CXCR3 Deficiency Exacerbates Liver Disease and Abrogates Tolerance in a Mouse Model of Immune-Mediated Hepatitis

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CXCR3 Deficiency Exacerbates Liver Disease and Abrogates Tolerance in a Mouse Model of Immune-Mediated Hepatitis

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The chemokine receptor CXCR3 is preferentially expressed by Th1 cells and critically involved in their recruitment to inflamed tissue. In a mouse model of immune-mediated liver injury inducible by Con A, we investigated the role of CXCR3 in acute IFN-γ–mediated hepatitis as well as in tolerance induction, which has been shown to depend on IL-10–producing CD4+CD25+Foxp3+ regulatory T cells (Tregs). Induction of Con A hepatitis resulted in increased intrahepatic expression of the CXCR3 ligands CXCL9, CXCL10, and CXCL11. CXCR3−/− mice developed a more severe liver injury with higher plasma transaminase activities and a more pronounced Th1/Th17 response compared with wild-type (wt) animals upon Con A injection. Moreover, CXCR3−/− mice did not establish tolerance upon Con A restimulation, although Tregs from CXCR3−/− mice were still suppressive in an in vitro suppression assay. Instead, Tregs failed to accumulate in livers of CXCR3−/− mice upon Con A restimulation in contrast to those from wt animals. Con A-tolerant wt mice harbored significantly increased numbers of intrahepatic CXCR3+T-bet+ Tregs that produced IL-10 compared with nontolerant animals. IFN-γ deficiency or anti–IFN-γ Ab treatment demonstrated that conversion to CXCR3+T-bet+ Tregs depended on a Th1 response. Accordingly, in an immunotherapeutic approach, CD4+ CD25+Foxp3+ Tregs from Con A-pretreated CXCR3-deficient mice failed to protect against Con A-induced hepatitis, whereas Tregs from Con A-tolerant wt mice allowed CXCR3-deficient mice to recover from Con A hepatitis. In summary, CXCR3+T-bet+ IL-10+ Tregs are generated in the liver in dependence of IFN-γ, then disseminated into the organism and specifically migrate into the liver, where they limit immune-mediated liver damage. The Journal of Immunology, 2011, 186: 000–000.

Chemokines mediate migration of immune cells into infected or inflamed tissue to initiate effective immune responses (1). T cell subsets express specific patterns of chemokine receptors, resulting in migration capacities that depend on T cell function. CD4+ Th1 cells are characterized by expression of CCR5, CXCR3, and CXCR6, whereas specific chemokine receptor expression by regulatory T cells (Tregs) is still obscure.

Tissue infiltration of T cells expressing high levels of CXCR3 depends on IFN-γ–induced release of the CXCR3 ligands CXCL9, CXCL10, or CXCL11 (1). CXCR3 and its ligands are involved in the pathogenesis of various autoimmune diseases (2), but also in acute allograft rejection (3). Accordingly, CXCR3-deficient mice show reduced trafficking of effector T cells into the kidney, resulting in less renal injury (4). However, studies using CXCR3-deficient mice have generated controversial results relating to disease severity in models of multiple sclerosis (2). Therefore, CXCR3-expressing T cells might exert either protective or deleterious functions, depending on which organ is affected.

Interestingly, CXCR3 is not only expressed on Th1 cells, but also on non-lymphoid tissue-homing CD4+CD25+ Tregs besides high levels of CD103, CCR5, CCR4, and CCR8 (5), suggesting that Tregs and CD4+ effector T cells might exhibit overlapping receptor expression profiles. Indeed, CXCR3 expression on Tregs induced by an acute systemic Th1 response has recently been shown to depend on the Th1-specific transcription factor T-bet, because T-bet−/− mice completely lack CXCR3+Foxp3+ Tregs (6). Hence, enhancement of Treg recruitment into inflamed tissue might improve the direct interaction with effector T cells or might modify APCs, thus limiting autoimmune-mediated tissue damage. In contrast to inflammation-seeking Tregs, lymphoid tissue-homing Tregs preferentially express CCR7 and CD62L, which allows them to control naïve CD4+ T cell proliferation and homing/recirculation (1).

With reference to liver pathologies, several studies on human hepatitis C virus infection have demonstrated that high CXCR3 expression correlates with viral clearance and liver damage (7). Moreover, intrahepatic CXCR3highCCR7low Tregs, representing an activated, tissue-infiltrating phenotype, were identified in patients with autoimmune liver diseases (8). However, the functional role of CXCR3 has not yet been investigated in experimental models of Th1-related liver disease. Therefore, we studied the impact of CXCR3 in the well-established mouse model of T cell-mediated liver injury inducible by Con A (9), which depends on activated CD4+ T cells, NKT cells, and Kupffer cells and is mediated by release of the proinflammatory cytokines IFN-γ (10) and TNF-α, whereas IL-10 is protective (11). Immune pathology in this model shares certain features with autoimmune hepatitis, specifically: relevance of CD4+ T cells, genetic prevalence of certain mouse...
strains in terms of susceptibility, gender-related differences, and immunosuppression during remission. We have recently described that IL-10–producing CD4+CD25+Foxp3+ Tregs, but not immunoregulatory NKT cells, mediate long-lasting tolerance against Con A restimulation that develops 8 d after the first Con A injection and is characterized by suppression of the Th1/Th17 responses and liver damage (12). Moreover, Tregs from tolerant mice have a higher immunosuppressive activity in vivo and in vitro compared with those from nonpretreated animals.

Taken together, the critical role of CXCR3 for recruitment of Th1 cells on the one hand and for immunoregulatory T cells on the other hand prompted us to investigate the role of CXCR3 in both Con A-induced Th1-mediated hepatitis and Con A tolerance. Using CXCR3-deficient mice, we have demonstrated in this study that the immune-regulatory effect of CXCR3 expressed by T-bet–positive IL-10–producing Tregs seems to overcome its function with respect to a liver-specific Th1 response, because CXCR3-deficient mice developed more severe liver injury and failed to develop Con A tolerance, which corresponded to reduced Treg recruitment into the liver.

In conclusion, CXCR3*T-bet*IL-10* Tregs specifically recruited to the liver might represent a novel option for improved therapy of autoimmune liver disease.

Materials and Methods

**Mice**

Male CD45.1-expressing congenic mice kindly provided by Dr. Andrea Horst (Clinical Chemistry), as well as male C57Bl/6 wild-type (wt), CD1d−/−, IFN-γ−/−, and CXCR3−/− (4) mice, all aged 6–10 wk, were obtained from the animal facilities of the University Medical Center Hamburg-Eppendorf. Animals received humane care according to guidelines of the National Institutes of Health in Germany. Experiments were approved by the institutional review board Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz.

**Animal treatments**

Con A (5–15 mg/kg; Sigma-Aldrich, Munich, Germany) or saline was administered i.v. followed by Con A restimulation 8 d later. For immunotherapeutic experiments, splenic CD4+CD25* Tregs or CD4*CD25* responder cells were injected i.v. into B6 wt, CXCR3-deficient, or CD45.1* mice. Rat anti-mouse IFN-γ (XGM1.2; 500 μg/mouse; purified by Julia Holzer/Stefanie Hueneemoerder, Institute of Immunology, University Medical Center Hamburg-Eppendorf) or rat IgG was injected i.p. 1 h prior to Con A challenge.

**Determination of plasma transaminase and cytokine levels**

Liver injury was quantified by measurement of plasma activities of alanineaminotransferase (ALT) 8 h after Con A administration using the COBAS Mira System (Roche Diagnostic, Mannheim, Germany). ELISAs (TNF-α, IFN-γ, IL-2, IL-6, IL-10, IL-17) were performed as described previously (4, 12).

**Expression analysis of chemokines/cytokines**

Total RNA was reversely transcribed, followed by RT-PCR using the Bio-Rad CFX96 real-time system (Bio-Rad) and ABsoluteQPCR SYBR mix (Thermo Fisher). Primer pairs were used as described previously (4, 12). Relative mRNA levels were calculated after normalization to b-actin or GAPDH, using the CFX96 Manager software.

**Isolation of various cell subpopulations**

Liver sinusoidal endothelial cells (LSECs) were isolated using anti-CD146 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Primary hepatocytes were isolated as described previously (13). LSECs and hepatocytes were incubated with 100 ng/ml rIFN-γ (PeproTech, Hamburg, Germany) in vitro for 24 h or isolated ex vivo 2 h upon Con A treatment. Spleen, liver, and lymph nodes (LN) were passed through 100-μm nylon meshes, which yielded in single-cell suspensions. Hepatocytes were removed by density gradient prior to erythrocyte lysis. To isolate splenic CD4+CD25* Tregs and responder T cells, splenocytes were sorted using a combination of MACS and FACS (BD FACSAria; BD Biosciences) as described previously (12) to achieve highly purified CD4+CD25* Tregs (∼98%).

**Flow cytometric analysis**

A total of 1 × 10⁶ leukocytes were stained using standard protocols. The following anti-mouse mAbs were used: CD45.2-biotin (clone 104), CD4-FITC (GK1.5), CD25-PE-Cy7 (PC61), NK1.1-PE (PK136), CD3e-CyChrome (145-2C11; all BD Pharmingen), CXCR3-allophycocyanin, Foxp3-PE-Cy5 (FJK-16s), T-bet-PE (ebio4B10); all ebioscience/Naturnec, Frankfurt, Germany), and streptavidin-Pacific Blue (Invitrogen, Karlsruhe, Germany). Data were recorded using the BD FACS Canto II system and BD FACS Diva software (BD Biosciences).

**Statistical analysis**

Data are presented as mean values ± SEM. Results were analyzed using Student’s t test, Tukey’s test, or log-rank test. All p values >0.05 were considered significant.

**Results**

**Increased intrahepatic expression of CXCR3 ligands upon Con A challenge**

Analysis of the basal expression of the CXCR3-binding ligands CXCL9, CXCL10, and CXCL11 revealed that all three chemokines were constitutively expressed in liver tissue in contrast to very low expression levels in kidney (except for CXCL10) and heart tissue (Fig. 1A). To correlate IFN-γ levels and intrahepatic expression of the IFN-γ–inducible CXCR3-binding ligands CXCL9–11 during Con A-induced inflammation, wt mice were treated with 12 mg/kg Con A or saline as control. Liver tissue samples and cardiac blood were taken after 1.5, 3, 6, and 8 h. IFN-γ expression in plasma (Fig. 1B) and liver tissue (Fig. 1C) correlated with increased intrahepatic expression of CXCL9, CXCL10, or CXCL11 upon Con A injection (Fig. 1D–F; time point = 0: expression level set to 1). To strengthen the chemoattractant function of resident liver cells (14–16), we either cultivated primary hepatocytes or LSECs isolated from wt mice 2 h upon Con A challenge ex vivo, or we incubated such cells from untreated wt mice with rIFN-γ in vitro. Both cell populations strongly expressed CXCL9–11 ex vivo (Fig. 1G) or in vitro compared with control cells (Fig. 1H), thus demonstrating their capacity to produce IFN-γ–inducible chemokines for recruitment of CXCR3-expressing T cells. As the liver constitutively expresses CXCL9–11, we compared the frequencies of CX3CR3*CD4+ naive T cells and CX3CR3* Tregs in liver with other peripheral lymphocyte-containing compartments of untreated wt mice. FACS analyses revealed a predominance of CX3CR3*CD4+Foxp3* Tregs (Fig. 2A) depicts the specificity of the anti-CX3CR3 Ab; Fig. 2B: representative dot plots of CX3CR3*CD4*Foxp3* Tregs, all Foxp3* cells were also CD25 positive; Fig. 2D: quantification of CX3CR3*CD4*Foxp3* Tregs) as well as of naive CX3CR3*CD4+ T and CX3CR3*CD4+ NKT cells (Fig. 2C: representative dot plots of CX3CR3*CD4*CD25* T cells, all CD25+ cells were also Foxp3 negative; Fig. 2E: quantification of CX3CR3*CD4+CD25* T cells) in the liver compared with secondary lymphoid organs and blood, suggesting a selective migration of these cells into liver tissue; this was most probably due to the constitutive hepatic expression of CXCR3-binding chemokines.

**Lethal aggravation of liver injury in CXCR3−/− mice upon Con A challenge**

The prevalence of intrahepatic naive CX3CR3*CD4+ T cells and CX3CR3*CD4+CD25*Foxp3+ Tregs prompted us to analyze induction of Con A hepatitis in CX3CR3−/− animals. Intriguingly, all CX3CR3−/− mice died within 48 h, even upon treatment with a low dose of 10 mg/kg Con A, whereas all wt mice survived, indicating...
a protective role of CXCR3 in immune-mediated liver injury (Fig. 3A). ALT activity was not increased per se in nontreated CXCR3-deficient mice (wt: 49 ± 6 units/l versus CXCR3<sup>−/−</sup>: 46 ± 2 units/l). Moreover, CXCR3-binding chemokines were still released in Con A-treated CXCR3<sup>−/−</sup> mice (data not shown). Hence, we decided to challenge CXCR3<sup>−/−</sup> mice with a sublethal dose of 5 mg/kg Con A in the following experiments. This treatment resulted in aggravated liver damage in CXCR3<sup>−/−</sup> mice compared with wt mice, as demonstrated by increased plasma transaminase activities (Fig. 3B). Aggravated liver damage was accompanied by a pronounced increase of plasma levels of the proinflammatory cytokines TNF-α, IL-17, IL-6, and IFN-γ as well as by reduced IL-10 production (Fig. 3C). Accordingly, intrahepatic mRNA expression of the proinflammatory cytokines IFN-γ, IL-17, and IL-6 was significantly upregulated in CXCR3<sup>−/−</sup> mice compared with wt mice upon Con A challenge (Fig. 3D). FACS analysis did, in fact, reveal significantly enhanced frequencies of CD3+NK1.1+NKT cells in untreated CXCR3<sup>−/−</sup> mice in comparison to wt mice (CXCR3<sup>−/−</sup>: 25.9 ± 2.0% versus wt: 18.1 ± 1.4%). The presence of increased numbers of NKT cells in CXCR3<sup>−/−</sup> mice might contribute to the higher sensitivity of these animals toward Con A (Fig. 3H: representative dot plot; Fig. 3I: quantification of NKT cell frequency), because NKT cells mediate Con A-induced liver injury by contributing to IFN-γ production (IFN-γ [pg/ml]: CXCR3<sup>−/−</sup>: 1410 ± 290 versus wt: 840 ± 210; Fig. 3C). The intrahepatic frequencies of NK cells, macrophages, CD4<sup>+</sup> responder T cells, and CD8<sup>+</sup> T cells was not significantly changed in CXCR3-deficient mice compared with wt mice (data not shown).

**Loss of Con A tolerance in CXCR3<sup>−/−</sup> mice correlates with decreased intrahepatic numbers of Tregs**

Recently, we reported that IL-10–producing Tregs mediate tolerance against Con A restimulation by suppressing the Th1 response and attenuating liver pathology (12). It has also been shown that CXCR3<sup>+</sup> Tregs preferentially migrate into the inflamed liver (8, 17, 18). We therefore investigated whether CXCR3<sup>−/−</sup> mice have defective immunoregulation by studying their ability to develop Con A tolerance. As shown in Fig. 3E, a sublethal dose of Con A (5 mg/kg) not only failed to induce tolerance toward Con A restimulation in CXCR3<sup>−/−</sup> mice but also even aggravated liver damage in these animals (Fig. 3E, right panel). Although low-dose pretreatment of wt mice with 5 mg/kg only induced a slight reduction of plasma transaminase activities (Fig. 3E, middle bars), pretreatment with 10 mg/kg significantly attenuated Con A-induced liver injury (Fig. 3E, left bars). These results suggest that a threshold of immune activation is necessary...
for the development of Con A tolerance in wt mice and that tolerance is not inducible in the absence of CXCR3.

According to the high plasma transaminase activities in CXCR3
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mice upon Con A restimulation, proinflammatory cytokine production was not reduced in either plasma or liver tissue compared with saline-pretreated CXCR3-deficient mice both in plasma and liver tissue (Fig. 3G). Also in accordance with our previous results (12), IL-2 production was still suppressed in plasma (Fig. 3F) and liver tissue (Fig. 3G) of Con A-pretreated CXCR3-deficient mice upon Con A rechallenge, indicating that impairment of IL-2 production occurred independently of CXCR3 and failed to correlate with suppression of immune pathology. The anti-inflammatory and protective cytokine IL-10 is mainly produced by Tregs in Con A-tolerant mice (12). Interestingly, CXCR3-deficient mice produced less IL-10 than wt mice upon a single i.v. Con A injection (Fig. 3C, black bar) and failed to establish tolerance-induced production of IL-10 both in plasma and liver tissue (Fig. 3F, 3G) upon Con A restimulation. These findings correlate very well with the reduced numbers of Tregs found in livers of both saline- and Con A-treated CXCR3-deficient mice compared with wt mice on day 8 (i.e., at the time point of tolerance induction) (Fig. 4A, 4B). However, the number
of CD4+CD25+Foxp3+ Tregs was significantly increased in the secondary lymphoid organs (spleen and portal LN) of CXCR3−/− mice, indicating a reduced ability of CXCR3−/− Tregs to migrate from spleen or liver-draining LNs into the liver (Fig. 4B: representative dot plots of CD4+Foxp3+ Tregs, all Foxp3+ cells were also CD25 positive; Fig. 4C: quantification of Treg numbers). Moreover, Treg frequencies were significantly increased in livers of Con A-tolerant wt mice 2 h after Con A restimulation, whereas this effect was not detectable in CXCR3−/− mice (Fig. 4D). In contrast, livers of both saline- and Con A-treated CXCR3−/− mice harbored considerably increased numbers of activated CD4+CD25−Foxp3+ effector T cells compared with wt animals (Fig. 4E: quantification of the frequency of CD4+CD25−Foxp3+ T effector cells; Fig. 4F: representative dot plots of CD4+CD25−Foxp3+ T effector cells in wt versus CXCR3-deficient mice 2 h upon Con A challenge). Besides the increased frequency of activated T cells, the mean fluorescence intensity (MFI: 344 versus 495) was also elevated in CXCR3-deficient mice, demonstrating an upregulation of CD25 on the single-cell level. These results explain both the higher susceptibility toward Con A hepatitis as well as loss of tolerance in CXCR3−/− mice. NKT cells do not seem to play any role in the recruitment of CD4+CD25+Foxp3+ Tregs in this model (Fig. 4G), because Treg frequencies were still significantly increased in Con A-tolerant NKT cell-deficient CD1d−/− mice upon restimulation, thus confirming our previous results that showed Con A tolerance develops independently of NKT cells (12). Impaired therapeutic potential of CD4+CD25+ Treg, derived from Con A-pretreated CXCR3−/− mice As recently demonstrated, Tregs from Con A-tolerant wt mice exhibit a higher immunotherapeutic potential against Con A-induced hepatitis compared with naturally occurring Tregs (12). Splenic Tregs from Con A-tolerant mice were CD62Llow, arguing for their preferential migration capacity to the inflamed liver upon Con A challenge (data not shown). Indeed, CD4+CD25+ Tregs that
were adoptively transferred from Con A-tolerant wt mice into wt mice 24 h prior to Con A challenge reduced liver injury in contrast to Tregs transferred from CXCR3-deficient mice (Fig. 5A).

Moreover, Tregs from Con A-tolerant wt mice also protected CXCR3-deficient mice against Con A hepatitis (Fig. 5B), again providing evidence for the high suppressive capacity of Con A-primed Tregs.

To confirm the impaired migration capacity of CXCR3-deficient Tregs, we performed an in vivo migration experiment using CD45.1-expressing congenic mice. To recover the injected CD45.2+ cells, it was necessary to transfer $10^6$ splenic Tregs from either wt or CXCR32/2 mice 24 h prior to Con A challenge. Purity and CD25/Foxp3 expression of the injected cells were controlled by FACS analysis (>90%). Eight hours upon Con A treatment, CD45.2+ cells were identified in spleen and portal LN, respectively, 2 h after Con A challenge (data not shown).

Increased expression of the transcription factor T-bet in Tregs isolated from Con A-tolerant mice

Recently, it has been shown that CXCR3 expression on Tregs depends on the Th1-specific transcription factor T-bet, because T-bet-deficient mice completely lack Foxp3CXCR3+ Tregs (6). To identify whether Tregs had been converted to T-bet+ Tregs following induction of the Th1 response in the liver, we analyzed T-bet protein expression by intrahepatic Tregs ex vivo using intracellular FACS staining 8 d after the first Con A injection. Tregs
from Con A-treated wt mice actually did exhibit significantly increased T-bet protein expression compared with saline-treated controls. Interestingly, almost all of these T-bet–positive Tregs from Con A-treated wt mice coexpressed CXCR3 (897%; Fig. 6A, representative contour plot; Fig. 6B: quantification of CXCR3+T-bet+ Tregs among CD4+CD25+Foxp3+ Tregs, 10.8 ± 1.0% versus 23.1 ± 3.9%). The MFI was nearly unchanged, demonstrating an increase in the number of double-positive CXCR3+ Tbet+ Tregs, but not an upregulation of T-bet and CXCR3 on a single-cell level.

In addition, T-bet mRNA expression was also measured in highly purified splenic CD4+CD25+ Tregs from saline- or Con A-treated wt animals and, as a control, in splenic CD4+ responder cells upon in vitro stimulation. Intracellular Foxp3 expression by Tregs was controlled by flow cytometry (>95%). Interestingly, therapeutically efficient Tregs (compare Fig. 5) primed in a Th1 environment, as a result of the in vivo Con A challenge, exhibited significantly increased T-bet expression upon in vitro restimulation, whereas T-bet expression in Tregs from naive mice was close to the detection limit (Fig. 6C, left graph). Therefore, Tregs from Con A-tolerant mice significantly overexpressed the immunosuppressive cytokine IL-10 compared with responder cells and Tregs from saline-treated mice (Fig. 6C, right graph).

To demonstrate the relevance of an intact Th1 immune response for inducing T-bet expression in Tregs from Con A-tolerant mice, we either injected a neutralizing Ab against IFN-γ 1 h prior to Con A challenge or we used IFN-γ−/− mice. The frequencies of CXCR3+T-bet+ Tregs isolated from Con A-anti–IFN-γ−treated wt or Con A-tolerated IFN-γ−/− mice 8 d upon Con A challenge were comparable to saline-treated mice and significantly reduced in comparison with Con A-tolerant mice (Fig. 6D: representative contour plot of CXCR3+T-bet+ Tregs isolated from Con A-tolerated wt or IFN-γ−/− mice, respectively; Fig. 6E: quantification of CXCR3+T-bet+ Tregs among CD4+CD25+Foxp3+ Tregs, WT: 17.2 ± 1.5% versus IFN-γ−/−: 8.3 ± 0.9% or versus anti–IFN-γ/WT: 10.3 ± 1.8%). These data demonstrate that an IFN-γ response is required for increased frequencies of CXCR3+T-bet+ Tregs in the inflamed liver. Successful anti–IFN-γ treatment was confirmed by the absence of increased plasma transaminase activities and IL-6 secretion 8 h after Con A challenge (data not shown). To confirm the redundancy of NKT cells for intrahepatic Treg recruitment in our model of Con A tolerance, we also analyzed the frequency of CXCR3+T-bet+ Tregs in CD1d-deficient mice. In agreement with the overall increased Treg frequency in Con A-tolerant CD1d−/− mice (compare Fig. 4G), we also detected a significantly increased frequency of double-positive CXCR3+T-bet+ Tregs despite the lack of NKT cells (Fig. 6F: representative contour plot of CXCR3+T-bet+ Tregs isolated from saline- or Con A-treated CD1d−/− mice, respectively; Fig. 6G: quantification of CXCR3+T-bet+ Tregs among CD4+CD25+Foxp3+ Tregs, 3.0 ± 0.5% versus 13.2 ± 2.0%), suggesting that IFN-γ release by conventional CD4+ T cells was responsible and sufficient for conversion to CXCR3+T-bet+ Tregs in our model of Con A tolerance.

Discussion

The liver appears to be a privileged organ regarding induction of immunological tolerance due to its special position linking the gastrointestinal tract and the systemic venous circulation. Hence, the liver assumes the function of a scavenger organ by clearance of foreign Ags from the gut without inflammation, and therefore, it probably harbors organ-specific or dominant immunoregulatory mechanisms (19, 20). Moreover, liver transplants are often not rejected despite MHC discrepancies and even in the absence of immunosuppression (21). However, hepatotropic pathogens have to be eliminated to prevent persistence and chronic infections (22). To fulfill these functions, the liver contains both resident and recruited lymphocytes. In the healthy liver, most lymphocytes are activated and express much higher levels of CXCR3 and CCR5 than circulating memory T cells (23). These T cells are not necessarily Th1 cells, because patients with autoimmune liver diseases harbor CXCR3hiCCR7low Tregs in the inflamed liver in contrast to a predominance of CXCR3loCCR7hi Tregs in the peripheral blood (8). Accordingly, as shown in this study, livers from untreated mice contain higher frequencies of both CXCR3hi CD4+ T cells and CXCR3+ Tregs compared with secondary lymphoid organs or blood.

In the current study, we have demonstrated a beneficial role for CXCR3 in Th1-mediated acute hepatitis as well as in Con A tolerance. It is well known that several hepatic cell populations...
secrete specific chemokines upon IFN-γ activation (23). We identified hepatocytes and LSECs as CXCR3 ligand-producing cells upon Con A challenge in vivo and thus demonstrated their involvement in CXCR3+ lymphocyte recruitment. In this context, Jaruga et al. (16) reported that IFN-γ treatment induces the expression of several chemokines in primary hepatocytes, LSECs, and Kupffer cells through activation of STAT1, STAT3, and IFN regulatory factor 1. Accordingly, the lack of IFN regulatory factor 1 resulted in protection against Con A-induced hepatitis due to suppressed chemokine expression (among others, CXCL9, CXCL10, and CXCL11) and decreased leukocyte infiltration into the liver (16).

To elucidate the role of CXCR3 during Th1-mediated Con A-induced liver injury, we used CXCR3-deficient mice. Interestingly, CXCR3-deficient mice were highly susceptible toward Con A in contrast to wt mice as a result of an increased intrahepatic frequency of disease-mediating NKT cells. This suggests that CXCR3 has a protective function in acute immune-mediated liver injury. In contrast to our findings, Jiang et al. (24) reported that NKT cells were virtually absent in CXCR3-deficient mice (wt: 20% versus CXCR3−/−: 0.8%). The reason for this discrepancy is unclear. One explanation might be that Jiang et al. (24) used mice backcrossed from 129J mice for five generations. In 129J mice, the marker NK1.1 is not expressed on NK and NKT cells. Hence, FACS analysis using anti-CD3 and anti-NK1.1 Abs for detection of NKT cells is not applicable in some mouse strains. We used mice backcrossed for more than seven generations to the C57/BL6 background (4) and compared knockout mice to age- and sex-matched wt controls. Moreover, in preliminary studies, Santodomingo-Garzon et al. (17) observed reduced hepatic NK+ cell numbers in CXCR3-deficient mice. However, they did not mention which background and Abs were used for the determination of intrahepatic NK+ cells in CXCR3-deficient mice. Interestingly, a further study demonstrated that the chemokine receptor CXCR3 is necessary to retain NKT cells in the thymus (25). Hence, CXCR3−/− mice harbored an increased frequency of NKT cells in the periphery, in line with our results.

FIGURE 6. Increased expression of T-bet, IL-10, and CXCR3 by Tregs from Con A-tolerant wt mice. A, The wt mice were treated with saline or Con A. On day 8, FACS analysis was performed to measure T-bet and CXCR3 protein expression by intrahepatic CD4+CD25+Foxp3+ Tregs. A representative contour plot is depicted. B, The intrahepatic frequency of CXCR3+T-bet+CD4+CD25+ Foxp3+ Tregs from saline-treated (white bar) and Con A-treated wt mice (black bar) was quantified (**p = 0.0095; mean ± SEM; n = 5). C, Spleenic CD4+CD25+ Tregs were isolated from Con A-pre-treated (Tregs [Con A]) or saline-pre-treated (Tregs [sal]) wt mice by FACS sorting. As a control, CD4+CD25+ responder cells were isolated from naive wt mice. Total of 1 × 10^6 cells/well were cultivated and stimulated with anti-CD3 mAb (5 μg/ml, plate-bound) for 72 h. x-fold induction was calculated, referring to mRNA levels of T-bet (p = 0.04) and IL-10 (p = 0.03) in responder cells from saline-treated wt animals using qRT-PCR (mean ± SEM; n = 3). D, Representative contour plot of CXCR3+T-bet+ Tregs isolated from Con A-treated wt or IFN-γ−/− mice, respectively. E, The wt mice (white bars) were pretreated with anti-IFN-γ mAb or control rat IgG 1 h prior to Con A challenge. Saline-treated wt mice represent the negative control. IFN-γ−/− mice (black bars) were treated with saline or Con A. After 8 d, intrahepatic frequencies of CXCR3+T-bet+CD4+CD25+Foxp3+ Tregs were measured by FACS analysis (**p = 0.001; mean ± SEM; n = 5). F, Representative contour plot of CXCR3+T-bet+ Tregs isolated from saline- or Con A-treated CD1d−/− mice. G, CD1d−/− mice were treated with saline (white bars) or Con A (black bars). Intrahepatic frequencies of CXCR3+T-bet+CD4+CD25+Foxp3+ Tregs were determined by FACS analysis 8 d after Con A challenge (**p < 0.001; mean ± SEM; n = 5).
Tregs (26). Indeed, α-galactosylceramide–induced, IFN-γ–mediated specific activation of NKT cells has been shown to induce CXCL10 release, followed by selective recruitment of CXCR3-expressing Tregs into the liver (17). This immediate recruitment of Tregs has also been observed in the Con A model as early as 24 h following the first injection (12) and 2 h upon Con A restimulation; however, it did not depend on NKT cells, because increased Treg frequencies were also detectable in Con A-tolerant CD1d−/− mice (Fig. 4F). Hence, upon Con A-induced inflammation, NKT cells are not required for Treg recruitment into the liver. Nevertheless, NKT cells might regulate Treg and T cell homeostasis in the liver under noninflammatory conditions.

The early Treg infiltration observed within the first 24 h upon Con A challenge (12) may regulate the acute inflammatory response. Hence, aggravated liver damage associated with increased mortality in CXCR3−/− mice might not only be the result of increased intrahepatic NKT cell frequency but also due to impaired early recruitment of CXCR3-expressing Tregs into the liver. Importantly, naive CXCR3−/− mice displayed significantly reduced numbers of CD4+CD25+Foxp3+ Tregs in the liver per se in contrast to naive wt mice (Fig. 4A, white bars), whereas the Treg frequency was increased in secondary lymphoid organs isolated from CXCR3−/− mice (Fig. 4C), suggesting a retention of Tregs in these organs. Therefore, the decreased numbers of intrahepatic Tregs that correspond to elevated frequencies of activated CD4+CD25+Foxp3+ responder cells in livers of untreated CXCR3−/− mice might also have exacerbated Con A hepatitis in these animals. Moreover, in an in vivo migration experiment, we clearly demonstrated that CXCR3-deficient Tregs were less frequent in the liver of congenic mice upon Con A challenge compared with wt Tregs, verifying an impaired migration capacity of Tregs lacking CXCR3. It has to be remembered that non-lymphoid tissue-homing Tregs also express further chemokine receptors beside CXCR3 (e.g., CCR5 and CCR6), which might also account for Treg recruitment to the inflamed site (27–30). Hence, we did find a 5-fold increase of the CXCR3+ Treg subpopulation in Con A-tolerant CD1d−/− mice (Fig. 6E), which might be due to the general lack of IFN-γ. This could lead to a decreased expression pattern of IFN-γ–inducible CXCR3-binding ligands CXCL9, CXCL10, and CXCL11 and hence a reduced recruitment of CXCR3+ T cells in IFN-γ−/− mice. The necessity of IFN-γ for inducing CXCR3 expression has also been demonstrated by Nakajima et al. (31), but only in an in vitro assay. Moreover, the increased frequency of CXCR3+T-bet+ Tregs in the liver of Con A-tolerant mice seems to be independent of NKT cells, because Con A-tolerant, NKT cell-deficient CD1d−/− mice also displayed elevated numbers of intrahepatic CXCR3+T-bet+ Tregs (Fig. 6F, 6G), confirming our previous results regarding NKT cell redundancy in our model of Con A tolerance (12). Because the initial frequency of CXCR3+T-bet+ Tregs was reduced in naive CD1d−/− mice compared with wt mice (3.0 ± 0.5% versus 10.8 ± 1.0%), one could expect that NKT cells are essential for Treg recruitment under steady-state conditions as also shown by Santodomingo-Garzon et al. (17). However, upon a strong inflammatory process, we detected a 5-fold increase of the CXCR3+ Treg subpopulation in Con A-tolerant CD1d−/− mice, despite the lack of NKT cells.

Hence, our study considers distribution and migration of Treg subsets between target organ and secondary lymphoid organs. These novel findings emphasize the special role of the liver in the development of tolerance mediated by conversion to T-bet+ CXCR3+ Tregs. Thus, CXCR3+ Tregs primed in a Th1 microenvironment are specialized to inhibit Th1-mediated liver inflammation in a kind of a feedback loop, resulting in immunosuppression and Con A tolerance.

Remarkably, in a murine acute graft-versus-host disease model, transfer of CXCR3-transfected Tregs ameliorated liver disease, due to more pronounced migration and prolonged persistence of highly suppressive CXCR3+ Tregs within the liver (32). These findings correlate very well with our data, because Tregs from Con A-pretreated CXCR3-deficient mice failed to protect against Con A-induced hepatitis in contrast to Tregs from Con A-tolerant wt mice, most probably because of the impaired migration capacity of CXCR3−/− Tregs. Intriguingly, Tregs from CXCR3-deficient mice even augmented the disease severity of Con A-induced hepatitis compared with Con A-challenged control mice, suggesting that firstly, Tregs from CXCR3-deficient mice might not be able to enter the inflamed liver, and, secondly, they might even block immunoregulatory functions mediated by APCs in the draining LN. However, CXCR3-deficient mice can be cured by Tregs isolated from Con A-tolerant wt mice, because these Tregs exhibit an intact and preferential migration capacity into the inflamed liver.

A very recent study has demonstrated that intrahepatic Tregs from chronically inflamed human tissue express high levels of CXCR3 and CCR4. In vitro adhesion assays argued for a role of CXCR3 in endothelial transmigration of isolated Tregs (18). However, a functional role of CXCR3 related to the severity of liver diseases in vivo has never been described. We have clearly demonstrated that functional impairment of CXCR3 is associated with higher intrahepatic frequencies of CD4+CD25+Foxp3+ responder T cells, reduced recruitment of CXCR3+ Tregs into the liver, to suppress the Th1 response, and loss of IFN-γ–dependent conversion to CD4+CD25+Foxp3+CXCR3+T-bet+ IL-10+ Tregs that maintain liver tolerance.

In the past, clinical trials using low m.w. chemokine receptor antagonists for treatment of autoimmune and inflammatory disease have failed, although beneficial effects have been observed in animal models (33). Our study clearly demonstrates that this might have been caused by the loss of immune regulation by Tregs via inhibition of their conversion and accumulation at the inflamed
site. Hence, targeting chemokine–receptor pathways as a therapeutic approach might have detrimental consequences and therefore requires precise investigation of the functional and local contribution of immune cell subsets, because the recruitment of either immunosuppressive Tregs or proinflammatory Th1-polarized cells to inflamed tissue and conversion of Tregs seems to be time and organ dependent.

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