Distinct Roles for CCR4 and CXCR3 in the Recruitment and Positioning of Regulatory T Cells in the Inflamed Human Liver

Ye H. Oo, Chris J. Weston, Patricia F. Lalor, Stuart M. Curbishley, David R. Withers, Gary M. Reynolds, Shishir Shetty, Jehan Harki, Jean C. Shaw, Bertus Eksteen, Stefan G. Hubscher, Lucy S. K. Walker and David H. Adams

*J Immunol* published online 17 February 2010

http://www.jimmunol.org/content/early/2010/02/17/jimmunol.0901216

---

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Regulatory T cells (T<sub>reg</sub>) are found at sites of chronic inflammation where they mediate bystander and Ag-specific suppression of local immune responses. However, little is known about the molecular control of T<sub>reg</sub> recruitment into inflamed human tissues. We report that up to 18% of T cells in areas of inflammation in human liver disease are forkhead family transcriptional regulator box P3 (FoxP3)+ T<sub>reg</sub>. We isolated CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>FoxP3<sup>+</sup> T<sub>reg</sub>s from chronically inflamed human liver removed at transplantation; compared with blood-derived T<sub>reg</sub>, liver-derived T<sub>reg</sub>s express high levels of the chemokine receptors CXCR3 and CCR4. In flow-based adhesion assays using human hepatic sinusoidal endothelium, T<sub>reg</sub>s used CXCR3 and α4β1 to bind and transmigrate, whereas CCR4 played no role. The CCR4 ligands CCL17 and CCL22 were absent from healthy liver, but they were detected in chronically inflamed liver where their expression was restricted to dendritic cells (DCs) within inflammatory infiltrates. These DCs were closely associated with CD8 T cells and CCR4<sup>+</sup> T<sub>reg</sub>s in the parenchyma and septal areas. Ex vivo, liver-derived T<sub>reg</sub>s migrated to CCR4 ligands secreted by intrahepatic DCs. We propose that CXCR3 mediates the recruitment of T<sub>reg</sub>s via hepatic sinusoidal endothelium and that CCR4 ligands secreted by DCs recruit T<sub>reg</sub>s to sites of inflammation in patients with chronic hepatitis. Thus, different chemokine receptors play distinct roles in the recruitment and positioning of T<sub>reg</sub>s at sites of hepatitis in chronic liver disease. *The Journal of Immunology*, 2010, 184: 000–000.
infiltrating Tregs (LITregs) use CXCR3 to undergo transendothelial migration across hepatic sinusoidal endothelium and use CCR4 to respond to CCL17 and CCL22 secreted by intrahepatic DCs. Thus, different chemokine receptors play distinct roles in the recruitment and positioning of Tregs at sites of hepatitis in chronic liver disease.

Materials and Methods

Tissues and blood

Venous blood was obtained from patients undergoing liver transplantation before induction of anesthesia and before any blood transfusion. Subsequently, explanted diseased liver was obtained from the same patients to allow the isolation of matched blood- and liver-derived cells. Nondiseased liver tissue was obtained from donor liver tissue surplus to clinical requirements or from uninvolved liver tissue removed at the time of resection for colorectal hepatic metastases. All samples were collected with appropriate patient consent and local research ethics committee approval.

Abs and reagents

The following primary Abs were used for immunohistochemistry, immunofluorescence, and confocal microscopy: mouse anti-human CCL22 mAb (MAB 336, R&D Systems, Minneapolis, MN), goat anti-human CCL17 mAb (AF 364, R&D Systems), mouse anti-human FoxP3 (Abcam, 236A/E7, Cambridge, U.K.), rabbit anti-human polyclonal to CCL22 amino terminal end (Ab 53002-100; Abcam), rabbit monoclonal to CD11c (Abcam, EP13474), rabbit monoclonal to phosphorylated STAT5 (phosphoSTAT5) (Abcam/E208), mouse anti-human CD4 IgG 2b (eBioscience, Hatfield, U.K.), anti-human CD3 IgG2a (eBioscience), and mouse anti-human CD8 IgG2a (eBioscience). Relevant secondary Abs used were anti-IgG1 FITC (Southern Biotech, Cambridge, U.K.), rabbit-anti FITC (Sigma-Aldrich, St. Louis, MO), goat-anti rabbit FITC (Southern Biotech), anti-IgG2b biotinylated (Southern Biotech), anti-IgG1 biotinylated (Southern Biotech), streptavidin 555 (Invitrogen, Paisley, U.K.), streptavidin 647 (Invitrogen), mouse anti-human CCL22 Cy-3 (Southern Biotech), and goat anti-mouse IgG1 FITC (Southern Biotech).

The following Abs were used for multicolor flow cytometry: mouse anti-human CD4 Pacific Blue (RPA-T4, BD Pharmingen, San Diego, CA), mouse anti-human CD25 PE Cy-5 (IM2646, Beckman Coulter, Fullerton, CA), mouse anti-human CCL3 PE-Cy7 (UCHT-1, eBioscience), mouse anti-human CD127 Alexa-488 (Biolegend, Cambridge, U.K.; HCD127), mouse anti-human APC Cy-7 (O323, Biolegend), mouse anti-human CCL39 PE (Clone eBio A1, eBioscience), mouse anti-human CD134/CD39 (CD134/CD39) (IM2646, Beckman Coulter, Fullerton, CA), mouse anti-human CD39 PE (Clone eBio A1, eBioscience), rat anti-human Foxp3 PE (UCHT-1, eBioscience), and CD45 RO PE Cy-5 (UCHL-1, BD Pharmingen). CCR1 mAb (53504/ FAB145P, 1/10), CCR2 mAb (48607/FAB151P), CCR3 mAb (550967; BD), CCR4 mAb (12G5/FAB170P), CCR5 mAb (51505/FAB190P), CCR6 mAb (56811/FAB699P)

FIGURE 1. Phenotype of LITregs freshly isolated from explanted human liver without expansion in vitro. A, Human LILs were stained for cell surface expression of CD4, CD25, and CD127. The stained cells were fixed, permeabilized, and stained intracellularly for FoxP3. For analysis, LILs were gated on forward scatter and side scatter, and LITregs were identified by gating on CD3highCD4high lymphocytes, followed by CD25high (y-axis) and CD127low (x-axis). The square box represents the population of LITregs. Graph shows the expression of transcription factor FoxP3, which was analyzed on LITregs (CD3highCD4highCD25highCD127low) by flow cytometry. B, Flow cytometry overlay histograms show the percentage of LITregs positive for CD45RO, CD27, CD39, or OX40. The percentage expression of relevant markers are provided. Representative data of one of six independent experiments. C, The suppressive function of freshly isolated human LITregs was assessed by [3H]thymidine incorporation. LITregs were isolated using the Dynal Treg isolation kit from freshly isolated liver-derived lymphocytes. Suppression of proliferation was done at ratios of 1:16 to 1:1 freshly isolated LITreg naïve lymphocytes activated by allogenic liver-derived DCs. Data represent mean ± SEM of three independent experiments. *p < 0.05; **p < 0.005 by Student t test.
FIGURE 2. Detection of Tregs in different liver diseases and portal lymph node. 
Ai, Single immunohistochemical staining of formalin-fixed, paraffin-embedded liver sections revealed FoxP3+ cells staining using mAb (Abcam clone 236A/E7) (detected as a brown pigment) in PT and parenchyma (P) of patients with NL and different chronic liver diseases: AIH, ALD, non-A non-B (seronegative) hepatitis, PBC, chronic hepatitis B (HBV), and chronic HCV. FoxP3+ cells in liver tissue are indicated by arrows. Control sections are stained with isotype-matched IgG (original magnification x100).
Aii, Expression
Liver-infiltrating lymphocyte and LITreg isolation

Liver-infiltrating lymphocytes (LILs) were isolated from fresh liver tissue, as previously described (18, 19). To maximize yield and viability, LITregs were isolated immediately after explantation of livers from patients with various liver diseases, including alcoholic cirrhosis, nonalcoholic steatohepatitis, primary biliary cirrhosis (PBC), and autoimmune hepatitis (AIH). Liver tissue was diced into 5-mm cubes and subjected to mechanical homogenization in a 400-μl cell harvester (230 rpm for 5 min), without the use of an enzymatic-digestion step, which removes some chemokine receptors. The resulting homogenate was filtered through fine gauze, and lymphocytes were separated using Lympholyte (VH Bio, Gateshead, U.K.) density gradient centrifugation (650 × g for 30 min). The lymphocyte band was removed and washed, and cell viability was assessed by trypan blue exclusion. Tregs were purified from the total lymphocyte population by negative selection to remove CD8+ cells and positive selection of CD4+CD25+ cells with a Treg isolation kit (Dynal, Erembodegem, Belgium). Treg purity was confirmed using CD4 and CD25 and intracellular staining expression of FoxP3 using a FoxP3 staining kit (eBioscience) was used to confirm the expression of FoxP3.

Liver-infiltrating DCs and monocyte-derived DCs

Liver-infiltrating DCs (LIDCs) were isolated from diseased liver using a method established in our laboratory (20). Briefly, after isolation of lymphocytes, followed by a 13.5% OptiPrep density gradient (Axis Shield, Cambridge, U.K.), CD11c-positive magnetic selection was used to obtain highly purified DCs. Monocyte-derived DCs (MoDCs) from peripheral blood were isolated according to established methods, as previously described (21). Briefly, OptiPrep was added touffy coats made from whole blood to increase its density to ~1.1 g/ml, overlaid by two different density solutions of OptiPrep (1.058 and 1.054 g/ml), and centrifuged at 750 × g for 20 min. The monocytes in the 1.068-g/ml layer were collected and cultured for 5 d in media containing RPMI 1640, 5% FCS, and 10 ng/ml IL-4 (BD Pharmingen) with 1000 U/ml recombinant human GM-CSF (Milenagen; Schering-Plough, Kenilworth, NJ), after which the immature DCs were matured with TNF-α. To obtain highly pure DCs, these cells underwent positive immunomagnetic selection with CD11c.

Isolation and culture of hepatic sinusoidal endothelial cells

Hepatic sinusoidal endothelial cells (HSECs) were isolated according to methods established in our laboratory (22). Explanted liver tissue (~30 g) was finely chopped and subjected to enzymatic digestion (1 mg/ml collagenase type IV; Sigma-Aldrich) and density gradient centrifugation (Percoll; Amersham Biosciences, GE Healthcare, Little Chalfont, U.K.). The nonparenchymal cell band was removed and further purified by immunomagnetic selection using mAb HEA-125 (PROGEN, Heidelberg, Germany) to remove cholangiocytes. CD31 (DAKO) was used to select HSECs, which were plated in collagen-coated 25-cm² tissue culture flasks (Corning Glass, Londonborough, U.K.) and cultured in human endothelial basal media (In Vitrogen) containing 10% human serum (H&D Supplies, Bucks, U.K.), penicillin, streptomycin (100 U/ml), gentamycin (2 mmol/l), hepatocyte growth factor (10 ng/ml, Peprotech, Peterborough, U.K.), and vascular endothelial growth factor (10 ng/ml, PeproTech). Cells were cultured to confluence and were used between passages two and four in all experiments.

Immunohistochemistry

Formalin-fixed paraffin-embedded liver specimens from seven cases each of normal liver (NL), AIH, alcoholic liver disease (ALD), PBC, fulminant seronegative hepatitis or non-A-non-B hepatitis, chronic hepatitis B virus, and chronic hepatitis C virus (HCV); five portal draining lymph nodes were examined. The tonsil/lymph node tissue sections were used as positive control, and negative controls were used for each staining as well. The liver tissue sections were dewaxed in xylene for 10 min and incubated through different steps of alcohol/ether/acetone/ethanol. Endogenous peroxidase (10% hydrogen peroxide in methanol), and Ag retrieval was achieved using EDTA buffer. Then slides were incubated in horse serum for 20 min, followed by incubation in primary Abs CD3 (clone F7.2.38; DAKO Cyto- mation) and FoxP3 (clone 236A/E7; Abcam) and rabbit mAb to phosphoSTAT5 (Y694; Abcam) in blocking buffer (2% horse serum) for 60 min at 37 °C in a humidified container for 1 h. The slides were washed in TBS/ Tween 20 (7.6) for 5 min and incubated in Vector Immunoperoxidase Universal secondary kit (mouse anti-human and rabbit anti-human, Vector Laboratories, U.K.) for 30 min and diaminobenzidine substrate to visualize staining. Slides were counterstained in hematoxylin and mounted in DPX. For double immunohistochemical staining, formalin-fixed paraffin-embedded tissue sections were dewaxed, rehydrated in TBS/Tween and blocked with endogenous peroxidase (as described above). Sections were subjected to Ag retrieval and incubated with 2% casein (Vector Laboratories) for 10 min. Primary mouse mAb, FoxP3 (clone 236A/E7; Abcam) was applied at a 1/50 dilution for 30 min. After a TBS/Tween wash, secondary ImmPRESS anti-mouse reagent (Vector Laboratories) was applied and visualized with ImmPACT diaminobenzidine (Vector Laboratories) for 5 min. Then sections were incubated again with 2% casein, followed by primary rabbit anti-human polyclonal CCL22 (Ab 53002, Abcam); secondary ImmPRESS rabbit anti-human reagent was applied, and visualization was performed with NovaRed substrate (both from Vector Laboratories). Sections were counterstained in hematoxylin, dehydrated through alcohol, placed into xylene, and mounted in DPX.

Sections for dual immunofluorescence were incubated with 10% normal mouse or rabbit serum for 30 min before adding the primary Abs against CCL22 or CD11c for 1 h. Control sections were incubated with IgG mouse mAb or rabbit IgG. Subsequently, sections were incubated with FITC or Texas Red secondary Abs (mouse anti-human and rabbit anti-human, Abcam), followed by a Texas Red secondary kit (mouse anti-human and rabbit anti-human, Abcam) for 5 min. Then sections were mounted in mounting medium (Dako). For double immunofluorescence staining, sections were incubated with Alexa Fluor 555 (Invitrogen). RPMI 1640, 10% fetal calf serum, streptomycin (100 U/ml), penicillin (100 U/ml), and l-glutamine (2 mmol/l) were used.

Multicolor confocal microscopy

Liver tissue sections for NLs, diseased livers, and portal lymph nodes were incubated with blocking buffer (1% horse serum in PBS/1% BSA). Primary Abs against FoxP3, CD3, and CD4 were applied for 1 h. FoxP3 primary Abs were amplified with anti-IgG1 biotin, followed by streptavidin Alexa Fluor 555 (Invitrogen). Rabbit monoclonal CD11c was used as a positive control for confocal microscopy.
visualized by goat-anti-rabbit FITC, then with rabbit anti-FITC, and, finally, with goat anti-rabbit FITC. CD3 and CD4 Abs were detected with anti-IgG2a Cy-3/IgG2b tetramethylrhodamine isothiocyanate (TRITC). All incubation steps were for 30 min, with 10-min washes in PBS, in a humidified chamber protected from the light. Quadruple immunofluorescence staining was visualized and analyzed by confocal microscopy using LSM software (ZEISS).

PCR and ELISA for CCL17 and CCL22

DCs and HSECs were stimulated with cytokines for 24 h, and cells and supernatant were stored for later analysis by RT-PCR and ELISA.

RT-PCR

LIDCs and MoDCs were stored in RNA later, and CCL17 and CCL22 gene expression was analyzed by RT-PCR amplification of RNA from LPS-stimulated and unstimulated LIDCs and MoDCs and from TNF-α + IFN-γ-stimulated and unstimulated HSEC low passage (passage two through four) and high passage (passage five and above).

Total RNA was extracted from cells using an RNeasy kit (Qiagen, Crawley, U.K.), and single-stranded cDNA was synthesized. The PCR reaction was conducted using hot start 95˚C for 5 min, denaturing at 95˚C for 3 min, annealing at 66˚C, extension at 72˚C for 33 cycles, and final extension at 72˚C for 3 min, annealing at 66˚C, extension at 72˚C for 3 min, and reverse, 5'-GCC TGC TTC ACC ACC TTC TTG ATG TC-3', reverse, 5'-GCC TGC TTC ACC ACC TTC TTG ATG TC-3'. Primers were designed from GenBank sequences for GAPDH, CCL17, and CCL22: GAPDH forward, 5'-GCC TGC TTC ACC ACC TTC TTG ATG TC-3', reverse, 5'-GCC TGC TTC ACC ACC TTC TTG ATG TC-3'; CCL17 forward, 5'-TAC ACT CGG GAG CAT AAC TTT GG-3', reverse, 5'-AGG ACA GAG CAT AAC TTT GG-3'; CCL22 forward, 5'-AGG ACA GAG CAT AAC TTT GG-3', reverse, 5'-ACT GCT CCA GGG ATG CCA TCG TTT TT-3'. Product sizes for each set of primers were 270 bp for CCL17 and 363 bp for CCL22. Positive PCR products were separated on 2% agarose gel containing ethidium bromide (Sigma-Aldrich).

ELISA

Chemokines in cell culture supernatants (normalized to cell numbers) were measured by sandwich ELISA using Quantikine Human Immunoassay kits (R&D Systems). We used freshly isolated LITregs and matched PBLs for phenotypic analysis. More than 90% of LITregs expressed the surface ectodomain 600 600 A 600 B 600 C 600 D 600 E 600 F 600 G 600 H 600 I 600 J 600 K 600 L 600 M 600 N 600 O 600 P 600 Q 600 R 600 S 600 T 600 U 600 V 600 W 600 X 600 Y 600 Z 600 [600]. Plasma was separated from blood samples, and serum was used for ELISA. Supernatants from stimulated cells were collected and stored at -30˚C until used. Samples were measured in duplicate and compared with control wells containing RPMI 1640 medium with 0.1% BSA alone. Supernatants were incubated with pertussis toxin (PTx; 100 ng/ml; Sigma-Aldrich) prior to the assay to inhibit chemokine-mediated signaling.

Flow-based transendothelial migration assay

We performed a flow-based adhesion assay incorporating HSECs grown in microslides to study interactions between human HSECs and freshly isolated Tregs under physiological blood flow (18, 22). HSECs were cultured to confluence in glass microslides, stimulated for 24 h with TNF-α and IFN-γ (both 10 ng/ml; PeproTech), and connected to the flow-based system, as described previously (22). Freshly isolated Tregs were perfused through microslides at flow rates of 0.182 ml/min, generating shear stresses of 0.05 Pa, which were comparable to wall shear stresses found in the liver sinusoids in vivo. Adherent cells were visualized microscopically under phase contrast, and a video monitor was used to record assays for off-line analysis to determine the percentage of rolling cells, stably adherent cells, and transmigrated cells. The number of adherent cells was converted to adherent cells/mm² corrected for the number of Tregs perfused (i.e., adherent cells/mm² per 10⁶ Tregs perfused). Cells appearing phase bright were above the endothelial monolayer, whereas those appearing phase dark had migrated through the monolayer. HSEC monolayers were incubated with blocking Abs against ICAM-1 (11C81: 10 μg/ml) and VCAM-1 (1B2: 10 μg/ml) (both from R&D Systems), or lymphocytes were incubated with a blocking Ab against CXCR3 (MAb160/49801; 10 μg/ml; R&D Systems) for 30 min. To inhibit chemokine-mediated signaling, Tregs were incubated with PTx (100 ng/ml; Sigma-Aldrich) 30 min prior to assay. We also blocked the individual CXCR3 chemokine ligands by incubating HSECs with anti-CXCL9 (49106; 50 μg/ml), anti-CXCL10 (30306; 5 μg/ml), or anti-CXCL11 (87328; 5 μg/ml) (all from R&D Systems) for 30 min. HSECs had been stimulated for 24 h with TNF-α and IFN-γ to induce the expression of CXCR3 ligands. All experiments were compared with control microslides in which isotype-matched IgG was used.

Statistical analysis

Paired or independent t tests were used to assess data that were normally distributed, whereas nonparametric data were compared using the Mann-Whitney U test.

Results

Definition of LITregs and peripheral blood-derived regulatory T cells

Liver and blood-derived Tregs were defined by the characteristic phenotype CD4⁺CD25⁺FoxP3⁺ (8, 9). Liver-derived lymphocytes were freshly isolated from explanted human liver tissue by mechanical digestion and density gradient centrifugation before being stained with Abs against CD3, CD4, CD25, CD127, and the intracellular transcription factor FoxP3. More than 95% of CD4⁺CD25⁺FoxP3⁺ LILs expressed FoxP3 (Fig. 1A). The vast majority of LITregs expressed CD45RO⁺, and >80% expressed high levels of CD27 (Fig. 1B), consistent with a tissue-infiltrating phenotype. More than 90% of LITregs expressed the surface ectoenzyme CD39, and 35% expressed OX-40 (Fig. 1B).
**LITregs suppress T cell activation in vitro**

Allogenic stimulation/suppression assays confirmed that LITregs suppress T cell activation in vitro at ratios of 1:4 Treg/responder cells, demonstrating that LITregs are functional (Fig. 1C).

**Chronically inflamed human liver tissue contains a higher proportion of LITregs than nondiseased liver**

Few Tregs were detected in NL tissue by immunohistochemistry, although they could be readily detected in the chronically inflamed livers (Fig. 2A) in septal and parenchyma areas (Fig. 2A, 2B). Multicolor confocal microscopy staining revealed them to be closely associated with CD8 T cells and CD11c DCs (Fig. 2B). Tregs were also detected in liver-draining portal lymph nodes in close association with CD8+ cells and CD11c+ DCs (Fig. 2C). T cells were readily isolated from normal and inflamed liver tissue (Fig. 2D), and the total numbers and the proportion of the infiltrate that was CD4+CD25high CD127lowFoxP3+ was greater in diseased tissue than in NL tissue (Fig. 2D). We used immunohistochemistry to quantify the proportion

---

**FIGURE 3.** Chemokine receptor expression on LITreg. A. Flow cytometry was used to analyze staining for chemokine receptors on liver-infiltrating CD4highCD25highCD127lowFoxP3+ Treg freshly isolated from diseased liver tissues. The chemokine receptors CCR1-10 and CXCR1-6 were stained together with Treg markers, and the percentage of cells expressing each chemokine receptor was analyzed. Data represent mean ± SEM of seven different experiments using livers from patients with ALD, PBC, cryptogenic cirrhosis, AIH, seronegative (non-A non-B) hepatitis, and chronic hepatitis C. B. Overlay graph of CCR4 and CXCR3 frequency on LITreg from inflamed liver tissue compared with peripheral blood Tregs (PBTregs) in matched samples from patients who underwent liver transplantation. Cells were gated on CD4highCD25highCD127lowFoxP3+. One representative graph of five experiments from a patient with PBC. C. Analysis of CXCR3 expression on freshly isolated CD4highCD25highLITreg compared with CD4highCD25low cells from diseased livers (top panel), CXCR3 expression on diseased CD4highCD25highLITreg compared with normal CD4highCD25high LITreg (middle panel), and CCR4 expression on CD4high CD25high LITreg compared with CD4highCD25low cells from diseased liver (bottom panel). LILs were gated on Treg markers, CD4, CD25, and CD4CD25high; CD4CD25low gating was applied to CXCR3 and CCR4 expression. Data represent mean ± SEM (n = 5). *p < 0.05, Student t test. D. CCR4 and CXCR3 are coexpressed on LITreg. LILs gated on CD3+CD4+CD127lowCD25high were stained for CCR4 (y-axis) and CXCR3 (x-axis), confirming the coexpression of these chemokine receptors on the same cell. One representative experiment of five is shown from a patient with AIH.
of the CD3 infiltrate that was FoxP3+ in a variety of liver diseases. In all diseases studied, the inflamed diseased liver contained a higher proportion of Tregs compared with nondiseased liver (Fig. 2E). Interestingly, greater numbers of Tregs were seen in the patients with the most severe inflammation, with the highest frequencies detected in patients who had undergone emergency liver transplantation for fulminant liver failure as a consequence of severe seronegative (non-A non-B) hepatitis (Fig. 2E). In seronegative hepatitis and AIH, FoxP3+ Tregs were found at higher frequencies in the parenchyma compared with PTs, whereas in the other liver diseases and in NL, FoxP3+ Tregs were detected at higher frequencies in PTs (Fig. 2F).

LITregs express high levels of CXCR3 and CCR4

The results of staining for chemokine receptor expression on freshly isolated liver-derived Tregs are shown in Fig. 3A. Approximately 20% ± 5% of LITregs expressed CCR7, but the majority expressed CXCR4 (74% ± 7%) and CXCR3 (67 ± 9% SD) (Fig. 3A). Furthermore, the levels of these receptors were higher on LITregs isolated from inflamed liver compared with NL tissue; 74% ± 7% versus 32% ± 7% for CXCR4 and 67% ± 9% versus 21% ± 6% for CXCR3 (p ≤ 0.05 for both) (Fig. 3C). We compared levels of CCR4 and CXCR3 in circulating and liver-derived Tregs of patients from whom we had collected matched blood and liver samples (Fig. 3B): 82% of LITregs stained for CCR4 versus 67% of matched blood Tregs, and 66% of LITregs stained for CXCR3 compared with 43% of matched blood Tregs (p < 0.05). Seventy-four percent of LITregs expressed CCR4, compared with 40% of liver-infiltrating CD4+ CD25low cells from diseased livers. Thus, CCR4 is preferentially expressed on LITregs. However, the levels of CXCR3 were similar for LITregs and liver-infiltrating CD4+CD25low cells (Fig. 3C).

We correlated CCR4 with CXCR3 expression on LITregs to determine whether the receptors were coexpressed on the same cells or whether there were two subpopulations of LITregs defined by CCR4 and CXCR3 expression (Fig. 3D). More than 75% of LITregs coexpressed CCR4 and CXCR3 (Fig. 3D), and 40% of the residual LITregs that were CCR4+ but CXCR3low expressed CCR4 and CCR5 (data not shown).

CXCR3 activation promotes stable adhesion and transmigration of LITregs across human HSECs under conditions of flow

Cultured HSECs express markers that are characteristic of sinusoidal endothelium in vivo, including CD31, lymphatic vessel...
endothelial receptor 1, liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin, and liver and lymph node sinusoidal endothelial cell C-type lectin (23–25). When stimulated by TNF-α, HSECs express ICAM-1 and VCAM-1 and support the adhesion of lymphocytes under conditions of flow (22). Furthermore, HSECs secrete the CXCR3 ligands CXCL9, CXCL10, and CXCL11 in response to IFN-γ; this is increased if TNF-α is used in combination with IFN-γ (18). Thus, we used HSECs treated with 10 ng/ml IFN-γ and TNF-α in the flow-based adhesion assays in the current study to allow us to model inflamed hepatic sinusoidal endothelium, which expresses ICAM-1, VCAM-1, and CXCR3 ligands in chronic hepatitis (18).

When flowed over cytokine-stimulated HSECs, some of the Tregs displayed brief rolling/tethering interactions followed by arrest and stable adhesion, whereas others arrested without rolling; this behavior was similar to that reported using unfractionated liver-derived lymphocytes (18). The numbers of cells undergoing stable adhesion from flow could be reduced by blocking CXCR3 and, to a lesser extent, VCAM-1 but not by blocking ICAM-1. Global G-protein inhibition with PTx reduced adhesion to levels seen with anti-CXCR3 blockade, suggesting that CXCR3 is the dominant chemokine receptor involved (Fig. 4A). The proportion of adherent cells that subsequently underwent transendothelial migration was also significantly reduced by CXCR3 block. Again, PTx had a similar inhibitory effect to CXCR3 blockade.

These findings suggest that CXCR3 is crucial for Treg adhesion to, and subsequent transmigration across, inflamed hepatic sinusoids (Fig. 4B). To determine which of the CXCR3 ligands is involved, we used HSECs treated with 10 ng/ml IFN-γ and TNF-α in the flow-based adhesion assays in the current study to allow us to model inflamed hepatic sinusoidal endothelium, which expresses ICAM-1, VCAM-1, and CXCR3 ligands in chronic hepatitis (18).

**FIGURE 5.** LITreg CXCR3 expression and liver-infiltrating CD4 and CD8 effector cell cytokine expression. To determine whether the levels of CXCR3 expression by LITreg are associated with secretion of TNF-α and IFN-γ by liver-infiltrating CD4 and CD8 effector cells, we correlated levels of CXCR3 expression on LITreg with the frequency of IFN-γ– and TNF-α–secreting effector cells in the same liver samples. The percentage of liver-derived CD8 (upper panel) and CD4 (lower panel) T cells expressing TNF-α (left panels) and IFN-γ (right panels) is shown on the x-axis, and the frequency of CXCR3+ Treg (CD4+CD25highCD127low) isolated from the same donor liver and analyzed without expansion is shown on the y-axis. Cells from four NLs and four diseased livers (AIH, PBC, ALD, and non-A non-B [seronegative] fulminant hepatitis) are shown. There was no clear correlation between CXCR3 frequencies in the Treg population and cytokine secretion by effectors. Intracellular cytokine and TNF-α and IFN-γ staining was done by incubating LILs with PMA and ionomycin for 5 h, with brefeldin A Golgi block in the last 2 h, followed by intracellular cytokine staining. The patient with the highest frequency of TNF-α– and IFN-γ–secreting effector cells had fulminant non-A non-B hepatitis. A representative flow cytometry dot-plot analysis of T cells isolated from a diseased liver (PBC) is shown to demonstrate IFN-γ and TNF-α secretion. Cells were gated on LILs and gated again on CD3 lymphocytes (one representative example of four experiments).
FIGURE 6. CCR4 ligands are detected in diseased liver tissue and secreted by LIDCs. A, LITregs migrate toward CCR4 ligands in vitro. Migration of LITregs to CCR4 ligands CCL22 and CCL17 was assessed using fibronectin-coated 5-μm-pore transwell migration chambers. The lower chambers were filled with 600 μl assay media (RPMI 1640 medium with 0.1% BSA) alone or supplemented with chemokines (100 ng/ml recombinant human CCL22 and CCL17). BSA was used as control. A total of 5 × 10⁵ Tregs isolated from inflamed livers (n = 4) were loaded per well in the upper chamber; Tregs were collected from the top and bottom chambers after 4 h of incubation at 37˚C in 5% CO₂. The proportion of transmigrated cells was calculated by fixed-volume counting and phenotyped for Treg markers and chemokine receptor expression by flow cytometry. To inhibit chemokine-mediated signaling, Tregs were incubated with PTx (100 ng/ml) prior to the assay. Results are expressed as mean ± SEM. LITregs showed significant migration responses to CCL22 and CCL17 compared with migration to BSA, and these were inhibited by PTx. p < 0.05, Student t test. B, To determine whether liver DCs secrete CCL17 and CCL22, we isolated and cultured myeloid DCs from human liver tissue and stimulated them with LPS (1 μg/ml) for 24 h. CCL17 and CCL22, measured by ELISA, were detected in cell culture supernatants of LPS-stimulated liver-derived DCs isolated from patients with AIH (n = 2) or PBC (n = 1) or from NL (n = 3). All DCs secreted detectable CCL17 and CCL22, with a 2-fold increase in secretion by DCs derived from inflamed liver tissue compared with NL. Data represent mean ± SEM of three experiments using supernatants from different DCs. Values represent the chemokine concentration (pg/ml) of three replicate wells relevant to a standard curve of known concentration. p < 0.05; p < 0.01. C, Expression of CCL22 in human liver tissue samples by immunohistochemistry. CCL22 staining (i) was detected on LIDCs within inflammatory infiltrates (positive staining cells brown) in a patient with AIH. No detectable CCL22 was found in NL or when control Abs were used (ii). (Original magnification ×200.) D, Dual staining of FoxP3-expressing cells (brown) and CCL22 (red) in liver tissue from a patient with AIH reveals CCL22+ cells in close association with FoxP3+ Tregs (green) in liver tissue. Serial liver sections from a patient with PBC were stained with CD11c (top panels) and CCL22 (bottom panels). Cells that coexpress CD11c and CCL22 can be determined by comparing staining of CD11c (top right panel) and CCL22 (bottom right panel) at higher power (original magnification ×400). E, Confocal image of dual staining of FoxP3-expressing cells (red/TRITC) and CD11c (green/FITC) in liver tissue from a patient with AIH reveals CD11c+ cells in close association with FoxP3+ Tregs within an inflammatory infiltrate (original magnification ×200). F, Colocalization of DCs and CCL22 in inflamed liver tissue. Serial liver sections from a patient with PBC were stained with CD11c (top panels) and CCL22 (bottom panels). Cells that coexpress CD11c and CCL22 can be determined by comparing staining of CD11c (top right panel) and CCL22 (bottom right panel) at higher power (original magnification ×200). Staining with isotype-matched control Abs was negative (left panels, original magnification ×100.) G, Colocalization of DCs and CCL22 in inflamed liver tissue. Staining of FITC-labeled CCL22 (green) with Texas Red-labeled CD11c (red). Areas of coexpression (top left panel) appear yellow. Nuclei were stained with DAPI (blue). Control Abs demonstrated minimal background tissue fluorescence. The findings are representative of the pattern of immunofluorescence staining seen in tissue sections from four donors each for PBC, ALD, AIH, and HCV.
we repeated the experiments using blocking Abs against the individual CXCR3-binding chemokines CXCL9 (monokine induced by IFN-γ), CXCL10 (IFN-γ inducible protein-10), and CXCL11 (IFN-inducible T cell α–chemoattractant). We observed a reduction in adhesion (Fig. 4C) and transmigration (Fig. 4D) with each individual blocking Ab treatment, but these were less than the inhibition seen with anti-CXCR3, which suggests that all three ligands may be involved. This is consistent with our previous work showing that HSECs secrete all three ligands in response to IFN-γ and TNF-α (18).

Correlation between CXCR3 expression on LITregs and cytokine secretion by liver-infiltrating effector T cells

To determine whether proinflammatory cytokines secreted by liver-infiltrating effector cells are responsible for activating the CXCR3 pathway, we investigated whether the levels of CXCR3 expression on Tregs correlated with IFN-γ and TNF-α secretion by effector cells in the same livers. NL and inflamed liver contained IFN-γ– and TNF-α–secreting CD4 and CD8 T cells (Fig. 5B). The proportion of cytokine-expressing cells was similar in normal and diseased tissue, although the total number of T cells was much higher in the inflamed livers. Once again, we detected higher frequencies of CXCR3+ Tregs in inflamed liver tissue (60–80% cells positive) compared with those isolated from noninflamed liver (15–28%) (Fig. 5A). However, there was no correlation between CXCR3 expression on Tregs and the proportion of CD4 or CD8 TNF-α– and IFN-γ–secreting cells in the infiltrate (Fig. 5A).

LITregs express functional CCR4

We used chemotaxis assays to confirm that CCR4 on LITregs is functional, as demonstrated by the ability of the cells to migrate to the CCR4 ligands CCL17 and CCL22 (Fig. 6A). The chemotactic indices for CCL17 and CCL22 were approximately three times greater than control. We phenotyped the LITregs before chemotaxis assays and confirmed that >75% expressed CCR4.

Localization and source of CCR4 ligands CCL17 and CCL22 in inflamed liver tissue

We were unable to detect CCL17 and CCL22 in NL tissue, but both chemokines were detected in chronically inflamed liver tissue by Western blotting (Fig. 6D) and immunohistochemistry (Fig. 6F). Immunohistochemistry demonstrated that CCL22 is present in cells with the morphology of DCs in inflamed liver tissue (Fig. 6F). Staining was most marked at areas of active inflammation, including portal infiltrates, interface hepatitis, and lobular hepatitis within the parenchyma (Fig. 6F). There was no detectable staining of DCs with isotype-matched control Abs (Fig. 6A). Confocal staining showed that intrahepatic CCL22+ DCs were closely associated with LITregs in PTs and inflamed parenchyma (Fig. 6E, Fig. 7) and in close proximity to CD4 and CD8 T cells (Fig. 7). Serial liver sections (Fig. 6F) and
dual staining (Fig. 6G) suggested that CCL22 expression was detected on CD11c DCs in inflamed liver.

**Liver-derived DCs secrete CCL17 and CCL22**

To confirm that hepatic DCs secrete CCR4 ligands, we isolated myeloid DCs from normal and inflamed human liver tissue and analyzed them for the expression of CCL17 and CCL22. mRNA (Fig. 6H) and protein (Fig. 6B) for both chemokines were detected in DCs isolated from diseased liver; neither mRNA (Fig. 6H) nor protein (Fig. 6D) could be detected in HSECs, even after cytokine stimulation consistent with the lack of detectable CCL17 and CCL22 on sinusoids by immunohistochemistry (Fig. 6Ci, 6D). Furthermore, we localized staining of CCL22 to LIDCs in liver tissue (Fig. 6B). These data are consistent with previous reports that myeloid DCs secrete CCL17 and CCL22 when stimulated with LPS (26).

The majority of LITregs do not express phosphoSTAT5

We looked for evidence of STAT5 activation in LITregs by staining liver tissue with an Ab that recognizes phosphoSTAT5 (Fig. 8). PhosphoSTAT5 was detected in <20% of the FoxP3+ cells, suggesting that not all FoxP3 Tregs are actively signaling through IL-2R (Fig. 8).

**Discussion**

Tregs operate at sites of inflammation in peripheral organs as well as in secondary lymphoid tissues (12, 27). At inflammatory sites, they mediate bystander and Ag-specific suppression of local immune responses to prevent collateral tissue damage in response to injury and infection (28–30). The outcome of chronic inflammation is the result of a balance between pro- and anti-inflammatory responses in which Tregs maintain stable chronic inflammation while preventing fulminant destructive inflammation (31). This is particularly important in liver disease, in which intrahepatic Tregs are detected in chronic hepatitis from a variety of causes (5, 32, 33). The frequency and function of intrahepatic Tregs were reported to affect the outcome of chronic infection with hepatitis B and C viruses and to be associated with suppression of the antitumor immune response in hepatocellular carcinoma (4, 33).

We found low numbers of Tregs in nondiseased human liver and a higher number in the liver of patients with a variety of autoimmune and chronic inflammatory liver diseases. Approximately 5% of the CD3+ T cells in NL tissue expressed FoxP3 by immunohistochemistry; this proportion was significantly greater in chronic inflammatory liver diseases. We also isolated CD4+CD25+FoxP3+CD127low cells from explanted liver tissue and again found that NL contained low frequencies of Treg and that the frequencies were increased in chronic inflammatory liver diseases. The proportion of liver-derived T cells that stained CD4+CD25+FoxP3+CD127low after isolation from liver tissue was lower than the proportion seen in situ using immunohistochemistry with Abs to FoxP3. This could be because FoxP3 stains other cells in liver tissue, although this seems unlikely because the majority of FoxP3+ liver-derived lymphocytes were CD4+CD25−FoxP3CD127low. Alternatively, our tissue-isolation methods may not remove all of the LITregs. However, although the proportions of Tregs varied using the two methods, the overall results were similar, with the highest frequencies detected in patients with fulminant seronegative (non-A non-B) hepatitis, the most florid and destructive form of hepatitis that leads to liver failure. These findings suggest that T cell-mediated hepatitis and liver destruction are not a consequence of the failure of Tregs to infiltrate the liver but rather are due to an inability of the recruited cells to control the effector arm of the response.

Analysis of the distribution of Tregs in liver tissue demonstrated them to be closely associated with CD11c+ DCs and CD8+ T cells at sites of inflammation in PTs and in the hepatic lobules. Thus, Tregs are ideally situated to mediate suppression of intrahepatic immune responses. To confirm that they were functional, we isolated CD4+CD25+FoxP3+CD127low cells from liver tissue and showed that they suppressed T cell activation in vitro. These cells were CD27high, consistent with previous reports that CD27 expression characterizes tissue-infiltrating Tregs in inflamed synovial tissue (13).

The migration and positioning of leukocytes in tissues during homeostasis and in response to inflammation are controlled by the coordinated expression of adhesion molecules and chemokine receptors that determine where and when cells will be recruited via endothelium, as well as their subsequent migration and positioning in tissue. Little is known about the molecular control of Treg recruitment to inflamed tissues, although this will be critical in defining their role in disease and in developing strategies for their therapeutic use. To determine the receptors responsible for the recruitment of Tregs to human liver, we compared chemokine receptor expression on liver-derived Tregs (analyzed without expansion straight from liver tissue) with blood-derived Tregs isolated from the same patient at the time of liver transplantation. In particular, two receptors were overexpressed
on liver-infiltrating cells: CXCR3 and CCR4; the majority of LITreg in chronic inflammatory liver diseases express both receptors.

CXCR3 was reported on circulating human Treg (34, 35), where its expression is presumed to favor homing to inflamed tissue sites. Our data confirm that this is the case because the majority of Treg in inflamed human liver expressed high levels of functional CXCR3, which promoted their transmigration across liver endothelium under flow. We found that only 15–20% of Treg in NL expressed CXCR3, which further supports its particular role in the recruitment of Treg to sites of inflammation. Levels of the CXCR3 ligands are low to undetectable in NL, but they are markedly increased in inflammatory liver diseases (18, 36).

The CXCR3 ligands CXCL9, CXCL10, and CXCL11 are increased in chronic inflammatory liver disease as a consequence of secretion by hepatocytes, stromal cells, and endothelium. They can be readily detected on the glycocalyx of sinusoidal vessels (37–39), where they promote the transendothelial migration of effector T cells (18). We developed an in vitro flow-based adhesion assay in which primary cultures of HSECs are grown in microslides under physiological levels of shear stress to model the hepatic sinusoid (18). Treatment of HSECs with TNF-α and IFN-γ induces the expression of CXCL9, CXCL10, and CXCL11, as well as the adhesion molecules ICAM-1 and VCAM-1, thereby recapitulating the phenotype of chronically inflamed sinusoids in vivo (18). We used this assay in the current study to determine the role of CXCR3 on LITreg. We found that signaling through CXCR3 activated integrin-mediated arrest from flow and promoted the transendothelial migration of Treg across inflamed hepatic sinusoidal endothelium. The fact that a similar degree of inhibition to both processes was seen when a blocking CXCR3 Ab or the G protein inhibitor PTx was used suggests that CXCR3 is the dominant chemokine receptor involved. All three CXCR3 ligands were involved in the in vitro flow assays, because blocking individual chemokines had a lesser effect than blocking the common receptor CXCR3. These findings are consistent with studies in murine models of inflammatory disease that report the requirement for CXCR3 expression to allow Treg to be recruited to inflamed tissues in graft-versus-host disease (40) and to the brain in experimental autoimmune encephalomyelitis (41).

Recently, CXCR3 has been implicated in the recruitment of Treg into the murine liver in response to inflammation and IFN-γ production from NKT cells (42), and Foxp3+ Treg could upregulate Th1 specifying transcription factor T-bet, which, in turn, promote the expression of the chemokine receptor CXCR3 on Treg in response to IFN-γ (43). Thus, IFN-inducible chemokines CXCL9 and CXCL10 expressed on inflamed hepatic sinusoidal endothelium promote the recruitment of CXCR3-expressing Treg under shear stress as they do the recruitment of effector cells (18). A difference between the behavior of effector cells reported in our previous study and Treg was the involvement of VCAM-1, but not ICAM-1, in the adhesion of Treg to HSECs, whereas effector cells use ICAM-1 (18).

CCR4 is the other chemokine receptor that was strongly over-expressed on LITreg; it was coexpressed with CXCR3 in nearly 80% of Treg from chronically inflamed livers. Liver-derived Treg migrated to the CCR4 ligands CCL17 and CCL22 in vitro, demonstrating that the receptor is functional. CCR4 expression has been associated with Treg and shown to control migration and immune suppression in the skin (44, 45) and in immunologically tolerant cardiac allografts (46). Until this report, CCR4 was not believed to play a role in the recruitment of T cells to the liver, and few effector cells in the liver express CCR4 (39). CCR4 is the receptor for two chemokines CCL17 and CCL22, both of which are secreted by DCs on maturation, and they recruit and retain Treg in contact with DCs in lymph nodes (44, 47–51). The interaction between Treg and DCs inhibits DC maturation and the expression of costimulatory molecules required for effector T cell activation (52–55). We were unable to detect either chemokine in normal human liver tissue by Western blotting of whole liver tissue or by immunohistochemistry. However, both chemokines were detected in inflamed liver tissue, and immunohistochemistry showed staining restricted to CD11c+ DCs within inflammatory infiltrates in septal areas and lobules. To confirm that LIDCs secrete CCR4 ligands, we isolated myeloid DCs from liver tissue and showed that they expressed and secreted CCL17 and CCL22.

Confocal microscopy of liver tissue revealed that LITreg were closely associated with DCs and CD8 T cells in chronic hepatitis (Fig. 7), suggesting that they are ideally positioned to maintain intrahepatic immune suppression. We propose that localization to these infiltrates occurs in response to sequential chemokine signals. Local proinflammatory cytokines, including IFN-γ, lead to the secretion of CXCL9 and CXCL10 by sinusoidal and parenchyma cells; these chemokines are presented on the glycocalyx of sinusoidal endothelium where they recruit CXCR3high circulating Treg into the liver. Subsequent migration within the inflamed liver is guided by CCR4, which allows tissue-infiltrating Treg to respond to CCL17 and CCL22 secreted by activated intrahepatic DCs, thereby resulting in their accumulation within DC-rich inflammatory infiltrates in liver tissue.

This begs the question of why inflammation persists in the presence of so many Treg. These cells are functional in vitro, but it is possible that their function is suppressed within the liver microenvironment in vivo. To investigate this possibility, we stained the intrahepatic Treg in situ for phosphoSTAT5. STAT5 phosphorylation is a consequence of IL-2R activation and, thus, provides an indication of whether the intrahepatic Treg are activated (56). We quantified the numbers of pSTAT5-positive cells in the liver and found that only 5–15% of the liver-infiltrating FOXP3+ cells also expressed pSTAT5. Thus, it is possible that these cells are suppressed in situ, possibly by programmed death receptor-1–dependent mechanisms, as has been proposed for chronic HCV infection (57), allowing the effector arm to drive persistent inflammation.

Acknowledgments

We thank the staff at the Liver Transplant Unit, Queen Elizabeth Hospital, for their help with sample collection and the patients for donating blood and tissue. We also thank Nicholas Graham, Rebecca Adams, and Janine Yous- ter for help with immunohistochemistry and Gill Murhead for help with HSEC isolation and culture.

Disclosures

The authors have no financial conflicts of interest.

References


