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*J Immunol* 2003; 171:2571-2580; doi: 10.4049/jimmunol.171.5.2571
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CD1d-Restricted NKT Cells Express a Chemokine Receptor Profile Indicative of Th1-Type Inflammatory Homing Cells

Seddon Y. Thomas,* Runhaua Hou,‡ Jonathan E. Boyson,§ Terry K. Means,* Christoph Hess,* Douglas P. Olson,* Jack L. Strominger,§ Michael B. Brenner,‡ Jenny E. Gumperz,‡ S. Brian Wilson,‡ and Andrew D. Luster2*  

CD1d-restricted T cells (NKT cells) are innate memory cells activated by lipid Ags and play important roles in the initiation and regulation of the immune response. However, little is known about the trafficking patterns of these cells or the tissue compartment in which they exert their regulatory activity. In this study, we determined the chemokine receptor profile expressed by CD1d-restricted T cells found in the peripheral blood of healthy volunteers as well as CD1d-restricted T cell clones. CD1d-restricted T cells were identified by Abs recognizing the invariant Vα24 TCR rearrangement or by binding to CD1d-Fc fusion tetramers loaded with α-GalCer. CD1d-restricted T cells in the peripheral blood and CD1d-restricted T cell clones expressed high levels of CXCR3, CCR5, and CCR6; intermediate levels of CXCR4 and CXCR6; and low levels of CXCR1, CCR1, CCR2, and CX3CR1, a receptor pattern often associated with tissue-infiltrating effector Th1 cells and CD8+ T cells. Very few of these cells expressed the lymphoid-homing receptors CCR7 or CXCR5. CXCR4 was expressed predominantly on CD4+, but not on double-negative CD1d-restricted T cells, which may indicate differential trafficking patterns for these two functionally distinct subsets. CD1d-restricted T cell clones responded to chemokine ligands for CXCR1/2, CXCR3, CXCR4, CXCR6, CCR4, and CCR5 in calcium flux and/or chemotaxis assays. These data indicate that CD1d-restricted T cells express a chemokine receptor profile most similar to Th1 inflammatory homing cells and suggest that these cells perform their function in peripheral tissue sites rather than in secondary lymphoid organs. *The Journal of Immunology, 2003, 171: 2571–2580.

The broad grouping of NKT cells has been used to define cell types ranging from T cells with NK-like markers to CD1d-restricted invariant T cells (reviewed in Refs. 1 and 2). In this work, we define NKT cells strictly as CD1d-restricted T cells, which includes both invariant and diverse T cells. Human CD1d-restricted invariant T cells express a semi-invariant TCR with a Vγ11 chain, which pairs with a restricted set of TCR β-chains, including Vβ11 (3). CD1d-restricted T cells have been referred to as natural memory cells because they are thought to bridge a gap between innate and acquired immune responses and are thought to have a limited Ag repertoire.

Although the endogenous ligands for CD1d-restricted T cells are mostly unknown, glycolipid or lipid Ag is presented to NKT cells through the nonpolymorphic MHC-like molecule, CD1d, which pairs with β2-microglobulin (4). The glycolipid produced by a marine sponge, α-galactosylceramide (α-GalCer),3 has been used as a surrogate for a natural CD1d ligand because it is presented by CD1d and activates the majority of invariant T cells (5).

CD1d-restricted T cells that are invariant or bind CD1d tetramer loaded with α-GalCer can be divided into CD4+ and double-negative (DN) subsets (6–8). DN NKT cells do not express CD4 or CD8αβ, but can still express CD8α as a homodimer, CD8αα. Upon stimulation, CD1d-restricted T cells rapidly produce cytokines, including IFN-γ, TNF-α, GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13 (5, 6, 7, 8). It has recently been shown that CD4+ and DN CD1d-restricted T cells are functionally distinct in their cytokine production using CD1d tetramer loaded with α-GalCer (7, 8). CD4+ CD1d-restricted T cells have a Th0-like cytokine profile, while DN CD1d-restricted T cells have a Th1-like cytokine profile. CD1d-restricted T cells influence the outcome of diverse immune responses, ranging from autoimmune conditions to host defense against pathogens (reviewed in Refs. 1 and 2).

CD1d-restricted T cells can be detected throughout the body, but seem to be preferentially enriched at specific tissue sites. In the mouse, NKT cells are found at high levels in the liver, bone marrow, and thymus; at intermediate levels in the spleen, blood, and lung; and at lowest levels in the lymph node (6, 10). Human peripheral blood (PB) CD1d-restricted T cells have been detected at much lower frequencies than in murine tissue or PB. In addition to PB, human CD1d-restricted T cells have been cloned from bone marrow, liver, and decidual tissue (11–13).

1 This work was supported by National Institutes of Health Grants A149999 and CA69212 to A.D.L., T32-HL07874 to T.K.M., and AI45051 and JDRF 1-2002-732 to S.B.W.
2 Address correspondence and reprint requests to Dr. Andrew D. Luster, Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital, Building 149, 13th Street, Charlestown, MA 02129. E-mail address: aluster@partners.org
3 Abbreviations used in this paper: α-GalCer, α-galactosylceramide; DN, double negative; ELC, EBV-induced molecule 1 ligand chemokine; GRO-α, growth-regulated oncogene α; IP-10, IFN-γ-inducible protein 10; I-TAC, IFN-inducible T cell α-che moattractant; MIP, macrophage-inflammatory protein; PB, peripheral blood; SLC, secondary lymphoid tissue chemokine; TARC, thymus and activation-regulated chemokine.
Although CD1d-restricted T cells exhibit activity in various immune models, the trafficking patterns of these cells are not well understood. Chemokine receptor expression profiles can define functional subsets of leukocytes and can provide insights into the tissue localization and functional interactions in cellular immunity. There are two overlapping models of chemokine-induced T cell trafficking. One trafficking model focuses on tissue-specific homing of T cells as part of basic immune surveillance. This is based on the concept that naive and central memory T cells express CCR7 and can home to lymph nodes. Effector memory T cells are thought to lose CCR7 and gain tissue-specific homing receptors, such as CCR4 and CCR10 for the skin and CCR9 for the gut (14) (reviewed in Ref. 15).

In addition to tissue-specific trafficking, an inflammation-specific model of T cell trafficking has been proposed, which focuses on effector T cell populations. This model does not exclude a homeostatic role for tissue-specific lymphocyte trafficking, but suggests that during inflammation, inflammatory chemokines and their receptors play an important role in effector T cell trafficking. Thus, in Th1-type inflammation, NF-κB-inducible and STAT1-inducible chemokines and the cognate receptors for these chemokines, which are found on Th1 cells and effector CD8 cells, such as CXCR3, CCR5, and CXCR6, play a dominant role in effector T cell recruitment (16–19). In contrast, in Th2-type inflammation, STAT6-inducible chemokines and the cognate receptors for these chemokines, which are found on Th2 cells, such as CCR3, CCR4, and CCR8, play a dominant role in effector T cell recruitment (20).

In this study, we have examined chemokine receptor expression profiles on CD1d-restricted T cells using Abs recognizing the invariant Vα24 TCR rearrangement or CD1d-Fc fusion tetramers loaded with α-GalCer. In addition, CD1d tetramer–NKT cells were sorted into CD4+ and DN populations for RNA isolation and quantitative PCR analysis of cytokine, chemokine, and chemokine receptor mRNA levels. PB CD4+, PB DN, and decidual CD4+ CD1d-restricted T cell clones were analyzed for chemokine receptor expression and functional responses measured by calcium flux and chemotaxis. Our data indicate that CD1d-restricted T cells express a chemokine receptor profile most similar to Th1 inflammatory homing cells and suggest that these cells perform their function mainly in the tissue rather than in secondary lymphoid organs.

Materials and Methods

α-GalCer

The stock solution of α-GalCer (220 μg/ml) was diluted in 0.5% Tween 20 in PBS (KRN7000; Kirin Brewery, Gunma, Japan). This stock solution was further diluted with PBS for tetramer loading.

Flow cytometry reagents

Abs to CCR1 (53504.111), CXCR1 (42705.111), CCR2 (48607.121), CXCR2 (48311.211), CCR3 (61828.111), CXCR5 (51505.111), CCR6 (53103.111), CXCR6 (56811), CCR7 (51505.03), and CCR9 (112509) were purchased from R&D Systems (Minneapolis, MN), CCR3 (1C6), CCR4 (1G1), CXCR4 (12G5), CCR5 (3A9), CXCR6 (11A9), CCR7 (2H4), CD3 (UCHT1), CD4 (SK3), CD8α (RPA-T8), and CD19 (SJ25C1) were purchased from BD PharMingen (San Diego, CA). CD8β (2ST8.5H7), Vα24 (C15), and Vβ11 (C21) were purchased from Beckman Coulter (Fullerton, CA). CXCR1 (2A9-1) was purchased from MBL (Naka-ku, Nagoya, Japan).

The mAb (6B11) to the invariant Vα24oHq junction used in our studies was generated by immunizing C57BL/6 CD1d knockout mice with cyclic peptides, representing the CDR3 loop of the invariant TCR Vα24 sequence (21, 22).

Tetrameric complexes of the CD1d–Fc fusion protein or the IgG2a isotype UPC10 negative control mAb (Sigma-Aldrich, St. Louis, MO) were formed using protein A fluorescently labeled with Alexa 488 dye molecules (7). Similar nonfluorescent complexes for use as a flow cytometry blocking reagent were prepared using the UPC10 mAb and unlabeled protein A (Sigma-Aldrich). To load the fusion protein with Ag, a 4:1 molar ratio of α-GalCer dissolved in 0.5% polysorbate 20 in PBS was incubated with the CD1d tetramer for 24–48 h at room temperature.

FACS staining

PBMCs were prepared by centrifugation through a density gradient on Histopaque-1077 (Sigma-Aldrich). For staining with 6B11 and Vα24 Abs, PBMC were blocked with 10% human serum before staining with Abs to individual chemokine receptors, CD4, CD8, and Vα24, in PBS with 1% FCS. For staining with CD1d tetramers, PBMC were blocked with a solution containing 100 μg/ml nonfluorescent UPC10 tetramer, 50 μg/ml MOPC21 IgG1 mAb (Sigma-Aldrich), 0.5 mg/ml OVA, and 0.05% NaN3, and stained with tetramer loaded with α-GalCer and Abs to individual chemokine receptors. CD4, and CD19 in PBS with 1% BSA. For both 6B11–Vα24 and tetramer + α-GalCer staining, ~750,000–2,500,000 cells were collected per tube to identify 500–2,000 CD1d-restricted T cells for chemokine receptor analysis. Samples were run on a BD FACS Calibur and analyzed with CellQuest software.

Sorted cells

Samples were B cell depleted with anti-CD19 magnetic beads to reduce nonspecific staining using the MACS system, according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). CD1d-restricted T cell clones were analyzed for chemokine receptor expression. Samples were run on a BD FACSCalibur and analyzed with CellQuest software.

Quantitative PCR

Total RNA was extracted from sorted cells using the RNeasy protocol (Qiagen, Valencia, CA). After DNase I (Invitrogen, San Diego, CA) treatment, total RNA from each sample was used as template for the reverse-transcription reaction. cDNA was synthesized using oligo(dT)18 and random hexamers. Oligonucleotide primers were designed using Primer Express software 1.0 (Applied Biosystems, Foster City, CA). The PCR was performed as described (23).

CD1d-restricted T cell clones

PB CD1d-restricted T cell clones were generated from healthy donors, as previously described (9). Decidual CD1d-restricted T cell clones were generated from healthy donors, as previously described (13). Chemokine receptor expression and function were determined for all CD1d-restricted T cell clones at 10–14 days following restimulation.

Calcium flux

Samples were run on a BD LSR equipped with a helium-cadmium laser tuned to 633 nm (BD Biosciences, San Jose, CA). Indo-1 fluorescent calcium probe indo-1/acetoxymethylester in DMSO for 30 min at 37°C and then washed twice in cold PBS (Molecular Probes, Eugene, OR). For each stimulation, an aliquot of PBL (0.5 ml total volume) was warmed at 37°C for 3 min. Cells were monitored before adding chemokine to establish a baseline. The cells were then stimulated with chemokine by removing the FACS tube from the cytometer, adding chemokine (usually in 2 μl vol), vortexing sample, and replacing tube on cytometer. Data were analyzed with CellQuest software.

The percentage of cells undergoing calcium flux in response to a specific chemokine was calculated by placing a threshold line at baseline. This baseline threshold is defined by placing the line at the height at which 4–5% of the cells are above the threshold in the time before chemokine is added. The percentage undergoing flux in response to each chemokine is then measured by the percentage of cells above the threshold in the time window before fluorescence ratio levels of indo-1 begin to decrease. The
Chemotaxis

CD1d-restricted T cell clones were suspended in RPMI with 1% BSA and loaded in triplicate into a disposable chemotaxis apparatus (ChemoTx; Neuroprobe, Gaithersburg, MD). Chemokines were placed in the lower wells at various concentrations. After incubation for 3 h at 37°C, cell counts were determined in the lower wells with Cyquant fluorescent dye (Molecular Probes) in a CytoFluor MultiWell Plate Reader Series 4000 (Applied Biosystems).

Results

6B11 and Va24 Abs and CD1d tetramer specifically identify CD1d-restricted T cells

CD1d-restricted T cells were identified directly from PB based on Ab double staining of a canonical TCR rearrangement using an Ab that recognizes the TCR Vα24 chain and another Ab that recognizes the invariant Vα24JoQ junction (6B11) or based on binding to a CD1d tetramer loaded with a surrogate ligand for NKT cells, α-GalCer. These methods are more specific for identifying CD1d-restricted T cells than staining with Va24 and Vβ11 Abs, which are often used as surrogate markers for human NKT cells.

To directly evaluate various methods for identifying NKT cells, PBMCs from 10 donors were stained with the respective reagents. Representative staining is shown with Va24 and Vβ11 Abs (0.17% of cells in lymphocyte gate), with Va24 and 6B11 Abs (0.084%), and with CD1d tetramer loaded with α-GalCer (0.082%) for a donor with high numbers of CD1d-restricted T cells (Fig. 1A). Overall, Va24 and Vβ11 Abs identified a larger percentage of lymphocytes than did either 6B11 and Va24 Abs or CD1d tetramer loaded with α-GalCer. Of 10 donors tested, the average number of Va24+ Vβ11+ T cells was 0.055 ± 0.027%.

FIGURE 1. Expression of chemokine receptors on CD1d-restricted NKT cells from human PB as identified by either 6B11 and Va24 double-positive Ab staining or CD1d tetramer loaded with α-GalCer. A, Representative comparison of various flow cytometry-based methods of identifying NKT cells. PBMCs were stained for Va24 and Vβ11, Va24 and 6B11, or CD1d tetramer loaded with α-GalCer. Data are representative of 10 donors tested. B, Representative flow cytometry analysis of chemokine receptor expression on 6B11+ Va24+ cells, CD1d tetramer + α-GalCer, and CD4+ T cells with CD4 on the x-axis and chemokine receptor on the y-axis. Flow cytometry data are shown on a logarithmic scale. Comparison is representative of three donors stained with 6B11 Va24 Abs or CD1d tetramer loaded with α-GalCer. C, Representative flow cytometry analysis of chemokine receptor expression on 6B11+ Va24+ cells with CD4 on the x-axis and CX3CR1 on the y-axis. Flow cytometry data are shown on a logarithmic scale. D, Summary of chemokine receptor expression on 6B11+ Va24+ NKT cells, α-GalCer-loaded CD1d tetramer-stained NKT cells, and CD4+ T cells. The first, second, and fifth columns represent n = 5 subjects, with the exception of the CX3CR1 and CCR7 data with n = 4 subjects and CCR6 and CXCR6 data with n = 3 subjects. The third and fourth columns represent n = 3 subjects.
Chemokine receptor expression is similar between 6B11+Va24+ and CD1d tetramer+ T cells

CD1d-restricted T cells were examined for their chemokine receptor expression using both 6B11+ Va24+ staining and CD1d tetramers loaded with α-GalCer. CD1d-restricted T cells were divided into either CD4+ or DN (CD4–CD8–) populations and chemokine receptor profiles examined in comparison with total CD4+ T cells (Fig. 1B). The expression of chemokine receptors on CD1d-restricted T cells was similar between the two populations of cells identified by CD1d tetramers and 6B11 Va24 Abs with a few notable exceptions. CCR1 was expressed on 13.6 ± 4.2% of CD4+ CD1d tetramer− cells, but was detected on <1.5% of other CD1d-restricted T cells. CCR2 was expressed at lower levels on CD1d tetramer− cells than on 6B11+ Va24+ cells. CXCR5 and CCR9 were slightly enriched on CD4+ CD1d tetramer− cells as compared with either 6B11+ Va24+ cells or CD4+ T cells. CXCR4 and CXCR1 were not examined on CD1d tetramer− cells. These data suggest that populations of CD1d-restricted T cells as defined by binding of 6B11 and Va24 Abs or CD1d tetramer loaded with α-GalCer would traffic in a similar manner with only minor differences. To simplify discussion of the results, comparison of chemokine receptor expression profiles between 6B11+ Va24+ CD1d-restricted T cells and CD4+ T cells will be emphasized.

CD1d-restricted T cells express memory Th1 inflammatory homing extralymphoid chemokine receptors

CXCR3, CCR5, and CXCR6 are Th1/Tc1-associated chemokine receptors (16, 17). On average, 6B11+ Va24+ cells express high levels of CXCR3 (81.5 ± 15.2%), CCR5 (88.4 ± 2.4%), and CXCR6 (50.1 ± 1.1%), while bulk CD4+ T cells express these receptors at lower levels (27.5 ± 6.0% for CXCR3, 13.8 ± 3.7% for CCR5, and 1.8 ± 0.3% for CXCR6) (Fig. 1D). However, it is interesting to note that CXCR6 expression is more highly enriched on DN (59.1 ± 4.7%) than on CD4+ (25.2 ± 11.0%) 6B11+ Va24− cell subsets.

CCR2 was expressed on 23.1 ± 10.8% of 6B11+ Va24+ cells, while CCR2 was expressed on only 2.0 ± 0.6% of CD4+ T cells. Similarly, CXCR1 was expressed on 8.5 ± 4.4% of 6B11+ Va24+ cells, while CXCR1 was expressed on 1.0 ± 0.6% of CD4+ T cells. Our data demonstrate that CXCR1 and CCR2 are expressed at low levels on both CD4+ and DN 6B11+ Va24+ cells as compared with almost undetectable levels on bulk CD4+ T cells.

CCR6 was also enriched on CD1d-restricted T cells (77.7 ± 5.7% for DN and 62.5 ± 5.7% for CD4+) as compared with CD4+ T cells (24.1 ± 2.2%). Because >90% of 6B11+ Va24+ cells are CD45RO+, it is not surprising that CCR6 would be enriched on these cells (data not shown).

CXCR4 was expressed on a lower percentage of DN CD1d-restricted T cells (13.7 ± 5.6%) than on either CD4+ CD1d-restricted T (32.5 ± 8.4%) or bulk CD4+ T cells (39.7 ± 3.8%). Thus, the differences in CXCR4 expression could allow for differential trafficking of CD4+ and DN CD1d-restricted T cell subsets.

Lymph node homing receptors, CXCR5 and CCR7, were low on CD1d-restricted T cells (1.2 ± 0.4% for CXCR5 and 10.1 ± 3.4% for CCR7) as compared with CD4+ T cells (9.0 ± 2.5% for CXCR5 and 85.4 ± 3.6% for CCR7). This suggests that CD1d-restricted T cells would be unlikely to home to lymph nodes unless inflammatory chemokines were expressed in reactive lymph nodes.

Low percentages of CD1d-restricted T cells (6.5 ± 1.1%) express CXCR1, while almost no CD4+ T cells (1.1 ± 0.2%) express this receptor. Finally, CXCR4 was not differentially expressed on 6B11+ Va24+ and CD4+ T cells, and CCR1, CCR3, and CCR9 were not significantly expressed on either 6B11+ Va24+ T or CD4+ T cells, even though as a positive control these Abs stained other cells, demonstrating they were functional (data not shown).

Quantitative RT-PCR analysis of sorted CD4+ and DN CD1d tetramer+ T cells and CD4+ and CD8+ T cells

To confirm flow cytometric studies and to further examine chemokine receptors that could not be studied with Abs, tetramer-positive cells were sorted into CD4+ and DN CD1d tetramer+ populations along with CD4+ and CD8+ T cells. After isolation, these cells were analyzed for mRNA levels of specific chemokine receptors, cytokines, and chemokines (data not shown). Overall, the quantitative PCR data support the expression patterns observed by flow cytometry. Because Abs to CCR8 and CCR10 were not available for our studies, mRNA levels of these receptors were especially interesting. CCR8 is associated with Th2, but not Th1 cells (24). CCR8 mRNA was at low levels on all CD1d tetramer+ and T cell subsets examined. CCR10 mRNA was detected from both CD1d tetramer+ and T cell subsets. Based on these CCR10 mRNA data combined with cutaneous lymphocyte Ag expression data previously observed, it is likely that a subset of both DN and CD4+ CD1d tetramer+ cells expresses CCR10 and has a skin-homing phenotype (7).

The mRNA levels of various chemokines and cytokines were also examined. By far, the highest level of chemokine mRNA was RANTES, followed by IL-8 (CXCL8) and macrophage-inflammatory protein-1α (MIP-1α, CCL3). The other chemokines examined, including MIP-3α (CCL20), EBV-induced molecule 1 ligand chemokine (ELC, CCL19), secondary lymphoid tissue chemokine (SLC, CCL21), IFN-γ-inducible protein 10 (IP-10, CXCL10), IFN-inducible T cell α-chemoattractant (I-TAC, CXCL11), and growth-regulated oncogene α (GRO-α, CXCL1) were expressed at low levels or below levels of detection.

Chemokine receptor expression patterns from CD4+ and DN PB and CD4+ decidual CD1d-restricted T cell clones

Studies of PB CD1d-restricted T cells were complemented with studies of chemokine receptor expression and function on clonally derived CD1d-restricted T cells (Fig. 2). Although both CD4+ and DN decidual CD1d-restricted T cells were detected ex vivo, for unknown reasons only CD4+ decidual CD1d-restricted T cells could be expanded (13). Thus, PB CD4+, PB DN, and CD4+ decidual CD1d-restricted T cell clones were studied for expression of chemokine receptors and functional activity of calcium flux and chemotaxis.

CD1d-restricted T cell clones displayed similar expression patterns to PB CD1d-restricted T cells examined ex vivo (Fig. 2). CD1d-restricted T cell clones expressed high levels of CXCR3 and more variable levels of CCR5 and CXCR6 (Fig. 2B). CXCR4 expression was more highly enriched on PB CD4+ and decidual CD4+ clones than on PB DN CD1d-restricted T cell clones. CXCR4 was expressed at low levels on the CD1d-restricted T cell clones, which may reflect down-regulation of the receptor following activation in culture. CXCR1, CXCR2, CXCR6, CCR7,
CCR9 were detected at low levels on all three types of CD1d-restricted T cell clones tested. CCR1, CCR2, CCR3, CXCR5, and CX3CR1 expression levels on CD1d-restricted T cell clones were not distinguishable from the isotype control.

**Calcium flux and chemotaxis responses from CD4+ and DN PB and CD4+ decidual CD1d-restricted T cell clones**

The functional consequences of chemokine receptor expression on CD1d-restricted T cell clones were examined by calcium flux and chemotaxis assays (Figs. 3 and 4). Overall, a larger percentage of decidual CD4+ clones fluxed in response to chemokine stimulation than did the PB CD1d-restricted T cell clones. I-TAC, a ligand for CXCR3, induced calcium flux and chemotaxis in PB CD4+, PB DN, and decidual CD4+ CD1d-restricted T cell clones. IP-10 also induced detectable, but less robust chemotaxis in these cells. This is in keeping with the observation that I-TAC is a more potent ligand for CXCR3 than IP-10 (25). Stromal cell-derived factor-1α (CXCL12), a ligand for CXCR4, also induced a calcium flux and chemotaxis in the CD1d-restricted T cell clones, although the calcium response was weak in the PB CD4+ CD1d-restricted T cell clones.
clones. From these data, it was apparent that I-TAC induced either greater than or similar levels of chemotaxis as stromal cell-derived factor-1/THP-1. Thymus and activation-regulated chemokine (TARC; CCL17), a ligand for CCR4, induced calcium flux in all three groups of CD1d-restricted T cell clones tested. Of note, only one of the three PB DN CD1d-restricted clones tested fluxed 15% in response to TARC, while this was true for two of three PB CD4+ CD1d-restricted T cell clones tested. TARC was chemotactic for the PB CD4+ clone, while macrophage-derived chemokine (CCL22) was chemotactic for both PB CD4+ and decidual CD4+ CD1d-restricted T cell clones. This supports the idea that CCR4 is preferentially expressed and active on CD4+ rather than DN CD1d-restricted T cells.

CD1d-restricted T cell clones were only slightly responsive to MIP-1β (CCL4), a ligand for CCR5. Only two of the five CD4+ decidual clones responded to MIP-1β with >15% calcium flux. RANTES and, to a lesser extent, MIP-1β induced chemotaxis in the decidual CD4+ CD1d-restricted T cell clone shown, while RANTES induced chemotaxis in some PB and DN CD1d-restricted NKT cell clones tested (data not shown). The variation in responsiveness to CCR5 ligands was not necessarily surprising because CCR5 expression was variable on the CD1d-restricted T cell clones and may be down-regulated in these cells.

CXCL16, a ligand for CXCR6, induced chemotaxis in members of all three subsets of clones tested. However, some PB CD4+ and decidual CD4+ CD1d-restricted T cell clones did not respond to the CXCR6 ligand (data not shown). The levels of chemotaxis were similar to those observed for I-TAC.

Few CD1d-restricted T cell clones responded in calcium flux assays and none of the clones tested migrated to SLC, a ligand for CCR7. Only one of the three PB CD4+, one of the three PB DN, and one of the five decidual CD4+ CD1d-restricted T cell clones responded to SLC with >15% calcium flux. This agreed with low levels of CCR7 expression data in both ex vivo CD1d-restricted T cells and clones.

Interestingly, GRO-α, a ligand for CXCR2, produced calcium flux, but no chemotaxis, in a small percentage of the CD1d-restricted T cell clones. Three of five decidual CD4+ clones and one of three DN CD1d-restricted T cell clones responded to GRO-α with >15% calcium flux. This was surprising because CXCR2 expression was at low to undetectable levels in these clones. This has been observed previously in NK cells that respond to GRO-α, but have no detectable level of CXCR2 expression (26).

Other chemokines, including monocytic chemotactic protein-1 (CCL2; CCR2 ligand), MIP-3β (CCL20; CCR6 ligand), I-309 (CCL1; CCR8 ligand), thymus-expressed chemokine (CCL25; CCR9 ligand), cutaneous T cell-attracting chemokine (CCL27; CCR10 ligand), SLC and ELC (CCR7 ligands), B cell-attracting chemokine-1 (CXCL13; CXCR5 ligand), IL-8 (CXCR1 and CXCR2 ligand), GRO-α (CXCR2 ligand), and soluble fractalkine (CX3CL1; CX3CR1 ligand), had little to no chemotactic activity on the CD1d-restricted T cell clones.

Discussion
We have found that CD1d-restricted T cells express a set of chemokine receptors that are most similar to activated, memory, Th1
inflammatory homing, extralymphoid lymphocytes. CD1d-restricted T cells express high levels of CXCR3, CCR5, and CCR6; intermediate levels of CXCR4, CXCR6, and CCR4; low levels of CCR7, CXCR1, and CXC1R1; and variably low levels of CCR1 and CCR2. Expression of CCR1, CCR2, CCR5, CCR6, CXCR1, CXCR3, CXCR6, and CXC1R1 was increased on CD1d-restricted...
T cells as compared with CD4+ T cells, while expression of CCR7 and CXCR5 was decreased on CD1d-restricted T cells as compared with CD4+ T cells. CCR4 was expressed on more CD4+ than DN CD1d-restricted T cells, whereas CXCR6 was expressed on more DN than CD4+ CD1d-restricted T cells. CCR8 and CCR10 mRNA levels from CD1d-restricted T cells were similar to those from T cells; thus, CCR8 and CCR10 may be expressed on a subset of CD1d-restricted T cells. PB and decidual CD1d-restricted T cell clones expressed a similar pattern of receptors as those identified from PB and responded to CXCR1/2, CXCR3, CXCR4, CXCR6, CCR4, and CCR5 ligands through calcium flux and/or chemotaxis. A summary of our expression data and a comparison of chemokine receptor expression on naive T, Th1, Th2, and effector CD8 T and NK cells are shown in Table I.

Based on the pattern of chemokine receptors expressed, we hypothesize that CD1d-restricted T cells would be recruited early to sites of infection and/or inflammation. Following Toll-like receptor activation of tissue macrophages or immature dendritic cells, specific chemokines are rapidly produced, including ligands for CCR1, CXCR1/2, CXCR3, CCR5, and CXCR6 (18, 19). CD1d-restricted T cells, which express these receptors, would be recruited to these tissue sites of inflammation. In a mouse model of tolerance induction, CXCR2 has been shown to play an important role in the trafficking NKT cells, defined by CD3 and NK1.1 coexpression (27). CXCR2, however, does not appear to be highly expressed on human CD1d-restricted T cells, while CXCR1 was expressed at low levels. This may reflect differences between the mouse and human NKT cells. Our data suggest that other chemokine receptors, such as CXCR3, CCR5, and CXCR6, may play a more significant role in recruiting CD1d-restricted T cells to tissue inflammatory sites. CCR6, which is expressed on a high percentage of CD1d-restricted T cells and immature dendritic cells, may promote the interaction of CD1d-restricted T cells and immature dendritic cells in the periphery, which could have an important influence on dendritic cell maturation (28). In addition, CXCR2 and CXCR1 could recruit a subset of CD1d-restricted T cells to specific inflammatory sites, where they could interact with recruited tissue macrophages and cytotoxic effectors, respectively (29, 30).

Although populations of CD1d-restricted T cells, as defined by binding to 6B11 and Va24 Abs or CD1d tetramer loaded with α-GalCer, most likely overlap, it is possible that there are minor differences in these populations. These differences could result from binding of the CD1d tetramer + α-GalCer to a small, diverse population of nonvariant T cells not recognized by 6B11 and Va24 Abs or from binding of 6B11 and Va24 Abs to invariant T cells that recognize a different lipid Ag than α-GalCer (31, 32).

Even with potential differences in these two CD1d-restricted T cell definitions, both populations seem to express similar levels of chemokine receptors and are likely to traffic in a similar manner.

A recent study by Kim et al. (33) examined chemokine receptor expression profiles on Va24+ Vb11+ cells as a surrogate for CD1d-restricted T cells. Although the combination of Va24 and Vb11 Abs does not recognize specifically CD1d-restricted T cells, CD1d-restricted T cells are enriched in this subset. On average, percentages of Va24+ Vb11+ T cells were 1.5-fold higher than 6B11+ Vo24+ NKT cells and 1.3-fold higher than CD1d tetramer loaded with α-GalCer. In our study, 6B11+ Vo24+ cells and CD1d tetramer+ cells expressed similar patterns of chemokine receptors, while Va24+ Vb11+ cells had a somewhat altered chemokine receptor profile. CCR7 was expressed at lower levels on 6B11+ Vo24+ and CD1d tetramer+ cells than on Va24+ Vb11+ cells. Although we found that CCR6 and CXCR6 were more highly expressed on DN CD1d-restricted T cells than on their CD4+ counterparts, our data actually narrowed the gap in expression of these receptors between the CD4+ and DN CD1d-restricted T cell subsets as compared with the expression of these receptors on Vo24+ Vb11+ subsets. Our study also included chemokine receptors that had not been examined on CD1d-restricted T cells previously, such as CXCR1, CXCR2, and CXCR1. CXCR1 and CXCR1 were detected at low levels on both CD4+ and DN CD1d-restricted T cells, while CXCR2 was almost undetectable on these subsets.

Surprisingly, we detected CCR1 expression only on CD4+ CD1d tetramer+ cells. This is in contrast to Kim et al., who found that 15% of CD4+ and 70% of DN Va24+ Vb11+ cells expressed CCR1. Additionally, while Kim et al. observed high levels of CCR2 on Va24+ Vb11+ T cells, we found CCR2 was expressed at low levels on 6B11+ Vo24+ cells and was almost undetectable on CD1d tetramer+ cells. We repeated the staining for CCR1 and CCR2 on Va24+ Vb11+ T cells and observed similarly low receptor levels to those observed for 6B11+ Vo24+ NKT cells (data not shown). Because the same mAbs were used in our study and by Kim et al., the differences in CCR1 and CCR2 staining are puzzling and may reflect differences in the use of directly conjugated Abs in our study and unconjugated Abs with secondary Abs in the study by Kim et al., or may reflect differences in cell preparation. These data suggest that CD1d-restricted T cells are most similar to Th1 inflammatory homing memory cells, that CD4+ and DN CD1d-restricted T cells differ less dramatically in chemokine receptor expression, and that CCR1 and CCR2 may play a lesser role in recruitment of CD1d-restricted T cells than previously predicted. Although trends in chemokine receptor expression were generally similar when comparing Va24+ Vb11+ T cells with

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**Table 1. Summary of chemokine receptor expression on human CD1d-restricted T cells and comparison with human naïve T, Th1, effector CD8 T, and NK Cells**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>CCR1</th>
<th>CCR2</th>
<th>CCR3</th>
<th>CCR4</th>
<th>CCR5</th>
<th>CCR6</th>
<th>CCR7</th>
<th>CCR8</th>
<th>CCR9</th>
<th>CCR10</th>
<th>CXCR1</th>
<th>CXCR2</th>
<th>CXCR3</th>
<th>CXCR4</th>
<th>CXCR5</th>
<th>CXCR6</th>
<th>CX3CR1</th>
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<td>Naive T</td>
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<tr>
<td>CD4+ NK T</td>
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<td>Effector CD8</td>
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<td>NK (CD56+CD16+)</td>
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<td>NK (CD56+CD16-)</td>
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* Chemokine receptor expression on human CD1d-restricted T cells is compared with data from the literature on chemokine receptor expression from other subsets of human T and NK cells on directly analyzed peripheral blood lymphocyte subsets. Chemokine receptors preferentially associated with Th1 and CD8 effector cells are marked in bold. Note similarities between NK, Th1, and CD8 effector T cells. +, Receptor present as measured by quantitative RT-PCR. NR, Not reported.
either 6B11+ Vα24+ or CD1d tetramer-stained cells, our study was restricted to the cells of interest, CD1d-restricted T cells, and defines more clearly the specific and exclusive expression of extralymphoid inflammatory homing receptors on these cells.

In addition to examining the chemokine receptor profile on ex vivo CD1d-restricted T cells, we determined both expression and function of chemokine receptors on PB and decisional CD1d-restricted T cell clones. Few differences were observed between chemokine receptor expression on clones and PB CD1d-restricted T cells. There were some notable exceptions, however. CCR1, CCR2, CCR6, CXCR4, and Cx3CR1 were present on PB CD1d-restricted T cells, while these receptors were not found on CD1d-restricted T cell clones. Because CCR1, CCR2, and Cx3CR1 were expressed on a low percentage of CD1d-restricted T cells ex vivo, it is not surprising then that most CD1d-restricted T cell clones did not express these receptors. In a separate experiment with bulk Vα24+ T cells cultured with PHA, IL-2, and irradiated feeder cells, we demonstrated that CCR6 is up-regulated at day 3 following stimulation, but is reduced to almost background levels at days 10 and 14 (data not shown). This implies that CD1d-restricted NKT clones lose expression of CCR6 in culture. CCR5 was more variably expressed on clones than on CD1d-restricted T cells observed ex vivo. The high levels of RANTES expression in CD1d-restricted T cells may have contributed to autocrine activation of CCR5 and internalization. It is interesting to note that CD1d-restricted T cells have an activated chemokine receptor profile at baseline that is maintained in culture, which is different from what we have observed for other types of T cell clones and their PB counterparts (unpublished observations).

CD1d-restricted T cell clones were used to determine the functionality of chemokine receptors expressed on CD1d-restricted T cells using calcium flux and chemotaxis assays. CD1d-restricted T cell clones fluxed calcium predominantly in response to CXCR3, CXCR4, and CCR4 ligands. CXCR3 and CXCR4 ligands were chemotactic for all CD1d-restricted T cell clones tested, while CCR4, CCR5, and CXCR6 ligands were chemotactic only for specific clones. Our functional data from CD1d-restricted T cell clones support the significance of the chemokine receptor expression patterns observed ex vivo.

The difference in homing properties of CD1d-restricted T cells and CD4+ and CD8+ T cells predicts differences in immune function at baseline. Although naive peptide-specific T cells home to lymph nodes to sample peptide Ag presented by mature dendritic cells, CD1d-restricted T cells traffic into inflamed tissue and are, thus, likely to respond to lipid Ag in the periphery. CD1d expression remains unchanged on immature and mature dendritic cells (34). This implies that immature dendritic cells present lipid Ag in the context of CD1d to NKT cells before MHC class II is up-regulated. Based on the pattern of chemokine receptors consistently expressed on NKT cells, CD1d-restricted T cells are likely to be recruited into the tissue early in infection and inflammation. These CD1d-restricted T cells could then influence and promote the conventional T cell response by acting at an earlier time point, inducing maturation of dendritic cells through cytokine production, and recruiting inflammatory homing cells through production of chemokines, such as IL-8, MIP-1α, and RANTES. Our studies of CD1d-restricted T cell clones identified the cells of interest specifically based on two different criteria and support the notion that CD1d-restricted T cells are likely to home to extralymphoid inflammatory tissue sites.

Acknowledgments

We thank Kirin Brewery for providing the KRN7000 (α-GalCer).

References


