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Deletion of IL-6 in dnTGFβRII Mice Improves Colitis but Exacerbates Autoimmune Cholangitis

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Abstract

The role of IL-6 in autoimmunity attracts attention because of the clinical usage of mAbs to IL-6R, designed to block IL-6 pathways. In autoimmune liver disease, activation of the hepatocyte IL-6/STAT3 pathway is associated with modulating pathology in acute liver failure, liver regeneration and in the murine model of Concanavalin A-induced liver inflammation. We have reported that mice expressing a dominant negative form of transforming growth factor β receptor (dnTGFβRII) under control of the CD4 promoter develop both colitis and autoimmune cholangitis with elevated serum levels of IL-6. Based on this observation, we generated IL-6 deficient mice on a dnTGF-βRII background (dnTGFβRII IL-6−/−) and examined for the presence of anti-mitochondrial antibodies, levels of cytokines, histopathology and immunohistochemistry of liver and colon tissues. As expected, based on reports of the use of anti-IL6R in inflammatory bowel disease, dnTGFβRII IL-6−/− mice manifest a dramatic improvement in their inflammatory bowel disease, including reduced diarrhea and significant reduction in intestinal lymphocytic infiltrates. Importantly, however, autoimmune cholangitis in dnTGFβRII IL-6−/− mice was significantly exacerbated, including elevated inflammatory cytokines, increased numbers of activated T cells and worsening hepatic pathology. The data from these observations emphasize that there are distinct mechanisms involved in inducing pathology in inflammatory bowel disease compared to autoimmune cholangitis. These data also suggest that patients with inflammatory bowel disease...
may not be the best candidates for treatment with anti-IL-6R if they have accompanying autoimmune liver disease and emphasize caution for therapeutic use of anti-IL6R antibody.

**Keywords**

Interleukin 6; TNF-α; Colitis; Autoimmune cholangitis; Cholangiocyte proliferation

IL-6 is a glycoprotein of 212 amino acids with pleiotropic effects that include modulating Th1/Th2 ratios (1), cell maturation and TH17 differentiation (1,2), generation of acute phase proteins, induction of inflammation and finally an oncogenic role in multiple myeloma (3), colorectal cancer (4) and hepatocellular carcinoma (5). In the liver, the major sources of IL-6 are Kupffer cells, liver-resident monocyte-derived macrophages and intra-hepatic biliary epithelial cells (BEC) (6). IL-6 mediates its biological effect by either binding to its cognate classical membrane-anchored IL-6R or by forming complexes with soluble IL-6R (sIL-6R) with subsequent binding of the complex to gp130 (7). sIL-6R is generated via proteolysis from the membrane bound receptor or by translation from alternatively spliced mRNA (8). The IL-6/sIL-6R complex is sufficient to bind to gp130 and thence induce intracellular signaling. The presence of two signaling pathways is important as it facilitates and expands the effects of IL-6 to both membrane-anchored IL-6R expressing and non-expressing cells (9–11).

Elevated levels of multiple pro-inflammatory cytokines, including IL-6, have been demonstrated in both the serum and liver of patients with PBC (12,13). Further, we have previously reported that several spontaneous murine models of PBC including IL-2Rα−/− mice (14), Scurfy mice (15) and dnTGFβRII mice (16) manifest elevated sera levels of IL-6 and that such levels increase with age, particularly in dnTGFβRII mice. A variety of therapeutic approaches are being utilized to block IL-6 in humans including the blockage of IL-6 binding to its receptor IL-6R, blockage of IL-6/IL-6R complex binding to gp130 and blocking intracytoplasmic signaling through gp130 (17–19). To directly address the role of IL6 in murine PBC, we introduced IL-6−/− onto the dnTGFβRII background. Since these mice on a normal diet develop both colitis and autoimmune cholangitis, the generation of dnTGFβRII IL-6−/− mice facilitated our ability to study the effect of an IL-6R knockout on both disease processes. Importantly, we report herein that while deletion of IL-6 leads to a dramatic improvement in inflammatory bowel disease, these mice develop worsened biliary pathology.

**Materials and Methods**

**Animals**

B6.129S6-Il6tm1Kopf mice were purchased from Jackson Laboratory (Bar Harbor, ME). dnTGFβRII mice were bred on a C57BL/6 background at the University of California Davis vivarium. To generate dnTGFβRII IL-6−/− mice, IL-6−/− mice were mated with dnTGFβRII mice to obtain an F1 generation (dnTGFβRII IL-6+/−). F1 male mice were subsequently backcrossed onto female IL-6−/− mice to derive dnTGFβRII IL-6−/− mice. Mice were screened for IL-6 and TGFβRII dominant negative genotype by PCR using prepared genomic DNA as previously described (16). All mice were maintained in individually ventilated cages under specific pathogen-free conditions. Experiments were performed following approval from the University of California Animal Care and Use Committee.
Experimental protocol

Groups of dnTGFβRII IL-6−/− mice, and control dnTGFβRII animals were followed and serially evaluated for the presence and levels of anti-mitochondrial antibodies and serum cytokines. At 22 weeks of age, animals were sacrificed and their liver and colon processed as below. In addition, liver mononuclear cells were isolated and subjected to phenotypic analysis by standard flow cytometry.

Anti-mitochondrial antibodies

Serum anti-mitochondrial antibodies (AMAs) were evaluated using recombinant PDC-E2 (14,20,21), including known positive and negative standards. Briefly, one μg recombinant PDC-E2 antigen in 100 μl carbonate buffer (pH 9.6) was coated onto 96-well ELISA plates at 4°C overnight. Plates were washed with PBS containing 0.05% Tween-20 (PBST) (Fisher Biotech, Fair Lawn, NJ), then blocked with 200 μl of 1% BSA in PBS for 1 hour at room temperature. 100 μl of diluted sera (1:250) was added to each well and incubated at room temperature for 1 hour. Plates were washed with PBST for at least 3 times. 100 μl of horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Zymed, San Diego, CA) diluted (1:3000) in PBS with 1% BSA was added into each well and incubated for 1 hour at room temperature. Plates were re-washed and 100 μl of TMB peroxidase substrate (BD Biosciences) was added to each well. Optical density (OD) was read at 450 nm.

Flow Cytometry

Mononuclear cells were isolated from liver tissue using density gradient centrifugation with Accu-Paque (Accurate Chemical & Scientific Corp., Westbury, NY). Anti-mouse CD16/32 (clone 93, Biolegend) was used to block the Fc receptor prior to staining. The mononuclear cells were stained with fluorochrome-conjugated antibodies including Alexa Fluor 750–conjugated anti-TCR-β (clone H57-597, eBiosciences), Alexa Fluor 647–conjugated anti-CD19 (clone eBio1 D3, eBiosciences), PerCP-conjugated anti-CD4 (clone RM4-5, Biolegend), FITC-conjugated anti-CD8α (clone 53-6.7, Biolegend), APC-conjugated anti-CD44 (clone IM7, Biolegend) and PE-conjugated anti-NK1.1 (clone PK136, BD-PharMingen, San Diego, CA). Stained cells were analyzed using a FACScan flow cytometer (BD Bioscience) that was upgraded by Cytec Development (Fremont, CA), which allows for five-color analysis. Data were analyzed utilizing CELLQUEST software (BD Bioscience). Appropriate known positive and negative controls were used throughout.

Serum and Hepatic Cytokine Assay

TNF-α, IFN-γ, IL-6, were measured quantitatively by the mouse inflammatory Cytometric Bead Array (CBA) kit and the mouse Th1/Th2 cytokine CBA kit (BD Biosciences, San Jose, CA). Serum and hepatic IL-12p40 was evaluated using mouse IL-12/IL-23 p40 allele-specific DuoSet ELISA development kit (DY499 R&D Systems, Minneapolis, MN).

Histopathology

Immediately after sacrifice, the liver was harvested, fixed in 4% paraformaldehyde (PFA) at room temperature for 2 days, embedded in paraffin, and cut into 4-mm sections. The liver sections were de-paraffinized, stained with hematoxylin and eosin (H&E), and evaluated using light microscopy. For evaluation of bile duct proliferation, 100 portal tracts were examined in each specimen and a score was given, as noted in Figure 3A. For example, based upon the blinded review of the pathologist, if there were no proliferating ductules then the score was zero. If the number were greater than 0 but less than 10%, the score was 1. If between 10 and 25%, the score was 2; between 25 and 50%, the score was 3 and if greater than 50%, the score was 4. Mice with scores between 1 and 2 were considered to have mild bile ductular proliferation. A score of 3 was considered to have moderate proliferation.
whereas a score of 4 was considered as severe. Mouse mAb CK22 against human cytokeratin (GeneTex, San Antonio, TX) is crossreactive with murine cytokeratin(s) expressed by biliary epithelial cells (22) and was used for immunohistochemical staining of liver sections as previously described (23). The M.O.M. kit (Vector, Burlingame, CA) was used for special blocking. The large bowel was removed and similarly evaluated by histology and immunohistochemistry as previously described (24).

Statistical Analysis
The data are presented as the mean ± SEM. Two-sample comparisons were analyzed using the two-tailed unpaired t-test. A value of \( p < 0.05 \) was considered statistically significant.

Results
Depletion of IL-6 prevents inflammatory bowel disease in dnTGFβRII mice
As expected, while sera from the dnTGF-βRII IL-6−/− mice showed no detectable levels of IL-6 (data not shown), sera from the dnTGFβRII mice showed significant levels of IL-6, which increased with age (Figure 1A). dnTGFβRII mice had clinical manifestations of inflammatory bowel disease, including diarrhea and loss of body weight. These changes were not seen in dnTGF-βRII IL-6−/− mice (Figure 1B). Histologic examination of the large bowel of dnTGFβRII mice disclosed chronic inflammation and granulomatous reactions, including the presence of multi-nucleated giant cells (Figure 1C). There was chronic and active inflammation with cryptitis and crypt abscess throughout the large intestine of dnTGFβRII mice. Importantly, there were no detectable histologic abnormalities in the intestinal tissues of the dnTGF-βRII IL-6−/− mice.

dnTGF-βRII IL-6−/− mice manifest exacerbated autoimmune cholangitis
Unlike the clinical improvement in the colon, dnTGF-βRII IL-6−/− mice demonstrate a significant \( p < 0.05 \) increase in absolute number of hepatic mononuclear cells as compared with dnTGF-βRII control mice at age of 22–24 weeks (Figure 2). Flow cytometric data demonstrated that the absolute number of TCRβ+NK1.1− T cell lineages and CD19+ B cells are also significantly \( p < 0.01 \) elevated in the liver of dnTGF-βRII IL-6−/− mice, associated with a significant increase in the absolute cell number of CD44-expressing T cells. Furthermore, there was a considerable increase (graded moderate to severe) in bile ductular proliferation in liver sections of dnTGF-βRII IL-6−/− mice (Figure 3A) as compared with liver sections from dnTGF-βRII mice that showed mild to moderate bile ductular proliferation (Figure 3B).

A characteristic histopathological feature of human PBC is granuloma formation. Indeed, dnTGFβRII mice frequently demonstrate hepatic granuloma formation (Figure 4). Interestingly, no granulomas were detected in dnTGF-βRII IL-6−/− mice. All histologic evaluations were performed in a blinded fashion.

Serum cytokine and AMA levels
The level of serum TNFα was significantly decreased in dnTGFβRII IL-6−/− mice compared to dnTGFβRII mice (19.5 ± 2.9 pg/ml versus 28.5 ± 2.2 pg/ml; \( p < 0.05 \)). There were no significant differences in serum IFNγ (dnTGFβRII IL-6−/− mice: 10.3 ± 1.4 pg/ml versus dnTGFβRII mice: 19.3 ± 5.0 pg/ml; \( p > 0.05 \)) or serum IL-12p40 (dnTGFβRII IL-6−/− mice: 664.0 ± 91.4 pg/ml versus dnTGFβRII mice: 865.7 ± 223.5 pg/ml; \( p > 0.05 \)) in the two groups of mice (Figure 5A). Although not statistically significant, the levels of both IFNγ and IL-12p40 were decreased in the serum of dnTGFβRII IL-6−/− compared to the dnTGFβRII mice. The levels in liver were comparable between dnTGFβRII IL-6−/− and dnTGFβRII mice (Figure 5A, B). However, the levels of hepatic TNFα and IFNγ were significantly \( p <
increased in dnTGFβRII IL-6−/− compared to dnTGFβRII mice (Figure 5B). There was also a significant decrease (p < 0.01) in serum titers of anti-mitochondrial antibodies in dnTGFβRII IL-6−/− mice (0.20 ± 0.02 at O.D. 450 nm; n=8) compared to the control dnTGFβRII mice (0.47 ± 0.06 at O.D. 450 nm; n=6).

Discussion

We have previously reported that autoimmune cholangitis in dnTGF-βRII mice has features strikingly similar to human PBC, including portal cell infiltrates and anti-mitochondrial antibodies with the identical specificity as humans (16). We have also demonstrated that depletion of IL-12p40 strongly inhibits the appearance of autoimmune cholangitis in dnTGFβRII mice, which indicates the critical obligatory requirement of IL-12p40 signaling in the pathogenesis of autoimmune cholangitis (25). Discussion of other anti-inflammatory and regulatory roles of mononuclear subsets in both patients and our animal models have been discussed elsewhere (13,26–32). Furthermore, autoimmune cholangitis can be transferred to Rag−/− recipients using splenic derived CD8 T cells from dnTGFβRII mice. In contrast, inflammatory bowel disease can be transferred in the identical system through the use of splenic derived CD4 T cells (33). We found the same pathological dichotomy here. Thus, depletion of IL-6 in this model leads to dramatic improvement of inflammatory bowel disease but is accompanied by a significant increase in hepatic inflammation.

IL-6 has attracted and continues to attract significant attention as a means to modulate immune function and reduce inflammation. This is best exemplified by the proposed usage of monoclonal antibodies to IL-6R in patients with inflammatory bowel disease (34). IL-6 was originally identified as an essential B cell differentiation factor that activates B cells to produce immunoglobulin (2,35) exemplified by the IL-6 dependent anti-DNA antibody production in a murine pristane-induced lupus model (36). Yet the data herein demonstrate that liver lymphocytic infiltration and biliary proliferation became worse in dnTGFβRII IL-6−/− compared with dnTGFβRII mice despite a decrease of anti-mitochondrial antibodies in the dnTGFβRII IL-6−/− mice. In this respect, it is important to note that our laboratory has also reported that depletion of B cells in dnTGFβRII mice, using another double construct animal, the dnTGFβRIIμ−/− mouse, led to reduced inflammatory bowel disease but exacerbated autoimmune cholangitis (22). Herein we also note that the liver of the dnTGFβRII IL-6−/− mice not only show significant increases in liver infiltrates but these mice also show an increase in biliary duct proliferation as compared to similarly aged dnTGFβRII mice. Nonetheless, it is interesting to note the absence of granuloma in the dnTGFβRII IL-6−/− mice.

Biliary ductular proliferation has been proposed as an important factor in the initiation and progression of biliary cirrhosis (37–39). Proliferating intrahepatic biliary epithelial cells are a prominent feature of autoimmune cholangitis in our NOD.c3c4 mouse model (40). However, the molecular mechanisms responsible for the pathogenesis of cholangiocyte proliferation and biliary cirrhosis are not well understood. Data from several studies have suggested the involvement of IL-6 on cholangiocyte proliferation, but the data have been conflicting. Firstly, IL-6-mediated activation of the STAT3 pathway and the p44/p42 mitogen-activated protein kinase pathways have been reported to be at least partially responsible for LPS-induced cholangiocyte proliferation (41). Rat cholangiocytes responded to LPS by a marked increase in cell proliferation and IL-6 secretion. Anti-IL-6 neutralizing antibody inhibits LPS-induced proliferation of cholangiocytes (42). In contrast, studies using IL-6 deficient mice suggests that animals that lack the IL-6/gp130/STAT3 signaling pathway develop more severe biliary cirrhosis following BDL (43). IL-6 is well known to have a mitogenic effect on BEC and therefore it is not surprising that the increased levels of IL-6 would promote DNA synthesis in quiescent normal BEC (6,44,45). However, IL-6 is
also reported to inhibit proliferation of hepatocytes (46,47). Hence, the effect of IL-6 on BEC will be dependent on the cell cycle and also upon the stage of disease. On the other hand, in the presence of chronic inflammation, elevated levels of IL-6 could induce cell cycle arrest.

We also note that there are multiple compensatory mechanisms associated with IL-6 deficiency. For example, another BEC mitogen, leukemia inhibitory factor (LIF), is increased in IL-6 \(^{-/-}\) mice after BDL (43). This finding is consistent with data that hepatic levels of TNFα are likely responsible for cholangiocyte damage, mutagenesis of biliary epithelial cells (48,49) and high levels of TNFα have been suggested to correlate with progression of PBC (50). In our study, hepatic expression of TNFα becomes significantly increased in the absence of IL-6. Thus, this may be the explanation as to why lack of IL-6 exacerbates cholangiocyte proliferation in dnTGFβRII II-6 \(^{-/-}\) mice. There is clearly a complex interrelationship between genetics and environment in inflammatory bowel disease and IL-6, and the IL-6 signaling pathways are critical factors in the effector mechanisms of inflammation (34,51,52). Thus, for example, levels of IL-6 in sera correlate with disease severity in inflammatory bowel disease and the blockage of IL-6 trans-signaling with a neutralizing antibody against IL-6R suppresses T-cell responses in experimental colitis (53). It is important to note that sIL-6R had been reported in the colonic mucosa of IBD patients (54). These data are consistent with our own report and provides further evidence for the pathogenic role of IL-6 in experimental colitis.

In addition to IBD, other autoimmune diseases are accompanied by elevated levels of sIL-6R. It is not surprising therefore that therapeutic approaches have been focused on blockage of the IL-6/IL-6R/gp130 pathway, including tocilizumab (INN, or atlizumab), a humanized monoclonal antibody against the IL-6 receptor proposed as a therapeutic agent for rheumatoid arthritis, systemic onset juvenile idiopathic arthritis (JIA), adult-onset Still’s disease, Castleman’s disease and Crohn’s disease (55,56). Amongst the adverse events reported with this therapy, are abnormal liver function tests (57). Based on our current study, we suggest that therapeutic usage of IL-6 for autoimmune diseases be used with caution in patients who have evidence of liver disease.

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**Abbreviations**

- **dnTGF-β RII**
  - dominant negative form of transforming growth factor β receptor
- **IL-6**
  - interleukin 6
- **AMAs**
  - anti-mitochondrial antibodies
- **PBC**
  - primary biliary cirrhosis
- **BEC**
  - biliary epithelial cells
- **H&E**
  - hematoxylin and eosin
- **PDC-E2**
  - pyruvate dehydrogenase-E2
- **LPS**
  - lipopolysaccharide
- **BDL**
  - bile duct ligation
References


Figure 1.
Knockout of IL-6 prevents inflammatory bowel disease in dnTGF-βRII IL-6−/− mice. (A) Levels of IL-6 in the sera from dnTGF-βRII mice at age of 12–14 weeks and 22–24 weeks using an inflammatory cytokine Cytometry Bead Array kit. (B) Body weights (gms) of dnTGF-βRII IL-6−/− as compared with dnTGF-βRII mice at 12–14 weeks and 22–24 weeks. (Mean ± SEM; dnTGF-βRII, n=6; dnTGF-βRII IL-6−/−, n=7; * p < 0.05, ** p < 0.01) (C–D) Representative H&E analysis of small and large intestinal tissue sections from dnTGF-βRII and dnTGF-βRII IL-6−/− mice at 22–24 weeks. (dnTGF-βRII, n=9; dnTGF-βRII IL-6−/−, n=10)
Increased liver lymphatic infiltration in dnTGF-βRII IL-6−/− mice compared with dnTGF-βRII mice. Mononuclear cells were isolated from 22–24 wk-old murine liver tissues pretreated with FcR blocking antibody and stained with a panel of fluorochrome-conjugated mAbs. The cells were subjected to flow cytometric analysis and absolute numbers of a) therapeutic mononuclear cells (MNC), TCRβ+ T cells, CD19+ B cells determined; b) absolute number of CD4+ and CD8+ T cells determined; c) absolute number of CD4+, CD44+ and CD8+ CD44+ cells determined. (Mean ± SEM; dnTGF-βRII, n=6; dnTGF-βRII IL-6−/−, n=7; * p < 0.05, ** p < 0.01)
dnTGF-βRII IL-6−/− mice developed severe bile ductular proliferation. Sections of liver sample were prepared from 22–24 wk old dnTGF-βRII and dnTGF-βRII IL-6−/− mice. (A) The degree of bile ductular proliferation in liver tissues from dnTGF-βRII and dnTGF-βRII IL-6−/− mice was scored. (Mean ± SEM; dnTGF-βRII, n=9; dnTGF-βRII IL-6−/−, n=10; ** p < 0.01) Score 0: None; 1: <10%; 2: 10–25%; 3: 25–50%; 4: >50%. Mice with scores between 1 and 2 were considered to have mild bile ductular proliferation; score of 3 was considered to have a moderate proliferation; score of 4 was considered as a severe proliferation.

(B) Immunostaining of liver sections using anti-cytokeratin cocktail (CK22) highlighting bile ducts showed more severe bile ductular proliferation in dnTGF-βRII IL-6−/− mice than dnTGF-βRII mice. Data shown are representative from a dnTGF-βRII and 10 dnTGF-βRII IL-6−/− mice.
Figure 4.
Deletion of IL-6 inhibits hepatic granuloma formation. H&E staining of mouse liver sections from 22–24 wk old dnTGF-βRII mice showing granuloma changes in both portal area and parenchyma area, while no such pathological changes detected in dnTGF-βRII IL-6<sup>−/−</sup> mice. Data shown are representative of 9 dnTGF-βRII and 10 dnTGF-βRII IL-6<sup>−/−</sup> mice.
Figure 5.
Elevated expression levels of TNF-α and IFN-γ in dnTGF-βRII IL-6−/− liver. Cytokine concentrations was measured using an inflammatory cytokine Cytometry Bead Array kit for TNF-α and IFN-γ and IL-12p40 levels using the IL-12/IL-23p40 allele-specific DuoSet ELISA development kit. (A) TNF-α and IFN-γ concentrations (Mean ± SEM, dnTGF-βRII n=9, dnTGF-βRII IL-6−/− n=9; * p < 0.05) and IL-12p40 levels (Mean ± SEM, dnTGF-βRII n=6, dnTGF-βRII IL-6−/− n=7; p > 0.05) in sera from 22–24 wk old mice. (B) Whole protein was extracted from liver of 22–24 wk old dnTGF-βRII mice and the following cytokine levels determined (Mean ± SEM; dnTGF-βRII, n=6; dnTGF-βRII IL-6−/−, n=7; * p < 0.05). Cytokine expression levels in 22–24 wk old mouse liver.