The Function of the Chemokine Receptor CXCR6 in the T Cell Response of Mice against Listeria monocytogenes

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Abstract

The chemokine receptor CXCR6 is expressed on different T cell subsets and up-regulated following T cell activation. CXCR6 has been implicated in the localization of cells to the liver due to the constitutive expression of its ligand CXCL16 on liver sinusoidal endothelial cells. Here, we analyzed the role of CXCR6 in CD8+ T cell responses to infection of mice with Listeria monocytogenes. CD8+ T cells responding to listerial antigens acquired high expression levels of CXCR6. However, deficiency of mice in CXCR6 did not impair control of the L. monocytogenes infection. CXCR6-deficient mice were able to generate listeria-specific CD4+ and CD8+ T cell responses and showed accumulation of T cells in the infected liver. In transfer assays, we detected reduced accumulation of listeria-specific CXCR6-deficient CD8+ T cells in the liver at early time points post infection. Though, CXCR6 was dispensable at later time points of the CD8+ T cell response. When transferred CD8+ T cells were followed for extended time periods, we observed a decline in CXCR6-deficient CD8+ T cells. The manifestation of this cell loss depended on the tissue analyzed. In conclusion, our results demonstrate that CXCR6 is not required for the formation of a T cell response to L. monocytogenes and for the accumulation of T cells in the infected liver but CXCR6 appears to influence long-term survival and tissue distribution of activated cells.

Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped bacterium with ubiquitous distribution in nature. Infection mainly occurs by contaminated food. Risk groups include immunocompromised and old persons, pregnant women and neonates. Infection of mice with L. monocytogenes causes rapid activation of the innate immune system, which is essential for the restriction of bacterial replication. Due to its intracellular growth, L. monocytogenes induces a strong CD8+ T cell response. These CD8+ T cells accumulate in spleen and liver and are mainly responsible for bacterial clearance and for effective protection after infection [1,2]. The mechanisms regulating CD8+ T cell accumulation in the infected liver are only partially understood [3]. Recruitment of T cells to sites of infection is controlled by the local expression of addressins, adhesion molecules and pro-inflammatory chemokines. On an mRNA level, activated CD8+ T cells in L. monocytogenes infection express relatively high levels of the chemokine receptors CCR2, CCR5, CXCR3 and CXCR6 which respond to pro-inflammatory chemokines (unpublished results). However, there are only a few studies on the role of these chemokine receptors in L. monocytogenes-infection. CXCR3 was shown to control migration of CD8+ T cells within different compartments of the spleen and to regulate differentiation processes of these cells. However, absence of CXCR3 did not reduce liver accumulation of CD8+ T cells during acute infection [4]. CCR5-deficient mice showed impaired control of L. monocytogenes [5], though a second study could not confirm enhanced susceptibility and further showed that CCR5-deficient mice mounted normal listeria-specific CD8+ T cell responses [6].

The chemokine receptor CXCR6 (CD186), also known as Bonzo, STRL3 or TYMSTR, was originally described as a co-receptor for SIV and HIV [7–9]. It is expressed on subsets of T cells, NK cells, NKT cells, and plasma cells [10]. CXCR6 is expressed at very low levels on naive CD8+ T cells [11], but it is up-regulated after activation [12–14]. The only known ligand for CXCR6 is CXCL16, also known as SR–PSOX (scavenger receptor that binds phosphatidyl serine and oxidized lipoprotein) [11,15,16]. Similar to CXCL1/CXCL6, CXCL16 is expressed with a transmembrane domain and can function as a membrane-bound chemokine or adhesion molecule [11,15]. Transmembrane CXCL16 is expressed by macrophages, monocytes, dendritic cells, B cells and sinusoidal endothelium cells of the liver [7,18]. Under certain conditions, soluble CXCL16 is generated by protease mediated shedding of the membrane-bound form by ADAM-10 or ADAM-17 [19].

In this study, we investigated the role of CXCR6 in CD8+ T cell activation, differentiation, and migration during the immune response of mice against L. monocytogenes. Listeria-specific CD8+ T cells in spleen and liver acquired CXCR6 expression on their
surface. However, CXCR6 deficiency did not impair control of L. monocytogenes infection and CXCR6-deficient mice generated normal CD4+ and CD8+ T cell responses and showed similar accumulation of these cells in the liver. In T cell transfer assays, early accumulation of activated Listeria-specific CD8+ T cells in the liver depended on the expression of CXCR6. However, CXCR6 strain expressing ovalbumin (LmOVA) 

Materials and Methods

Mice

C57BL/6 mice (The Jackson Laboratory), CD90.1-congenic C57BL/6 mice (B6.PL-Thy1a/CyJ; The Jackson Laboratory), RAG1−/− mice (The Jackson Laboratory), OT-I mice [20], and CXCR6GFP/GFP mice [21] were bred under specific-pathogen-free conditions at the animal facility of the University Medical Center Hamburg-Eppendorf. Experiments were conducted according to the German animal protection law. Experiments were approved by the Behörde für Gesundheit und Verbraucherschutz of the City of Hamburg under the permits 56/12 and 99/10. Animals were housed in individually ventilated cages under 12 h light/dark cycles and constant temperature. Water and food was provided ad libitum. During acute infection, mice were controlled daily. Animals with overt symptoms of disease were euthanatized to avoid suffering. Animals were euthanatized with CO2.

Infection of mice with L. monocytogenes

Age (>8 weeks) and sex-matched mice were infected intravenously (i.v.) or intraperitoneally (i.p.) with indicated doses of the wild type L. monocytogenes strain EGD (Lm) or of a L. monocytogenes strain expressing ovalbumin (LmOVA) [22]. Bacterial inocula were controlled by plating serial dilutions on tryptic soy broth (TSB) agar plates.

Isolation of cells

Lymphocytes from spleen and lymph nodes were obtained by mashing the organs through a cell sieve into PBS followed by erythrocyte lysis with lysing buffer (155 mM NH4Cl, 10 mM KHCO3, 100 m M EDTA [pH 7.2]). Liver and lung were perfused with PBS. For purification of lymphocytes from the liver, the liver cell suspension was additionally separated using a 40%/70% Percoll gradient (Biochrom AG, Berlin, Germany) before erythrocyte lysis. Lungs were cut into small pieces and digested with 2 mg/ml collagenase D (Roche, Penzberg, Germany) and 100 μg/ml DNase I (Roche) in complete RPMI1640 medium (RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 μg/ml gentamycin) before lysing red blood cells. Lymphocytes from bone marrow were isolated out of one femur and 50 mg/ml gentamycin) before lysing red blood cells. Lymphocytes from spleen and lymph nodes were obtained by mashing the organs through a cell sieve into PBS followed by erythrocyte lysis with lysing buffer (155 mM NH4Cl, 10 mM KHCO3, 100 m M EDTA [pH 7.2]). Liver and lung were perfused with PBS. For purification of lymphocytes from the liver, the liver cell suspension was additionally separated using a 40%/70% Percoll gradient (Biochrom AG, Berlin, Germany) before erythrocyte lysis. Lungs were cut into small pieces and digested with 2 mg/ml collagenase D (Roche, Penzberg, Germany) and 100 μg/ml DNase I (Roche) in complete RPMI1640 medium (RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 μg/ml β-mercaptoethanol, and 50 mg/ml gentamycin) before lysing red blood cells. Lymphocytes from bone marrow were isolated out of one femur and erythrocytes were lysed.

Determination of listeria titers

Bacterial burdens in spleen and liver were determined by homogenization of organs in 0.1% Triton X-100. Serial dilutions of homogenates were plated on TSB agar plates and colonies were counted after 24 h of incubation at 37°C.

In vitro stimulation of T cells

For the determination of cytokine production, 2×106 lymphocytes were incubated with ovalbumin peptide (OVA257-264, SIIIFEKL, JPT Peptide Technologies GmbH, Berlin) for specific stimulation of CD8+ T cells and with listeriolysin O peptide (LLOH9-291, NEKVAQAYPANV; JPT Peptide Technologies GmbH, Berlin) for specific stimulation of CD4+ T cells in complete RPMI1640 medium for 4 h at 37°C. To prevent protein secretion, 10 μg/ml Brechelin A (BFA; Sigma) was added for the final 3.5 h of culture.

For the analysis of proliferation, 4×105 cells from spleen were incubated for 3 d at 37°C with increasing concentrations of CXCL16 (3–300 ng/ml), with increasing concentrations of IL-15 (3–300 ng/ml) or with 2 μg/ml anti-CD3 mAb and 2 μg/ml anti-CD28 mAb.

Flow cytometry

For extracellular cell-staining, lymphocytes were incubated with 10 μg/ml anti-CD16/CD32 mAb (anti-FcyRII/III; BioXCell, West Lebanon) and 1:100 normal rat serum (NRS; Jackson Laboratories, Bar Harbor) to minimize unspecific antibody binding and then stained with specific fluorochrome-conjugated mAbs for 20 min at 4°C. MAb to CD8α (53–67), CD4 (RM4–5), CD90.1 (His51), CD90.2 (53–21), CD44 (IM7), CD62L (MEL–14), KLRG1 (2F1), IFN–γ (XMG1.2), TNF–α (MP6–XT22, TN3–19), PD–1 (J43), LAG3 (eBioC9B7W) and CD244 (BD4) were purchased from BioLegend (San Diego), eBioscience (San Diego), or BD (Heidelberg). Cells were measured with a FACS CantoII flow cytometer (BD, Heidelberg). Results were analyzed with the DIVA Software (BD, Heidelberg) and the FlowJo Software (Tree Star, Ashland). Debris, doublets, and dead cells were excluded from analysis. Dead cells were identified by staining with Dapi (4′,6-diamidino-2-phenylindole; Merck, Darmstadt) or AlexaFluor–750 succinimidyl ester (AF750; Invitrogen, Karlsruhe).

Intracellular staining

For intracellular cytokine staining, cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and washed with 0.2% BSA in PBS. Cells were blocked with anti-CD16/32 mAb and NRS in PBS containing 0.1% BSA and 0.3% saponin (Sigma) and stained with fluorochrome-conjugated mAb in the same buffer.

Flow cytometry-based assays

In vivo proliferation was determined by BrdU (Bromodeoxyuridine; BD, Heidelberg) incorporation. Mice received 1 mg BrdU i.p. 1 d before analysis. Anti-BrdU mAb was used according to the manufacturer’s protocol. In vitro proliferation was measured by staining of cells with the proliferation dye eFluor 670 (eF670; eBioscience, San Diego). 5×107 cells were incubated in PBS containing 5 μM eFluor670 and 0.2% BSA for 15 min at 37°C. Apoptosis was determined by using the FLICA Kit (Immunochemistry Technologies, Bloomington) according to the manufacturer’s protocol.

Competitive T cell transfer experiments

CD8+ T cells from spleens of CXCR6GFP/GFP OT–I mice and CD90 congenic wt OT–1 mice were purified by negative magnetic activated cell sorting (MACS; Miltenyi Biotech, Bergisch Gladbach) according to the manufacturer’s protocol. Wt OT–1 (CD90.1+ CD90.2+) and CXCR6GFP/GFP OT–1 (CD90.1+ CD90.2+) CD8+ cells were mixed in a 1:1 ratio and a total of...
5 × 10⁴ cells were injected i.v. into congenic wt (CD90.1⁺ CD90.2⁻) or RAG1⁻/⁻ mice. Recipient mice were infected with 1 × 10⁵ L. OVA i.p. 4 h before transfer.

Statistical analysis
All statistical analysis was performed with GraphPad Prism software (La Jolla). Differences between two groups of mice were analyzed by Mann-Whitney U test (bacterial burden) or student t-test. Differences between three or more groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett post-test. A p-value of 0.05 was considered significant (*, p<0.05; **, p<0.01; ***, p<0.001).

Results

CXCR6 expression on CD8⁺ T cells during L. monocytogenes infection
Expression of the chemokine receptor CXCR6 has been described for activated CD8⁺ T cells [12–14]. We therefore tested whether the infection of mice with L. monocytogenes causes changes in the expression level of CXCR6 on CD8⁺ T cells. For the determination of CXCR6 expression, we used heterozygous GFP knock-in mice, in which one allele of the CXCR6 gene has been replaced by enhanced GFP [21]. In naive mice, we observed CXCR6 expression on approx. 20% of CD8⁺ T cells in spleen and liver. After infection of mice with L. monocytogenes, CD8⁺ T cells from spleen and liver showed up-regulation of CXCR6 (Fig. 1A, 1B). Up-regulation of CXCR6 was particular prominent on CD8⁺ T cells with an activated CD4⁴⁺CD62L⁻ phenotype (Fig. S1). In the liver, even in the non-infected state, most of CD4⁴⁺CD62L⁻ CD8⁺ T cells were CXCR6⁺. Mice were infected with a L. monocytogenes strain recombinant for ovalbumin (LmOVA). Short-term stimulation with the immunodominant peptide OVA257-264 enabled detection of CD8⁺ T cells generated in response to infection via their expression of IFN–γ (Fig. 1C, 1D). At d5, the first time point of detection of specific CD8⁺ T cells, 50% of IFN–γ⁺ cells co-expressed CXCR6. CXCR6 reached a high expression level on IFN–γ⁺ CD8⁺ T cells at the peak of the CD8⁺ T cell response at day 9 and remained at this high level at d14, when the cells was delayed.

CXCR6 is not essential for a protective immune response against L. monocytogenes
Control of L. monocytogenes depends on innate immune mechanisms as well as on effective CD4⁺ and CD8⁺ T cell responses. To investigate the role of CXCR6 in the control of infection, CXCR6-deficient (CXCR6⁶/⁶/GFP) and wt mice were infected with L. monocytogenes and the bacterial burdens in spleen and liver were determined (Fig. 2). In the spleen, CXCR6⁶/⁶/GFP and wt mice had similar bacterial titers at all analyzed time points. Interestingly, CXCR6⁶/⁶/GFP mice showed lower listeria titers in the liver at d5 and d7 post infection. Slightly better protection was also observed in the liver of CXCR6⁶/⁶/GFP mice after infection with a higher bacterial dose, although differences did not reach a level of significance (Fig. S2). Thus, control of listeria-infection occurred independent of CXCR6 and in the liver, absence of CXCR6 even improved clearance of bacteria.

To determine the role of CXCR6 in the induction of CD4⁺ and CD8⁺ T cell responses, we infected CXCR6⁶/⁶/GFP and control (CXCR6²/²/GFP) mice with LmOVA. On day 8, cells from spleen and liver were stimulated in vitro with the immunodominant peptides LLO₁₈₉-₂₀₁ (CD4⁺ T cells) and OVA₂₅₇-₂₆₄ (CD8⁺ T cells), and frequencies of IFN–γ⁺ cells were determined. We detected enhanced CD4⁺ T cell responses in spleen and liver of CXCR6⁶/⁶/GFP mice when compared to control mice (Fig. 3A–C). CD8⁺ T cell responses in spleen and liver tended to be higher in CXCR6⁶/⁶/GFP mice as well, however, differences did not reach a significant level. Overall, CXCR6-deficient mice were not impaired in the generation of CD4⁺ and CD8⁺ T cell responses to L. monocytogenes.

CXCR6 controls early accumulation of activated CD8⁺ T cells in the liver
To restrict the analysis to the function of CXCR6 on CD8⁺ T cells, competitive T cell transfer assays were conducted. We transferred small numbers of an 1:1 mixture of wt and CXCR6⁶/⁶/GFP OVA-specific OT-I CD8⁺ T cells into LmOVA-infected wt recipients. Differential expression for CD90.1 and CD90.2 allowed the distinction of wt OT-I cells (CD90.1⁺CD90.2⁻), CXCR6⁶/⁶/GFP OT-I cells (CD90.1⁺CD90.2⁻) and recipient CD8⁺ T cells (CD90.1⁻CD90.2⁺) (Fig. 4A). At day 3 after transfer and infection, we found different percentages of wt and CXCR6⁶/⁶/GFP OT-I cells in spleen and liver of recipient mice. Whereas the portion of CXCR6⁶/⁶/GFP OT-I cells was higher in the spleen, the portion of wt OT-I cells was higher in the liver. The normalized ratio of transferred CXCR6⁶/⁶/GFP to OT-I cells between spleen and liver was significantly different (Fig. 4B and Fig. S3A). Characterization of transferred CD8⁺ T cells revealed that the majority of wt OT-I cells in the liver displayed an activated CD44⁺CD62L⁻ phenotype with enhanced KLRG1 expression (Fig. 4C, 4D and Fig. S3B, S3C). In contrast, CXCR6⁶/⁶/GFP OT-I cells were largely CD44⁺CD62L⁻. However, five days after transfer the populations of wt and CXCR6⁶/⁶/GFP OT-I cells had equalized in spleen and liver of recipient mice and both T cell populations displayed a similar phenotype. These results demonstrate that early after infection with L. monocytogenes, CXCR6 was important for migration of activated CD8⁺ T cells to the liver but CXCR6 became dispensable at later time-points of infection.

CXCR6 controls tissue distribution and survival of pre-activated CD8⁺ T cells
CXCR6 has been shown to be required for the long-term persistence of memory NK cells [23]. To determine the role of CXCR6 for CD8⁺ T cell persistence and tissue distribution over extend time periods, we used the competitive T cell transfer assay. Although CXCR6⁶/⁶/GFP mice were backcrossed to the C57BL/6 background, we cannot fully exclude rejection of transferred T cells in wt recipients, particularly when transferred cells express high GFP levels [24]. Therefore, competitive transfers were conducted with RAG1⁻/⁻ mice as recipients (Fig. 5A and Fig. S4A). Five weeks after transfer and LmOVA infection, we observed similar frequencies of wt and CXCR6⁶/⁶/GFP OT-I cells in spleen, blood, lymph node, lung and liver. Compared to wt OT-I cells, CXCR6⁶/⁶/GFP OT-I cells remained relatively stable in bone marrow and spleen of recipient mice. In contrast, the proportion of CXCR6⁶/⁶/GFP OT-I cells steadily declined in blood, lymph node, lung and liver, and 30 weeks after transfer, wt OT-I cells largely outnumbered CXCR6⁶/⁶/GFP OT-I cells in these tissues.

Transferred T cells were analyzed at different time point for their activation status. For all time points analyzed, we observed similar expression profiles of CD44 and CD62L on wt and CXCR6⁶/⁶/GFP OT-I T cells, and following peptide stimulation both cell populations showed comparable expression of IFN–γ and TNF-α (Fig. S4B–E). The rate of proliferation of transferred T
Cells was measured by determination of BrdU incorporation (Fig. 5B). Five and 15 weeks after co-transfer and infection, we observed significantly reduced BrdU incorporation in CXCR6GFP/GFP OT-I T cells. In parallel, apoptosis of cells was analyzed with FLICA (fluorescent labeled inhibitor of caspases), which irreversibly binds activated caspases. With this assay, CXCR6GFP/GFP T cells showed reduced apoptosis when compared to wt T cells (Fig. 5C). These results indicate that transferred wt OT-I T cells and CXCR6GFP/GFP OT-I T cells differ in their turnover, with increased proliferation but also enhanced apoptosis in wt cells. Transferred T cells were also characterized for the expression of the exhaustion markers PD-1, LAG3 and CD244 (Fig. S5A, S5B). Expression of PD-1 and CD244 were largely similar on wt and CXCR6GFP/GFP OT-I cells, although we observed some variability at different time points, and LAG3 was consistently lower on CXCR6GFP/GFP cells. Thus there was no indication for increased exhaustion of transferred CXCR6GFP/GFP cells.

Finally, we tested whether the CXCR6 ligand CXCL16 could directly induce proliferation and survival in CD8+ T cells. Purified CD8+ T cells were eF670 labeled and incubated with different concentrations of CXCL16 or as controls with IL-15, anti-CD3 mAb plus anti-CD28 mAb or a combination of CXCL16 and IL-15 (Fig. S6A). IL-15 caused a dose dependent proliferation as determined by the loss of eF670 staining. In contrast, CXCL16 failed to cause T cell proliferation and did not enhance the
CXCR6 is not essential for a protective secondary response against *L. monocytogenes*

Acquired protection against secondary *L. monocytogenes* infection mainly depends on specific CD8+ T cells generated during primary infection. To test whether the alterations in the maintenance of CD8+ T cells impair the control of *L. monocytogenes* following secondary infection, wt and CXCR6GFP/GFP mice were challenged 60 days after primary infection with a high dose of *L. monocytogenes* (Fig. 6). Compared to naive mice, both control and CXCR6GFP/GFP mice showed profoundly reduced listerial titers in spleen and liver two days post challenge infection. Although we observed slightly higher titers in the liver of secondary infected CXRC6GFP/GFP mice, the difference did not reach the level of significance.

**Discussion**

A population of CXCR6+ CD8+ T cells was detected in naive mice and mainly confined to CD68+ cells with a pre-activated CD44+CD62L+ phenotype. In the liver, the majority of CD44+CD62L+ CD8+ T cells was CXCR6+, indicating that under homeostatic conditions, CXCR6+ CD8+ T cells preferentially accumulate in the liver, probably due to the constitutive expression of CXCL16 on liver sinusoidal endothelium cells (LSEC) [11,25]. Following *L. monocytogenes* infection, CXCR6 was up-regulated on activated CD8+ T cells and the majority of CD8+ T cells responding to listerial antigens acquired a CXCR6+ phenotype. Interestingly, CXCR6 expression was delayed when compared to the expression of IFN-γ, which would indicate that expression of CXCR6 and IFN-γ are independently regulated in CD8+ T cells. However, it has to be considered that although GFP expression is under the control of the Cxcr6 promoter, GFP and CXCR6 could still be differently regulated on mRNA and protein level. Thus, GFP might not fully reflect surface expression of the CXCR6 protein.

Surprisingly, CXCR6-deficient mice showed better control of *L. monocytogenes* infection in the liver and equal or even stronger CD4+ and CD8+ T cell responses in spleen and liver. The reason for this improved response is currently unclear. We consider it unlikely that the CXCL16/CXCR6 interaction is directly suppressive to cells involved in the control of *L. monocytogenes*. However, CXCR6-deficient mice could either lack suppressive cells or show a compensatory response to the absence of a protective cell population, which might then even exceed the response observed in wt mice. CXCR6-deficient mice show profoundly reduced numbers of NKT cells in the liver [26] and might also lack certain NK cell subsets in this organ [23]. NKT cells are regarded as pro-inflammatory cells and as protective in bacterial infections [27]. However, in *L. monocytogenes* infection the role of NKT cells is unclear with studies showing better as well as diminished control of listeria in the absence of NKT cells [28–30]. In addition, there is also evidence for a suppressive role of NKT cells [31–33] and the CXCL16/CXCR6 axis might be a central regulatory element of this function. We could recently demonstrate that absence of CXCR6 causes aggravation of autoimmune kidney inflammation.
which was mainly due to diminished recruitment of regulatory NKT cells to the kidney [34]. Thus, reduced numbers of NKT cells in CXCR6-deficient mice could be responsible for the altered response to \textit{L. monocytogenes}. For a hepatic NK cell population, it was proposed that CXCR6 is required to locate these cells to a suppressive environment (e.g. in the close proximity to LSEC) and thereby restrict their reactivity [23]. If a similar mechanism suppresses T cell activity, absence of CXCR6 might result in enhanced T cell responses in the liver.

CXCR6-deficient mice showed equal or even slightly enhanced accumulation of listeria-specific CD4$^+$ and CD8$^+$ T cells in the liver, when compared to wt mice. T cell transfer assays confirmed this result for CD8$^+$ T cells. Thus CXCR6 was not necessary for the massive recruitment of T cells to the infected liver. In this respect, our result from the \textit{L. monocytogenes} infection differs from studies in a graft-versus-host disease model, in which absence of CXCR6 causes reduced accumulation of CD8$^+$ T cells in the inflamed liver [14]. Activated CD8$^+$ T cells express an array of chemokine receptors for pro-inflammatory chemokines such as CCR5 or CXCR3 [21,35–37], and these receptors could compensate for the absence of CXCR6, particularly in the context of strong hepatic inflammation during \textit{L. monocytogenes} infection. On the other hand, the liver is easily accessible even for naive T cells [38] and up-regulation of integrin ligands and addressins could be sufficient for recruitment of activated T cells even in the absence of chemokine receptor signaling. Such a scenario was recently proposed for the recruitment of inflammatory monocytes to the \textit{L. monocytogenes}-infected liver [39]. Interestingly, we observed impaired hepatic accumulation of CXCR6-deficient CD8$^+$ T cells at early time points after transfer and infection which mainly affected CD8$^+$ T cells with a highly activated CD44$^+$ CD62L$^2$ KLRG1$^+$ phenotype. Thus, CXCR6 appears to play a role in the early phase of infection but is subsequently replaced by other chemokine receptors or chemokine independent mechanisms of T cell recruitment up-regulated with ongoing infection.

\textbf{Figure 4. CXCR6 controls early accumulation of activated CD8$^+$ T cells in the liver.} Purified CD8$^+$ T cells from wt OT–I and CXCR6$^{GFP/GFP}$ OT–I mice were mixed in a ratio of 1:1 and a total of $5 \times 10^6$ cells were injected into congenic wt mice i.v. infected with $1 \times 10^5$ LmOVA. Transferred cells from spleens and livers of recipient mice were analyzed at day 3 and day 5 p.i. (A) Representative CD90.1 and CD90.2 staining of purified and mixed OT–I cells before (dc, donor cells) and three and five days after transfer. Numbers show percentages of positive cells. (B) Normalized ratio of transferred OT–I cells in spleens and livers of recipient mice on indicated time points. (C, D) Activation status of transferred OT–I cells in spleens and livers of recipient mice on indicated time points. (C) Bars show distribution of CD44$^+$ CD62L$^+$, CD44$^+$ CD62L$^-$, and CD62L$^-$ cells within wt and CXCR6$^{GFP/GFP}$ OT–I cells. (D) Mean fluorescence intensity (MFI) of KLRG1 staining on transferred OT–I cells. Bars give mean ± SEM, n=3. The experiment was repeated twice with consistent results. **, p<0.01; ***, p<0.001. doi:10.1371/journal.pone.0097701.g004
Our long-term transfer studies revealed that CXCR6-deficient CD8$^+$ T cells were outnumbered over time by wt CD8$^+$ T cells. However, stability of the CXCR6-deficient cells differed in the analyzed tissues. The proportion of CXCR6-deficient CD8$^+$ T cells had significantly declined by 10 weeks in blood, peripheral lymph nodes, lung and liver but remained relatively stable for several additional weeks in spleen and bone marrow. We did not observe significant changes in the activation status of wt and CXCR6-deficient CD8$^+$ T cells as well as in their cytokine response to in vitro stimulation. In addition, compared to wt cells CXCR6-deficient CD8$^+$ T cells expressed similar or even lower levels of surface markers associated with CD8$^+$ T cell exhaustion.

A role of CXCR6 in the maintenance of a cell population has been described for hepatic NKT cells and subsets of NK cells [23,26]. Similar to our observation for CD8$^+$ T cells, these studies also show that CXCR6 is necessary for maintaining the NK or NKT cell populations but does not affect the function of the cells.

Measuring the rates of proliferation and apoptosis of transferred CD8$^+$ T cells revealed that wt cells showed both higher proliferation and enhanced apoptosis in comparison to CXCR6-deficient cells. Thus, enhanced stability of wt cells was associated with a higher turn-over rate of these cells.

In vitro, CXCL16 was not able to induce proliferation in CD8$^+$ T cells. However, CXCR6-deficient CD8$^+$ T cells showed reduced survival when compared to wt cells. The addition of CXCL16 did not further enhance survival of wt cells, however, it is well possible that cells present in the culture, such as DCs, already provide saturating amounts of the chemokine [11,26]. CXCR6 might also provide survival signals for CD8$^+$ T cells in vivo. It is also possible that CXCR6 does not directly induce survival but directs cells to sites where these signals are available. Such a function of CXCR6 was proposed for the maintenance of NK cell subsets in the liver [23]. In a similar way, CXCR3 can direct activated CD8$^+$ T cells to areas in the spleen where further differentiation signals are provided [4]. Such an indirect mechanism might also explain why CXCR6-deficient CD8$^+$ T cells show different stability in different tissues.

Despite alterations in tissue distribution and long-term stability of CXCR6-deficient CD8$^+$ T cells in the competitive T cell transfer assay, CXCR6-deficient mice were not significantly impaired in the control of secondary L. monocytogenes infection. Thus, despite the alterations, the CD8$^+$ memory T cell compartment was still
sufficient for substantial protection, at least at day 60 post primary infection. Protection assays were conducted in wt and CXCR6-deficient mice and not in the transfer model. Since CXCR6-deficient mice mount stronger primary CD8⁺ T cell responses, it is possible that they also generate a larger memory cell population, which might compensate for impaired survival of memory CD8⁺ T cells. In agreement with this hypothesis, we observed similar frequencies of L. monocytogenes-specific CD8⁺ T cells in wt and CXCR6GFP/GFP mice at day 5 post secondary infection (data not shown). Finally, it is also possible that the stronger CD8⁺ T cell response of CXCR6GFP/GFP mice might result in enhanced CD4⁺ T cell mediated protection.

In conclusion, our results demonstrate that CXCR6 is neither essential for the generation of T cell responses following L. monocytogenes infection nor for the recruitment of activated CD8⁺ T cells to infected tissues. However, CXCR6 appears to regulate the frequencies of L. monocytogenes cells. In agreement with this hypothesis, we observed similar possible that they also generate a larger memory cell population,

Supporting Information

Figure S1 CXCR6 expression on CD8⁺ T cells during L. monocytogenes infection. CXCR6⁺/GFP mice were infected with $1 \times 10^5$ LmOVA i.v. and cells from spleens and livers were analyzed at indicated time points. The figure shows the expression of CXCR6 on activated CD44⁺/CD62L⁻ CD8⁺ T cells. Symbols give the mean ± SEM, n=5, and are representative for two experiments.

Figure S2 Control of L. monocytogenes in CXCR6-deficient mice. Wt and CXCR6GFP/GFP mice were infected with $5 \times 10^4$ Lm i.v. and listeria titers in spleens and livers were determined at indicated time points. Colony forming units (CFU) for individual mice and the median of one experiment are shown, n=7. Detection limit was 20 CFU. †, one mouse died before determination of titers.

Figure S3 CXCR6 controls early accumulation of activated CD8⁺ T cells in the liver. Purified CD8⁺ T cells from wt OT-1 and CXCR6GFP/GFP OT-I mice were mixed in a ratio of 1:1 and a total of $5 \times 10^4$ cells were injected into congenic wt mice i.v. infected with $1 \times 10^5$ LmOVA. Transferred cells from spleens and livers of recipient mice were analyzed at day 3 and day 5 p.i. (A) Percentage of transferred OT-I cells before (dc, donor cells) and three and five days after transfer in spleens and livers of recipient mice. (B) Representative dot plots of CD44 and CD62L expression on transferred OT-I cells. (C) Representative histograms of KLRL1 expression on transferred OT-I cells. Bars give mean ± SEM, n=3. The experiment was repeated twice with consistent results. MFI, mean fluorescence intensity.

Figure S4 Distribution, phenotype and cytokine profile of transferred wt and CXCR6GFP/GFP CD8⁺ T cells. Purified CD8⁺ T cells from wt OT-I and CXCR6GFP/GFP OT-I mice were mixed in a ratio of 1:1 and a total of $5 \times 10^4$ cells were injected into RAG1⁻/⁻ mice i.v. infected with $1 \times 10^5$ LmOVA. Transferred cells of recipient mice were analyzed at 5, 10, 15 and 30 weeks p.i. (A) Percentage of transferred OT-I cells in bone marrow, spleens, lungs, blood, lymph nodes and livers on indicated time points. (B) Representative dot plots of CD44 and CD62L expression on transferred OT-I cells in spleens of recipient mice. Numbers show percentages of positive wt (black) and CXCR6GFP/GFP (red) cells. (C) Activation status of transferred OT-I cells in spleens of recipient mice. Bars show distribution of CD44⁺/CD62L⁺, CD44⁺/CD62L⁻, and CD62L⁻ cells within wt and CXCR6GFP/GFP OT-I cells. (D,E) Cytokine-production of transferred OT-I cells in spleens of recipient mice after in vitro stimulation with OVA257-264 peptide. (D) Representative histograms of IFN-γ and TNF-α expression of transferred OT-I cells. (E) Percentage of IFN-γ⁺ and TNF-α⁺ transferred OT-I cells. Bars give mean ± SEM of combined results from two independent experiments, n=6, dc, donor cells.

Figure S5 Transferred CXCR6GFP/GFP CD8⁺ T cells show similar or reduced expression of exhaustion markers. Purified CD8⁺ T cells from wt OT-I and CXCR6GFP/GFP OT-I mice were mixed in a ratio of 1:1 and a total of $5 \times 10^4$ cells were injected into RAG1⁻/⁻ mice i.v. infected with $1 \times 10^5$ LmOVA. Transferred cells were analyzed at 5, 10, 15 and 30 weeks p.i. For every sample, unstained cells were measured (FMO, fluorescence minus one). (A) Representative histograms of PD-1, LAG3 and CD244 expression on transferred OT-I cells. (B) Difference between MFI (mean fluorescence intensity) of antibody staining and FMO-staining for PD-1, LAG3 and CD244. Bars give mean ± SEM of combined results from two independent experiments, n=6, dc, donor cells.

Figure S6 CXCL16 induces survival but no proliferation of CD8⁺ T cells in vitro. (A) $2 \times 10^5$ cells from wt spleen were stained with the proliferation dye eFluor70 and incubated for three days with indicated amounts of CXCL16 and IL-15 or anti-CD3 plus anti-CD28 mAbs. For the combination of IL-15 and CXCL16 100 ng/ml of both cytokines were used. Bars give mean MFI (mean fluorescence intensity) ± SEM of eFluor70 of CD8⁺ T cells. (B) $2 \times 10^5$ cells from wt and CXCR6GFP/GFP splenocytes were mixed and incubated for three days without stimuli (none), with 300 ng/ml CXCL16 or with anti-CD3 plus anti-CD28 mAbs. Bars give normalized ratios of co-cultured wt and CXCR6GFP/GFP CD8⁺ T cells. Bars give combined results from two independent experiments, n=4.

Acknowledgments

We thank Dr. Hao Shen for providing a recombinant L. monocytogenes strain.

Author Contributions

Conceived and designed the experiments: KH UP HWM. Performed the experiments: KH FR VS SH. Analyzed the data: KH FR VS SH HWM. Contributed reagents/materials/analysis tools: UP. Wrote the paper: KH HWM.

References

1. Pamer EG (2004) Immune responses to Listeria monocytogenes. Nat Rev Immunol 4: 812–823.
2. Gellin BG, Broome CV (1989) Listeriosis. JAMA 261: 1313–1320.
3. Kursar M, Janner N, Pfeffer K, Brinkmann V, Kaufmann SHE, et al. (2008) Chemokine receptor CXCR3 facilitates CD8⁺ T cell differentiation into short-lived effector cells leading to memory degeneration. J Exp Med 208: 1605–1620.
5. Zhou Y, Kurilcara T, Ryeck RP, Yang Y, Ryan C, et al. (1998) Impaired macrophage function and enhanced T cell-dependent immune response in mice lacking CCR5, the mouse homologue of the major HIV-1 coreceptor. J Immunol 160: 4018–4025.

6. Zhong MX, Kantel WA, Pamer EG, Serbinova N V (2004) Chemokine Receptor 5 Is Dispensable for Innate and Adaptive Immune Responses to Listeria monocytogenes Infection. Infect Immun 72: 1057–1064.

7. Loetscher M, Amara a, Oberlin E, Brass N, Legler D, et al. (1997) TYMSTR, a putative chemokine receptor selectively expressed in activated T cells, exhibits HIV-1 coreceptor function. Curr Biol 7: 632–640.

8. Liao F, Akhatib G, Peden KWC, Sharma G, Berger E, et al. (1997) STRL33, A Novel Chemokine Receptor-like Protein Functions as a Fusion Cofactor for Both Macrophage-tropic and T Cell Line-tropic HIV-1. J Exp Med 185: 2015–2023.

9. Deng H, Unutmaz D, Kewalramani VN, Litman DR (1997) Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature 380: 296–300.

10. Deng L, Chen N, Li Y, Zheng H, Lei Q (2010) CXCR6/CXCL16 functions as a regulator in metastasis and progression of cancer. Biochim Biophys Acta 1806: 42–49.

11. Matloubian M, David a, Engel S, Ryan JE, Cyster JG (2000) A transmembrane chemokine with a CX3C motif. Nature 385: 640–644.

12. Kim CH, Rott L, Kunkel EJ, Genovese MC, Andrew DP, et al. (2001) Rules of coreceptor function. Curr Biol 7: 652–660.

13. Matsumura S, Wang B, Kawashima N, Braunstein S, Badura M, et al. (2008) Role for CXCR6 in Recruitment of Activated CD8+ Lymphocytes to Inflamed Liver. J Immunol 181: 277–283.

14. Sato T, Thorlacius H, Johnston B, Tracy L, Campbell JJ, et al. (2001) Bonzo/STRL33, both Macrophage-tropic and T Cell Line-tropic HIV-1. J Exp Med 198: 5913–5918.

15. Wilbanks A, Zondlo SC, Murphy K, Mak S, Soler D, et al. (2001) CXCR6 expression defines type 1-polarized T-cell subsets with extralymphoid tissue homing potential. J Clin Invest 107: 595–601.

16. Matsunuma S, Wang B, Kawashima N, Braunstein S, Badura M, et al. (2008) Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. J Immunol 181: 3099–3107.

17. Sato T, Thorlacius H, Johnston B, Tracy L, Xiang W, et al. (2005) Role for CXCR6 in Recruitment of Activated CD8+ Lymphocytes to Inflamed Liver. J Immunol 174: 277–283.

18. Wilbanks A, Zondlo SC, Murphy K, Mak S, Soler D, et al. (2001) Expression Cloning of the STRL33/BONZO/TYMRIG Ligand Reveals Elements of CC, CXC, and CX3C Chemokines. J Immunol 166: 5145–5154.

19. Shirasaka T, Kunze N, Minami M, Hayashi K, Kataoka H, et al. (2000) Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages. J Biol Chem 275: 40663–40666.

20. Bazan JF, Bacon KB, Harliman G, Wang W, Sod K, et al. (1997) A new class of membrane-bound chemokine with a CX3C motif. Nature 385: 640–644.

21. Bjordahl RL, Gapin L, Marrack P, Refaeli Y (2012) iNKT cells suppress the CD1d+ T cell response to a murine Burkitt’s-like B cell lymphoma. PLoS One 7: e42635.

22. Shi C, Vela´zquez P, Hohl TM, Leiner I, Dustin ML, et al. (2010) Monocyte NKT cells suppress CD8+ T cell-mediated allergic contact dermatitis independently of regulatory CD4+ T cells. J Invest Dermatol 133: 980–987.

23. Paust S, Gill HS, Wang R, Flynn MP, Ashley E, et al. (2010) Critical role for CXCR6 in NK cell-mediated antigen-specific memory to hapten and viruses. Nat Immunol 11: 1127–1135.

24. Bubnic SJ, Nagy A, Kreating A (2005) Donor hematopoietic cells from transgenic mice that express GFP are immunogenic in immunocompetent recipients. Hematology 10: 289–295.

25. Van der Voort R, Verweij V, de Witte TM, Lasonder E, Adema GJ, et al. (2010) An alternatively spliced CXCL16 isoform expressed by dendritic cells is a secreted chemotractant for CXCR6+ cells. J Leukoc Biol 87: 1029–1039.

26. Geissmann F, Cameron TO, Sidobre S, Manlongat N, Kronenberg M, et al. (2005) Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. PLoS Biol 3: e113.

27. Kronenberg M (2005) Toward an understanding of NKT cell biology: progress and paradoxes. Annu Rev Immunol 23: 877–900.

28. Arrunategui-Corraa V, Sil Kim H (2004) The role of CD1d in the immune response against Listeria infection. Cell Immunol 227: 109–120.

29. Szalay G, Ladef GH, Blum C, Brossay L, Kronenberg M, et al. (1999) Cutting edge: anti-CD1 monoclonal antibody treatment reverses the production patterns of TGF-beta 2 and Th1 cytokines and ameliorates litters in mice. J Immunol 162: 6955–6958.

30. Emoto M, Yoshizawa I, Emoto Y, Miamoto M, Hurwitz R, et al. (2006) Rapid development of a gamma interferon-secreting glycolipid/CD1d-specific Valpha14+ NK1.1- T-cell subset after bacterial infection. Infect Immun 74: 5903–5913.

31. Bjordahl RL, Gapin L, Marrack P, Refaeli Y (2012) iNKT cells suppress the CD1d+ T cell response to a murine Burkitt’s-like B cell lymphoma. PLoS One 7: e42635.

32. Geissmann F, Cameron TO, Sidobre S, Manlongat N, Kronenberg M, et al. (2005) Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. PLoS Biol 3: e113.

33. Mattarollo SR, Yong M, Gosmann C, Choyce A, Chan D, et al. (2011) Invariant NKT cells inhibit antigen-specific effector CD8+ T-cell induction to skin viral proteins. J Immunol 187: 1601–1608.

34. Riedel J-H, Paust H-J, Turner J-E, Tittel AP, Krebs C, et al. (2012) Immature renal dendritic cells recruit regulatory CXCR6+ invariant natural killer T cells to attenuate crescentic GN. J Am Soc Nephrol 23: 1987–2000.

35. Bouwiet J, Kantel EJ, Campbell JF, Keefli EB, Butcher EC, et al. (2003) Liver-infiltrating lymphocytes in end-stage hepatitis C virus: subsets, activation status, and chemokine receptor phenotypes. J Hepatol 38: 67–75.

36. Kim CH, Rott L, Kantel EJ, Genovese MC, Andrew DP, et al. (2001) Rules of chemokine receptor association with T cell polarization in vivo. J Clin Invest 106: 1331–1339.

37. Shield PL, Morland CM, Salmon M, Qin S, Hubscher SG, et al. (1999) Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. J Immunol 163: 6236–6243.

38. Cose S, Brammer C, Khanna KM, Masepout D, Lefrancois L (2006) Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway. Eur J Immunol 36: 1423–1433.

39. Shi C, Velázquez P, Hohl TM, Leiner I, Dustin ML, et al. (2010) Monocyte trafficking to hepatic sites of bacterial infection is chemokine independent and directed by focal intercellular adhesion molecule-1 expression. J Immunol 184: 6266–6274.