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Chemokine Receptor CXCR3 Deficiency Exacerbates Murine Autoimmune Cholangitis by Promoting Pathogenic CD8+ T Cell Activation

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Abstract

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Author contributions:

Hong-Di Ma, Yun-Yun Fei and Zhe-Xiong Lian designed the experiments. Hong-Di Ma conducted most of the experiments and analyzed the data. Wen-Tao Ma, Qing-Zhi Liu, Zhi-Bin Zhao and Mu-Zi-Ying Liu contributed to some of the experiments. Koichi Tsuneyama scored the H&E-stained liver sections. Jin-Ming Gao provided important experiment materials. William M. Ridgway, Aftab A. Ansari and Eric M. Gershwin helped to discuss about research design and manuscript writing. Hong-Di Ma, Eric M. Gershwin and Zhe-Xiong Lian wrote the manuscript.

CXC Chemokine Receptor 3 (CXCR3) is functionally pleiotropic and not only plays an important role in chemotaxis, but also participates in T cell differentiation and may play a critical role in inducing and maintaining immune tolerance. These observations are particularly critical for autoimmune cholangitis in which CXCR3 positive T cells are found around the portal areas of both humans and mouse models of primary biliary cholangitis (PBC). Herein, we investigated the role of CXCR3 in the pathogenesis of autoimmune cholangitis. We have taken advantage of a unique CXCR3 knockout dnTGF β RII mouse to focus on the role of CXCR3, both by direct observation of its influence on the natural course of disease, as well as through adoptive transfer studies into Rag $^{-/-}$ mice. We report herein that not only do CXCR3 deficient mice develop an exacerbation of autoimmune cholangitis associated with an expanded effector memory T cell number, but also selective adoptive transfer of CXCR3 deficient CD8 $^{+}$ T cells induces autoimmune cholangitis. In addition, gene microarray analysis of CXCR3 deficient CD8 $^{+}$ T cells reveal an intense pro-inflammatory profile. Our data suggests that the altered gene profiles induced by CXCR3 deficiency promotes autoimmune cholangitis through pathogenic CD8 $^{+}$ T cells. These data have significance for human PBC and other autoimmune liver diseases in which therapeutic intervention might be directed to chemokines and/or their receptors.

Keywords

Interferon-gamma Induced Chemokines; Primary Biliary Cholangitis; CD8T cell gene expression profile; T-bet; KLRG1

1. Introduction

CXCR3 is a chemokine receptor highly expressed on effector T cells and critically involved in both T cell trafficking and the differentiation of T cells into distinct functional subsets [1]. CXCR3 is upregulated following CTL and Th1 cell differentiation [2]. CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 are the natural ligands of CXCR3 [3–5]. CXCR3 is functionally pleiotropic; it plays an important role in the chemotaxis of Tregs to sites of inflammation following binding [6, 7], but also participates in T cell differentiation [5, 8]. CXCR3 expression on T cells alters the balance between effector and memory CD8 $^{+}$ T cell generation [9]. Finally, the CXCL11-CXCR3 chemokine axis polarizes naive T cells and repolarizes effector CD4 $^{+}$ T cells into IL-10 $^{\text{hi}}$ Tr1 cells to induce an immune-tolerizing state [10]. Our lab has been studying the pathogenesis of human PBC and during the course of these studies reported significant increases in the levels of soluble IP-10 (CXCL10) and MIG (CXCL9) in PBC and also a marked increase in the frequency of CXCR3 expressing T cells in both blood and liver [11]. An epigenetic study of DNA from PBC revealed aberrant demethylation of the CXCR3 promoter in CD4 $^{+}$ T cells [12]. DnTGF β RII mice, transgenic for directed expression of a dominant-negative form of TGF β receptor type II (dnTGF β RII), under the direction of the CD4 promoter [13], develop autoimmune cholangitis [14]. DnTGF β RII mice mimic several key phenotypic features of human PBC, including spontaneous production of AMAs, lymphocytic liver infiltration with periportal inflammation and an inflammatory cytokine profile. Furthermore, using adoptive transfer studies we have demonstrated that the CD8 $^{+}$ T cell is pathogenic effector population [15]. In this study, we have advanced our work by developing CXCR3 gene deleted dnTGF β RII

(TG) mice. We report herein that knocking-out CXCR3 significantly worsens autoimmune cholangitis and that the mechanism is secondary to generation of a hyper-activated CD8⁺ T cells which promote biliary pathology. These data are significant for human autoimmune disease in which blockade of CXCR3 or its ligands is proposed.

2. Materials and methods

2.1 Mice

dnTGFβRII (TG) mice on a C57BL/6 back-ground (B6.Cg-Tg(Cd4-TGFBR2)16Flv/J) [13] were initially derived from Jackson Laboratory. The maintenance and genetic monitoring of this colony has been previously reported [14]. CXCR3 knockout mice on a C57BL/6 background were previously established by gene targeting [16] and were kindly provided by Dr. Bao Lu (Harvard Medical School, Boston, MA, USA). dnTGFβRII CXCR3^{-/-} (TGC3) mice were obtained by selectively backcrossing TG mice with CXCR3 knockout mice. 8–14 week old female TGC3 mice were used in all experiments. B6/Rag1^{-/-} mice (Ly5.2) were obtained from Jackson Laboratory. 8–9 week old female Rag1^{-/-} mice were used as recipients in our CD8⁺ T adoptive transfer model. All mice were housed in a specific pathogen-free and controlled environment (22°C, 55% humidity, and 12-h day/night rhythm) and care provided according to the regulations of animal care at University of Science and Technology of China (Hefei, Anhui, China).

2.2 RNA Preparation, Reverse Transcription and Quantitative Real-Time PCR

Total RNA was extracted from the liver of wide type B6 and TG mice using Trizol (Invitrogen, Carlsbad, CA, US). M-MLV Transcriptase (Invitrogen) was used for reverse transcription. Quantitative real-time PCR was performed using Premix Ex Taq (Takara, Kusatsu, Shiga, Japan). The expression levels of target genes (Cxcl9 and Cxcl10) were normalized to the housekeeping gene β2-microglobulin and the results calculated by Ct [17]. Known standards were included throughout. Primer sequences were based on Primer-Bank (<http://pga.mgh.harvard.edu/primerbank>) and blasted to confirm the target genes using Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). The primer sequences used were as follows: β2-microglobulin, 5′-CCGAACATACTGAACTGCTACGTAA-3′ and 5′-CCCGTTCTTCAGCATTTGGA-3′; Cxcl9, 5′-AATGCACGATGCTCCTGCA-3′ and 5′-AGGTCTTTGAGGGATTTGTAGTGG-3′; Cxcl10, 5′-GCCGTCATTTTCTGCCTCA-3′ and 5′-CGTCCTTGCGAGAGGGATC-3′. The primers were synthesized by Sangon Biotech (Shanghai, China).

2.3 Enzyme-Linked Immunosorbent Assays (ELISA)

TG and TGC3 mice livers were homogenized in PBS and centrifuged for 5 minutes at 5000 × g; supernatants were assayed using a CXCL9/MIG Quantikine ELISA Kit or Mouse CXCL10/IP-10/CRG-2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, US). Serum anti-PDC-E2 (AMA) was measured by ELISA with purified recombinant PDC-E2 as previously described [18] and including positive and negative controls.

2.4 Serum cytokines

Serum concentration of IFN- γ , TNF- α and IL-6 from TG and TGC3 mice were quantitated using a mouse Th1/Th2/Th17 CBA kit (BD Biosciences, Franklin Lakes, NJ, US), using FACSVerse flow cytometer (BD Bioscience). Data were analyzed by FCAP Array™ v3.0.1 (BD Bioscience) Software.

2.5 Liver tissue preparation and histological scoring

Liver from TG and TGC3 mice were fixed with 4% paraformaldehyde, embedded in paraffin and cut into 4 μ m sections for hematoxylin-eosin (H&E) staining. Liver portal inflammation scores were “blindly” performed on H&E-stained liver sections using a set of 4 indices by a “blinded” pathologist (K.T.), based on the levels of inflammation surrounding middle-large portal tract; the indices were scored as 0, none; 1, mild; 2, moderate; and 3, severe inflammation.

2.6 Preparation of hepatic mononuclear cells and Flow Cytometric Analysis

Hepatic mononuclear cells were isolated as described [19]. For flow cytometry analysis, hepatic mononuclear cells were blocked by incubation with anti-mouse CD16/32 (Biolegend, San Diego, CA, US) and stained with fluorochrome-conjugated antibodies (Abs) at 4°C in PBS with 0.2% bovine serum albumin for 20 minutes. All fluorochrome-conjugated Abs, unless otherwise noted, were purchased from Biolegend. To identify liver CD8⁺ T cells and different T cell subsets, hepatic mononuclear cells from TG and TGC3 mice were stained with PacificBlue-CD3 (17A2), APC-Cy7-CD8 α (19517), V500-CD4 (RM4-5, BD Bioscience), PE-Cy7-NK1.1 (PK136), PE-CXCR3 (CXCR3-173), FITC-CD44 (IM7), PerCP/Cy5.5-CD62L (MEL-14), APC-KLRG-1 (2F1/KLRG-1). Intracellular transcriptional factor T-bet was stained by PerCP/Cy5.5-T-bet (4B10) and Alexa 647-Foxp3 (MF14). Normal IgG isotype controls (Biolegend) were used as controls.

For intracellular cytokine staining, hepatic mononuclear cells were stimulated in vitro with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience, San Diego, CA, US) in RPMI-1640 (Life Technology, Carlsbad, CA, US) with 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, US) at 37°C, 5% CO₂ for 4 hours. Cells were thence fixed and permeabilized with BD Cytotfix/Cytoperm™ Fixation/Permeabilization Kit (BD Bioscience), and stained with PE-IFN- γ (XMG1.2). Stained cells were analyzed using a FACSVerse (BD Bioscience) flow cytometer. Flow data were calculated using Flow Jo software (Tree Star, Ashland, OR, US).

2.7 Adoptive transfer

Adoptive transfer was performed as described by our laboratory [15]. Splenic cells were collected from 7–8 week old female TG and TGC3 mice and CD8⁺ T cells were purified by positive selection with anti-CD8 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). 8–9 week old female Rag1^{−/−} mice were used as recipients. Specifically, 1 $\times$ 10⁶ purified CD8⁺ T cells were injected i.v. into Rag1^{−/−} recipients. 8 weeks after transfer, the livers from recipients were harvested and the total number of hepatic mononuclear cells were calculated and donor-derived CD8⁺ T cells were analyzed by flow cytometer.

2.8 Gene-expression profiling analysis of liver CD8⁺T cells

CD8⁺ T cells from the livers of 13–14 week old female TG and TGC3 mice were first enriched by positive selection with anti-CD8 magnetic microbeads (Miltenyi Biotech) and then purified by FACS Aria (BD Bioscience). The CD8⁺ T cell sorting purity was greater than 95%. Sorted CD8⁺ T cells were suspended in Trizol (Invitrogen). Total RNA was extracted and purified using an miRNeasy Mini Kit (Cat.#217004, QIAGEN, GmBH, Germany) and checked for a RIN number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Cat.# 5190-2305, Agilent technologies). Labeled cRNA, using an RNeasy mini kit (Cat.# 74106, QIAGEN), were hybridized with a Gene Expression Hybridization Kit (Cat.# 5188-5242, Agilent technologies). Slides were scanned using an Agilent Microarray Scanner (Cat.# G2565CA) and data extracted with Feature Extraction software 10.7. Raw data were normalized using the Quantile algorithm, Gene Spring Software 12.6.1. The transcription profile chip service was provided by Shanghai Biotechnology Cooperation (Shanghai, China). Gene classification was based on the annotation in the Kyoto Encyclopedia of Genes and Genomes (KEGG) data base. Heat map and red-green scale schemes were designed using Multiple Experiment Viewer 4.8 software. The GEO accession number of the data is GSE84218. The access to the GEO data is <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ovoleiikzhiprad&acc=GSE84218>.

2.9 Statistical analysis

All data were presented as the mean $\pm$ standard deviation (SD). The significance of differences was determined using a two-tailed unpaired Student's t test in GraphPad Prism. All experiments were replicated at least three times. The significance levels are marked * P < 0.05; **P < 0.01; *** P < 0.001.

3. Results

3.1 Increased CXCR3 ligands are associated with increased frequencies of Hepatic CXCR3 expressing T cells

As noted in Figs. 1A and 1B, the levels of Cxcl9 and Cxcl10 were significantly upregulated in the liver of 12–14 week old female dnTGF β RII (TG) mice compared with age and sex-matched wild type (WT) B6 littermates both at the message (Cxcl9 P=0.0026, Cxcl10 P=0.0060) and the protein level (P < 0.0001). These increased chemokine levels were associated with increased frequencies (Figure 1D,E and Figure S1) and absolute numbers of CXCR3 expressing CD8⁺ T (P< 0.0001) and CD4⁺ T cells (P< 0.0003) in the liver of 16 week old female TG mice as compared with WT littermates as determined by flow cytometry (P < 0.0001) (Figure 1C). In addition, there was an age associated increase in the frequencies (Figure 1D,E and Figure S1) and numbers of both CXCR3 positive CD8⁺ T (P < 0.0001) and CD4⁺ T (P=0.007) cells (Figure 1C).

3.2 CXCR3 deficiency promotes autoimmune cholangitis

Histological studies of liver from 13–14 week old female dnTGF β RII CXCR3 $^{-/-}$ (TGC3) mice revealed a significant increase of inflammatory infiltrates within the portal tract (Figure 2A) compared with age-matched WT dnTGF β RII (P=0.0005, Figure 2C). The increased total number of hepatic mononuclear cells in TGC3 mice correlated with histological score (P=0.0011, Figure 2B). Further, deletion of the CXCR3 gene led to the spontaneous development of elevated levels of anti-mitochondrial Abs (AMAs)-PDC-E2 (P=0.0370) in TGC3 mice (OD of PDC-E2: 0.301 $\pm$ 0.152) compared with age matched TG mice (OD of PDC-E2: 0.180 $\pm$ 0.047) (Figure S2A). The increased inflammatory response in the liver of TGC3 mice was associated with increased levels of the inflammatory cytokines IL-6, IFN- γ and TNF- α compared with age matched TG mice (IL-6 P=0.0153, IFN- γ P=0.0157, TNF- α P=0.0275, Figure 2D). .

3.3 CXCR3 deficiency expands the effector memory T cell subset with pathogenic potential

The levels of absolute numbers of total, CD4 $^{+}$ and CD8 $^{+}$ T cells were all increased in liver from TGC3 mice compared with age matched TG mice (Total T cell P=0.0111, CD8 $^{+}$ T P=0.0143, CD4 $^{+}$ T P=0.0249), Figures 3A and 3B. Subset analysis of the CD8 $^{+}$ and CD4 $^{+}$ T cells in livers of TGC3 mice revealed a selective increase in the effector memory population (CD8Tem, P=0.0007; CD4Tem, P=0.0308) compared with age-matched TG mice (Figure 3C). However, the frequency of Foxp3 $^{+}$ Treg cells did not significantly change (Figure S3A). Further, there were higher percentages (Figure 4A) and absolute numbers (Figure S2B) of KLRG1 expressing CD8 $^{+}$ T and CD4 $^{+}$ T cells in TGC3 mice. These data suggest that CXCR3 deficiency in TGC3 mice led to selective differentiation of CD8 $^{+}$ T and CD4 $^{+}$ T cells into cytotoxic KLRG1 $^{+}$ CD8 $^{+}$ T (P=0.0016) and terminally differentiated KLRG1 $^{+}$ CD4 $^{+}$ T (P < 0.0001) cells (Figure 4B). These phenotypic changes were associated with increased levels of T-bet and IFN- γ expressing CD8 $^{+}$ T and CD4 $^{+}$ T cells in the livers of TGC3 mice compared with age-matched TG mice (Figures S3C and 4D). The relative fluorescence intensity (RFI) of T-bet (CD8 $^{+}$ T P < 0.0001, CD4 $^{+}$ T P=0.0001, Figure 4C) and the frequencies of IFN- γ $^{+}$ producing (CD8 $^{+}$ T P < 0.0001, CD4 $^{+}$ T P=0.0002, Figure 4E) hepatic CD8 $^{+}$ T and CD4 $^{+}$ T cells in TGC3 mice were significantly increased compared to TG mice.

3.4 CXCR3 deficient CD8 $^{+}$ T cells Promote Autoimmune Cholangitis

1 $\times$ 10 6 splenic CD8 $^{+}$ T cells from TG mice or TGC3 mice were intravenously transferred into Rag1 $^{-/-}$ recipients (Figure 5A). 8 weeks later, livers from recipients were harvested and mononuclear cells analyzed for the absolute numbers of different donor derived CD8 $^{+}$ T cells by flow cytometry. As seen in Fig.5B, the adoptive transfer of CD8 $^{+}$ T cells from TGC3 mice led to a marked increase in cellular infiltrates compared with adoptive transfer of CD8 $^{+}$ T cells from TG mice (P=0.0244). Phenotypic analysis of the cellular infiltrates demonstrated that the predominant phenotype of the donor infiltrates were composed of CD8 $^{+}$ T cells (P=0.0242, Figure 5C) that expressed the memory phenotype (P=0.0224, Figure 5D and Figure S4A) and increased frequencies of IFN- γ producing cells compared with Rag1 $^{-/-}$ recipients of CD8 $^{+}$ T cells from TG mice (P=0.0049, Figure 5E).

3.5 CXCR3 deficient CD8+ T cells display a pro-inflammatory and hyper-activated profile

Based on the data we obtained we next focused on potential intrinsic changes of CD8+ T cells. Gene expression profiles of CD8+ T cells isolated from livers of both the TG and TGC3 mice were analyzed by gene microarray. Genes coding for cytokine-cytokine receptor interaction related genes were over-expressed in CD8+ T cells from CXCR3 deficient TG mice (Figure 6A). The lack of CXCR3 led to the over-expression of a number of pro-inflammatory cytokines and cytokine receptors including Cxcl14, Tnfrsf25, Ifng and Csf2rb (Figure 6A). The genes related to cell cycling were also upregulated in CD8+ T cells from CXCR3 deficient TG mice (Figure 6B). Finally CXCR3 deficiency significantly suppressed the activity of leukocyte transendothelial migration and downregulated the expression of chemokine receptors (Figure 6C).

4. Discussion

Primary biliary cholangitis (PBC) is a progressive autoimmune liver disease characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts [20–24]. The etiology of PBC remains enigmatic, but is linked by a combination of genetic susceptibility and environmental factors [25–28]. Clearly, the immune response and biliary destruction is mediated by a multi-lineage innate and adaptive immune response [20, 23, 29]. In both humans and mouse models, there is a specific enhancement of autoantigen-specific CD4+ T and CD8+ T cells [15, 23, 30, 31]. We have also recently reported that intrahepatic terminally differentiated (KLRG1+) CD8+ T cells from dnTGFβRII mice are cytotoxic to cholangiocytes in vitro [32]. Definition of the earliest events that lead to biliary destruction will be critical for the identification of therapeutic targets.

We demonstrate herein that CXCR3 deficiency promotes the activation and proliferation of pathogenic CD8+ T cells and is accompanied by intrinsic alteration of the T cell gene expression profile. Our proposed model is illustrated in Figure 7 reflecting CD8+ T cell activation in the presence (up) and absence (down) of CXCR3 in autoimmune cholangitis. When CXCR3 is absent, the frequency of KLRG1+ terminal differentiated effector memory CD8+ T cells is upregulated, the expression of T-bet is increased and IFN-γ production enhanced in activated CD8+ T cells. At the same time, CXCR3 deficiency promotes the proliferation of CD8+ T cells, which results in more severe liver inflammation.

Chemokines and chemokine receptors guide the migration of effector cells into inflammatory sites, which are critical for the pathogenesis of autoimmune diseases [3, 4, 33, 34]. However, the observation in TGC3 mice that inflammatory cells accumulate within the inflammatory sites of liver in the absence of CXCR3 suggests that the pathogenic role of CXCR3 deficient CD8+ T cells does not require the chemotaxis of CXCR3 and is likely mediated either by other redundant chemokine(s) mediated chemotaxis or due to the absence of inhibitory signals normally provided by CXCR3 ligation. Thus, instead of reducing the inflammatory infiltration, CXCR3 deficiency aggravates autoimmune cholangitis by promoting pathogenic CD8+ T cell activity and proliferation. It is generally thought that chemokines participate in the process of T cell differentiation two ways. First, APCs, like DCs, may use chemokines to promote encounters with antigen-specific T cells [35, 36]. Second, chemokines expressed in specific lymphoid compartments may influence more

globally the positioning of T cells in particular microenvironments, to bring these cells in contact with the appropriate APCs or accessory cells important for their differentiation [9, 37, 38]. Chemokine receptors are 7-transmembrane GPCRs, and thus may transmit diverse signaling cascades upon binding to different ligands to determine T cell fate [10]. Specifically, CXCL10/CXCR3 interactions drive effector Th1 polarization via STAT1, STAT4, and STAT5 phosphorylation, while CXCL11/CXCR3 binding induces an immune-tolerizing state characterized by IL-10^{hi} (Tr1) and IL-4^{hi} (Th2) cells, mediated via p70 kinase/mTOR in STAT3- and STAT6-dependent pathways [10].

CXCR3 deficiency expands effector memory T cell number and promotes their pathogenic potential. Gene microarray data demonstrates that CXCR3 deficient CD8⁺ T cells differentially display an increased number of pro-inflammatory genes that likely contributed to hyper-activation and proliferation, suggesting that CXCR3 deficiency affects CD8⁺ T cell activation and proliferation in an intrinsic way. We hypothesize that CXCR3 deficiency alters the signaling pathway in CD8⁺ T cells associated with their activation and proliferation. Further, gene profile analysis suggests that a lack of CXCR3 leads to the over-expression of pro-inflammatory cytokines and cytokine receptors, i.e. Cxcl14, Tnfrsf25, Ifng and Csf2rb (Figure 6A), and influences the activation of key signaling pathways in CD8⁺ T cells. These effects promote the pathogenic character and increases the number of CD8⁺ T cells.

Since CXCR3 deficiency promotes pathogenic CD8⁺ T cell activity and proliferation, why is there a high level of CXCR3 and its ligands in not only the murine models of PBC but also patients with PBC? We note that CD8⁺ T cells accumulate in the livers of TG mice and in the CD8⁺ T cell transfer model even without CXCR3 expression, implying that the infiltration of pathogenic liver CD8⁺ T cells may be due to the activation and proliferation of intrahepatic CD8⁺ T cells unique to the specific microenvironment in PBC. Activated CD8⁺ T cells expand in PBC liver and produce more IFN- γ and therefore a positive feedback loop for inflammation. The upregulation of CXCR3 expression on CD8⁺ T cells by inflammation suppresses further activation and proliferation of CD8⁺ T cells in this feedback loop. Thus, CXCR3 expression on CD8⁺ T cells becomes an important regulator of liver inflammation.

Clearly our data supports the view that CXCR3 is functionally pleiotropic. CXCR3 does exist in allelic forms. Human CXCR3 has three splice variants: CXCR3-A, CXCR3-B and CXCR3-alt [39, 40]; CXCR3 ligands have distinct and non-redundant biological roles and likely explain the diversity of cellular behavior triggered by CXCR3 activation [41]. Activation of CXCR3-A appears to induce chemotaxis and proliferation [42, 43], while CXCR3-B activation inhibits migration and proliferation and induces apoptosis [44]. Since one form appears to confer cell activation and the other appears to inhibit cellular proliferation, it is possible that the inhibitory form of CXCR3 is critical for normal liver homeostasis and contributes to tolerance induction. In its absence the migratory cells mediate effector function unchecked. Thus, one needs to distinguish trafficking properties (chemotactic function) from signaling and inhibitory/activation function.

5. Conclusion

We report herein that CXCR3 deficient TG mice have a significant enhancement of autoimmune cholangitis. Further, the mechanism of this aggravation is secondary to hyper-activated CD8+ T cells which promote biliary pathology. Our data has significance not only in human PBC, but in other autoimmune liver diseases in which therapeutic intervention of chemokines and/or their receptors are contemplated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PBC	Primary biliary cholangitis
dnTGFβRII, TG	dominant negative transforming growth factor β receptor II
CXCR3	CXC Chemokine Receptor 3
WT	wide type
TGC3	dnTGFβRII CXCR3 ^{-/-}
MNCs	mononuclear cells
CTL	cytotoxic lymphocyte
APCs	antigen-presenting cells
DCs	dendritic cells
IFN-γ	interferon-γ
TNF-α	tumor necrosis factor alpha
IL-6	interleukin-6
MIG	monokine induced by gamma-interferon
IP-10	interferon-induced protein of 10 kDa
I-TAC	interferon-inducible T cell alpha chemoattractant

CXCL9	chemokine (C-X-C motif) ligand 9
CXCL10	chemokine (C-X-C motif) ligand 10
ELISA	enzyme-Linked Immunosorbent Assay
AMAs	anti-mitochondrial antibodies
Th1	T helper 1
Tem	effector memory T cells
KLRG1	killer cell lectin-like receptor G1
CAMs	cell adhesion molecules
KEGG	Kyoto Encyclopedia of Genes and Genomes
GPCRs	G protein-coupled receptors

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Highlights

- Chemokine receptor CXCR3 deficiency promotes autoimmune cholangitis.
- CXCR3 deficiency expands the effector memory T cell subset with pathogenic potential in the murine PBC model.
- CXCR3 deficient CD8⁺ T cells display a pro-inflammatory and hyper-activated profile in gene expression.

Figure 1. CXCR3 ligands-CXCL9, CXCL10-and CXCR3 expressing T cell number increased in liver of autoimmune cholangitis model

(A) mRNA levels of *Cxcl9*, *Cxcl10* in liver of control B6 (WT) mice (n=5) and dnTGFβRII (TG) mice (n=5). (B) CXCL9 and CXCL10 levels in liver homogenates were determined by ELISA in WT (n=8) and TG (n=7) mice. (C) Number of CXCR3 expressing CD8⁺ T cells (CXCR3⁺CD3⁺CD8⁺NK1.1⁻) and CXCR3 expressing CD4⁺ T cells (CXCR3⁺CD3⁺CD4⁺NK1.1⁻) in liver of 16 week old WT mice (n=4), 8 week old TG mice (n=5) and 16 week old TG mice (n=5) by flow cytometry. Representative FACS plots show

CXCR3 expression patterns of CD8+ T (D) and CD4+ T cells (E). Graphs reflect mean $\pm$ SD. **P <0.01, ***P <0.001.

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Figure 2. CXCR3 deficiency exacerbates autoimmune cholangitis

(A) H&E-stained livers of female TG and dnTGF β RII CXCR3 $^{-/-}$ (TGC3) mice at 13 to 14 weeks of age. (B) Absolute number of hepatic mononuclear cells from TG mice (n=7), TGC3 mice (n=7). (C) Scores of portal inflammation in TG (n=7) and TGC3 mice (n=9). (D) Concentrations of inflammatory cytokines in serum of TG (n=10) and TGC3 mice (n=8). Graphs reflect mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. CXCR3 deficiency increases effector memory T cell numbers

Total cell numbers of T (A), CD8+ and CD4+ cells (B), CD8Tem (CD3+NK1.1-CD8+CD44+CD62L-) and CD4Tem (CD3+NK1.1-CD4+CD44+CD62L-) cells (C) in liver of TG (n=7) and TGC3 (n=7) mice by flow cytometry. Graphs reflect mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4. CXCR3 deficiency promotes T cell activation

(A) Representative FACS plots demonstrate KLRG1 expression patterns of CD8⁺ T (upper panel, gated on CD3+NK1.1-CD8⁺ hepatic MNCs) and CD4⁺ T cells (lower panel, gated on CD3+NK1.1-CD4⁺ hepatic MNCs) from TG and TGC3 mice. (B) The frequency of KLRG1⁺ cells in liver CD8⁺ T and CD4⁺ T cells from TG (n=7) and TGC3 (n=7) mice are shown. (C) Relative fluorescence intensity (RFI=MFI/MFI_{iso}) of T-bet in liver CD8⁺ and CD4⁺ T cells from TG (n=4) and TGC3 (n=5) mice by intracellular staining. (D) Representative FACS plots demonstrating IFN- γ production in liver CD8⁺ T and CD4⁺ T cells from TG and TGC3 mice. (E) Percentage of IFN- γ producing cells in liver CD8⁺ T and CD4⁺ T cells from TG (n=4) and TGC3 (n=5) mice. Graphs reflect mean $\pm$ SD. **P < 0.01, ***P < 0.001.

Figure 5. CXCR3 deficient CD8⁺ T cells promote autoimmune cholangitis in adoptive transfer
 (A) Schematic diagram shows the strategy of CD8⁺ T cell transfer. (B) The total number of liver mononuclear cells from TG derived CD8⁺ T (TG-CD8T) recipients (n=4) and TGC3 derived CD8⁺ T (TGC3-CD8T) recipients (n=4) was compared. The absolute numbers of liver CD8⁺ T cells (C), CD8 Tem cells (D) and IFN- γ producing ability of liver CD8⁺ T cells (E) in TG-CD8T recipients (n=4) and TGC3-CD8T recipients (n=4) by flow cytometry.

Figure 6. Gene expression profile of CXCR3 deficient CD8⁺ T cells

Differentially expressed genes from TG-CD8T (left) and TGC3-CD8T (right) cells were classified by functional category. Heat maps reflect signal values of the listed genes. Cytokine-cytokine receptor interaction related genes are shown in panel (A). Cell cycle associated genes are displayed in panel (B). Genes involved in cell chemotaxis and migration including leukocyte transendothelial migration (up), chemokine signaling pathway (middle) and cell adhesion molecules (CAMs) (bottom) are exhibited in panel (C).

Figure 7. CXCR3 deficiency promoted pathogenic CD8+ T cell proliferation and activity to aggravate autoimmune cholangitis

A schematic diagram of CD8+ T cell activation in the presence (up) and absence (down) of CXCR3 in autoimmune cholangitis. When CXCR3 is absent, the frequency of KLRG1+ terminal differentiated effector memory CD8+ T cells is upregulated, the expression of T-bet is increased and the ability of IFN-γ production is enhanced in activated CD8+ T cells. At the same time, CXCR3 deficiency also promotes the proliferation of CD8+ T cells, which exacerbates inflammation.