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Critical Role for the Chemokine Receptor CXCR6 in Homeostasis and Activation of CD1d-Restricted NKT Cells

Elitza Germanov,2* Linnea Veinotte,2* Robyn Cullen,† Erin Chamberlain,* Eugene C. Butcher,§ and Brent Johnston3*‡

NK T (NKT) cells play important roles in the regulation of diverse immune responses. However, little is known about the mechanisms that regulate homeostasis and activation of these cells. Thymic NKT cells up-regulated the chemokine receptor CXCR6 following positive selection and migrated toward CXCL16 in vitro. However, CXCR6 was not essential for thymic development or maturation. In contrast, liver and lung NKT cells were depleted in CXCR6+ and CXCR6−/− mice. The reduction in liver and lung NKT cells coincided with an increase in bone marrow NKT cells, suggesting a redistribution of NKT cells in CXCR6−/− animals. In wild-type mice, CXCL16 neutralization reduced accumulation of mature NK1.1+ but not immature NK1.1− NKT cell recent thymic emigrants in the liver. Given that thymic NKT cells are preferentially exported as NK1.1− cells, this suggests an additional role for CXCR6/CXCL16 in maturation or survival of immature liver NKT cells. CXCL16 blockade did not deplete resident NK1.1+ NKT cells, indicating that CXCR6/CXCL16 are not required to retain mature NKT cells in the liver. Cytokine production by liver and spleen NKT cells was impaired in CXCR6−/− mice following in vivo stimulation with α-galactosylceramide, implicating a novel role for CXCR6 in NKT cell activation. Reduced IFN-γ production was not due to an intrinsic defect as production was normal following PMA and ionomycin stimulation. Preformed transcripts for IL-4, but not IFN-γ, were reduced in CXCR6−/− liver NKT cells. These data identify critical roles for CXCR6/CXCL16 in NKT cell activation and the regulation of NKT cell homeostasis. The Journal of Immunology, 2008, 181: 81–91.

The Journal of Immunology
and CD28-B7 interactions are important for generation of both IFN-γ and IL-4 (33, 36). It is likely that other signals contribute to the regulation of cytokine responses generated by Ag-stimulated NKT cells. Chemokines induce leukocyte adhesion and migration via interactions with seven transmembrane-spanning G-protein coupled receptors, facilitating differential homing of specific leukocyte subsets to normal and inflamed tissues (37, 38). However, chemokines also play important roles in lymphocyte development, differentiation, and effector functions including cytokine polarization (39, 40). Human and mouse NKT cells express several chemokine receptors, including high levels of CXCR6 (41–44). CXCR6, and its ligand CXCL16 have been shown to mediate the accumulation of NKT cells in the liver (45) and localization of NKT cells to cardiac allografts (46). CXCR6 and CXCL16 have also been implicated in lymphocyte accumulation in several inflammatory diseases (47–51). In addition to being expressed in inflamed tissues (49–52), CXCL16 is expressed at high levels in the liver and lungs, with lower levels in the spleen, thymus, and other tissues (44, 45, 53). Importantly, CXCL16 is expressed as a transmembrane protein on the surface of APCs including DCs, macrophages, and B cells (44, 53). Therefore, in addition to mediating tissue homing, CXCR6-CXCL16 interactions could facilitate activation or costimulation of NKT cells.

To investigate the role of CXCL16 and CXCR6 in mediating NKT cell development, localization, and function, we examined NKT cell populations in CXCR6-deficient mice and wild-type mice treated with anti-CXCL16 Abs. CXCR6−/− mice exhibited reductions in liver and lung NKT cells, whereas NKT cells were

**FIGURE 1.** Functional expression of CXCR6 on thymic NKT cells. A. NKT cells were stained with loaded CD1d tetramers and fluorochrome-conjugated Abs against CD4 and CD8 to distinguish CD4+CD8− (DN), CD4+CD8+ (DP), CD4−, and CD8− subsets. Expression of CXCR6 on NKT cells subsets was determined by eGFP expression in CXCR6-eGFP knock-in heterozygotes, or binding of a CXCL16-Fc chimera in wild-type mice. Nonspecific staining were excluded using unloaded tetramers and doublets were excluded by FSC-H vs FSC-A gating (data not shown). B and C. Chemotactic migration assays of thymic NKT cells to CXCL16. Fresh unfractionated thymocytes were allowed to migrate through Transwell inserts (5-μm pore size) for 2 h. B. Wild-type NKT cells were stained with loaded CD1d tetramers and fluorochrome-conjugated Abs against TCRβ, CD4, and CD8 to distinguish subsets. The proportion of migrated cells in each population was calculated as a fraction of the input population (43, 55). C. NKT cell migration of age- and sex-matched CXCR6−/−, CXCR6−/−/, and CXCR6+/+ littermates in response to CXCL16. D. NKT cells were stained with loaded CD1d tetramers, CXCL16-Fc, and fluorochrome-conjugated Abs against CD44 and NK1.1 to distinguish sequential stages of NKT cell development: CD44−NK1.1−, CD44−NK1.1+, and CD44+NK1.1+. E. NKT cells in the liver, lung, and spleen were identified with loaded CD1d tetramers and Ab against TCRβ. CXCL16-Fc was used to examine CXCR6 expression. FACS plots are representative of at least three independent experiments. Chemotaxis assays show the mean ± SEM of six individual experiments. *, p < 0.05 compared with basal migration. †, p < 0.05 compared with CXCR6−/−.
increased in the bone marrow. CXCR6/CXCL16 mediated the accumulation of recent thymic emigrants in the liver and also played a role in their maturation and/or survival. However, CXCL16 was not required for retention or survival of NKT cells that had previously matured in the liver. Stimulation with \( \alpha \)-GalCer revealed a novel role for CXCR6/CXCL16 in mediating Ag-dependent NKT cell activation as the production of IL-4 and IFN-\( \gamma \) and other cytokines was impaired in CXCR6\(-/-\) mice. This study has provided evidence that CXCR6 and CXCL16 play a critical role in NKT cell activation and cytokine generation in addition to their role in NKT cell localization.

**Materials and Methods**

**Mice**

CXCR6-enhanced GFP (eGFP) knock-in mice, generated by replacing the coding exon for CXCR6 with eGFP (52), were backcrossed against C57BL/6J mice for 10 generations. Heterozygotes were bred together, and litters were genotyped by PCR and FACS. Sex- and age-matched wild-type (CXCR6\( ^{+/+} \)), heterozygote (CXCR6\( ^{+-} \)), and knockout (CXCR6\( ^{-/-} \)) littermates were compared in these studies. Additional male and female C57BL/6J mice were obtained from The Jackson Laboratory. Experiments were conducted when mice were 6–12 wk of age. Mice were housed in the Carleton Animal Care Facility at Dalhousie University. Experiments were performed with approval from University Committee on Laboratory Animals.

**Screening of CXCR6-deficient mice**

DNA was isolated from tail clippings using a DNeasy Tissue Kit (Qiagen). PCR amplification was performed using an Eppendorf Mastercycler ep thermocycler with specific primers for CXCR6 (forward, 5'-TACGATGGGCACTAGGAGGGAG; reverse, 5'-GCAAAAGAAACCCAACAGGGAGACAC) and eGFP (forward, 5'-TGAAGCAGCACGACTTCTAAGTC; reverse, 5'-TCGTCATGCGAGTGATGCA). Amplification was conducted for 40 cycles with: denaturation at 94°C for 45 s, annealing at 60°C for 60 s, and extension at 72°C for 330 s. PCR results correlated with the mean fluorescence intensity of eGFP expressed by peripheral blood CD8\( ^{+} \) T lymphocytes obtained by submandibular venous puncture (data not shown).

**Cell isolation**

Mice were anesthetized with a ketamine-xylazine mixture and sacrificed by cervical dislocation. Blood was drawn into a heparinized syringe by cardiac puncture. Leukocytes were isolated from thymus, spleen, bone marrow...
(femur and tibia), and peripheral lymph nodes (pooled inguinal, axillary, brachial, and superficial cervical nodes) by mechanical dispersion through a wire mesh followed by hypotonic erythrocyte lysis. Liver lymphocytes were obtained by mincing the tissue, mechanically dispersing it through a wire mesh, and isolating the lymphocytes on a 33% Percoll (GE Healthcare Bio-Sciences) gradient. Lung lymphocytes were isolated by digesting lungs in 300 U/ml type VIII collagenase (Sigma-Aldrich) for 30 min as described previously (54). Lymphocytes were resuspended in RPMI 1640 with 10% 

FIGURE 4. CXCL16 mediates accumulation of FITC-labeled recent thymic NKT cell emigrants (RTE) in the liver. Wild-type mice received intrathymic injections of 10 μl of FITC (filtered 1 mg/ml solution) or saline. Animals were treated daily with a blocking CXCL16 mAb (i.p. 250 μg) or an isotype-matched control Ab. Cells were isolated from the thymus (A), spleen (B), and liver (C) 72 h after thymic injection and analyzed by flow cytometry using loaded CD1d tetramers and fluorochrome-conjugated Abs against TCRβ and NK1.1. The frequency of NKT cells (CD1d tetramer+) and the proportion of NK1.1+ and NK1.1− NKT cells were assessed in the FITC+ and FITC− gates. FACS plots are representative of six individual mice per group. The frequency of NK1.1+ and NK1.1− CD1d tetramer+ NKT cells was determined in the FITC+ gate of the thymus (D), spleen (E), and liver (F) and presented as the mean ± SEM of six individual mice per group. *, p < 0.05 compared with isotype Ab.
FCS and allowed to recover in a CO₂ incubator for >1 h before chemotaxis experiments.

Chemotaxis assays

Chemotactic migration assays were performed as previously described (43, 55). Briefly, 1.0 × 10⁶ lymphocytes were placed in the upper chamber of Transwell inserts (5-μm pore size; Corning Costar). Inserts were placed in wells containing medium alone (basal) or medium plus chemokine. The chemokine CXCL16 was purchased from R&D Systems and used at 5 nM, the optimal concentration determined by titration. After 2 h of migration, inserts were removed, and polystyrene beads (Polysciences) were added to each well as an internal standard. Three wells were pooled for each condition. Migrated leukocyte populations were stained with Abs and CD1d tetramer to identify cell populations by flow cytometry. Chemotaxis was determined by comparing the bead to cell ratios in the migrated and input populations (43, 55).

Flow cytometry

The following conjugated mAbs were used in various staining protocols: FITC, PE, PerCP, or allophycocyanin-labeled NK1.1 (clone PK136); Cy-Chrome or allophycocyanin-labeled TCRβ (clone H57-597); FITC, PerCP, APC, or allophycocyanin-Cy7-labeled CD4 (clone RM4-5); FITC, PE, PE-Cy7, or allophycocyanin-labeled CD8α (clone 53-6-7); FITC-labeled CD44 (clone 1M7). A CXCL16-Fc chimera was used to detect expression of CXCR6 (44). The chimera was detected using a PE-labeled F(ab')₂ goat anti-human Fcγ polyclonal Ab (Jackson ImmunoResearch Laboratories). PE- and allophycocyanin-labeled CD1d tetramers loaded with the α-GalCer analog PBS57 were obtained from the National Institutes of Health Tetramer Core Facility (Emory Vaccine Center at Yerkes, Atlanta, GA). Unloaded tetramers were used as a control. Four-color flow cytometry was performed using a two-laser FACSCalibur with BD CellQuest Pro software (BD Biosciences). Isotype-matched control Abs were used to establish placement of gates and quadrants. Five-color flow cytometry was performed using a two-laser BD FACSaria sorters with BD FACSDiva software (BD Biosciences). Doublet discrimination was determined by analysis of forward light scatter (FSC-H vs FSC-A).

Labeling and tracking of recent thymic emigrants

In some mice, 10 µl of FITC (filtered 1 mg/ml solution; Sigma-Aldrich) was injected into each lobe of the thymus to detect recent thymic emigrants in the periphery (14, 15). Animals were treated daily, starting 1 day before thymic injections, with 250-µg i.p. injections of a blocking CXCL16 mAb (clone 142417) or an isotype-matched control Ab (clone 54447), both from R&D Systems. Cells were isolated from thymus and peripheral tissues 72 h after thymic FITC injection and analyzed by flow cytometry.

Activation with α-GalCer

Mice were injected i.p. with 4 µg of α-GalCer (Alexis Biochemicals; in 0.9% saline with 0.5% Tween 20) or vehicle alone. After 2 and 24 h, blood was collected for measurement of serum cytokine levels using the BD Biosciences Cytometric Bead Array (CBA) kit for mouse Th1/Th2 cytokines (IL-2, IL-4, IL-5, IFN-γ, and TNF). Samples were processed according to the manufacturer’s protocols and analyzed using BD CBA software (version 1.4). In some animals, liver, spleen, and blood were harvested at 2 h for determination of intracellular IL-4 (clone 11B11) and IFN-γ (clone XMG1.2) cytokine staining levels using PE conjugated Abs (eBioscience). Samples were processed using the BD Cytofix/Cytoperm kit without in vitro restimulation.

PMA and ionomycin stimulation

Liver and spleen lymphocytes were surface stained with CD1d-tetramers and Ab against TCRβ. Cells were then stimulated in culture for 2 h with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) followed by intracellular staining for IL-4 and IFN-γ.

NKT cell apoptosis

Tetramer-positive NKT cells were purified from liver and spleen lymphocytes using a BD FACSaria cell sorter. Cells were cultured in 96-well plates with RPMI 1640 with 10% FCS. Cells were stained at different times with annexin V and 7-aminoactinomycin D (BD Biosciences) to differentiate apoptotic and necrotic NKT cells.

Real-time PCR

Tetramer-positive NKT cells were purified from liver lymphocytes using a BD FACSaria cell sorter. RNA was isolated using a RNeasy kit (Qiagen) with the following modifications. Cells were lysed in TRIZOL reagent (Invitrogen) and mixed 4:1 with chloroform. After centrifugation, the aqueous phase of the TRIZOL-chloroform mixture was applied to an RNeasy column (Qiagen). The subsequent steps of the RNeasy kit were followed according to manufacturer’s instructions. Reverse transcription was performed with SuperScript II (Invitrogen). Quantitative real-time PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). IFN-γ, IL-4, and GAPDH transcripts were measured using TaqMan Gene expression assays (IFN-γ, Mm00445259; IL-4, Mm00445259) and a TaqMan Rodent GAPDH endogenous reference kit (Applied Biosystems). Data were analyzed with ABI Prism 7000 SDS software using a relative standard curve method according to the manufacturer’s protocol. IFN-γ and IL-4 expression levels were normalized to the endogenous GAPDH control and expressed relative to mRNA levels in NKT cells isolated from the liver of wild-type mice.

Statistical analysis

Values are reported as means ± SEM except for real time PCR which is reported as means ± SD. A Mann-Whitney U test was used for comparisons between two groups. Comparisons between multiple data groups were performed by nonparametric ANOVA (Kruskal-Wallis test) followed by Dunn’s posttest for multiple comparisons. Statistical significance was set at p < 0.05.

FIGURE 5. Reduced NK1.1 expression on CXCR6⁻/⁻ NKT cells is not due to differential survival. A and B, Tissue lymphocytes were stained with loaded CD1d tetramers and fluorochrome-conjugated Abs against TCRβ and NK1.1 to identify immature (NK1.1⁺) and mature (NK1.1⁻) NKT cell populations. A, Representative NK1.1 staining of CXCR6⁻/⁻ and CXCR6⁺/⁺ liver NKT cells. B, Frequency of NK1.1 expression on liver, spleen, bone marrow, and thymic NKT cell populations in CXCR6⁻/⁺ and CXCR6⁺/⁺ mice. C, Tetramer-positive NKT cells were purified from the livers of CXCR6⁻/⁺ and CXCR6⁺/⁺ mice and cultured for 18 h. Cells were stained with annexin V and 7-aminoactinomycin D to examine the proportion of apoptotic NKT cells. Data are presented as the mean ± SEM of four individual experiments. *, p < 0.05 compared with CXCR6⁺/⁺.
Results

Thymic NKT cells up-regulate CXCR6 following positive selection

We and others have reported that NKT cells in blood and peripheral tissues express high levels of the chemokine receptor CXCR6 (41–44). To examine the expression of CXCR6 on CD1d-restricted NKT cells in the thymus, heterozygous mice with a targeted replacement of CXCR6 by eGFP were analyzed by flow cytometry. The CXCR6-eGFP reporter was not detected on CD4+H11001 CD8+H11001 DP NKT cells (Fig. 1A), which are thought to be undergoing positive selection (9–11). However, CXCR6-eGFP was detected at high levels on positively selected CD4+H11001 and CD4+H11002 CD8+H11002 double-negative (DN) NKT cells. In mice, CD8+H11001 CD1d-restricted NKT cells are absent due to negative selection or down-regulation of CD8 (3, 7). The few residual CD8+H11001 NKT cells that could be detected did not express CXCR6-eGFP. Expression of eGFP in heterozygotes correlated with staining in wild-type mice using a CXCL16-Fc chimera that binds CXCR6 (Fig. 1A). As reported by Pellicci et al. (15), >80% of the DP NKT cell gate consisted of doublets and cells binding tetramer nonspecifically. These cells were excluded from our analysis using unloaded tetramers and doublet-exclusion gating (FSC-H vs FSC-A). Very few CD4+H11001 or DN NKT cells were present as doublets or bound unloaded tetramers (data not shown). In functional chemotaxis assays, CD4+H11001 and DN NKT cells from wild-type mice migrated in response to the CXCR6 ligand, CXCL16 (Fig. 1B). In addition, migration to CXCL16 was absent in CXCR6+/- mice, indicating that CXCR6 is the only receptor for CXCL16 expressed by these cells (Fig. 1C). Furthermore, we were able to demonstrate tight regulation between CXCR6 and eGFP expression as eGFP+H11001 NKT cells in heterozygous mice migrated in response to CXCL16, whereas eGFP+H11002 NKT cells did not (data not shown). To further elucidate the timing of CXCR6 up-regulation following positive selection, we examined binding of the CXCL16-Fc chimera to NKT cells at different stages of development. NKT cells acquired CXCR6 expression coincident with the up-regulation of CD44, but before acquisition of NK1.1 expression (Fig. 1D). Following

FIGURE 6. CXCR6−/− NKT cells are impaired in their generation of cytokines after stimulation with α-GalCer. Mice were treated i.p. with the NKT cell stimulator α-GalCer (4 μg/mouse). A and B, Two hours after α-GalCer treatment, spleen and liver lymphocytes were isolated and stained with loaded CD1d tetramers and fluorochrome-conjugated Abs against TCRβ and NK1.1 to identify CD1d-restricted NKT cells. Cells were fixed and permeabilized for intracellular staining with Abs against IFN-γ, IL-4, or an isotype control (IgGl). A, Representative intracellular cytokine staining of liver NKT cells (gated on TCRβ+ CD1d tetramer+ cells) from CXCR6+/- and CXCR6−/− mice. B, Proportion of NK1.1+ and NK1.1− liver and spleen NKT cells positive for intracellular staining with IL-4 and IFN-γ. Data are presented as mean ± SEM of six individual experiments. *, P < 0.05 compared with CXCR6+/- mice. C, Serum levels of IL-2, IL-4, IFN-γ and TNF were measured 2 and 24 h after α-GalCer stimulation using a cytometric bead array assay. Data are presented as the mean ± SEM of seven individual experiments. *, P < 0.05 compared with control. †, P < 0.05 compared with CXCR6+/-.
export into the periphery, CXCR6 expression was maintained at high levels on NKT cells (Fig. 1E).

**CXCR6 is not required for thymic NKT cell development**

Previously, CXCL16, the ligand for CXCR6, has been detected in the medullary regions of the mouse thymus, suggesting that it could play a role in thymocyte development (44). However, analysis of age- and sex-matched littermates from the mating of CXCR6-eGFP heterozygotes did not reveal differences in either the proportion or total number of NK1.1+ (NK1.1+TCRβ+) or CD1d-restricted NKT cells present in the thymus of CXCR6+/+, CXCR6+/−, or CXCR6−/− animals (Fig. 2). This indicates that CXCR6 is not required for NKT cell development in the thymus, and that it could be more important for NKT cell homing or function in the periphery.

**CXCR6 mediates NKT cell localization in the liver**

Analysis of NK1.1+ (NK1.1+TCRβ+) and CD1d-restricted NKT cells in the periphery of CXCR6-deficient mice revealed a significant reduction in the proportion and total number of NKT cells in the liver of CXCR6−/− and CXCR6−/− mice (Fig. 3). NKT cell populations were reduced in the liver of heterozygotes by 40–50%, whereas CXCR6−/− mice exhibited reductions of 75–85%. CXCR6+/− and CXCR6−/− mice also exhibited a reduction in the number of NKT cells in the lung (Fig. 3B). These results are consistent with the liver and lung containing high levels of CXCL16 message and protein (44, 45, 53). NKT cell numbers in CXCR6−/− mice were increased in the bone marrow (Fig. 3B), suggesting a redistribution of cells from the liver and/or lung. There were no significant differences in NKT cell numbers in the spleen and lymph nodes (Fig. 3B).

**CXCL16 mediates accumulation of thymic NKT cells in the liver**

Expression of CXCL16 has been reported in the spleen and liver (44, 45, 53). To examine the role of CXCL16 in the homing of NKT cells to the periphery, we used a function blocking mAb against CXCL16. Mice were treated with intrathymic FITC injections to enable detection of recent thymic emigrants in the periphery (14, 15). Animals were given daily i.p. injections of neutralizing CXCL16 Ab or an isotype control starting 1 day before FITC injection. Tissues and cells were isolated 72 h after thymic injection for determination of the effects of Ab treatment on NKT cell distribution. ELISA revealed that high levels of CXCL16 Ab remained in the serum 24 h after the last Ab treatment (data not shown). FITC labeling of thymic NKT cells was equivalent in both the anti-CXCL16 and isotype-treated mice, and there were no effects of anti-CXCL16 treatment on thymic NKT cell levels (Fig. 4, A and D). Similarly, there were no differences in accumulation of recent NKT cell emigrants in the spleen (Fig. 4, B and E). In contrast, treatment with anti-CXCL16 Ab significantly reduced the accumulation of FITC+ NKT cells in the liver (Fig. 4, C and F). The reduction in FITC+ liver NKT cells was observed in the NK1.1+ population of CD1d-restricted NKT cells, which is more mature than the NK1.1− subset that is exported from the thymus (14, 15). In contrast, anti-CXCL16 treatments had no significant effect on the resident FITC− NKT cells in the liver (Fig. 4C and data not shown), suggesting that CXCR6/CXCL16 interactions are not required for retention or survival of resident NKT cells that have already matured in the liver. Furthermore, CXCR6/CXCL16 interactions do not appear to be required for maintenance of NK1.1 expression given that NK1.1 expression was maintained on resident FITC− NKT cells following anti-CXCL16 treatment (Fig. 4C and data not shown). These results indicate that CXCL16 is important for the accumulation of NKT cells that have recently emigrated from the thymus but likely plays a role in the maturation or survival of immature NKT cells in the liver rather than a role in homing. An alternate possibility is that CXCR6/CXCL16 selectively mediates homing of NK1.1+ thymic emigrants. However, mature NK1.1+ NKT cells represent a minority of thymic emigrants in young mice (16).

**CXCR6 deficiency impairs maturation of NKT cells**

In our thymic emigration experiments, it became apparent that there was a preferential defect in the accumulation of mature CD1d-restricted NK1.1+ NKT cells in the liver of mice treated with anti-CXCL16 mAbs. We then examined the expression of NK1.1 on the residual NKT cells present in the liver of CXCR6−/− mice. Consistent with the anti-CXCL16 treatment experiments, the remaining NKT cells in the liver of CXCR6−/− mice were primarily NK1.1−. In wild-type mice, 75–85% of the liver NKT cells were NK1.1+ (Fig. 5, A and B). In contrast, only 25% of the residual NKT cells in CXCR6-deficient mice expressed NK1.1. Impaired maturation was not limited to the liver NKT cells as the proportion of NKT cells expressing NK1.1 was also reduced in the spleen and bone marrow (Fig. 5B) even though NKT cell numbers were not reduced (Fig. 3B). Unlike Geissmann et al. (45), we did not observe differences in the apoptosis of cultured liver NKT cells from CXCR6+/+ and CXCR6−/− mice (Fig. 5C), suggesting that differential NKT cell survival does not mediate the reduced number of NKT cells in the liver of CXCR6−/− mice.

**CXCR6 deficiency impairs cytokine production by activated NKT cells**

As cytokine production capacity of thymic NKT cells has been shown to change with maturity (14, 15), we investigated intracellular IL-4 and IFN-γ production as well as serum cytokine levels following in vivo stimulation with α-GalCer (i.p. 4 μg). Two hours after α-GalCer treatment, a large proportion of wild-type NK1.1+ and NK1.1− NKT cells from liver and spleen stained positive for the generation of intracellular IL-4 and IFN-γ (Fig. 6, A and B).
FIGURE 8. Examination of preformed cytokine mRNA transcripts in CXCR6−/− and CXCR6+/− NKT cells. Preformed mRNA transcripts were measured by quantitative real-time PCR in populations of liver NK cells purified from CXCR6−/− and CXCR6−/+ mice by FACS. IFN-γ and IL-4 expression levels were normalized to the endogenous GAPDH control and expressed relative to mRNA levels in NKT cell samples isolated from the liver of CXCR6+/+ mice. Data are presented as the mean ± SD of four independent experiments performed in duplicate. *p < 0.05 compared with CXCR6+/+.

This is consistent with recent findings demonstrating that peripheral NK1.1− NKT cells exhibit a mature cytokine profile (56). In contrast, the proportion of NK1.1+ and NK1.1− NKT cells generating IL-4 and IFN-γ was significantly reduced in CXCR6−/− mice (Fig. 6, A and B). Similarly, serum levels of IL-2, IL-4, and IFN-γ were significantly reduced in CXCR6−/− mice 2 h after α-GalCer treatment (Fig. 6C). At 24 h, serum levels of IL-2 and IL-4 had reduced to baseline levels in all mice. Serum IFN-γ increased further at 24 h, but CXCR6−/− mice continued to exhibit impaired IFN-γ production compared with wild-type mice. Serum levels of TNF (Fig. 6C) and IL-5 (data not shown) increased to similar levels in CXCR6−/− and CXCR6+/− mice.

To determine whether CXCR6 deficiency results in an intrinsic defect in NKT cell activation, we stimulated CXCR6−/− and CXCR6+/− splenocytes and liver lymphocytes in culture with PMA and ionomycin (Fig. 7). Intracellular cytokine staining for IFN-γ was equivalent in CXCR6−/− and CXCR6+/− NKT cells, implicating a direct role for CXCR6 in induction of this cytokine. Intracellular staining for IL-4 was reduced in liver but not spleen NKT cells from CXCR6+/− mice, suggesting a direct role for CXCR6 as well as an intrinsic developmental defect in CXCR6−/− liver NKT cells.

NKT cells contain preformed cytokine transcripts that contribute to their ability to rapidly generate cytokines after stimulation (17, 57). To investigate whether impaired cytokine production by CXCR6−/− NKT cells was related to reduced levels of constitutive cytokine mRNA, we examined IL-4 and IFN-γ transcripts in wild-type and CXCR6−/− NKT cells using quantitative real-time PCR (Fig. 8). There were no significant differences in the expression of preformed IFN-γ transcripts by CXCR6−/+ and CXCR6−/− NKT cells. In contrast, liver CXCR6−/− NKT cells expressed lower levels of preformed IL-4 transcripts.

Discussion

In this study, we examined the role of CXCR6 in the development, distribution, and activation of CD1d-restricted NKT cells. Functional CXCR6 expression was up-regulated on developing thymic NKT cells following positive selection but before acquisition of NK1.1 expression. Although CXCR6 was not required for thymic NKT cell development, it played a vital role in the accumulation of NKT cells in the liver and lungs. The redistribution of NKT cells to bone marrow of CXCR6−/− mice suggests a role for CXCR6 in NKT cell homing or retention in the liver and lungs. However, CXCR6 and CXCL16 also appear to be important in mediating maturation and/or survival of liver NKT cells soon after their localization to the liver. Anti-CXCL16 treatment did not block the homing of immature thymic NK1.1− NKT cells to the liver but significantly reduced the accumulation of mature NK1.1− recent thymic emigrants and/or acquisition of the mature NK1.1− NKT cell phenotype by recent thymic emigrants. The potential roles for CXCR6/CXCL16 in homing, retention, maturation, or survival of NKT cells do not need to be mutually exclusive, and it is quite likely that CXCR6/CXCL16 are playing roles in several or all of these processes. Our experiments also identified a novel role for CXCR6 in Ag-dependent NKT cell activation. After in vivo treatment with the NKT cell Ag α-GalCer, NKT cells in the liver and spleen of CXCR6−/− mice exhibited a significant impairment in their ability to generate intracellular IL-4 and IFN-γ, and the levels of serum cytokines were severely reduced at 2 and 24 h posttreatment. The reduction in IL-4 production could be partly explained by a reduction in the number of preformed IL-4 mRNA transcripts in CXCR6−/− NKT cells. However, there were no significant differences in the quantity of preformed IFN-γ transcripts in CXCR6−/− NKT cells. These results implicate a critical role for CXCR6/CXCL16 in Ag-dependent NKT cell stimulation and cytokine production in addition to their role in NKT cell homeostasis.

We found that the expression of CXCR6 on thymic NKT cells is up-regulated following positive selection (Fig. 1A). CXCR6 was not detected on many DP NKT cells but was expressed on 60–80% of CD4+ and DN NKT cells. Although, NKT cells proceed through a DP developmental step (9–11), the population detected by flow cytometry may consist of doublets of NKT cells bound to DP thymocytes or cells that bind tetramer nonspecifically (15). Indeed, the majority of DP NKT cells were detected as doublets or bound to unloaded CD1d tetramers. However, few of the specific DP singlets expressed CXCR6 compared with high levels on the CD4+ and DN NKT cells, suggesting that CXCR6 is up-regulated after the DP step. Further support for the up-regulation of CXCR6 after thymic NKT cell selection comes from the observation that CXCR6 is up-regulated concomitantly with CD44 expression. NKT cells up-regulate CD44 following selection but before NK1.1 is expressed (11, 14). Importantly, CXCR6 was up-regulated before final maturation into NK1.1− NKT cells (Fig. 1D). Because the majority of NKT cells exported from the thymus leave before up-regulating NK1.1 (14, 15), the expression of CXCR6 before this step would be consistent with a role for CXCR6 in mediating homing or function of recent thymic emigrants in the periphery.

We and others have shown previously that CXCR6 is expressed on subsets of T cells in addition to NKT cells (41–44, 47–53). However, chemotaxis of peripheral NKT cells to CXCL16 is very low (43), and migration of CXCR6+ T cells to CXCL16 requires cell activation (44). In contrast, we now show that resting thymic NKT cells exhibited significant migration in response to CXCL16, suggesting that these cells are more likely to respond to ligand in vivo and CXCR6/CXCL16 could mediate their peripheral tissue distribution before responses are down-regulated. The basal accumulation of NKT cells in the liver and lungs of wild-type mice is consistent with the high levels of CXCL16 expression in these tissues (44, 45, 53). In addition to playing a role in basal NKT cell localization, Jiang et al. (46) have reported that CXCL16 mediates accumulation of NKT cells in cardiac allografts. However, it is unclear whether the infiltrating NKT cells in this allograft model reflect the preferential accumulation of recent thymic emigrants expressing functional CXCR6 receptor, or results from activation signals that mobilize NKT cells from the periphery and up-regulate CXCR6 function, leading to CXCL16-dependent homing to the allograft. A role for CXCR6/CXCL16 in basal NKT cell tissue distribution is confirmed by the reduction of NKT cells in the liver.
and lung of CXCR6−/− mice (Fig. 3). Furthermore, the redistribution of NKT cells to the bone marrow in CXCR6−/− mice supports a role for CXCR6 in NKT cell homing or retention in the liver and lungs.

In contrast to our findings, Geissmann et al. (45) reported that NKT cell numbers were normal in the liver of CXCR6−/− mice. The reason for this discrepancy is unclear because animals used in both studies were generated from the same founder mice (52). One possibility is that Geissmann et al. (45) used mice backcrossed as few as three generations. We used mice backcrossed for 10 generations and compared age- and sex-matched littermates. Our studies indicate that CXCR6+−/− mice are not equivalent to wild-type mice and should not be used in the place of wild-type animals.

We found that CXCR6 also played a role in basal NKT cell accumulation in the lungs. In contrast, Meyer et al. (58) demonstrated that CCR4 mediates NKT cell recruitment to the lung and airways following intranasal stimulation with α-GalCer. CCR4 also mediated basal localization of NKT cells to the airways, but not the lung. Therefore, it is likely that CXCR6 and CCR4 mediate homeostasis of NKT cells in different lung compartments under basal conditions. However, in the inflamed lung, CCR4 appears sufficient for NKT cell recruitment given that lung inflammation was ablated in CCR4−/− but not CXCR6−/− mice (58).

In addition to alterations in NKT cell tissue distribution, there were differences in the number of mature NK1.1+ NKT cells in CXCR6−/− mice. This is most likely due to a role for CXCR6/CXCL16 in NKT cell maturation. We observed a reduction in NK1.1 expression on the residual liver NKT cells in CXCR6−/− mice (Fig. 5). Also, the NKT cell populations in the spleen and bone marrow of CXCR6−/− mice exhibited small reductions in the proportion of NK1.1+ expressing cells even though NKT cell numbers were normal or increased, respectively. Reduced NK1.1 expression on the residual liver NKT cells in CXCR6−/− mice were small reductions in the proportion of NK1.1+ expressing cells even though NKT cell numbers were normal or increased, respectively. Reduced NK1.1 expression on the residual liver NKT cells in CXCR6−/− mice were small reductions in the proportion of NK1.1+ expressing cells even though NKT cell numbers were normal or increased, respectively.

With the above explanation, it is possible that CXCR6/CXCL16 interactions mediate cell-cell adhesion and/or provide costimulatory signals that up-regulate NKT cell activation. Production of IL-4 by splenic NKT cells was also dependent on a direct role for CXCR6. However, there was an intrinsic deficiency in CXCR6−/− liver NKT cells as IL-4 induction was still reduced following stimulation with PMA and ionomycin. This indicates that CXCR6 is required for maturation/survival in the liver environment but dispensable in other tissues.

CXCR6/CXCL16 were also found to play a critical role in NKT cell activation. NKT cells are potently activated by CD1d-dependent presentation of the glycolipid Ag α-GalCer, resulting in the rapid production of immunoregulatory cytokines (6, 17–19). After α-GalCer stimulation, we observed deficiencies in both intracellular cytokine staining in NKT cells and serum cytokine levels in CXCR6−/− mice (Fig. 6). To determine whether CXCR6 plays a direct role in NKT cell stimulation vs an intrinsic developmental defect in CXCR6−/− NKT cells, we also stimulated NKT cells with PMA and ionomycin. Induction of IFN-γ was not impaired in CXCR6−/− NKT cells following stimulation with PMA and ionomycin (Fig. 7), implicating a direct role for CXCR6 in Ag-dependent NKT cell activation. Production of IL-4 by splenic NKT cells was also dependent on a direct role for CXCR6. However, there was an intrinsic deficiency in CXCR6−/− liver NKT cells as IL-4 induction was still reduced following stimulation with PMA and ionomycin. This is likely related to a reduction in preformed IL-4 transcripts in CXCR6−/− liver NKT cells (Fig. 8), and suggests an additional role for CXCR6 in the developmental regulation of preformed IL-4 transcripts in liver NKT cells. The recent work of Shimaoka et al. (59) also supports a direct role for CXCR6/CXCL16 in NKT cell activation. This group found similar deficiencies in liver NKT cell numbers and α-GalCer-induced cytokine production in CXCL16−/− mice. The expression of CXCL16 on DCs was critical for optimal cytokine production by cultured NKT cells in response to α-GalCer stimulation.

α-GalCer needs to be presented via CD1d on APCs to activate NKT cells (19, 25, 61). Because CXCL16 is expressed as a transmembrane molecule on activated DCs and macrophages (44, 53), it is possible that CXCR6/CXCL16 interactions mediate cell-cell adhesion and/or provide costimulatory signals that up-regulate NKT cell function. Indeed, optimal NKT cell activation requires bidirectional signals between NKT cells and DC. In previous studies, CD40/CD40L interactions and DC-derived IL-12 have been shown to be critical for the production of IFN-γ by NKT cells (33, 34), whereas CD28/B7 interactions are required for the generation of both IL-4 and IFN-γ (33, 36). The requirement for CXCR6 to obtain optimal NKT cell activation is consistent with the observation that chemokine receptor interactions help facilitate activation of T cells during Ag presentation (40, 62).
In conclusion, we have characterized the contribution of CXCR6 in NKT cell development, homeostasis and activation. We revealed an important role for CXCR6 not only in the control of NKT cell accumulation in the liver and lungs but also in the activation of NKT cells and regulation of cytokine production. In the future, it will be important to characterize the exact role of CXCR6/CXCL16 in NKT cell interactions with APCs. Because interactions between CXCR6 and CXCL16 appear to be critical for NKT cell function, they could act as therapeutic targets to enhance NKT cell responses against tumors and pathogens or regulate tolerance induction and autoimmunity.

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Disclosures

Eugene C. Butcher is a Director of Bio-Save Inc., Burlingame, CA.

References


