Research Article

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CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver

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Background & Aims: IL-17 secreting CD4 (Th17) and CD8 (Tc17) T cells have been implicated in immune-mediated liver diseases, but the molecular basis for their recruitment and positioning within the liver is unknown.

Methods: The phenotype and migratory behaviour of human liver-derived Th17 and Tc17 cells were investigated by flow cytometry and chemotaxis and flow-based adhesion assays. The recruitment of murine Th17 cells to the liver was studied *in vivo* using intra-vital microscopy.

Results: IL-17$^+$ T cells comprised 1–3% of the T cell infiltrate in inflammatory liver diseases and included both CD4 (Th17) and CD8 (Tc17) cells. They expressed RORC and the IL-23 receptor and included subsets that secreted IL-22 and interferon-γ. Th17 and Tc17 cells expressed high levels of CXCR3 and CCR6, Tc17 cells also expressed CXCR6. Binding to human sinusoidal endothelium from flow was dependent on β1 and β2 integrins, CXCR3, and, in the case of Th17 cells, VAP-1. Th17 recruitment via sinusoids in mice with liver inflammation was reduced by treatment with antibodies against CXCR3 ligands, confirming the role of CXCR3 in Th17 recruitment *in vivo*. In human liver, IL-17$^+$ cells were detected in portal infiltrates close to inflamed bile ducts expressing the CCR6 ligand CCL20. Cytokine-treated human cholangiocytes secreted CCL20 and induced CCR6-dependent migration of Th17 cells suggesting that local cholangiocyte chemokine secretion localises Th17 cells to bile ducts.

Conclusions: CXCR3 promotes recruitment of Th17 cells from the blood into the liver in both human and murine liver injury. Their subsequent positioning near bile ducts is dependent on cholangiocyte-secreted CCL20.

Introduction

Th17 are a distinct subset of CD4 effector cells [1,2] that develop under control of the nuclear receptor RORc in humans (RORγt in mice) [3,4], in response to antigen priming in an environment rich in IL-6 and TGF-β [5,6]. Th17 cells secrete cytokines IL-17A, IL-17F, IL-22, TNF-α, and IFN-γ [2] and provide protection against pathogens at mucosal sites [7,8]. Stimulation with IL-6, IL-21 or IL-1β and TGF-β increases expression of the IL-23 receptor [9] through which IL-23 stabilises the Th17 phenotype [2,9,10]. Both Th1 and Th17 cells have been implicated in inflammation and autoimmunity [11] and IL-23 shares the p40 subunit with the classical Th1 cytokine IL-12 [12]. In some antigen-driven models of autoimmunity, diseases can be transferred by Th17 cells alone although it is not yet certain how effective targeting IL-17 will prove in clinical disease [13,14].

Th17 cells play a role in chronic inflammatory liver diseases [2]. Numbers of circulating and intra-hepatic Th17 cells correlate with viral load and histological inflammation in chronic viral hepatitis [15,16] and the frequency of intra-hepatic Th17 cells correlates with disease severity in alcoholic liver disease [17]. Pro-inflammatory Th17 cells accumulate in hepatocellular carci-

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Abbreviations: Th17, interleukin-17 secreting CD4 T helper cells; Tc17, interleukin-17 secreting CD8 T helper cells; LIL, liver infiltrating lymphocytes; HSEC, hepatic sinusoidal endothelial cell; BEC, biliary epithelial cells; RORC, retinoic acid-related orphan receptor c; AIH, autoimmune hepatitis; HCV, chronic hepatitis C; PBC, primary biliary cirrhosis; ALD, alcoholic liver disease; NANB, non-A non-B acute hepatitis; NASH, non-alcoholic steato-hepatitis; NL, normal liver; CCL_4, carbon tetrachloride; ConA, concanavalin A; TNF-α, tumour necrosis factor-α; IFN-γ, interferon gamma; CFSE, carboxyfluorescein succinimidyl ester.

noma and promote disease progression [18]. In primary biliary cirrhosis, Th17 cells are detected in the portal tracts near damaged bile ducts [19]. Conversely, the Th17 cytokine IL-22 is hepato-protective in acute inflammation and ameliorates alcoholic liver injury [20,21]. We have recently reported high frequencies of Tc17 in human HCV-infected livers that correlate with control of disease progression [22].

Although both Th17 and Tc17 are detected in chronic hepatitis, little is known about the molecular basis of their recruitment and subsequent positioning within the liver. We had previously shown that both effector and regulatory T cells [23,24] used the chemokine receptor CXCR3 and its ligands to enter the liver via sinusoidal endothelium [23,25]. In the present study, we show that CXCR3 is also critical for Th17 recruitment from the blood into the inflamed liver and that CCR6 is involved in subsequent positioning at epithelial interfaces.

Materials and methods

Human blood and liver tissue were collected with informed consent at liver transplantation. C57BL/6 mice were obtained from existing colonies at the University of Birmingham.

Isolation of peripheral blood lymphocytes (PBL), liver-infiltrating lymphocytes (LIL), and biliary epithelial cells (BEC) was carried out using published methods described in Supplementary Materials and methods [23,24,26].

Human and murine Th17 cells isolation

IL-17 cells were isolated using IL-17 Enrichment & Detection Kit (Miltenyi; purity >95%). Tc17 and Th17 cells were generated from $CD4^+/CD8^+$ cells, murine splenocytes or human PBL stimulated with anti-CD3/CD28 beads in Iscove's Modified Dulbecco's Medium supplemented with Th17 polarising cytokines and anti-cytokine mAbs (see Supplementary Materials and methods).

CCL20 measurement

CCL20 chemokine was measured in human BEC supernatants by ELISA (Supplementary Materials and methods) and *CCL20* and *IL-17RA* mRNA was extracted from BEC and quantified by RT-PCR (Supplementary Materials and methods).

Immunohistochemistry and confocal microscopy

Human paraffin liver tissues were stained for immunohistochemistry and images captured with a Zeiss microscope (Supplementary Materials and methods) [23].

Flow cytometry

Freshly isolated LIL from human and murine livers were stained for surface and chemokine receptors before *in vitro* stimulation followed by intracellular cytokine and transcription factors staining (Supplementary Materials and methods).

Th17 chemotaxis

Primary BEC cultures were stimulated with IL-17A or medium alone for 24 h, supernatants collected and placed in the bottom wells of 5-μ pore transwells (Corning) with Th17 cells in the upper chamber in the presence or absence of blocking antibodies (Supplementary Materials and methods).

Flow-based adhesion assays

Recruitment of Th17/Tc17 by the hepatic endothelium *in vitro* was studied using a flow-based adhesion assay in which HSEC were cultured in micro-capillaries, stimulated for 24 h with TNF-α & IFN-γ prior to perfusion of cells at a wall shear stress of 0.05 Pa. Adherent cells were visualised by phase contrast microscopy (10× objective) (Supplementary Materials and methods).

Murine liver injury models and intra-vital microscopy

In vitro generated Th17 cells were labelled with 5 μM CFSE (Molecular Probes, Invitrogen) and 5×10^6 cells injected into mice with either ConA hepatitis or CCL_4-induced liver injury. Th17 interactions with hepatic vessels were imaged using intravital microscopy and a Sensicam CCD camera (Supplementary Materials and methods).

Statistical analysis

Data were analysed with Student's *t*-test when comparing numerical variables between two groups. One-way ANOVA analysis followed by Newman–Keul *post hoc* analysis or Bonferroni correction was used for comparisons between more than two groups. Statistical analyses were performed using GraphPad Prism software. A value of $p < 0.05$ was considered statistically significant. Data are presented as mean ± SEM.

Results

Distribution, frequency and subsets of IL-17 cells in human liver

Immunohistochemistry revealed that the normal human liver contained very few IL-17 cells (Fig. 1B and G) whereas numbers of intra-hepatic $IL\text{-}17^+$ cells increased in all chronic liver diseases studied (Fig. 1C–F, and G). $IL\text{-}17^+$ cells were detected in portal infiltrates (Fig. 1F) with preferential localization around bile ducts, particularly in PBC (Fig. 1D).

$IL\text{-}17^+$ cells comprised around 2–3% of the CD3 T cell infiltrate in liver disease, as defined by frequencies generated by immunohistochemical analysis (Fig. 1G) and by calculating the frequencies of $CD3^+$ IL-17 secreting cells, in cells freshly isolated from liver tissue by flow cytometry (Fig. 1H). $IL\text{-}17^+$ cells in tissue analysed using confocal microscopy revealed both CD3 and RORc expressing cells (Fig. 1I). These CD3 IL17 cells are composed of both Th17 ($CD4\ IL\text{-}17^+$) and Tc17 ($CD8\ IL\text{-}17^+$) cells (Fig. 2A and B). A proportion of Th17 also secreted IL-22. A $CD4^+$IL-22 producing population was detected consistent with previous reports on Th22 [28] (Fig. 1J and Table 1). Th17 cells included poly-secreting populations that expressed TNF-α and IFN-γ in addition to IL-17 (Fig. 1J and Table 1). Because T regulatory cells have been shown to secrete IL-17 at sites of inflammation, we looked for co-localisation of IL-17 and FoxP3. Although both $IL\text{-}17^+$ and $FoxP3^+$ cells were detected, no co-expression was observed, suggesting that regulatory T cells in the inflamed liver do not secrete IL-17 (Fig. 1K).

Frequency, phenotype, and chemokine receptors expression of human intra-hepatic Th17/Tc17 cells

Lymphocytes were freshly isolated from explanted human livers and analysed. Th17 and Tc17 cells were present in all liver diseases studied (Fig. 2A and B). Tc17 were also present in inflamed livers at slightly lower frequencies than Th17 (Fig. 2A and B). Very few liver-infiltrating Th17 and Tc17 cells were present in the normal liver. There was no significant difference between diseases (Fig. 2A and B).

Both Th17 and Tc17 expressed high levels of RORC and were found within the $CD161^{high}$ population, as previously reported (Supplementary Fig. 1C) [22]. Human liver-infiltrating Th17 expressed high levels of chemokine receptors, CCR6 68 ± 11% (mean ± SD), CXCR3 47 ± 11%, and CCR4 44 ± 13%, irrespective of the cause of liver disease (Fig. 2C). We have recently reported that