consequently, *il13*$^{-/-}$ and *il-13Rα1*$^{-/-}$ mice survive infection much longer than their WT cohorts.[15,16] We have been investigating the mechanisms that regulate IL-13 activity[17] and have identified 3 distinct negative regulatory pathways. These include IL-13Rα2, a high-affinity decoy receptor for IL-13[14]; IL-12p40, a key driver of Th1 and Th17 responses[18]; and IL-10, a potent immunosuppressive cytokine.[19] Here, we intercrossed IL-13Rα2$^{-/-}$, IL-12/23(p40)$^{-/-}$, and IL-10$^{-/-}$ mice to generate a triple knockout (TKO) mouse and examined whether the progression to liver fibrosis was accelerated in the absence of 3 negative regulatory mechanisms.

Materials and Methods

Mice

Female BALB/c, BALB/c IL-13Rα2$^{-/-}$, BALB/c IL-10$^{-/-}$, BALB/c IL-12/23(p40)$^{-/-}$, BALB/c IL-10$^{-/-}$IL-13Rα2$^{-/-}$, BALB/c IL-10$^{-/-}$IL-12/23(p40)$^{-/-}$, and BALB/c IL-10$^{-/-}$ IL-12/23(p40)$^{-/-}$ IL-13Rα2$^{-/-}$ (all gene knockout [KO] mice were backcrossed ≥10 generations to BALB/c) mice 6 to 8 weeks of age were maintained at National Institute of Allergy and Infectious Diseases animal facilities at Taconic (Germantown, NY). All mice were housed under specific pathogen-free conditions at the National Institutes of Health in an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility in accordance with the procedures in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Infections and Treatments

Mice were infected percutaneously via the tail with 25 to 35 cercariae of a Puerto Rican strain of *S mansoni* (Naval Medical Research Institute) (Biomedical Research Institute, Rockville, MD). Anti–IL-13 treatment was performed with rat anti-mouse IL-13 monoclonal antibody (CNTO 134; immunoglobulin [Ig] G2a isotype; Centocor, Inc, Horsham, PA).

RNA Isolation, Purification, and Real-Time Polymerase Chain Reaction

Total RNA was prepared from whole liver tissue samples as previously described.[16] Real-time polymerase chain reaction was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA).[16]

Histopathology and Fibrosis

Tissues were fixed in Bouin–Hollande fixative and embedded in paraffin for sectioning and staining as described previously.[16] Liver collagens were measured as hydroxyproline after hydrolysis of 200 mg of liver in 5 mL of 6N HCl. The same individual scored all histologic features and had no knowledge of the experimental design.

Hematology

EDTA-treated blood was processed at the National Institutes of Health Clinical Center for automated counting using a Vista Analyzer (Siemens, Deerfield, Ill).

Occult Fecal Blood

Seracult Single Slide (Propper Manufacturing Co, Inc, Long Island City, NY) were used as a diagnostic tool to measure Fecal Occult Blood. Fecal pellets from individual mice were obtained at 8 weeks postinfection and dispersed in saline using a Precellys 24 tissue homogenizer (Bertin Technologies, Rockville, MD). A total of 0.10 mL of fecal specimen was applied to the slide and read for positive peroxidase activity within 60 seconds.

Intracellular Cytokine Staining

Leukocytes isolated from the livers of infected mice were stimulated for 3 hours with phorbol 12-myristate 13-acetate (10 ng/mL), ionomycin (1 mg/mL), and brefeldin A (10 mg/mL). Cell surfaces were stained with phycoerythrin-indodicarbocyanine–conjugated antibody to CD4 (anti-CD4; H129.19), fixed for 20 minutes at 25°C in 2% (wt/vol) paraformaldehyde, made permeable for 30 minutes with 0.1% saponin buffer, and further stained with fluorescein isothiocyanate–conjugated anti–interferon (IFN)-γ (XMG1.2), phycoerythrin-conjugated anti–IL-13 (C531; Centocor, Horsham, PA), Alexa Fluor 647–conjugated anti–IL-4 (11B11), and allophycocyanin-conjugated anti–IL-5 (TRFK5) before being analyzed on a FACSCalibur (BD, Franklin Lakes, NJ). Antibodies were from BD PharMingen (San Diego, CA) except where noted otherwise.

Statistics

All data were analyzed with GraphPad Prism (GraphPad software version 5, La Jolla, CA), and statistical significance ($P <$ .05) was determined using a 2-tailed unpaired Student *t* test with a 95% confidence interval. Unless specified in the figure legends, all experiments and analyses were performed at least twice.

Results

S mansoni*–Induced Inflammation, Fibrosis, and Mortality Are Increased in TKO Mice*

Mice infected with *S mansoni* develop liver disease due to abundant egg deposition in the liver from long-lived worm pairs. Despite the heavy liver damage, *S mansoni* infection in WT mice rarely causes death. This low mortality is attributed to host immunomodulatory mechanisms that regulate potentially harmful aspects of the immune response.[12] We were interested in examining the outcome of *S mansoni* infection in mice for which specific down-modulatory mechanisms were genetically deleted. Groups of BALB/c mice and mice with targeted deletions of IL-10, IL-12/23(p40), and IL-13Rα2 (IL-10/IL-12/23(p40)/IL-13Rα2$^{-/-}$ designated "TKO") were exposed to 25 to 35 *S mansoni* cercariae. As shown previously, BALB/c mice survived *S mansoni* infection through week 12 (Figure 1*A*).[20] In contrast, TKO mice displayed 100% mortality at acute infection. All TKO mice died by week 10 postinfection, 3 to 4 weeks after the onset of egg deposition in the liver. We examined whether the mortality observed in TKO mice correlated with an increase in liver fibrosis. Groups of BALB/c and TKO