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Conserved and Heterogeneous Lipid Antigen Specificities of CD1d-Restricted NKT Cell Receptors[1]

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CD1d-restricted NKT cells use structurally conserved TCRs and recognize both self and foreign glycolipids, but the TCR features that determine these Ag specificities remain unclear. We investigated the TCR structures and lipid Ag recognition properties of five novel Vα24-negative and 13 canonical Vα24-positive/Vβ11-positive human NKT cell clones generated using α-galactosylceramide (α-GalCer)-loaded CD1d tetramers. The Vα24-negative clones expressed Vβ11 paired with Vα10, Vα2, or Vα3. Strikingly, their Vα-chains had highly conserved rearrangements to Jα18, resulting in CDR3α loop sequences that are nearly identical to those of canonical TCRs. Vα24-positive and Vα24-negative clones responded similarly to α-GalCer and a closely related bacterial analog, suggesting that conservation of the CDR3α loop is sufficient for recognition of α-GalCer despite CDR1α and CDR2α sequence variation. Unlike Vα24-positive clones, the Vα24-negative clones responded poorly to a glucose-linked glycolipid (α-glucosylceramide), which correlated with their lack of a conserved CDR1α amino acid motif, suggesting that fine specificity for α-linked glycosphingolipids is influenced by Vα-encoded TCR regions. Vα24-negative clones showed no response to isoglobotrihexosylceramide, indicating that recognition of this mammalian lipid is not required for selection of Jα18-positive TCRs that can recognize α-GalCer. One α-GalCer-reactive, Vα24-positive clone differed from the others in responding specifically to mammalian phospholipids, demonstrating that semi-invariant NKT TCRs have a capacity for private Ag specificities that are likely conferred by individual TCR β-chain rearrangements. These results highlight the variation in Ag recognition among CD1d-restricted TCRs and suggest that TCR α-chain elements contribute to α-linked glycosphingolipid specificity, whereas TCR β-chains can confer heterogeneous additional reactivities. ***The Journal of Immunology,*** **2006, 176: 3625–3634.**

Natural killer T cells recognize lipid and glycolipid Ags presented by CD1d molecules and can contribute to a wide variety of immunological processes (1). Most CD1d-restricted T cells use unusually nonheterogeneous or "canonical" TCRs consisting of a nearly invariantly rearranged TCR α-chain paired with TCR β-chains that use a restricted set of Vβ gene segments (2–5). The TCR β-chains are diversely recombined with a variety of different D and J segments and include substantial N region changes (2–5). Hence, canonical CD1d-restricted TCRs are semi-invariant, and the main region of diversity is the third CDR (CDR3) of the TCR β-chain. In humans, the TCR α-chain contains the Vα24 gene segment rearranged in a germline configuration with Jα18 (formerly called JαQ), whereas in mice a highly homologous Vα gene, *Vα14*, is joined to an almost identical J segment, Jα18 (formerly called Jα281). TCR β-chains of human canonical CD1d-restricted T cells use the Vβ11 gene segment; the murine TCRs predominantly use either Vβ8 (homologous to human Vβ11), Vβ7, Vβ2, or Vβ6 (2–5).

Sequence analysis of human and murine canonical CD1d-restricted TCR α-chains has revealed that non-germline nucleotides are sometimes present near the V/J junctional region, presumably as a result of DNA trimming followed by N region additions during the process of TCR recombination, but the overall length of the CDR3 loop and the sequence of the J chain remain conserved (2, 3, 5, 6). Thus, canonical NKT cell TCR α-chains are probably formed by the same recombination mechanisms used in the rearrangement of other TCRs, but there is apparently strong selection pressure against diversification of the Vα-Jα rearrangement. CD1d-restricted TCRs have also been identified that have diversely rearranged TCR α-chains using a variety of Vα and Jα segments and containing unique N region changes at the junctional regions (7–10). Hence, the canonical TCR is not required for binding to CD1d molecules, and the conservation of the semi-invariant TCR α-chain may instead reflect a critical role in Ag specificity. The significance of the highly diverse CDR3β sequences of canonical CD1d-restricted TCRs remains unclear.

The physiological Ags that stimulate NKT cells include both self and foreign lipids. NKT cells can recognize cell surface CD1d molecules in the absence of added Ags, and this ability has been shown to be dependent on the presentation of cellular lipids (9, 11–13). Canonical NKT cells also specifically recognize certain members of an unusual class of glycolipids called α-linked glycosphingolipids (α-GSLs),[4] which includes the synthetic Ags

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[4] Abbreviations used in this paper: α-GSL, α-linked glycosphingolipid; α-GalCer, α-galactosylceramide; α-GlcCer, α-glucosylceramide; iGb3, isoglobotrihexosylceramide; DC, dendritic cell; ER, endoplasmic reticulum.

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α-galactosylceramide (α-GalCer) and α-glucosylceramide (α-GlcCer) (14, 15). Known mammalian glycolipids differ from α-GSLs in that sugars are attached to the lipid moiety by a β-anomeric linkage rather than the α-anomeric linkage found in α-GSLs. Mammalian cells are thought not to produce α-GSLs and, therefore, these lipids probably do not function as self Ags. However, members of this class of lipids are produced by certain bacteria, and these bacterial compounds have been shown to activate NKT cells in a CD1d-dependent manner (16–18). Thus, NKT cells can respond to both mammalian and bacterial glycolipids as Ags, but the molecular basis for TCR recognition of self and foreign Ags remains unclear.

Previous studies have suggested that reactivity to α-GalCer is conferred by the TCR α-chain, because transfection of the canonical murine Vα14-positive TCR α-chain was sufficient to confer recognition when it was paired with a variety of TCR β-chains (19, 20). Additionally, there was no evidence of CDR3β sequence selection in an extensive study of α-GalCer-reactive NKT cells, suggesting that this region was not important for recognition (21). However, the specific TCRα features that confer recognition of α-GalCer are not known.

The role of the TCR α-chain in recognition of self Ags is less clear. Murine and human canonical NKT cells have recently been shown to recognize isoglobotrihexosylceramide (iGb3), a mammalian globoside biosynthetic intermediate that consists of the trisaccharide Galα1-3Galβ1-4Glc in a β-anomeric linkage to a ceramide lipid (22). Mice deficient in β-hexosaminidase b, a lysosomal enzyme that can cleave the mature globoside iGb4 to generate iGb3, are severely deficient in α-GalCer-reactive NKT cells (22). Hence, iGb3 may be the major self Ag responsible for thymic selection of NKT cells that express canonically rearranged TCR α-chains, and the α-GSL reactivity of these TCRs could be due to cross-reactivity with features of iGb3, such as its terminal α-linked galactose.

However, canonical NKT cell clones vary in their autoreactive responses to CD1d-positive APCs, suggesting that these responses are not always conferred by the invariant TCR α-chain (13, 23). NKT cells have also been shown to vary in their ability to recognize classes of lipids that are distinct from α-GSLs. Some canonical murine NKT cell hybridomas were found to respond specifically to the mammalian phospholipids phosphatidylinositol and phosphatidylethanolamine presented by recombinant CD1d molecules, whereas others showed no detectable response to these lipids (13). Additionally, small populations of murine T cells have been shown to specifically bind CD1d molecules loaded with mycobacterial phosphatidylinositol mannosides or with *Leishmania* phosphoglycans (24, 25). Similarly, a small subset of the α-GalCer-reactive NKT cells in tumor-immunized mice was stained specifically by CD1d tetramers loaded with the mammalian ganglioside lipid GD3 (26). Thus, certain NKT cell Ag specificities, including some self Ag responses, could be conferred by the presence of unique TCRβ sequences.

The TCR features of CD1d-restricted T cells that determine glycolipid Ag specificity remain unknown. The extraordinary conservation of the TCR α-chains of canonical NKT cells and their shared α-GSL specificities have prevented correlation of TCR α-chain structural features with glycolipid recognition differences. Here we investigate a series of novel Vα24-negative/Vβ11-positive human CD1d-restricted T cell clones that recognize α-GalCer. Because these clones use noncanonical TCR α-chains and differ in their lipid Ag specificity compared with canonical NKT cells, they shed new light on TCR features that influence lipid Ag recognition.

Materials and Methods

Preparation of lipid Ags

The glycolipid Ags α-GalCer, α-GlcCer, and α-mannosylceramide (α-ManCer) were prepared from D-lyxose as described (27). The *Sphingomonas wittichii* Ag GSL-1′sA was synthesized using a protected galacturonic acid and ceramide lipid moiety as described (16). The end products and synthetic intermediates of all lipids were characterized by ^{1}H- and ^{13}C-nuclear magnetic resonance and electrospray mass spectrometry. The iGb3 glycolipid Ag was a gift from Drs. A. Bendelac and D. Zhou of the University of Chicago (Chicago, IL). Purified and synthetic phospholipids were purchased from Matreya and Avanti Polar Lipids. All lipids were dissolved in DMSO at a concentration of 100 μg/ml and stored frozen at −20°C. Before use, the lipids were sonicated in a heated water bath for 15 min at 37°C.

Preparation of soluble CD1d fusion proteins and tetramers

Soluble human and murine CD1d-Fc fusion proteins and tetramers were produced as described previously (13, 28). Briefly, single-chain β2m-CD1d-Fc fusion proteins were produced in mammalian cells and purified from culture supernatants by protein A affinity chromatography. The resulting dimeric CD1d-Fc fusion proteins were formed into complexes with fluorescently labeled soluble protein A molecules and purified by size exclusion chromatography on a Superose 6 column (Amersham Biosciences).

Derivation of CD1d-restricted T cell clones

T cell clones were established from human tissue samples as described previously (29), and protocols were approved by the Brigham and Women's Hospital (Boston, MA) Institutional Review Board and the University of Wisconsin Medical School (Madison, WI) Minimal Risk Institutional Review Board. Briefly, T cells sorted using CD1d-tetramers were cultured at 37°C with 5% CO_2 in RPMI 1640 culture medium containing 10% FBS, 2% human AB serum, 1% penicillin and streptomycin, and 1% L-glutamine in the presence of irradiated allogeneic PBMC and PHA. After 5–10 days of culture, 200 U/ml recombinant human IL-2 (Chiron) was added to the medium.

Determination of TCR sequences

TCRβ sequences were obtained by RT-PCR using a Cβ domain primer (5′-GCTGCCTTCAGAAATCCTTTC-3′) paired with a primer specific for Vβ11 (5′-GCCCCAACTGTGCCATG-3′). TCRα sequences were obtained using a Cα domain primer (5′-TCAGCTGGACCACAGCC-3′) paired with a primer specific for Vα24 (5′-CTCTGCAGAATAAAAAT GAAAAAGC-3′). To amplify the TCRα sequences of the Vα24-negative TCRs, the constant domain primer was paired with mixes of primers specific for individual TCRα variable genes (30) or with one of the following degenerate primers: 1) 5′-CCGCGGCCGCGTCAYGGTCTCCCYGT GTCTTG-3′; 2) 5′-CCGCGGCCGCTTGGTGATCYTGTGGCTKC-3′; or 3) 5′-CCGCGGCCGCAGRTGATTTTTACSCTGGG-3′. The resulting PCR products were subcloned into the TOPO-TA vector (Invitrogen Life Technologies), and the inserts were sequenced with standard primers located in the vector. A minimum of three subclones were sequenced for each PCR product. To verify the sequences obtained using degenerate primers or primer mixes, nucleotide sequences of the initial PCR products were analyzed and compared with the GenBank database to identify the putative Vα gene segment used. Based on this analysis, the following specific PCR primers were designed and used to amplify complete V/J TCRα products: 1) 5′-CATGTGATAGAAAGACAAGATGGTC-3′ (Vα10 forward); 2) 5′-GGGCAGAAAAGAATGATGAAATC-3′ (Vα2S2 forward); 3) 5′-GGAAGAAGAATGGAAACTCTCC-3′ (Vα3S1 forward); and 4) 5′-TCAGCTGGACCACAGCC-3′ (Cα reverse).[5]

Flow cytometric analysis

CD1d tetramers were prepared as described above and loaded at a 40:1 molar ratio with glycolipid Ags dissolved in DMSO or mock treated with DMSO alone and used to stain cells freshly purified from human tissue samples or T cell clones, as described previously (28). Briefly, the cell samples were incubated for 20 min at 4°C with tetramers (10 μg/ml) or directly conjugated Abs in a PBS buffer containing 1 mg/ml BSA and 0.01% NaN_3. The cells were then washed, stained with propidium iodide to identify dead cells, and analyzed by flow cytometry. The mAbs used for

[5] The sequences presented in this article have been deposited in the GenBank accession numbers DQ31444, DQ31445, DQ31446, DQ31447, DQ31448, DQ31449, DQ31450, DQ31451, DQ31452, DQ31453, DQ31454, DQ31455, DQ31456, DQ31457, DQ31458, DQ31459, DQ31460, DQ31461, DQ31462, DQ31463, DQ31464, DQ31465, DQ31466, DQ31467, 341468, DQ358118, DQ358119, DQ358120, DQ358121, and DQ358122.

costaining were the following: anti-Vα24 (clone C15; Beckman Coulter); anti-Vβ11 (clone C21; Beckman Coulter); anti-CD4 (clone RPA-T4; BD Pharmingen/BD Immunocytometry Systems); and anti-CD3 (clone UCHT1, BD Pharmingen/BD Immunocytometry Systems). Where indicated, CD1d-restricted T cell clones were preincubated with 10 μg/ml unlabeled anti-Vα24 mAb and then washed and stained, or the CD1d tetramer was preincubated before use with 20 μg/ml CD1d42 anti-CD1d-specific mAb (provided by Drs. S. Porcelli, Albert Einstein College of Medicine (Bronx, NY) and M. Exley, Harvard Medical School (Boston, MA).

Human CD1d-Fc dimers were prepared by protein A affinity chromatography as described above and then further purified and desalted into PBS by size exclusion chromatography on a Superose 6 column (Amersham Biosciences). The dimers were loaded at a concentration of 100 μg/ml in PBS containing 100 μg/ml BSA (Sigma-Aldrich) with a 40:1 molar ratio of lipid Ags dissolved in DMSO or mock treated with DMSO alone by incubating for 24 h at 37°C. CD1d-restricted T cells were incubated with lipid-loaded or mock-treated dimers for 30 min at 4°C in a PBS buffer containing 1 mg/ml BSA and 0.05% NaN_3 and then washed and stained with 10 μg/ml phosphatidylethanolamine-labeled goat anti-mouse IgG (BD Biosciences) and detected by flow cytometry.

T cell responses to APCs

APCs were either CD1d-transfected 721.221 cells generated as described (31) or in vitro-derived immature dendritic cells (DCs) generated from human peripheral blood monocytes purified by CD14-positive magnetic bead selection (Miltenyi Biotec) that were cultured for 3 days with 200 U/ml and 300 U/ml rIL-4 and GM-CSF, respectively. DCs were pulsed for 12–16 h with lipid Ags at the indicated concentrations or with vehicle (DMSO) alone and then washed and coincubated with a 1:1 ratio of CD1d-restricted T cells (5×10^4 per well) in sterile 96-well plates in RPMI 1640 culture medium lacking IL-2 (i.e., RPMI 1640 containing 10% iron-supplemented bovine calf serum and penicillin/streptomycin) for 24 h at 37°C and 5% CO_2. Culture supernatants were then withdrawn and tested for the indicated cytokines using a standard, commercially available ELISA and purified recombinant cytokines as standards. Anti-CD1d mAb blocking experiments were performed by coincubating the CD1d59 murine IgM mAb (a gift from Dr. S. Porcelli, Albert Einstein College of Medicine (Bronx, NY)) or the MOPC-104E negative control IgM mAb (Sigma-Aldrich) with the APCs and T cells. The *Griffonia simplicifolia* I-B_4 lectin (Vector Laboratories; dissolved in 10 mM HEPES, 0.15 M NaCl, 0.08% NaN_3, and 0.1 mM Ca^{2+}) was coincubated with the APCs and T cells at the indicated concentrations and compared with mock-treated (diluent buffer alone) APCs.

T cell responses to lipid Ags presented by plate-bound CD1d molecules

CD1-restricted T cell clones were tested for responses to lipid Ags presented by plate-bound CD1d fusion proteins, as described previously (13, 29). Briefly, the CD1d-Fc fusion protein or an isotype-matched negative control mAb was coated in a 10:1 ratio with an anti-LFA-1 mAb onto 96-well microtiter plates. Lipid Ags dissolved in DMSO or vehicle alone were diluted into PBS and incubated with the CD1d fusion protein or negative control mAb at 37°C for 24–72 h. The plates were then thoroughly washed, and CD1d-restricted T cell clones were added in RPMI 1640 culture medium lacking IL-2. The plates were incubated for 24 h at 37°C and 5% CO_2, and the supernatants were then harvested and analyzed for cytokine content using a standard, commercially available ELISA.

Results

Generation of α-GalCer-reactive, Vα24-negative, CD1d-restricted T cell clones

CD1d-restricted T cells were cloned from human PBMCs by single cell flow cytometric sorting using α-GalCer-loaded human CD1d tetramers as described previously (28, 29). The tetramer-derived clones were tested for staining by mAbs specific for TCR Vα24 or Vβ11. Of eight CD1d-restricted T cell clones derived in an initial experiment, seven (J3N.4, J3N.5, J3N.11, C1N.4, C1N.5, C1H.3, and CAD1.1) stained positively using both the anti-Vα24 and anti-Vβ11 mAbs (Fig. 1*A* and data not shown), whereas one clone (J3N.1) stained

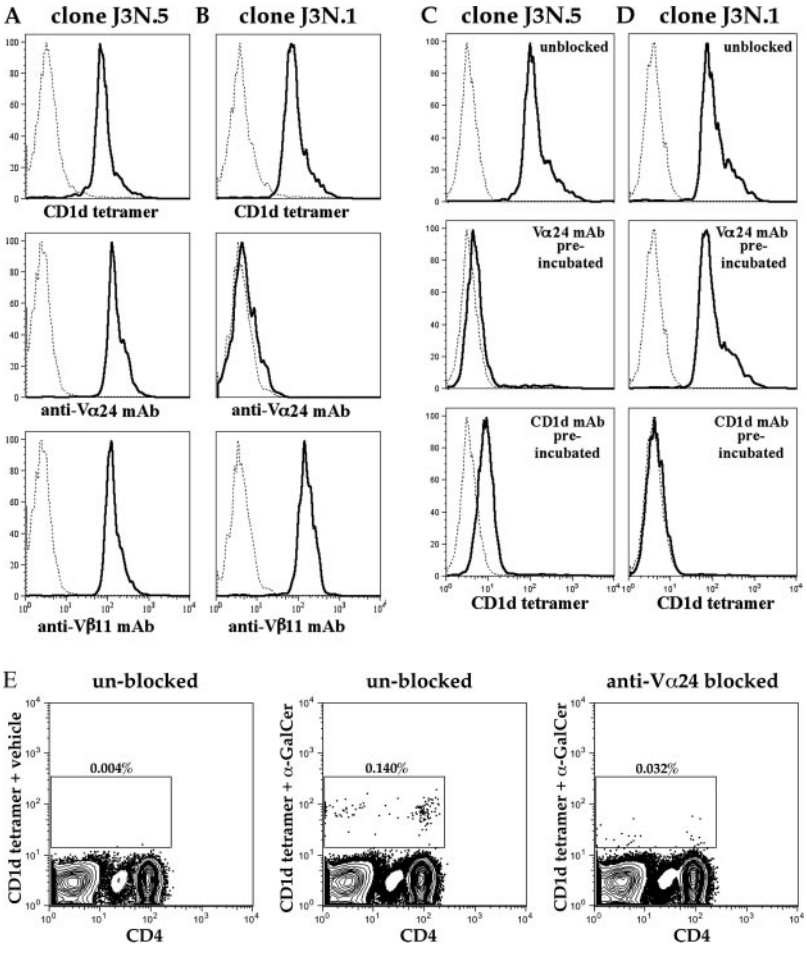

FIGURE 1. *A–D*, Flow cytometric staining of CD1d-restricted T cells. Staining of clone J3N.5 (*A*) and clone J3N.1 (*B*) with the reagent shown on the *x*-axes (solid lines) or with negative control tetramers or Abs (dashed lines). Clone J3N.5 (*C*) and clone J3N.1 (*D*) were stained with an α-GalCer-loaded CD1d tetramer (solid lines) or vehicle-treated CD1d tetramer (dashed lines). *Top panels* show staining of unblocked samples, *middle panels* show staining after the T cells were preincubated with an anti-Vα24 Ab, and *bottom panels* show staining with an α-GalCer-loaded tetramer that was preincubated with an anti-CD1d mAb. *E*, Staining of a B cell-depleted PBMC sample gated on the lymphocyte subset by forward and side scatter. The *left panel* shows the staining using vehicle-treated CD1d tetramer, the *middle panel* shows staining using α-GalCer loaded tetramer, and the *right panel* shows staining using α-GalCer-loaded tetramer after blocking with anti-Vα24 mAb.

positively with the anti-Vβ11 mAb and showed no specific staining with the anti-Vα24 mAb (Fig. 1*B*). Hence, this clone appeared to possess a Vα24-negative, Vβ11-positive TCR. To confirm that the staining was specific, we investigated whether the CD1d tetramer staining could be blocked by Ab binding. The anti-Vα24 mAb completely prevented binding of the α-GalCer-loaded CD1d tetramer to a clone that appeared Vα24-positive (Fig. 1*C*) but had no effect on tetramer binding to clone J3N.1 (Fig. 1*D*). In contrast, prebinding an anti-CD1d mAb to the CD1d tetramer inhibited its binding to clone J3N.1 as well as to a Vα24-positive clone, indicating that the staining was CD1d-dependent (Fig. 1, *C* and *D*).

A previous analysis reported polyclonal Vα24-negative T cell lines that stained with α-GalCer-loaded CD1d tetramers, but these cells were detected after expansion in vitro by stimulation with α-GalCer (10). To investigate the presence of such cells in unstimulated PBMC samples directly ex vivo, 20 healthy donors were tested to evaluate staining by α-GalCer-loaded CD1d tetramers after blocking with the anti-Vα24 mAb. For almost all donors the percentage of CD1d tetramer-stained events was significantly reduced in samples blocked with the anti-Vα24 mAb; however, some tetramer-positive events remained and usually included both CD4-positive and CD4-negative T cells (Fig. 1*E*). There was substantial donor-to-donor variation in the percentage of CD1d tetramer-stained events detected after anti-Vα24 mAb blocking, ranging from 3 to 100% of the unblocked percentage with a mean of 22% (SD of 23%) and a median of 14%. In most cases, after blocking with the anti-Vα24 mAb some tetramer-positive cells had reduced fluorescence intensity compared with unblocked samples, whereas the fluorescence intensity of some cells was similar to that of unblocked samples (Fig. 1*E*). This result suggested that in some cases the anti-Vα24 mAb did not compete at all with the tetramer for TCR binding, and in other cases it either competed inefficiently or facilitated the detection of increased numbers of T cells that have low affinity for α-GalCer-loaded CD1d. Thus, this analysis demonstrated that most donors possessed a significant fraction of CD1d tetramer-positive T cells for which staining was not efficiently blocked by the anti-Vα24 mAb, but it was not clear what fraction were Vα24-negative T cells as opposed to Vα24-positive T cells, that have low affinity for the anti-Vα24 mAb.

To further investigate the characteristics of the inefficiently blocked population, CD1d tetramer-stained T cells were cloned from an anti-Vα24 mAb-pretreated PBMC sample. Four CD1d-restricted T cell clones were obtained that, upon subsequent analysis, showed no positive staining with the anti-Vα24 mAb (J24N.16, J24N.22, J24N.43, and J24N.70), and three clones (J24L.10, J24L.17, and J24L.28) were obtained that stained weakly positive with the anti-Vα24 mAb (data not shown). All of the clones showed clearly positive staining with the anti-Vβ11 mAb (data not shown). Thus, all together the panel of T cell clones generated using α-GalCer-loaded human CD1d tetramers consisted of five clones that showed no staining with an anti-Vα24 mAb, but were stained with anti-Vβ11, and thirteen that were stained positively by both mAbs. Two of the Vα24-negative clones (J3N.1 and J24N.70) were negative for both CD4 and CD8β (double negative) and the other three were CD4-positive, whereas 12 of the 13 Vα24-positive clones were CD4-positive and one was double negative (data not shown).

TCR sequences of CD1d-restricted T cell clones

TCR α-chain and β-chain sequences of the Vα24-positive and Vα24-negative clones were determined by RT-PCR. Analysis of the TCR α-chain sequences of the CD1d-restricted T cell clones revealed three types (see Table I): 1) nine clones with "invariant" TCR α-chains that use the previously described canonical rearrangement of Vα24 with Jα18 (2–5); 2) four clones having "variant" Vα24-positive TCR α-chains that consist of Vα24 rearranged with Jα18 but containing single amino acid substitutions at position 92 or 93 at the end of the V gene segment; and 3) five clones that do not stain with the anti-Vα24 mAb and use V genes other than Vα24. These clones were found to use one of three Vα genes: AV10S1, AV2S2, or AV3S1. Three of the five Vα24-negative clones used AV10S1, but, notably, two different alleles were used, and each clone also had unique substitutions at the end of the V gene segment, presumably resulting from N region changes (Table I). Remarkably, all of the Vα24-negative T cell clones use the same Jα segment (Jα18) that is used by the Vα24-positive clones and maintain identical CDR3 length. Hence, the primary sequence of the CDR3 region of the TCR α-chains is highly conserved among all of the clones (Table I).

Analysis of the TCR β-chain sequences confirmed the use of the Vβ11 gene segment paired with a variety of Jβ segments (see Table I). Most clones contained unique TCRβ junctional rearrangements, but two independently derived clones were identical, and one clone appeared to have two different Vβ11-positive rearrangements (Table I). Because of the usage of different D and Jβ segments and substantial N region alterations, the TCR β-chains of the clones are predicted to have CDR3 loops that are highly heterogeneous in length, charge, polarity, and inclusion of aliphatic residues. Notably, the CDR3 regions of the TCR β-chains from the Vα24-negative clones did not appear distinct from those of the Vα24-positive clones in any of these parameters.

Conserved recognition of bacterial α-GSL

As expected from their specific binding of α-GalCer-loaded CD1d tetramers, the Vα24-negative T cell clones resembled Vα24-positive T cells by responding functionally to CD1d-mediated presentation of α-GalCer (see Figs. 2 and 3). The Vα24-positive and Vα24-negative clones showed similar dose-response curves to α-GalCer presented by CD1d-positive APCs or by plate-bound recombinant CD1d molecules, suggesting that they have a similar sensitivity to this Ag (Figs. 2 and 3). For both types of clone, the responses to α-GalCer could be inhibited by addition of an anti-CD1d mAb, and neither type showed significant responses to CD1d-negative APCs that were pulsed with α-GalCer (data not shown), demonstrating the CD1d-dependence of the α-GalCer recognition.

Bacterially produced α-GSLs have recently been identified that are structurally similar to α-GalCer but contain modifications of the sugar and lipid moieties (16). These compounds were significantly less potent activators of canonical CD1d-restricted NKT cells than α-GalCer, suggesting that the modifications reduce the efficiency of CD1d loading or TCR binding (16–18). We investigated the responses of Vα24-negative and Vα24-positive CD1d-restricted T cell clones to GSL-1′sA, a bacterial α-GSL related to α-GalCer, that has a keto group attached to carbon 6 of the galactose sugar head group and lacks one hydroxyl group on the lipid's sphingosine chain (16, 18). DCs (GM-CSF and IL-4 cultured human monocytes) were pulsed with α-GSL or α-GalCer or vehicle alone and then washed and cocultured with the T cell clones. The Vα24-negative and Vα24-positive clones had dose-response curves essentially identical to that of the GSL-1′sA lipid (Fig. 2). Thus, in addition to their shared reactivity for α-GalCer, Vα24-negative and Vα24-positive clones appear equivalently able to respond to a bacterial α-GSL.

Discrimination of α-linked sugar moieties

CD1d-restricted T cells with Vα14- or Vα24-invariant TCR α-chains have been previously characterized as responding similarly to α-GalCer and α-GlcCer but failing to respond to α-ManCer (14, 15). The Vα24-negative and Vα24-positive T cell clones were tested in parallel for their ability to respond to the

Table I. *CD1d-restricted T cell clone sequencing results*[a]

Clone	TCRα V gene	TCRα J gene	TCRα V/J junction peptide sequence	TCRβ V gene	TCRβ J gene	Vβ peptide sequence	N/D/N peptide sequence	Jβ peptide sequence
J3N.4	AV24S1	JA18	CVVS DRGS	BV11	BJ1S5	CASS	VGGD	QPQHFGDGTRLSIL
J3N.5	AV24S1	JA18	CVVS DRGS	BV11	BJ2S1	CASSE	SQYGRAAY	NEQFFGPGTRLTVL
J3N.11	AV24S1	JA18	CVVS DRGS	BV11	BJ2S3	CASS	ARGVRSGNS	STDTQYFGPGTRLTVL
BM2a.3	AV24S1	JA18	CVVS DRGS	BV11	BJ2S7	CASS	GQGNH	EQYFGPGTRLTVT
BM2a.5	AV24S1	JA18	CVVS DRGS	BV11	BJ2S6	CASS	SPLGGTP	GANVLTFGAGSRLTVL
CAD1.1	AV24S1	JA18	CVVS DRGS	BV11	BJ2S1	CASS	TAPPRGR	NEQFFGPGTRLTVL
C1N.4	AV24S1	JA18	CVVS DRGS	BV11	BJ2S1	CASSE	WGERTSGGAG	NEQFFGPGTRLTVL
J24L.10	AV24S1	JA18	CVVS DRGS	BV11	BJ2S5	CASSE	EGALKESVG	TQYFGPGTRLLVL
J24L.17	AV24S1	JA18	CVVS DRGS	BV11	BJ2S5	CASSE	EGALKESVG	TQYFGPGTRLLVL
J24L.28	AV24S1	JA18	CVVG DRGS	BV11	BJ2S1	CASS	GGRPGLAGGP	NEQFFGPGTRLTVL
C1N.5	AV24S1	JA18	CVAS DRGS	BV11	BJ1S6	CASS	VDRGD	SPLHFGNGTRLTVT
C1H.2[b]	AV24S1	JA18	CVVG DRGS	BV11	ND[c]	ND[c]	ND[c]	ND[c]
BM2a.4	AV24S1	JA18	CVVN DRGS	BV11	BJ2S1	CASSE	WGEGY	NEQFFGPGTRLTVL
J3N.1	AV10S1a2	JA18	CAGV DRGS	BV11	BJ2S1	CASSE	LMASMH	EQYFGPGTRLTVT
J24N.16	AV10S1a1	JA18	CAAL DRGS	BV11	BJ2S7	CASSE	IRESY	NEQFFGPGTRLTVL
J24N.43	AV10S1a1	JA18	CAGI DRGS	BV11	BJ1S3	CASSE	NSGTGRI	YEQYFGPGTRLTVT
J24N.22	AV3S1	JA18	CATY DRGS	BV11	BJ2S5	CASSE	FWDG	SGNTIYFGEGSWLTVV
J24N.70	AV2S2a1t	JA18	CAIT DRGS	BV11	BJ2S7	CASSE	LLAGGGG	TQYFGPGTRLLVL

[a] Shown are TCR V and J gene usages and predicted amino acid sequences of the V(D)J junctional regions of the panel of human Cd1d-restricted T cell clones.
[b] Clone C1H.2 gave clear sequencing results for the Vβ11 gene segment and the constant domain, but the sequence became unreadable in the V(D)J junctional area, suggesting that two differently rearranged Vβ11-positive sequences were present.
[c] ND, not determined.

α-GalCer and α-GlcCer lipids presented by plate-bound recombinant CD1d molecules (29). All of the Vα24-positive CD1d-restricted T cell clones responded robustly to both α-GalCer and α-GlcCer, whereas all of the Vα24-negative clones responded strongly to α-GalCer but showed markedly lower responses to α-GlcCer (Fig. 3). There was little or no cytokine secretion in response to α-ManCer or to vehicle-treated CD1d molecules by any of the clones, and no detectable response was observed to a negative control protein treated with α-GalCer, demonstrating the specificity and CD1d-dependence of the α-GalCer and α-GlcCer responses (data not shown). These results indicated that the Vα24-negative and Vα24-positive T cell clones differ in their specificity for sugar residues of α-GSLs.

To investigate this sugar specificity difference further, three Vα24-positive and three Vα24-negative T cell clones were tested for staining by human CD1d-Fc dimers loaded with either α-GalCer or α-GlcCer or treated with vehicle alone. The Vα24-positive T cells stained similarly with α-GalCer- and α-GlcCer-loaded CD1d dimers, whereas the Vα24-negative T cells showed significant positive staining only with the α-GalCer-loaded CD1d dimer (Fig. 4). The titration curves for clones J3N.1 and J24N.70 appeared to approach saturation at a similar concentration of the α-GalCer-loaded CD1d dimer as that observed for the Vα24-positive clones tested, suggesting that the affinity of these Vα24-negative TCRs for α-GalCer may be close to that of canonical Vα24-positive TCRs (Fig. 4). In contrast, the titration curve for clone J24N.22 reproducibly appeared to require higher dimer concentrations to reach saturation, suggesting that the TCR of this clone may have a lower affinity for α-GalCer (Fig. 4). Thus, variation in Vα-encoded TCR α-chain regions may affect the strength of the interaction with α-GalCer, but the most significant effect appears to be on the ability to bind α-GlcCer. These results suggest that the weaker functional response of the Vα24-negative T cell clones to α-GlcCer is due to lower TCR affinity for this glycolipid compared with α-GalCer, whereas Vα24-positive TCRs appear to have similar affinity for both glycolipids.

Autoreactive responses

Many CD1d-restricted T cells are autoreactive to CD1d-positive APCs in that they respond functionally to contact with such APCs in the absence of exogenously added Ags. A number of studies have shown that this effect is apparently due to recognition of specific cellular glycolipids presented at the cell surface by CD1d (9, 11–13). The autoreactive responses of selected Vα24-positive and Vα24-negative clones were investigated by testing cytokine secretion in response to CD1d-transfected 721.221 cells compared with the untransfected parent cells in the absence of added Ags, and the CD1d dependence was confirmed by blocking with an anti-CD1d mAb. A CD1d-dependent response could be detected for all of the clones tested, but the magnitude of the response varied markedly (Fig. 5*A*). The Vα24-negative clones J3N.1 and J24N.70 reproducibly showed only modest cytokine secretion in response to the CD1d-transfected APCs, whereas clone J24N.22 showed a somewhat higher cytokine secretion in response to the transfectants that was similar to that of the Vα24-positive clone J3N.4 (Fig. 5*A*). Two other Vα24-positive clones, J3N.5 and J24L.17, had progressively greater cytokine secretion responses, respectively (Fig. 5*A*). The level of the autoreactive responses did not correlate with cytokine secretion stimulated by the nonspecific stimulator PHA (Fig. 5*A*), suggesting that the differences among the clones were not simply due to differing activation states or cytokine production capacity.

The *G. simplicifolia* I-B_4 isolectin has previously been shown to inhibit autoreactive responses by a murine NKT cell hybridoma and by a human Vα24-positive NKT cell line, but not by murine CD1d-restricted T cells with diversely rearranged TCRs (22). This effect was thought to be due to the binding of the lectin to terminal α-linked galactose moieties of cellular glycolipids presented by CD1d molecules, which blocks Ag recognition by the T cells (22). We therefore investigated whether I-B_4 could block the responses of the Vα24-positive and Vα24-negative CD1d-restricted T cell clones to the CD1d/721.221 transfectants. One clone, J24L.17, reproducibly showed a slight but statistically significant decrease in cytokine secretion in the presence of the I-B_4 lectin, but none of the other clones tested showed any specific inhibition by the lectin (Fig. 5*B*). Cytokine secretion by clone J24L.17 was diminished by ~15% in the presence of 20 ng/ml I-B_4 lectin, and titrating the concentration of I-B_4 up to 1 mg/ml resulted in no further inhibition of cytokine secretion (Fig. 5*B* and data not shown). Hence, a

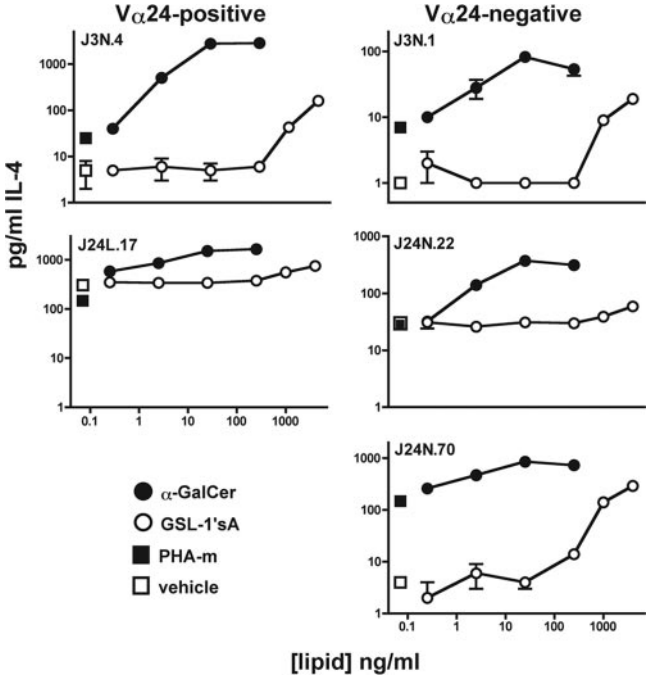

FIGURE 2. Cytokine secretion by Vα24-positive and Vα24-negative CD1d-restricted T cell clones in response to APCs pulsed with the indicated concentrations of α-GalCer (filled circles), α-GSL (open circles), or vehicle alone (open squares). Filled squares show the responses of each T cell to the nonspecific stimulator PHA-m. Values that were below the detection limit of the IL-4 ELISA were assigned a value of 1 pg/ml for plotting on the logarithmic scale. Assays were performed in triplicate, and error bars represent the SD values of the means (in some cases these values are too small for the bars to be visible on the plots). Similar results were obtained in three independent experiments.

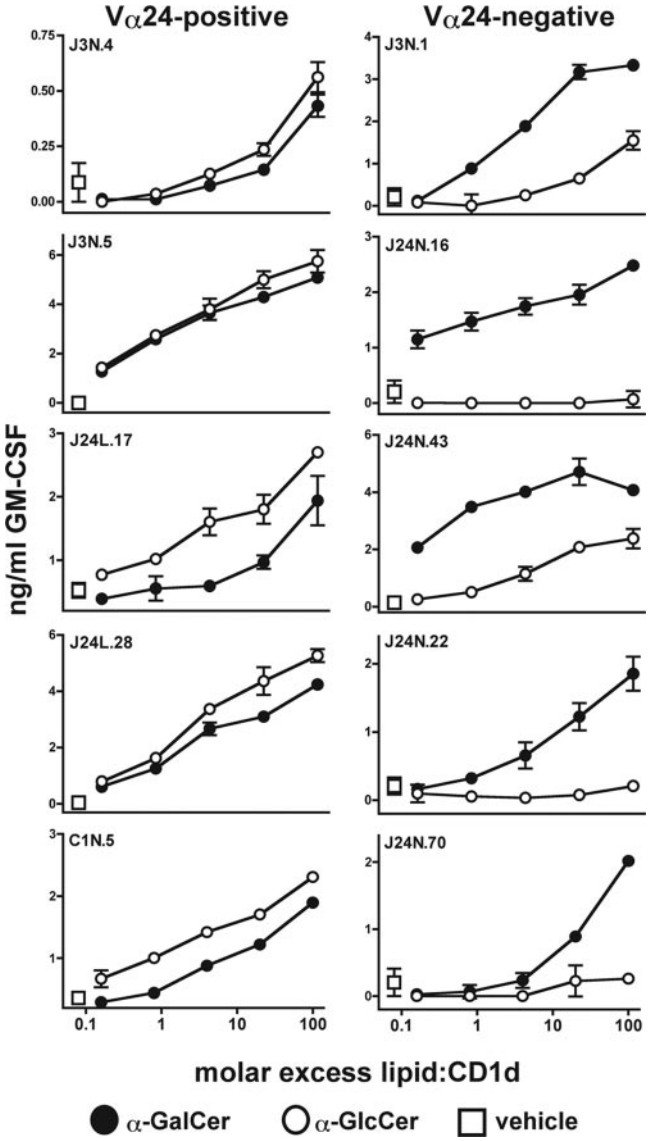

FIGURE 3. Cytokine secretion by Vα24-positive and Vα24–negative clones in response to plate-bound recombinant CD1d molecules pretreated with α-GalCer (filled circles), α-GlcCer (open circles), or vehicle alone (open squares). Assays were performed in triplicate, and error bars show the SD values of the mean. Similar results were obtained in three independent experiments.

fraction of the CD1d-presented self Ags recognized by clone J24L.17 may contain terminal α-linked galactose residues that are available for binding by the I-B_4 lectin, but the self Ags recognized by the five other clones tested in these experiments are not sensitive to this method of blocking.

Recognition of iGb3

The iGb3 glycolipid has recently been identified as a candidate self Ag that may select canonical murine CD1d-restricted T cells in the thymus and may also be responsible for their autoreactive responses (22). Three Vα24-negative and three canonical Vα24-positive clones were tested in parallel for responses to iGb3 presented by CD1d-transfected 721.221 cells. The Vα24-negative clones showed no responses to the iGb3 lipid despite responding strongly to the α-GalCer used as a positive control (Fig. 6, *right panels*). One Vα24-positive clone, J3N.4, showed marked responses to iGb3, indicating that this Ag was presented by the APCs (Fig. 6, *top left panel*). Surprisingly, two other Vα24-positive clones showed no significant responses to iGb3 (Fig. 6, *middle* and *bottom left panels*). These two Vα24-positive clones had strong autoreactive responses to the CD1d-transfected APCs (compare cytokine production in response to vehicle alone with that in response to PHA or α-GalCer; Fig. 6), and, therefore, it is possible that their responses to iGb3 were not detectable above the self Ag signal. Hence, it is not clear from these experiments whether only one or all three of the Vα24-positive clones tested were capable of recognizing iGb3. However, the Vα24-negative clones tested were not highly autoreactive and were functionally active, as demonstrated by their robust responses to α-GalCer. Therefore, these results suggest that the Vα24-negative TCRs are not able to recognize iGb3, and, hence, their positive selection in vivo was likely to have been mediated by a different self Ag.

Phospholipid recognition

We have shown previously that an autoreactive Vα14-positive, murine CD1d-restricted T cell specifically responded to certain phospholipids and that cellular Ags recognized by this T cell include a form of phosphatidylethanolamine (13, 32). Therefore, we investigated the reactivity of twelve Vα24-positive and Vα24-negative T cell clones to a series of purified phospholipids presented by plate-bound recombinant CD1d molecules. One Vα24-positive clone (BM2a.5) responded specifically to purified phosphatidylethanolamine and phosphatidylinositol but not to other phospholipids (Fig. 7*A*). The other Vα24-positive or Vα24-negative clones showed no detectable responses to any of the purified phospholipids (data not shown). In addition to showing modest but

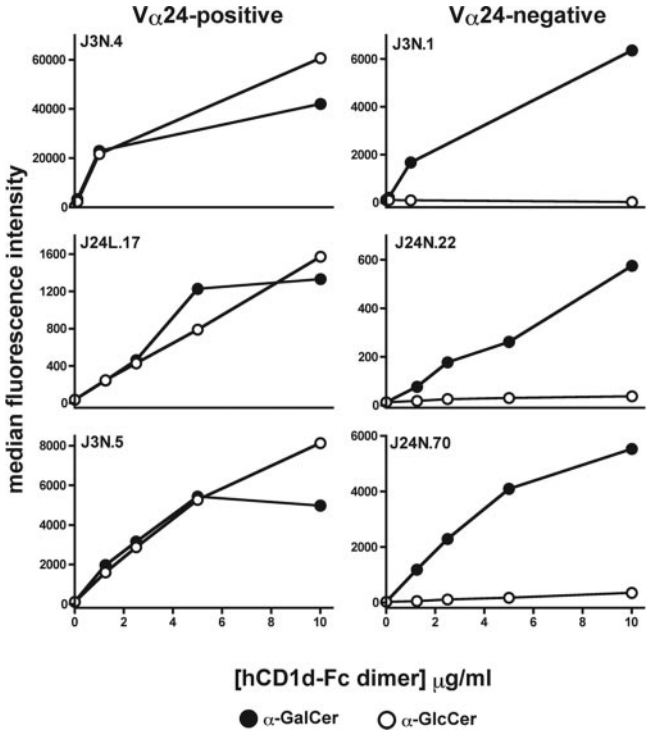

FIGURE 4. Flow cytometric staining of Vα24-positive and Vα24-negative T cell clones by CD1d-Fc dimers loaded with α-GalCer (filled circles) or α-GlcCer (open circles). Similar results were obtained in three independent experiments.

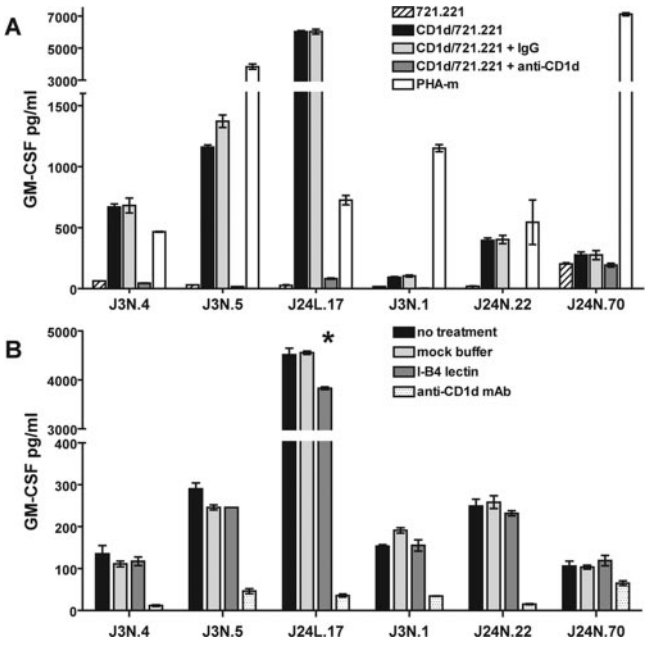

FIGURE 5. *A*, Cytokine secretion by the indicated T cell clones in response to untransfected 721.221 cells (hatched bars), CD1d-transfected 721.221 cells (black bars), CD1d-transfected 721.221 cells in the presence of a negative control mAb (light gray bars), the CD1d59 blocking mAb (dark gray bars), or the nonspecific stimulator PHA-m (open bars). *B*, Cytokine secretion by the indicated clones in response to CD1d-transfected 721.221 cells alone (black bars) or with mock buffer (light gray bars), 200 ng/ml I-B_4 (dark gray bars), or an anti-CD1d mAb (stippled bars). Assays were performed in triplicate, and error bars represent the SD values of the means. Similar results were obtained in three independent experiments.

clearly detectable responses to phosphatidylinositol and phosphatidylethanolamine, clone BM2a.5 responded strongly to CD1d molecules treated with α-GalCer, suggesting that the phospholipid reactivity is an additional lipid specificity that does not replace the ability to recognize CD1d-presented α-GalCer (Fig. 7*A*).

Further analysis showed that cytokine secretion in response to phosphatidylethanolamine-treated CD1d was dependent on the presence of the recombinant CD1d molecules, because a plate-bound negative control IgG2a molecule similarly pretreated with phosphatidylethanolamine elicited no significant response (Fig. 7*B*). Additionally, the response was highly specific, because synthetic phosphatidylethanolamine molecules containing two or three unsaturations in their acyl chains were recognized, but phosphatidylethanolamine containing no unsaturations was not recognized (Fig. 7*C*). A similar specific reactivity to phosphatidylethanolamine containing two or three acyl chain unsaturations was observed previously for an autoreactive murine NKT cell (32). Moreover, none of the synthetic phosphatidylethanolamine preparations stimulated a different Vα24-positive clone, J3N.5, demonstrating that these compounds do not nonspecifically activate NKT cell clones (Fig. 7*C*). Thus, clone BM2a.5 was able to specifically respond to two phospholipids presented by CD1d, whereas 11 other CD1d-restricted clones appeared unable to recognize these lipids. These results demonstrate directly that lipid reactivity can vary among canonical Vα24-positive NKT cells that share an ability to respond to α-GalCer and indicate that the ability to recognize CD1d-presented phospholipids may be a feature of a subset of human as well as murine CD1d-restricted T cells.

Correlations with TCR sequences

Both the Vα24-positive and Vα24-negative clones use Jα18 and, thus, the sequences of their CDR3α loops are nearly identical, but the CDR1, CDR2, and fourth hypervariable loops of the TCR α-chain differ substantially (Table II). Additionally, the Vα24-positive and Vα24-negative clones all use Vβ11, so the sequences of their TCR β-chain CDR1 and CDR2 loops are the same, but each clone has a unique TCRβ CDR3 (Table I). Hence, the specificity differences among the clones could be explained by Vα-encoded sequence differences, sequence differences in the TCR β-chain CDR3, or conformational differences in other critical TCR regions (e.g., the TCRα CDR3 loop) resulting from differences in these regions.

Clone BM2a.5 was the only one that used Jβ2.6 (Table I). Hence, the phospholipid reactivity of this clone could be due to its use of this Jβ segment or could result from residues within its unique N region-encoded CDR3β sequence. None of the other specificity differences among the clones showed any readily discernible correlation with the CDR3β sequence, suggesting that these are more likely to be due to differences in the TCR α-chain.

The presence of a conserved amino acid motif in the CDR1α loop appeared to correlate with the ability to respond robustly to both α-GalCer and α-GlcCer. Murine Vα14 and human Vα24 share expression of valine at position 26 and proline at position 28 of the CDR1α loop, and this motif is not present in the Vα24-negative TCRs (Table II). Because murine Vα14-positive and human Vα24-positive NKT cells share the ability to respond to both α-GalCer and α-GlcCer, whereas the Vα24-negative cells showed reduced responses to α-GlcCer, this observation suggests that the presence of this CDR1 motif could be important for the ability of canonical NKT TCRs to accommodate α-GSLs containing either glucose or galactose. Notably, one of the Vα24-negative TCRs (J24N.70) has a CDR1α loop that is predicted to have a similar structure to that of Vα24 (33), and, therefore, the correlation appears to be specific to the amino acids in the CDR1α loop rather than to its overall structure.

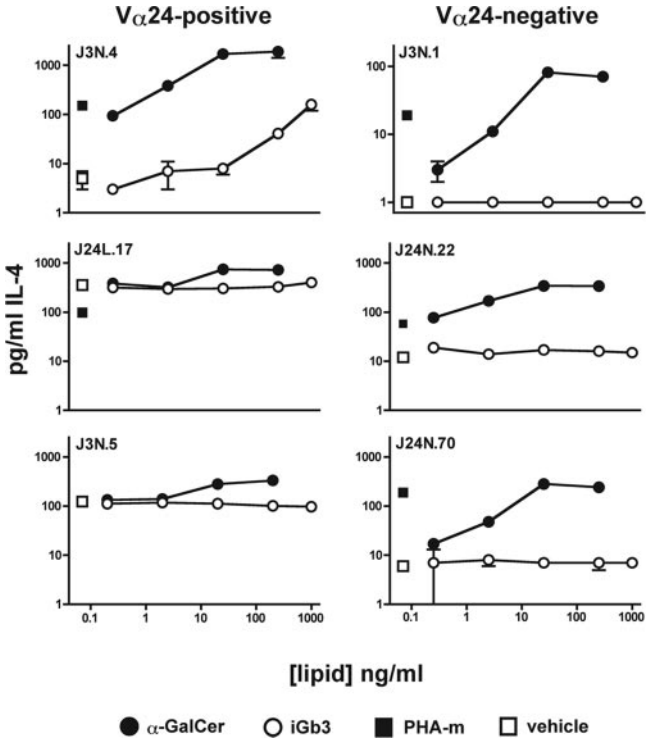

FIGURE 6. Cytokine secretion by Vα24-positive and Vα24-negative clones in response to APCs pulsed with the indicated concentration of α-GalCer (filled circles), iGb3 (open circles), or vehicle alone (open squares). Filled squares show the response of the T cell clones to the nonspecific stimulator PHA-m. Values that were below the detection limit of the ELISA were assigned a value of 1 pg/ml for plotting on the logarithmic scale. Assays were performed in triplicate, and error bars represent the SD values of the means. Similar results were obtained in three independent experiments.

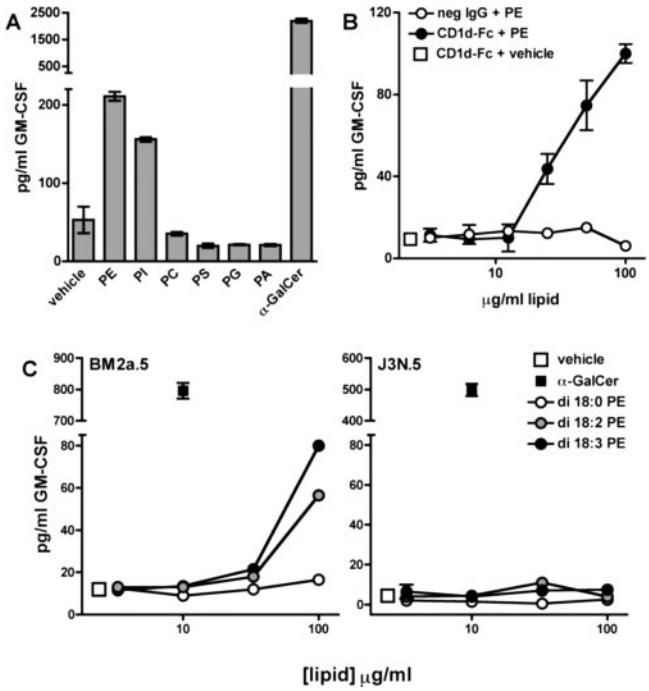

FIGURE 7. *A*, Cytokine secretion by clone BM2a.5 (Vα24-positive) in response to the CD1d-Fc fusion protein treated with DMSO (vehicle), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), or α-GalCer. *B*, Cytokine secretion by clone BM2a.5 in response to plate-bound CD1d-Fc fusion protein (filled circles) or a negative control mAb (open circles) that was pretreated with purified phosphatidylethanolamine (PE), or vehicle alone (open square). *C*, Cytokine secretion by clone BM2a.5 (*left panel*) and clone J3N.5 (*right panel*) in response to CD1d-Fc molecules treated with the indicated concentrations of synthetic phosphatidylethanolamine (PE) preparations containing acyl chains that have no unsaturations (di 18:0), two unsaturations (di 18:2), or three unsaturations (di 18:3). Assays were performed in triplicate, and error bars represent the SD values of the means.

Discussion

The results described here provide new insights into TCR elements that determine lipid Ag specificity. Analysis of the five Vα24-negative T cell clones that we have isolated suggests that conservation of the CDR3α loop may be sufficient to permit recognition of α-GalCer and a bacterial α-GSL despite substantial variation in the sequences and predicted conformations of the CDR1α and CDR2α loops. The finding that the Vα24-negative clones differed from their Vα24-positive counterparts in showing a reduced response to α-GlcCer suggests that Vα-encoded TCR features affect glycan specificity. These results suggest a dominant role for the TCR α-chain in recognition of, and specificity for, α-GSLs.

In contrast, the simplest explanation for the observation that the canonical Vα24-positive clone BM2a.5 responded to phospholipids, whereas the other clones did not, is that the phospholipid reactivity is conferred by the TCR β-chain CDR3 sequence because this is the only unique region of its TCR. We have previously characterized a canonical murine NKT cell hybridoma (24.8.A) that showed strong reactivity to the phospholipids phosphatidylinositol, phosphatidylethanolamine, and phosphatidylglycerol (13, 32). However, in contrast to clone BM2a.5, which recognizes α-GalCer as well as the phospholipids, the murine 24.8.A NKT hybridoma showed little or no response to α-GalCer (13). Thus, whereas the phospholipid reactivity of the 24.8.A hybridoma appeared to replace its specificity for α-GalCer, clone BM2a.5 shows phospholipid responses in addition to strong reactivity to α-GalCer. Taken together, these results are consistent with a model in which structurally conserved TCR α-chains confer shared reactivity for certain Ags and individual TCR β-chain CDR3 rearrangements can contribute private specificities for additional Ags. Hence, a single CD1d-restricted TCR may have the ability to recognize more than one molecularly unrelated type of Ag, perhaps because different Ags presented by CD1d contain epitopes that make contact with distinct TCR regions.

Crystal structures have recently been solved for murine CD1d containing phosphatidylcholine, human CD1d complexed with α-GalCer, and murine CD1d with a short-chain form of α-GalCer (34–36). In these structures, the polar head group of α-GalCer emerges from the CD1d binding pocket in a relatively central location, and the sugar ring is angled toward the C-terminal end of the CD1d α_2-helix (Fig. 8, *A* and *C*, and see Refs. 35 and 36). Previous crystal structures of TCRs complexed with MHC class I and II molecules have shown that the TCR α-chain docks over this end of the Ag-presenting molecule (37, 38). Because analysis of NKT cell responses to mutagenized CD1d molecules has suggested that their TCRs bind the Ag-presenting molecule in an overall similar manner to that observed for MHC class I and II-restricted TCRs (39), this positioning is consistent with the possibility that TCR α-chains play a dominant role in recognizing α-GSLs.

The crystal structures also show that the positioning of a key hydroxyl group of the galactose sugar is consistent with our finding that the Vα24-negative, CD1d-restricted TCRs could distinguish between α-GalCer and α-GlcCer. The conformation of the

Table II. *CD1d-restricted T cell clone TCRα amino acid sequences of the CDR1, CDR2, and HV4 loops*[a]

TCRα	α-GlcCer	CDR1	CDR2	HV4
Vα14.1 (murine)	+	S **V** T **P** D N H	V L V D Q K D K	A T L D K D A K H
Vα24.1 (human)	+	T **V** S **P** F S N	I M T F S E N T	A T L D A D T K O
Vα2.2	−	S N S A F Q Y	Y T Y S S G N	A Q V D K S S K Y
Vα3.1	−	K T S I N N	L I R S N E R E	V T L D T S K K S
Vα10.1	−	S S V F S S	T V V T G G E V	F Q F G D A R K D

[a] Comparison of the predicted amino acid sequences of the human and murine Vα-encoded CDR1α, CDR2α, and HV4α loops. The Vα24-positive clones use Vα24.1, which is largely homologous to the murine Vα14.1 gene segment. The Vα24-negative clone J24N.70 uses Vα2.2, clone J24N.22 uses Vα3.1, and clones J3N.1, J24N.16, and J224N.43 all use Vα10.1 (see Table I). Letters in boldface represent valine at position 26 and proline at position 28 of the CDR1α loop.

4′-OH of the sugar ring is the only structural difference between galactose and glucose; in galactose it is in the axial position, whereas in glucose it is in the equatorial position. This hydroxyl is seen in the crystal structures to be at the apex of the galactose sugar ring pointing "up" and, thus, appears highly accessible for TCR recognition (Fig. 8*A*). Because the position of this prominent hydroxyl would be altered in α-GlcCer, it seems reasonable that this difference might be able to affect TCR binding. Our data suggest that the presence of a conserved amino acid motif at the beginning of the CDR1α loop may allow canonical NKT TCRs to accommodate the difference caused by the change in the positioning of this hydroxyl in galactose vs glucose sugars, whereas the Vα24-negative TCRs cannot.

Interestingly, in the structure of murine CD1d with bound phosphatidylcholine, the choline head group of the lipid is angled toward the N-terminal end of the α_2 helix (Fig. 8, *B* and *D*, and see Ref. 34), opposite to the orientation of the sugar moiety of α-GalCer. Thus, the head groups of CD1d bound phospholipids might be oriented in such a way that they are mainly accessible for recognition by TCR β-chains, in contrast to the orientation of α-GSLs. This would be consistent with our observation that NKT cell reactivity to phospholipids appears clonally distributed, unlike the shared ability to recognize α-GSLs.

We observed that certain Vα24-positive T cell clones reproducibly showed significantly greater CD1d-dependent autoreactive responses than others. We hypothesize that this variation is due at least in part to heterogeneous specificities for self Ags conferred by clonal CDR3β rearrangements that provide differing reactivities to the pool of self Ags presented by cell surface CD1d molecules. A contrasting model of NKT cell autoreactivity is that it results from the recognition of self Ags such as iGb3, which contain terminal α-linked galactose moieties and are recognized by canonical TCR α-chains. It is not clear from our results whether the ability to recognize iGb3 is shared by all canonical human NKT cells, because iGb3-dependent responses were observed for one Vα24-positive NKT cell clone but not for two others. However, the discordance of Vα24-positive NKT cell autoreactivity and observable responses to iGb3 supports the possibility that this is not the only mammalian lipid that can significantly activate human NKT cells.

The finding that the Vα24-negative T cell clones did not respond to iGb3 also shows that the ability to recognize α-GSLs is separable from the ability to respond to iGb3. This observation raises intriguing questions about which ligands drive the positive selection of human TCRs that can recognize α-GSLs. iGb3 has been proposed to be the major self Ag responsible for thymic selection of canonical Vα14-positive murine NKT cells (22), but whether this compound plays a similar role in selecting human NKT cells remains unknown. Because the Vα24-negative T cells were all isolated from peripheral blood of healthy adult donors, they are presumed to have undergone positive selection and may have been expanded in vivo because of their Ag-recognition properties. Moreover, because these clones resemble canonical Vα24-positive clones by using a TCRα rearrangement that has strictly maintained the germline Jα18 sequence and conserved the overall CDR3α loop length, they may have been selected in vivo by a process similar to that which selects Vα24-positive NKT cells. Thus, although the requirement for iGb3 in the positive selection of canonical human Vα24-positive NKT cells remains unclear, our results suggest there are additional mammalian Ags that can select human CD1d-restricted TCRs that are specific for α-GSLs.

The possibility that multiple self Ags select CD1d-restricted T cells could explain the observation that NKT cell clones differ in their autoreactive responses. An ability to monitor a heterogeneous selection of self lipids could also have important implications for the physiological functions of NKT cells. For example, different NKT cell clones could be sensitive to lipids loaded in distinct

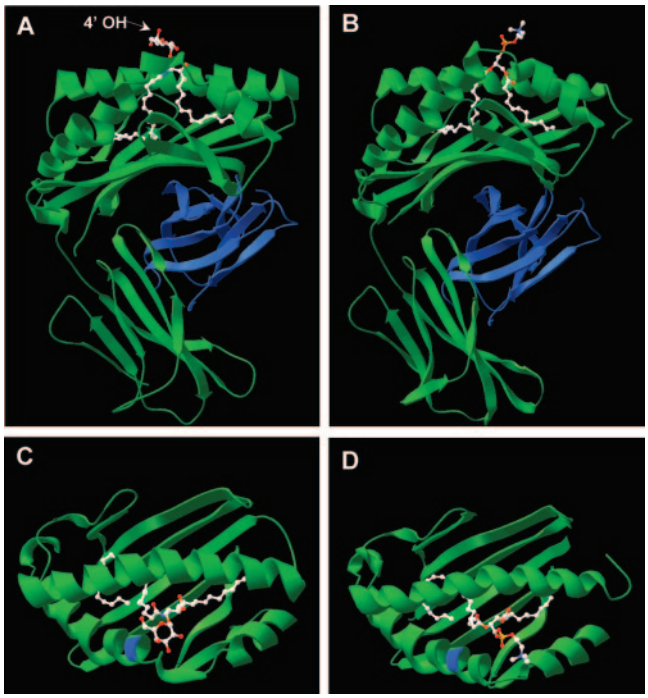

FIGURE 8. Views of the crystal structures of human and murine CD1d molecules with bound lipid ligands. *A* and *C*, Side and top view, respectively, of human CD1d complexed with α-GalCer (36). *B* and *D*, Side and top view, respectively, of murine CD1d complexed with phosphatidyl choline (34). CD1d heavy chains are shown as green ribbons, and the bound lipids are shown as ball-and-stick representations with carbon atoms shown in white, oxygen in red, nitrogen in blue, and phosphorus in orange. *A* and *B*, β_2-microglobulin is shown as a blue ribbon. *C* and *D*, for clarity only the α_1 and α_2 domains are shown, and Thr^{157} on the α_2 helix has been colored blue to provide a point of reference. The figures were created using the Swiss Protein Database software DeepView.

intracellular compartments, allowing them to become activated under differing circumstances. The finding that a human NKT cell clone exhibits a similar reactivity to phospholipids as that observed previously for a murine NKT cell hybridoma (13) suggests that this lipid Ag specificity is evolutionarily conserved. Because CD1d has been shown to bind phospholipids in the endoplasmic reticulum (ER), this specificity may permit NKT cell monitoring of ER-derived lipids and, thus, perhaps monitoring of the integrity of ER biosynthetic processes. Such an ability could be particularly valuable in viral infections, because NKT cells that have autoreactive specificity for ER-derived lipids may be broadly sensitive to changes that occur upon viral subversion of cellular processes.

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Disclosures

The authors have no financial conflict of interest.

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