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Distinct from its canonical effects, deletion of IL-12p40 induces cholangitis and fibrosis in interleukin-2Rα$^{-/-}$ mice

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ABSTRACT

The IL-12 family modulates T cell mediated autoimmune diseases and GWAS in PBC have suggested a critical role of IL-12 and its subunits in modulating portal inflammation. We have taken advantage of an aggressive model of portal inflammation and colitis in IL-2Rα$^{-/-}$ mice to study the specific role of IL-12 and, in particular, the immunobiology of p40$^{-/-}$IL-2Rα$^{-/-}$ mice. Colonies of IL-2Rα$^{+/-}$, IL-2Rα$^{-/-}$ and p40$^{-/-}$IL-2Rα$^{-/-}$ mice were studied for the natural history of immunopathology in liver and colon using histology and immunohistochemistry. Further, to focus on mechanisms, liver, spleen and mesenteric lymph node flow cytometry was employed to identify specific phenotypes; cytokine analysis on inflammatory cell populations was compared between groups. Finally, Real-Time PCR was used to focus on the genes involved in hepatic fibrosis. Surprisingly, p40$^{-/-}$IL-2Rα$^{-/-}$ mice manifest more severe portal inflammation and bile duct damage, including signs of portal hypertension and liver fibrosis, but a significant reduction in colitis. Indeed, p40$^{-/-}$IL-2Rα$^{-/-}$ mice reveal a profound hepatic CD8^{+} T cell infiltrate, whose major component are effector memory cells as well as enhanced hepatic Th1 but reduced Th17 responses. These observations were confirmed by Real-Time PCR analysis of fibrosis-related genes in the liver. Distinct from its canonical effects, IL-12p40 plays a critical role in autoimmune cholangitis, including hepatic fibrosis. These data take on striking significance for any proposed human trials that modulate the IL-12p40 pathway in human PBC.

1. Introduction

Primary biliary cirrhosis (PBC) is an autoimmune disease characterized by loss of tolerance to mitochondrial autoantigens, destruction of small bile ducts, and in some patients the development of fibrosis and cirrhosis [1–3]. We have previously reported that IL-2Rα$^{-/-}$ mice develop spontaneous autoimmune cholangitis with serologic findings and histopathology that resemble human PBC [4]. Importantly, however, these mice also develop inflammatory bowel disease (IBD); deletion of CD4 reduces colonic inflammation, whereas deletion of CD8 attenuates bile duct damage and portal inflammation [5]. Enhanced Th1 and Th17 responses are

Abbreviations: ALT, serum alanine aminotransferase; ANOVA, one-way analysis of variance; CCL2, chemokine (C–C motif) ligand 2; dnTGFβRII, dominant negative form of transforming growth factor beta receptor type II; GWAS, genome-wide association studies; HSC, hepatic stellate cell; IFN-γ, interferon γ; IL-2Rα$^{-/-}$, interleukin-2 receptor alpha knockout; mLNs, mesenteric lymph nodes; MNCs, mononuclear cells; p40$^{-/-}$, interleukin-12p40 knockout; PBC, primary biliary cirrhosis; Th1, T helper 1; TNF-α, tumor necrosis factor alpha.

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likewise found in IL-2Rα$^{-/-}$ mice compared to wild type controls [6]. Finally, our earlier work demonstrated increased serum levels of IL-12p40 in IL-2Rα$^{-/-}$ than in IL-2Rα$^{+/-}$mice [4].

There has been considerable interest in the IL-12 family in a variety of autoimmune diseases, and in particular, in human PBC based on GWAS (genome wide association studies) [7,8]. The interleukin 12 family, including IL-12, IL-23, IL-27 and IL-35, are heterodimeric cytokines consisting of an α-chain and a β-chain [9]. The p40 chain can pair with p35 or p19 chains to form IL-12 or IL-23; the latter is critical for Th1/Th17 cells, respectively [10]. In fact, IL-12p40 and its expression modulates a number of T cell mediated autoimmune diseases, including autoimmune myocarditis (AMC), collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveitis (EAU), and systemic lupus erythematosus (SLE) [11–15]. We report herein the striking data that deletion of p40 exacerbates autoimmune cholangitis, induces liver fibrosis, yet reduces colitis. In PBC, we propose that in the liver, IL-12p40 acts as a negative regulator of inflammation.

2. Material and methods

2.1. Mice

IL-2Rα$^{-/-}$ (B6.129S4-Il2ratm1Dw) and p40$^{-/-}$ (B6.129S1-Il12btm1Jm) on a C57BL/6J background were initially obtained from The Jackson Laboratory (Bar Harbor, ME), and thence bred at the University of California Davis Animal Vivarium. The mice studied herein were maintained in individually ventilated cages under specific pathogen-free conditions. As IL-2Rα$^{-/-}$ mice are infertile, IL-2Rα$^{+/-}$mice were crossed with p40$^{-/-}$ mice to generate p40$^{+/-}$IL-2Rα$^{+/-}$mice, which were then backcrossed with p40$^{-/-}$ mice to generate p40$^{-/-}$IL-2Rα$^{+/-}$mice. IL-2Rα$^{-/-}$ mice and IL-2Rα$^{+/-}$mice were bred using IL-2Rα$^{+/-}$mice, and p40$^{-/-}$IL-2Rα$^{-/-}$ mice were bred using p40$^{-/-}$IL-2Rα$^{+/-}$mice. As both the IL-12p40 mutant gene and the IL-2Rα mutant gene contain a neo gene, we identified the IL-12p40 wild type gene by PCR and the IL-2Rα genotype by flow cytometry at 3–4 weeks of age. Identification of the IL-2Rα genotype was based on mean fluorescent intensity of CD25. All mice were studied from 12 to 18 weeks of age and animal experiments conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals by the Laboratory Animal Center, University of Science and Technology of China.

2.2. Histology

Liver and colon sections were prepared and immediately fixed with 4% paraformaldehyde for 1–2 days and then embedded in paraffin. Paraffin-embedded liver tissues were cut into 4-μm slices; paraffin-embedded colon tissues were cut into 6-μm slices. All slices were deparaffinized, and stained with hematoxylin and eosin. Quantification of histology was based on the previous reports [16–18]. Histological scores were evaluated by a "blinded" pathologist using the following scales: First, for portal inflammation: 0, no inflammatory cells; 1, several mononuclear cells infiltration; 2, mild lympho-plasmacytic infiltration without interface hepatitis; 3, moderate lympho-plasmacytic infiltration with/without interface hepatitis or mild lympho-plasmacytic infiltration with interface hepatitis; 4, moderate-severe lympho-plasmacytic infiltration with interface hepatitis. Second, the scoring of bile duct damage was as follows: 0, no bile duct damage; 1, bile duct showing mild degeneration with surrounding lymphocytes; 2, bile duct showing clear degeneration with surrounding lymphocytes, or bile duct loss. Third, fibrosis was scored as follows: 0, no fibrosis; 1, fibrous expansion of portal areas with short fibrous septa; 2, fibrous expansion of portal areas with occasional bridging fibrosis; 3, fibrous expansion of portal areas with marked bridging fibrosis with occasional nodules (incomplete cirrhosis); 4, cirrhosis, probable or definite. Finally, colon histopathology was scored as follows: 0, no significant changes; 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with mild to moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; 3, moderate inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; and 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion, and loss of intestinal glands. Azan staining was also used for visualizing fibrosis on liver tissue sections.

Table 1
Real Time PCR primers used in this study.

Genes	Forward (5′-3′)	Reverse (5′-3′)
Acta2	AAGAGCATCCGACACTGCTGAC	AGCACAGCCTGAATAGCCACATAC
Col1a1	CAGGGTATTGCTGGACAACGTG	GGACCTTGTTTGCCAGGTTCA
CCL2	TAAAAACCTGGATCGGAACCAAA	GCATTAGCTTCAGATTTACGGGT
Ebi3	GGAACAGAGCCACAGAGCAT	AGAGCCACGAGAGCTGTTTC
IFNγ	TAGCCAAGACTGTGATTGCGG	AGACATCTCCTCCCATCAGCAG
IL4	ACAGGAGAAGGGACGCCAT	GAAGCCCTACAGACGAGCTCA
IL6	CCACTTCACAAGTCGGAGGCTTA	CCAGTTTGGTAGCATCCATCATTTC
IL7	CTTGTTCTGCTGCCTGTCAC	CTTGCGAGCAGCACGATTTAG
IL10	GCCAGAGCCACATGCTCCTA	GATAAGGCTTGGCAACCCAAGTAA
IL15	CATCCATCTCGTGCTACTTGTGTT	CATCTATCCAGTTGGCCTCTGTTT
IL17A	ACTACCTCAACCGTTCCACG	TTCCCTCCGCATTGACACAG
IL17F	CAGCCATTGGAGAAACCAGC	CTGCTTTGGGGTTCTTCCGA
IL18	CAGGCCTGACATCTTCTGCAA	TCTGACATGGCAGCCATTGT
IL21	CTTCGTCACCTTATTGACATTGTTG	CCAGGGTTTGATGGCTTGA
IL22	GGTGACGACCAGAACATCCA	GACGTTAGCTTCTCACTTTCCTT
IL27a	TCGATTGCCAGGAGTGAACC	AAGTGTGGTAGCGAGGAAGC
Timp1	TGAGCCCTGCTCAGCAAAGA	GAGGACCTGATCCGTCCACAA
TGFβ1	GTGTGGAGCAACATGTGGAACTCTA	CGCTGAATCGAAAGCCCTGTA
TNF	AAGCCTGTAGCCCACGTCGTA	AGGTACAACCCATCGGCTGG
Gapdh	CATGGCCTTCCGTGTTCCTA	CCTGCTTCACCACCTTCTTGAT

2.3. Immunohistochemistry

All tissues were handled using our standardized microwave protocol [19]. After deparaffinization and microwave heating for antigen retrieval, rabbit polyclonal antibodies against alpha-smooth muscle actin (SMA) (ABBiotech, San Diego, CA), collagen type 1 (Novotech, Sydney, Australia), or desmin (DAKO, Glostrup, Denmark) were applied and incubated under intermittent microwave irradiation. After washing with TBS, Envision-peroxidase for rabbit polyclonal antibodies (DAKO, Glostrup, Denmark) was applied and incubated under intermittent microwave treatment. As a substrate of peroxidase, 3,3′-Diaminobenzidine (DAB, Vector, Burlingame, CA) was applied for 5 min. Hematoxylin was used as a counter staining.

2.4. Hepatic hydroxyproline

Briefly, frozen liver tissue was weighed and homogenized in distilled H_2O. Hydroxyproline was released from tissue homogenates by acid hydrolysis, thence the free hydroxyproline was oxidized by chloramine T; the addition of Ehrlich's reagent resulted in the formation of a product with a wavelength maximum at 558 nm. Total hydroxyproline content was measured and data presented per gram of wet weight liver tissue [20].

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