Distinct Subsets of CD1d-restricted T Cells Recognize Self-antigens Loaded in Different Cellular Compartments

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Summary

Although recent studies have indicated that the major histocompatibility complex-like, β2-microglobulin-associated CD1 molecules might function to present a novel chemical class of antigens, lipids and glycolipids, to α/β T cells, little is known about the T cell subsets that interact with CD1. A subset of CD1d-autoreactive, natural killer (NK)1.1 receptor–expressing α/β T cells has recently been identified. These cells, which include both CD4+CD8+ and CD4+ T cells, preferentially use an invariant Vα14-Jα281 T cell receptor (TCR) α chain paired with a Vβ8 TCR β chain in mice, or the homologous Vα24-JαQ/Vβ11 in humans. This cell subset can explosively release key cytokines such as interleukin (IL)-4 and interferon (IFN)-γ upon TCR engagement and may regulate a variety of infectious and autoimmune conditions. Here, we report the existence of a second subset of CD1d-restricted CD4+ T cells that do not express the NK1.1 receptor or the Vα14 TCR. Like the Vα14+ NK1.1+ T cells, these T cells exhibit a high frequency of autoreactivity to CD1d, use a restricted albeit distinct set of TCR gene families, and contribute to the early burst of IL-4 and IFN-γ induced by intravenous injection of anti-CD3. However, the Vα14+ NK1.1+ and Vα14+ NK1.1− T cells differ markedly in their requirements for self-antigen presentation. Antigen presentation to the Vα14+ NK1.1+ cells requires endosomal targeting of CD1d through a tail-encoded tyrosine-based motif, whereas antigen presentation to the Vα14+ NK1.1− cells does not. These experiments suggest that the existence of two phenotypically different subsets of CD1d-restricted T cells that survey self-antigens loaded in distinct cellular compartments.

Key words: CD1 • self-antigen • endosome • interleukin 4 • interferon γ

Classic MHC class I and class II molecules in vertebrates capture pathogen-derived peptides in the endoplasmic reticulum or in the endosomal compartment, and present them on the cell surface for recognition by CD8+ or CD4+ α/β T lymphocytes, respectively (1). In contrast, CD1 molecules, a family of MHC-like, non-MHC-encoded molecules, seem to present a novel antigenic universe, made of lipids rather than peptides, to T cells (2). Human CD1b and CD1c molecules can present various lipid and glycolipid components of mycobacterial cell walls (3–5). CD1d, a conserved isotype that is expressed by all mammals studied to date, and the only one that is expressed by mice and rats, may also bind various glycolipids, including glycosyl phosphatidyl inositols (6) and glycosylated ceramides (7).

Although little is known about the frequency and phenotype of T cells that use CD1 as a presenting molecule, or about the antigen presentation pathways associated with CD1, several observations suggest that they differ from those defined in the classical MHC system. In humans, a few CD1b- and CD1c-restricted T cell lines have been reported. Most of them have an unusual CD4/CD8 double negative phenotype (3–5), while some are CD8+ (8). Their recognition of mycobacterial lipid antigens depends on a tyrosine-based motif encoded in the cytoplasmic tail of CD1b itself that targets CD1b to the endosome, a different mechanism of endosomal trafficking than that of the MHC class II pathway (9, 10). CD1d, the only CD1 isotype in mice, interacts with a prominent subset of CD1d-restricted T cells that has been identified in vivo on the basis of its unique phenotype and functional properties. This subset comprises the NK1.1 receptor–expressing α/β T cells (NK T cells) that preferentially use an invariant Vα14-Jα281 TCR α chain paired with a Vβ8 TCR β chain in mice.
presenting functions of CD1 molecules. These results have significant implications for the antigen together, these experiments suggest that there are two phe-
gon (7) suggests that these cells might survey a single, yet unidentified family of self-glycolipids with homology to al-
a component of marine sponges, can specifically stimulate Thus, a recent report showing that alpha galactosyl ceramide,
with gene (16, 17), in tumor rejection (18), and in autoim-
immune diseases (19, 20). A large fraction of these cells can be
thought to be the basis for their role in various intracellular
infections (16, 17), in tumor rejection (18), and in autoim-
une diseases (19, 20). A large fraction of these cells can be
shown to be autoreactive to CD1d-expressing cells (12).

Materials and Methods

Mice. C57BL/6 and C57BL/6 I Aβb−/− (MHC II−/−) mice were obtained from Taconic Farms. Vα14-α281 transgenic mice in a C57BL/6 or C57BL/6 Cα knockout background were described previously (22). All mice were raised in a specific patho-

CD1 Tail–mutant Constructs and Transfectants. Complement-
dary DNA for CD1-TD was generated by PCR using full-length CD1d cDNA (25) as template. First, primers S1 (5′-CCCTGG-
AGATGCTTCCG-3′) and Tr1 (5′-GGCCAGGTTAAGGAG-
GATCATCTCTCTGCAATAGA-3′) were used to amplify the 600-bp fragment A, and primers Tn oligo (5′-AAAA-
AGCGCGCGGAGTACGACATTTGCAGTT-3′) and Tm1 (5′-TACTATATCTGGAGGAGGAGAATCTCTCTTACTTTATT-
CACCTGCC-3′) were used to amplify the 240-bp fragment B.

In Vitro T Cell Stimulation Assay. T cells were cultured for 18 h in the presence of CD1d-expressing cells (5 × 10^6 responders and 5 × 10^5 thymocyte or splenocyte sti-
tors, unless otherwise stated) in 100 μl of a 1:1 mixture of Click’s medium and RPMI (Biofluids) enriched with 10% heat-inacti-
vated FCS, glutamine, antibiotics, and 5 × 10−5 2-ME. IL-2 or IL-4 released in the supernatant was measured using the CTLL or CT45 bioassays, respectively, as described (23).

T Cell Hybridoma Generation. Purified T cell subsets were cultured for 5 d with anti-CD3 and IL-2 and fused with BW5147αβ− as described (11). Vα14-α281-positive hybridomas were identified by PCR (11). CD1d-autoreactive hybridomas specifically secreted IL-2 (as well as IL-4) upon culture with CD1d-transfected RBL or C57SV cells but not with untrans-

Competitive Reverse Transcription PCR. Quantification of Cytokine mRNA. Messenger RNA was purified from 10^5 FACs®-sorted cells using the RNaseasy Mini Kit (QIAGEN), reverse transcribed, and PCR amplified using IL-4, IFN-γ, and hypoxanthine phosphoribosyl transferase (HPRT)J-specific primers in the presence of titrated amounts of competitor plasmids as described (24). T Cell Gene Sequencing. Reverse transcription (RT)-PCR, primers, and methods were as described previously (11).
were incubated for 45 min with FITC-labeled anti-CD1d mAb 19G11 (27). After three washes in permeabilizing buffer and one wash in PBS, the cells were mounted in 100 μg/ml 1,4 diacylxylopropane (2,2,2) octane (Dabco; Sigma Chemical Co.), 100 μg/ml mowiol (Calbiochem Corp.), 25% (vol/vol) glycerol, 100 mM Tris-HCl, pH 8.5. The samples were examined under an LSM 510 confocal microscope attached to an axiovert microscope equipped with an argon and a helium-neon laser (Carl Zeiss, Inc.). The Rhodamine and FITC emissions were recorded sequentially. Optical sections were recorded with a 63× lens and a pinhole aperture such that the thickness of the sections was ~0.7 μm. No immunofluorescence staining was observed when second antibodies were used without the first antibody or with an irrelevant first antibody.

**Results**

Two subsets of CD1d-restricted T cells. To determine whether there are CD1d-restricted CD4 cell subsets other than the NK T cell subset, we reexamined the residual CD4 cell population found in C57Bl/6 mice bearing a targeted mutation of MHC class II (IAb<sup>–</sup>/–, referred to as MHC II<sup>–/–</sup>). Fig. 1 shows that the residual 2 or 3% CD4<sup>+</sup> cells in MHC II<sup>–/–</sup> mice could be divided into CD4<sup>+</sup> NK1.1<sup>+</sup> (one third) and CD4<sup>+</sup>NK1.1<sup>−</sup> (two thirds) cells, as reported previously (21, 28–30). To examine these cells at the single cell level, we generated separate panels of T cell hybridomas from these sorted cell subsets. The hybrids were screened for autoreactivity to CD1d-transfected cells or to cells that naturally express CD1d, such as thymocytes and splenocytes, and for expression of the canonical V<sub>α</sub>14-J<sub>α</sub>281 TCR α chain. Significant numbers of CD1d-autoreactive cells were found in both NK1.1<sup>+</sup> (27%) and NK1.1<sup>−</sup> (32%)–derived subsets (Fig. 1). Most of the hybrids derived from NK1.1<sup>+</sup> cells used the V<sub>α</sub>14-J<sub>α</sub>281 TCR α chain (81%), as expected, whereas most of those derived from NK1.1<sup>−</sup> cells (86%) did not. Though the NK1.1<sup>−</sup> CD1d-autoreactive subset does not use the canonical V<sub>α</sub>14-J<sub>α</sub>281 TCR, it is nevertheless not a highly heterogeneous set but expresses a rather restricted set of TCR genes. For example, out of 13 such V<sub>α</sub>14-negative CD1d-autoreactive hybridomas collected in several independent fusion experiments and used in the experiments depicted below in Fig. 4, 5 used V<sub>α</sub>8, and 3 of them, derived from 2 different mice, had the V<sub>α</sub>8 gene rearranged to the same j<sub>α</sub>19 segment. Studies in progress on larger panels of hybridomas confirm that CD4<sup>+</sup>NK1.1<sup>−</sup> cells have a biased use of V<sub>α</sub> gene families (data not shown). In addition, a majority of the V<sub>α</sub>14-negative CD1d-autoreactive hybrids (9 out of 13), like the V<sub>α</sub>14-positive hybrids (11), used VB<sub>β</sub>8.2 (see legend to Fig. 4) with diverse junctional regions (not shown). Thus, the results demonstrate the existence of a second novel, phenotypically distinct set of CD1d-restricted T cells that are NK1.1<sup>−</sup>, use limited sets of TCRs, are autoreactive to CD1d, and whose frequency is comparable to that of V<sub>α</sub>14<sup>+</sup>NK1.1<sup>+</sup> cells.

Evidence for an Endosomal Pathway of Antigen Presentation by CD1d to V<sub>α</sub>14<sup>+</sup> T Cell Hybridomas. The use of a limited set of TCR families by both sets of CD1d-autoreactive cells suggests that they might recognize a limited number of CD1d-associated self-antigens. To examine the origin of self-molecules potentially presented by CD1d, we took advantage of the existence of a targeting motif in the cytoplasmic tail of CD1d that gives it access to the endosomal compartment (9, 31). We constructed a tail-deleted variant (CD1-TD) lacking the SAYQD1R COOH-terminal end of the cytoplasmic tail which contains the endosomal targeting motif (underlined), and generated stable transfectants expressing CD1-TD or the wild-type CD1 (CD1-WT) using two different cell lines, mouse C57SV fibroblasts (32) and rat RBL basophils (33). CD1-TD was well expressed on the plasma membrane (Fig. 2 a) in a highly glycosylated form similar to that of CD1-WT (not shown). Fig. 2 b shows that CD1-WT exhibited a prominent intracytoplasmic distribution with a diffuse vesicular pattern that colocalized extensively with LAMP-1, indicating that a significant fraction of the CD1 molecules pass through a late endosome/lysosome location. A similar distribution was found in BCL-1, a B cell line that naturally expresses CD1d (not shown). In contrast, most LAMP-1-positive vesicles were devoid of CD1d in CD1-TD–transfected cells, despite the matched expression levels of surface CD1d. These results, shown in Fig. 2 for C57SV, a mouse fibroblast cell line, are identical to those recently reported for a mouse B cell line, A20, transfected with CD1-WT or CD1-TD (31), and are similar to those established previously in the human CD1b system (9).

To determine whether the CD1d molecules trafficking through the endosome pick up a distinct set of self-antigens, we compared the ability of CD1-WT and CD1-TD transfectants to stimulate CD1d-autoreactive T cells. To achieve a dose titration of the CD1d molecules, we used multiple rounds of FACS<sup>®</sup> sorting to select several sublines expressing different surface levels of CD1-WT or CD1-TD. Fig. 3 shows that DN32.D3, a canonical V<sub>α</sub>14-J<sub>α</sub>281/V<sub>β</sub>8.2 hybridoma, responded 10-fold less well to CD1-TD than to CD1-WT, whether the CD1d molecule was expressed by rat RBL basophils or by mouse C57SV fibroblasts. In contrast, IC8 DC1, a V<sub>α</sub>14-negative hybridoma, showed identical responses to CD1-WT and CD1-TD over a wide range of surface concentrations, indicating that the CD1d-associated antigens recognized by IC8 DC1 were essentially unaffected by the drastic changes in intra-
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Cellular trafficking associated with the tail truncation, and therefore that they are most likely loaded in the secretory pathway. Again, identical results were obtained for both C57SV and RBL transfectants, as shown in Fig. 3, left and right, respectively, and confirmed in independent transfection experiments. An additional mutant of CD1d, CD1-Phe, which contains a Tyr to Phe mutation in the cytoplasmic tail motif, reproduced the CD1-TD phenotype (not shown, and see Table I below).

These results suggested the existence of two separate pathways of antigen presentation by CD1d, one dependent on and one independent of endosomal trafficking, and therefore the existence of two separate pools of self-antigens loaded in distinct intracellular compartments. To test whether the differences in recognition exhibited by DN32.D3 and 1C8.DC1 were characteristic of their representative subsets, we tested an extended panel of CD1d-autoreactive T cell hybridomas generated from normal or MHC II−/− splenocytes or thymocytes over the course of 3 yr in seven fusion experiments. Fig. 4 is a compilation of several experiments comparing their recognition of C57SV and RBL transfectants expressing matched levels of CD1-TD and CD1-WT. We found that none of the 13 Vα14-negative hybridomas discriminated between CD1-WT and CD1-TD, whereas 10 out of 14 (71%) Vα14-positive hybridomas clearly did, exhibiting a 3–15-fold impaired recognition of CD1-TD. These patterns of recognition of CD1-WT and CD1-TD suggest that there is a systematic difference between the two T cell subsets in their antigen presentation requirements.

Fresh NK1.1+ Vα14+ T cells require endosomal trafficking of CD1d for efficient recognition. A few (4 out of 14) Vα14-positive hybridomas were able to recognize CD1-TD as well as CD1-WT, possibly because of residual access of CD1-TD to the endosomal compartment, or because they crossreacted with other antigens. To assay a larger population of Vα14-positive T cells, and to rule out potential biases associated with studies of in vitro–derived hybridomas, we studied cells from a transgenic mouse where the invariant Vα14-Jα281 TCR α chain is expressed by all T cells, in association with endogenous, polyclonal TCR β chains (22). These T cells constitute a fresh polyclonal population of Vα14-Jα281-positive CD1d-autoreactive T cells. Table I shows that Vα14-Jα281 transgenic thymocytes responded strongly to CD1-WT- and poorly to CD1-TD-transfected cells. The response of 5 × 10⁴ transgenic cells to RBL.CD1-TD was much lower than the response of 10⁴ cells to RBL.CD1-WT, suggesting that >80% of fresh Vα14-positive cells specifically see endosomally loaded antigens. The control Vα14-negative T cell hybridoma 1C8.DC1 used in this experiment responded equally to both. We next compared fresh NK1.1− and NK1.1− CD4+ cells purified from the spleens of MHC
N K1.1+ and N K1.1− cells conclusively demonstrate that the ability to discriminate CD1-TD from CD1-WT is the common, characteristic pattern of the Vα14 T cell subset. Both N K1.1+ and N K1.1− T cells contribute to the early burst of IL-4 induced by anti-CD3 injection in vivo. A hallmark of N K T cells is their extraordinary ability to synthesize and secrete large amounts of cytokines, especially IL-4, at peak levels very quickly after anti-CD3 injection in vivo, a unique property that is likely to influence the outcome of the responses in which they are involved (22, 34). To determine whether CD4+/N K1.1− cells can contribute to this early cytokine burst, we purified CD4+/N K1.1− and CD4+/N K1.1+ splenocytes 1.5 h after intravenous injection of 1 μg of 2C11 anti-CD3 antibody to MHC II−/− mice, and measured their IL-4 and IFN-γ mRNA using a competitive RT-PCR procedure (24). Fig. 5 shows that both subsets contributed significantly to the early cytokine burst, although N K1.1+ T cells tended to produce two to three times more IL-4 and IFN-γ mRNA than N K1.1− T cells within this short time frame. In conventional in vitro mitogen stimulation assays, both subsets released the same amount of both IL-4 and IFN-γ proteins after a period of 48 h (not shown). Thus, we conclude that both subsets can produce IL-4 and IFN-γ upon primary stimulation, although the kinetics of in vivo production may be faster for N K T cells.

Discussion

We have identified the in vivo counterpart of the non-Vα14-expressing CD1d-autoreactive α/β T cell hybridomas reported previously by several laboratories, and showed that they belong to a novel subset of T cells that shares some characteristics with the Vα14+N K1.1+ T cells but differs in many others. Like Vα14-positive N K T cells, these T cells exhibit a high frequency of autoreactivity to CD1d, use restricted families of TCRs, and contribute to the early burst of IL-4 and IFN-γ induced by intravenous injection of anti-CD3. However, they do not express the N K1.1 receptor. In addition, they recognize different subsets of CD1d molecules. We showed using a large panel of hybridomas as well as polyclonal fresh populations of Vα14-positive and -negative T cells that the Vα14+N K1.1+ T cells require endosomal targeting of CD1d through a tail-encoded tyrosine-based motif for recognition of CD1d, whereas the newly discovered Vα14−N K1.1− T cells do not. This dichotomy between N K1.1-positive and -negative T cells is independent of the expression of N K1.1, because N K T cell-derived hybridomas fail to express N K1.1 (35; and data not shown). Altogether, these experiments clearly establish that there are two phenotypically different subsets of CD1d-restricted T cells that survey antigens loaded in distinct cellular compartments.

The results imply that CD1d may load self-antigens in the two cellular compartments that are sampled separately by the classical MHC class I and class II molecules. To sample the endosome, it uses an endosomal targeting motif...
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without which it can efficiently only sample the secretory pathway. In support of the existence of a secretory pathway of antigen loading is the recent report that a soluble, secretory form of CD1d could be loaded with endogenous cellular glycosyl phosphatidyl inositols (6). The existence of a second, endosomal pathway has also been previously suggested by the finding that presentation of alpha galactosyl ceramide, a mimic of the self-antigen recognized by Vα14 cells, is chloroquine dependent (7). As suggested (9), CD1d may differ from MHC class II in that it might reach the cell surface first and only secondarily be internalized, using its tail-encoded tyrosine-based motif to access the endosome, load new antigens, and recycle to the cell surface. Indeed, experiments in progress in our laboratory indicate that the rate of internalization of CD1-TD is significantly reduced compared with that of CD1-WT (data not shown).

Therefore, like MHC class I and class II–restricted CD8 and CD4 T cells, CD1d-restricted Vα14-negative and Vα14-positive cells may survey different pathways of antigen presentation. This dichotomy is reinforced by the distinct phenotypes of the two subsets, in particular with respect to the expression of the NKL1 receptor. The emerging picture is that CD1d-restricted T cells constitute at least two subsets of α/β T lymphocytes, comparable in numbers to the NK cells (on the order of 1–20% of the lymphocyte compartment in various tissues), that are particularly enriched in some tissues, such as the liver, spleen, and bone marrow, and that use a limited number of TCRs to focus on a limited number of distinct self- and foreign antigens. Although the self-antigens recognized by the two subsets of CD1d-restricted T cells described here remain to be characterized, the evidence that the T cells can recog-

**Table I.** Fresh CD1-WT–reactive Vα14-Jα281 T cells respond poorly to CD1-TD

| No. of cells | RBL.CD1-WT | RBL.CD1-TD |
|--------------|-------------|-------------|
| Vα14 Tg thymocytes | ×10⁴ | IL-4 (U/ml) |
| (100% Vα14) | 5 | 975 | 32 |
| 1C8.DC1 | 1 | 306 | 22 |
| (Vα14-neg) | 5 | 240 | 320 |
| 1 | 103 | 87 |

| | RBL.CD1-WT | RBL.CD1-Phe |
|CD4 NK1.1⁺ | 1 | 370 | 49 |
| (81% Vα14) | 1 | 130 | 5 |
| Vα14 Tg thymocytes | 1 | 695 | 400 |
| (100% Vα14) | 1 | 120 | 85 |
| CD4 NK1.1⁻ | 1 | 130 | 5 |
| (14% Vα14) | 1 | 695 | 400 |
| 1C8.DC1 | 1 | 120 | 85 |
| (Vα14-neg) | 1 | 130 | 5 |

Thymocytes from Vα14-Jα281 TCR α transgenic, Cα−/−:B6 mice or sorted CD4⁺NK1.1⁺ or CD4⁺NK1.1⁻ MHC II−/− splenocytes (same as in Fig. 1, containing an estimated frequency of 81 and 14% Vα14-positive TCRs, respectively) were cultured for 5 d with 2.25 μg/ml Con A and IL-4 (10 ng/ml), washed, and restimulated for 20 h at indicated cell numbers with 10⁴ RBL.CD1-WT, RBL.CD1-TD, or RBL.CD1-Phe cells expressing similar levels of surface CD1d, in the presence of 20 U/ml IL-2. IL-4 release was measured using the CT4S bioassay. 1C8.DC1 is a control Vα14-negative CD1d-autoreactive hybridoma. Untransfected R BL cells elicited <10 U/ml IL-4 in all cases. Results are representative of seven experiments with Vα14-Jα281 TCR α transgenic cells of either thymic or splenic origin, and of two experiments with sorted CD4⁺NK1.1⁺ or CD4⁺NK1.1⁻ MHC II−/− splenocytes. Tg, transgenic.
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