A Naive-Like Population of Human CD1d-Restricted T Cells Expressing Intermediate Levels of Promyelocytic Leukemia Zinc Finger

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A Naive-Like Population of Human CD1d-Restricted T Cells Expressing Intermediate Levels of Promyelocytic Leukemia Zinc Finger

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Rare CD1d-α-galactosylceramide–specific T cells that do not express the invariant Vα24 chain of human NKT cells were recently identified after expansion in vitro with the lipid Ag, but their phenotype and frequency in vivo and lineage relationship with NKT cells could not be elucidated. By using a CD1d tetramer-based method to enrich these cells from fresh peripheral blood, we demonstrated their naive-like CD62LhighCD45RO− phenotype and relatively high frequency of ~10−5 in several healthy individuals. Notably, these cells expressed the NKT lineage-specific transcription promyelocytic leukemia zinc finger (PLZF), indicating a developmental relationship with NKT cells and ruling out the possibility that they were conventional MHC-restricted T cells cross-reacting against CD1d-α-galactosylceramide. Although PLZF is known to direct the effector program of NKT cells, we show in this study that the naive-like cells expressed it at a significantly lower amount than NKT cells. Further, we present mouse studies demonstrating a sharp PLZF expression threshold requirement for induction of the effector phenotype. These findings directly demonstrate in vivo the existence of naive-like CD1d-restricted human T cells marked by intermediate levels of PLZF. The Journal of Immunology, 2011, 187: 309–315.

Cell lines recognizing various self- and foreign lipid Ags presented by glycoproteins of the CD1 family, CD1a, -b, -c, and -d in vitro have been described, but their lineages remain poorly defined in vivo (1–3). One exception is the prominent and well-characterized NKT cell population of CD1d-restricted T cells, which express conserved semi-invariant TCRs, mostly mVα14-Jα18/Vβ2, -7, and -8 in mice and hVα24-Jα18/ Vβ11 in humans (h). These cells are readily identified in vivo using CD1d tetramers loaded with the synthetic Ag α-galactosylceramide (αGalCer), a mimetic of α-branched microbial glycolipids. They constitutively express innate-like effector properties that are naturally induced during thymic development, in the absence of foreign Ags, by the signature transcription factor promyelocytic leukemia zinc finger (PLZF) (4, 5).

Attempts to directly identify other CD1-restricted T cells, particularly naive unexpanded T cells, using tetramers loaded with foreign lipids have been unsuccessful to date. There are inherent difficulties with this approach. Estimates of the frequency of naive T cells specific for any given Ag are on the order of 10−7 for human MHC-restricted T cells, a frequency at which FACS analysis alone is not considered reliable. MACS-enrichment methods using beads coated with Abs against the fluorochrome attached to the tetramers have dramatically improved the ability to identify such rare cells, because their frequency can be enriched up to 1000-fold, reaching percentages of 0.1–10% that are more suitable for FACS studies. Nevertheless, it often remains difficult to provide appropriate positive and negative controls to validate these analyses. The T cells obtained after tetramer staining and MACS sorting tend to rapidly die in culture, likely because of extensive TCR cross-linking, and cannot be tested functionally or expanded efficiently to confirm their reactivity to Ag. As the cells represent only a few percent of the cell population recovered after tetramer-based enrichment in vitro, it is also important to have independent markers to support their bona fide representation of the rare in vivo precursors.

In this study, we have sought to overcome these difficulties by examining a rare population of CD1d-restricted T cells specific for the same αGalCer Ag as NKT cells but lacking the canonical invariant Vα24 chain. Previous studies have shown that these cells readily expanded in cultures of human PBMC stimulated with αGalCer and could generate αGalCer-responsive clones. Because these lines exhibited a diverse TCR repertoire and lacked expression of CD161, one of the NKT lineage markers, their precursors might resemble more naive MHC-restricted T cells than innate-like effector NKT cells. Of interest, a majority of these T cell clones expressed the same Vβ11 gene as NKT cells. As only a few percent of mainstream T cells express Vβ11, we reasoned that this singular feature would provide a robust independent method to confirm the tetramer-based identification of the fresh in vivo precursors of Vα24-negative αGalCer-specific T cells.

Using these methods, our studies of healthy human PBMC revealed a sizeable frequency, ~10−3, and a naive-like CD62Lhigh...
CD45RO<sup>-</sup>CD45RA<sup>-</sup>CCR7<sup>-</sup>CD161<sup>-</sup>CD4<sup+</sup> cell-surface phenotype for these V<sub>α</sub>24-negative CD1d<sup+</sup>-aGalCer<sup+</sup>-specific T cells, apparently undistinguishable from conventional naive MHC class II-restricted T cells. Surprisingly, however, these cells expressed PLZF, the NKT lineage-specific transcription factor, indicating a lineage relationship with NKT cells and ruling out the possibility that they were merely conventional MHC-restricted T cells with a CD1d<sup+</sup>-aGalCer cross-reactive TCR. Interestingly, these naive-like CD1d-restricted T cells expressed significantly lower amounts of PLZF than NKT cells. Further studies in the mouse system established that a high threshold of PLZF expression was required for effector differentiation.

Thus, our studies establish the existence in human peripheral blood of naive-like CD1d-restricted T cells expressing intermediate levels of PLZF and provide strong support to the notion that CD1 molecules can select both naive and effector T cells during thymic development.

**Materials and Methods**

**Human peripheral blood**

Blood was drawn from 22–54-yr-old healthy volunteers in accordance with a protocol approved by the University of Chicago’s Institutional Review Board.

**Mice**

PLZF<sup>−/−</sup> mice carrying an induced deletion of exon 2 of the Zbtb16 gene (6) also in the B6 background were a gift from Dr. P.P. Pandolfi (Beth Israel Deaconess Cancer Center, Boston, MA). The previously described B6. PLZF-transgenic line #1797 (driven by the CD4 promoter) (4) was produced in the same injection series as the other founder lines #1960 and #1963. Animals were 4–8 wk of age when studied and compared with littermate controls. Mice were housed in a specific pathogen-free environment at the University of Chicago, and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

**Cell isolation and flow cytometry**

CD1d<sup+</sup>-aGalCer tetramers were prepared as described previously (7, 8) using the aGalCer analog PBS-57 (8). Fluorochrome-labeled mAbs (clone indicated in parentheses) against human CD3 (HTI3A or UCHT1), CD4 (RPA-T4 or OKT4), CD8<sup</sup> (RPA-11 or 53-6.7), CD24 (M1/69), CD44 (IM7), CD62L (MEL-14), and TCR<sup</sup> (H57-597) were produced by BD Biosciences, BioLegend, eBioscience, or Pierce Thermo Scientific. PBMC were labeled with allophycocyanin-conjugated hCD1d-<sup</sup> and anti-mouse CD16/32 (clone 93; BioLegend), respectively. For enrichment, PBMC were obtained from thymus, spleen, and peripheral lymph nodes (inguinal and cervical) and plated in 96-well plates. Sorted single cells were stimulated with feeder cells consisting of irradiated (2500 rad) allogenic PBMC (50,000/well) and irradiated (5000 rad) EBV-transformed cells (5000/well) in medium containing 1 μg/ml PHA and 100 U/ml human IL-2. Proliferating clones were maintained by restimulation every 3 wk, and reactivity to CD1-d<sup+</sup>-aGalCer was verified by tetramer staining.

**TCR sequencing**

Total RNA was extracted from PBMC clones with a combination of TRIzol (Invitrogen) and the RNeasy Micro Kit (Qiagen) and reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen) and oligo(DT). TCR<sup</sup> sequences were PCR amplified from cDNA using Platinum Taq DNA polymerase (Invitrogen) with the oTC4<sup</sup>a region primer paired with a V region primer other than oV24, as described previously (10). TCR<sup</sup> sequences of TCR<sup</sup> clones were amplified with the CB C region primer and a V region primer other than BV11, whereas the TCR<sup</sup> sequences of TCR<sup</sup> clones were amplified with the C region primer and BV11 (11). Following amplification, PCR products were gel purified and subcloned using the TOPO TA Cloning Kit (Invitrogen). Single colonies were inoculated, DNA-prepared, and sequenced by the University of Chicago DNA Sequencing Facility using a 3730xl DNA Analyzer (Applied Biosystems). Sequencing results were aligned to known V and J genes using the ImMunoGeneTics V-QUEST program (http://www.imgt.org/IMG_T_vquest/share/textes/). (12).

**Quantitative real-time PCR**

Total RNA was isolated from resting PBMC clones using a combination of TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen) and reverse-transcribed with random primers using the AffinityScript qPCR cDNA Synthesis Kit (Stratagen). Transcripts for human PLZF were quantified with primers spanning intron 3 (forward primer: 5'-TAGTTTGCGGCTGAGAATGC-3'; reverse primer: 5'-ACCGACTGATCAGACAAAAG-3') and normalized to hypoxanthine phosphoribosyltransferase (forward primer: 5'-GAAGGCTTTCTATCCATGG-3'; reverse primer: 5'-ATCCTTCGCTGCCATCCCT-3'). Quantitative PCR was performed on an Mx3005p system (Stratagen) using Brilliant SYBR Green PCR Master Mix (Stratagen).

**Microarray**

Total RNA was isolated from either resting (18 d following stimulation) or stimulated (3 d following stimulation) PBMC clones using a combination of TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen). The resulting RNA was processed by the University of Chicago Functional Genomics Facility and hybridized to GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix). Arrays were scanned by a GeneChip Scanner 3000 (Affymetrix), and intensity values were generated by MicroArray Suite 5.0 software (Affymetrix) and scaled to a median intensity of 500 prior to analysis. Gene expression data are available at the Gene Expression Omnibus under the accession number GSE28726 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28726).

**Statistical analysis**

The unpaired, two-tailed t test was used for all statistical calculations and performed with Prism (GraphPad).

**Results**

In vitro expansion of V<sub>α</sub>24<sup+</sup> CD1d<sup+</sup>-aGalCer<sup+</sup>-specific PBMC

In vitro expansion of CD1d<sup+</sup>-aGalCer<sup+</sup>-specific PBMC

All in vitro experiments were performed using RPMI 1640 medium containing 2 mM l-glutamine (Cellgro), 1 mM sodium pyruvate (Invitrogen), 1 mM nonessential amino acids (Invitrogen), 55 μM 2-ME, 8% FCS (Biowest), 2% AB human serum (Atlanta Biologicals), and penicillin-streptomycin (Sigma-Aldrich). A total of 2 × 10<sup</sup> freshly isolated PBMC were plated in 24-well plates at a density of 1 × 10<sup</sup> cells/ml and cultured in the presence of 100 ng/ml aGalCer analog PBS-57 (8) and 100 U/ml human IL-2. Cultures were split when necessary and fed every 3 to 4 d with fresh medium containing IL-2.

Generation of PBMC clones

CD3<sup</sup>CD1d<sup+</sup>-aGC<sup</sup>-V<sub>α</sub>24<sup</sup>+, CD3<sup</sup>CD1d<sup+</sup>-aGC<sup</sup>-V<sub>α</sub>24<sup</sup>-+, and CD4<sup</sup>CD3<sup</sup>-aGC<sup</sup> cells were single-cell sorted from PBMC of three healthy donors using a FACSaria (BD Biosciences) or MoFlo (DakoCytometry) and plated in 96-well plates. Sorted single cells were stimulated with feeder cells consisting of irradiated (2500 rad) allogenic PBMC (50,000/well) and irradiated (5000 rad) EBV-transformed cells (5000/well) in medium containing 1 μg/ml PHA and 100 U/ml human IL-2. Proliferating clones were maintained by restimulation every 3 wk, and reactivity to CD1-d<sup+</sup>-aGalCer was verified by tetramer staining.
from three healthy individuals (#1, #2, and #4) are presented in Table I as specific background for the fresh precursor characterization presented below. The findings confirmed the expression of a diverse set of Vα-chains, the overrepresentation of other components of the canonical NKT TCR, including the Jo18 segment and the Vß11 chain, and the predominant CD4 phenotype.

**MACS enrichment and characterization of fresh Vαa CD1d-aGalCer+ PBMC**

To directly examine the fresh precursors of these CD1d-aGalCer-specific cells in vivo, we used a method of MACS enrichment based on tetramer staining to identify the rare cells that bound the tetramers, yet lacked Vα24 (Fig. 2A). In four individuals (#1, #2, #3, and #4) examined, these cells could be sufficiently enriched from PBMC for meaningful analyses. They represented a variable proportion of the total tetramer-positive population, but were present at a relatively stable frequency between 4 \times 10^{-6} \text{ and } 1 \times 10^{-5} among total CD3+ PBMC in repeat examinations over a time period ranging from 1 mo to 2 y (Table II). In contrast, as previously reported (15), the frequency of Vαa CD1d-aGalCer+ NKT cells in circulating blood varied widely among these individuals, ranging between 1 \times 10^{-4} and 5 \times 10^{-3}, although it remained stable in each individual over time.

In agreement with studies of cultured cells (Table I) (13, 14), the fresh Vαa CD1d-aGalCer+ cells exhibited an overrepresentation of Vß11, reaching 47–63% on average, unambiguously confirming that they were the bona fide counterparts of the Vαa-negative clones (Fig. 2A, Table II). In marked contrast with the Vαa CD1d-aGalCer+ NKT cells, the majority of Vαa CD1d-aGalCer+ expressed a CD45RA+CCR7+CD62Lhigh phenotype characteristic of naive lymph node-seeking T cells, and they conspicuously lacked the CD161 (NKR-P1A) receptor. Thus, although only a limited number of key surface markers could be examined due to the rarity of these cells, their predominantly naive phenotype stood in stark contrast with the effector phenotype of NKT cells. The Vαa-negative cells mainly expressed CD4, as previously reported for their in vitro-derived clones, although in some individuals, a substantial fraction was found to express CD8α (Table II).

**PLZF expression in fresh Vαa CD1d-aGalCer+ PBMC**

The expression of the transcription factor PLZF in Vαa CD1d-aGalCer+ cells was directly examined by intranuclear FACS staining. Because of the cell losses associated with the permeabilization/fixation procedure in samples containing few cells, the human Vαa CD1d-aGalCer+ cells were FACS-sorted after MACS enrichment and mixed with Je18 mouse filler cells prior to intranuclear staining of PLZF. In three out of three individuals examined, PLZF expression was detected well above the background level of control CD4 cells (Fig. 2B, 2C). Notably, however, the amount of PLZF was significantly reduced by ~50% when compared side by side with the Vαa-positive NKT cells from the same individuals in the same experiments.

**PLZF expression in cultured Vαa CD1d-aGalCer+ PBMC**

PLZF mRNA was quantitated in clones simultaneously derived from Vαa CD1d-aGalCer+ (n = 16), Vαa CD1d-aGalCer+ (n = 12), and control CD4 (n = 15) PBMC from three individuals shown in Table I. Fig. 3A shows PLZF levels measured by quantitative RT-PCR in resting clones harvested 18 d after stimulation with PHA and IL-2. All CD1d-aGalCer+ cell clones, whether Vαa- or Vαa+, expressed high PLZF mRNA, whereas 13 out of 15 control CD4 cell clones derived from the same three healthy donors did not. As in the fresh cell experiments, the Vαa CD1d-aGalCer+ clones tended to express less PLZF than the Vαa CD1d-aGalCer+, although the 37% average difference did not quite reach statistical significance (p = 0.084). As shown below, this may be due to the natural variations of PLZF levels in cultured cells over time.

To test the stability of PLZF expression in culture, we examined a randomly selected set of clones 3 d after PHA and IL-2 stimulation when the cells were blasting or 18 d after they were resting. In these experiments, the differences between the Vαa CD1d-aGalCer+ and the Vαa CD1d-aGalCer+ clones reached statistical significance in both the resting and stimulated states. Note, however, that the clones showed a uniform tendency to decrease their levels of PLZF by 2-fold on average after stimulation, as shown by the significant deviation from the diagonal in Fig. 3B, indicating that PLZF expression fluctuated according to the rested or stimulated status of cells in culture. These experiments were performed on clones derived immediately after the first round of PBMC stimulation with aGalCer in vitro, and it is not known whether the cultured cells would maintain PLZF expression after multiple rounds of stimulation.

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** In vitro expansion of CD1d-aGC–specific PBMC. Fresh human PBMC before and after stimulation (day 18) with 100 ng/ml aGalCer analog PBS-57. Plots are prepared on CD3+ cells, and the percentages of hCD1d-aGC+ Vα24+ and hCD1d-aGC-Vα24+ cells are indicated. Data are representative of four individuals in six independent experiments.

<table>
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<th>Clone</th>
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<th>Vß11</th>
<th>TRAV</th>
<th>TRAJ</th>
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<td>+</td>
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<td>-</td>
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<td>-</td>
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V and 1 regions of TCRa-chains and V regions of TCRß-chains of 18 CD1d-aGC+Vα24+ clones obtained from three individuals were determined by sequencing. CD4, Vα24, and Vß11 expression were assessed by flow cytometry. Note that Vß11 corresponds to TRBV 25-1 in the gene nomenclature. NI, not identified.
FIGURE 2. Phenotype of CD1d-αGalCer\textsuperscript{+} \textit{V}o\textit{a}24\textsuperscript{*} PBMC after MACS enrichment. \textit{A}, Fresh human PBMC were MACS-enriched with CD1d-αGalCer tetramers prior to FACS analysis. The \textit{middle} and \textit{bottom panels} present the phenotype of the CD1d-αGalCer-positive and –negative populations, respectively, with numbers representing frequencies among upper quadrants (Vo\textit{a}24\textsuperscript{*}) and lower quadrants (Vo\textit{a}24\textsuperscript{−}). Summary data of four individuals are presented in Table II. \textit{B}, Fresh human PBMC were MACS-enriched with CD1d-αGalCer tetramers and then FACS-sorted into tetramer\textsuperscript{+}Vo\textit{a}24\textsuperscript{*} and tetramer\textsuperscript{−}Vo\textit{a}24\textsuperscript{+} populations, as indicated, prior to intranuclear staining for PLZF. The CD4 population represents tetramer\textsuperscript{+}CD4\textsuperscript{+} cells from the same individual. The MFI of PLZF is indicated for each population. \textit{C}, Summary data as in \textit{B} for three different individuals.

Altogether, these findings establish the existence of a T cell population with a naïve-like phenotype and a diverse TCR repertoire specific for CD1d associated with the foreign synthetic Ag αGalCer. These cells also expressed PLZF, the master regulator of the effector phenotype in the innate-like NKT cell lineage, albeit at a reduced level.

Manipulation of PLZF levels in mouse T cells
Although seemingly modest, the 2-fold reduction of PLZF protein might explain the lack of effector phenotype in the Vo\textit{a}24\textsuperscript{−} CD1d-αGalCer\textsuperscript{+} cells by comparison with their Vo\textit{a}24\textsuperscript{*} counterparts. Indeed, in the mouse system, we observed haploinsufficiency at a reduced level. In heterozygous 1797 line on average, brought about a mean fluorescence intensity (MFI) equivalent to 34% of the level expressed in the heterozygous 1797 line, the CD62L/CD44 phenotype was comparable to WT. Interestingly, homozygous expression of the PLZF transgene in the same line, which induced a 5-fold reduction of NKT cell frequency compared with wild-type (WT) (Fig. 5A). Thus, <1% of transgenic CD4 cells expressed the naive CD62L\textsuperscript{high}CD44\textsuperscript{low} phenotype compared with 80% of WT. In line 1963, where heterozygous expression of PLZF reached 25% on average of the level expressed in the heterozygous 1797 line, the CD62L/CD44 phenotype was comparable to WT. Interestingly, homozygous expression of the PLZF transgene in the same line, which induced a mean fluorescence intensity (MFI) equivalent to 34% of the level expressed in heterozygous 1797 line on average, brought about a very modest downregulation of CD62L and upregulation of CD44. In heterozygous 1960 mice, expression showed a variate phenotype. One fraction expressed 62% of the PLZF levels of the heterozygous 1797 on average and displayed full conversion to CD4 T cells had 1% of transgenic fresh NKT cells (16). In line 1797, where heterozygous expression exactly matched the natural levels of PLZF expressed by NKT cells during their effector conversion at development stages 1 and 2, as previously reported (4), all splenic CD4 T cells had downregulated CD62L, and many also upregulated CD44 compared with wild-type (WT) (Fig. 5A).

We next examined the degree of effector conversion imparted by various levels of PLZF in transgenic lines expressing PLZF under the control of the CD4 promoter. A previous report suggested a dose effect of a PLZF transgene but did not directly compare the level of PLZF protein expression achieved with that of fresh NKT cells (16). In line 1797, where heterozygous expression exactly matched the natural levels of PLZF expressed by NKT cells during their effector conversion at development stages 1 and 2, as previously reported (4), all splenic CD4 T cells had downregulated CD62L, and many also upregulated CD44 compared with wild-type (WT) (Fig. 5A).

Table II. Frequency and phenotype of CD1d-αGalCer\textsuperscript{+}Vo\textit{a}24\textsuperscript{−} cells in vivo

<table>
<thead>
<tr>
<th>Individual</th>
<th>Population</th>
<th>CD3\textsuperscript{+} (%)</th>
<th>CD4\textsuperscript{+} (%)</th>
<th>CD6α\textsuperscript{+} (%)</th>
<th>CD45RA\textsuperscript{+} (%)</th>
<th>CD62L\textsuperscript{+} (%)</th>
<th>CCR7\textsuperscript{+} (%)</th>
<th>CD161\textsuperscript{+} (%)</th>
<th>Vβ11\textsuperscript{+} (%)</th>
<th>Vβ2\textsuperscript{+} (%)</th>
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<td>Tetr\textsuperscript{+}Vo\textit{a}24\textsuperscript{*}</td>
<td>0.012 ± 0.0038</td>
<td>ND</td>
<td>43</td>
<td>13 ± 3.4</td>
<td>43 ± 7.2</td>
<td>ND</td>
<td>60 ± 15</td>
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<td>Tetr\textsuperscript{−}Vo\textit{a}24\textsuperscript{*}</td>
<td>0.0011 ± 0.00051</td>
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<td>26</td>
<td>73 ± 20</td>
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<td>41</td>
<td>72 ± 9.2</td>
<td>65 ± 11</td>
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<td>9.7 ± 6.6</td>
<td>5.1 ± 5.6</td>
<td>8.3 ± 0.08</td>
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<td>2</td>
<td>Tetr\textsuperscript{+}Vo\textit{a}24\textsuperscript{*}</td>
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<td>ND</td>
<td>12</td>
<td>41 ± 4.5</td>
<td>68 ± 4.5</td>
<td>13</td>
<td>91 ± 3.1</td>
<td>91 ± 2.8</td>
<td>0.044 ± 0.068</td>
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<td>ND</td>
<td>72 ± 35</td>
<td>7.16 ± 7.1</td>
<td>59 ± 7.1</td>
<td>73 ± 4.2</td>
<td>63</td>
<td>9.6 ± 0.62</td>
<td>6.2 ± 3.5</td>
</tr>
<tr>
<td>3</td>
<td>Tetr\textsuperscript{+}Vo\textit{a}24\textsuperscript{*}</td>
<td>0.016 ± 0.0032</td>
<td>58 ± 3.3</td>
<td>31</td>
<td>14</td>
<td>35 ± 17</td>
<td>21</td>
<td>73 ± 2</td>
<td>100 ± 0.085</td>
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<td>Tetr\textsuperscript{−}Vo\textit{a}24\textsuperscript{*}</td>
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<td>28</td>
<td>64</td>
<td>79 ± 11</td>
<td>67</td>
<td>18 ± 17</td>
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<tr>
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<td>Tetr</td>
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<td>66 ± 17</td>
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<td>6.2 ± 3.4</td>
<td>0.97 ± 0.044</td>
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<td>1.2</td>
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| ND, not determined; Tetr, tetramer.
Thus, a partial drop of the frequency of CD4 T cells from 59 to level of PLZF expression and extent of CD62L downregulation. These mice. The frequency of CD4 T cells varied according to their heterozygous 1797, did not change its naive phenotype. Fig. 5 shows how CD4 T cells populated the peripheral lymph nodes of expressed only 34% on average of the PLZF amount found in line 1960 compared with WT, whereas the highest PLZF-expressing line 1797 had only 3% CD4 cells on average among lymph node T cells. These data establish that, as judged by changes in surface expression of CD62L and CD44, a high threshold of PLZF is required for acquisition of the effector phenotype, with drastic functional consequences on the recirculation pattern of CD4 T cells.

**Discussion**

We have used a tetramer-based MACS enrichment strategy to identify, enumerate, and characterize the rare Vo24^CD1d-a-GC^+ T cells circulating in human peripheral blood. The identification of these cells in fresh blood was unambiguously validated by their characteristic overuse of Vβ11, similar to their Ag-stimulated progeny recently described by others (13, 14).

Our study revealed novel features of profound significance for our understanding of immune responses to lipid Ags. First, these cells predominantly expressed a naive CD45RA^CCR7^CD62L^high phenotype that predicted recirculation properties of lipid node-seeking T cells. Such properties are a prerequisite for the initiation of adaptive-like immune responses in lymph node draining sites of microbial invasion and are consistent with studies documenting the adaptive-like dynamics of CD1-restricted mycobacterial lipid-specific responses in mice and guinea pigs (17, 18). The functional properties of these naive-like cells could not be assessed in this study because the tetramer-MACS method used for enrichment impaired the survival and expansion of cells in culture. Our Vo24^-negative T cell clones expanded from cultures stimulated with αGalCer produced IL-4 and IFN-γ upon stimulation, similar to previous reports, but a similar profile was observed in control clones derived from CD1d-αGalCer-negative CD4 T cells or from Vo24^NKT cells (not shown), consistent with the ability of human T cells to acquire a broad range of cytokines after in vitro culture. Thus, the cytokine properties of the naive-like cells remain to be determined.

Secondly, the naive-like cells expressed PLZF, the signature transcription factor that induces NKT cells to developmentally acquire their innate-like effector program (4, 5). This observation is remarkably consistent with the predominant expression of CD1 isotypes by thymocytes and with the emerging understanding that PLZF is induced upon selection of thymocytes by bone marrow-derived rather than epithelial ligands. Indeed, when MHC class II expression was experimentally redirected from epithelial to thymocytes, CD4 thymocytes acquired a CD62L^low/CD44^high/Int phenotype. The other fraction, which expressed only 34% on average of the PLZF amount found in heterozygous 1797, did not change its naive phenotype. Fig. 5B shows how CD4 T cells populated the peripheral lymph nodes of these mice. The frequency of CD4 T cells varied according to their level of PLZF expression and extent of CD62L downregulation. Thus, a partial drop of the frequency of CD4 T cells from 59 to 34% on average was observed in line 1960 compared with WT.
MHC ligands and cross-react against CD1. They follow distinct developmental rules or because they are primarily restricted cells may express little or no PLZF, perhaps because of the avidity of TCR–ligand interactions during thymic development. Although they lack Vα24 but often express Jα18 and Vβ11, the other components of the NKT TCR, the Vα24–CD1d–αGalCer+ thymocytes may have lower avidity than NKT cells for a common set of self-lipid Ags involved in their thymic selection. For example, they did not respond to iGb3 (14), a self-Ag recognized as a weak agonist by most human NKT cells (21).

The frequency of the naive recirculating CD1d–αGalCer–specific T cells was on the order of $1 \times 10^{-5}$ and was stable over repeated samplings in the individuals studied. This surprisingly high frequency, which is similar to superior to estimates for MHC-peptide–specific T cells (22), may be explained by the existence of two germline gene segments, Jα18 and Vβ11, with intrinsic recognition properties for αGalCer and CD1d, respectively (23). As cells expressing PLZF represent a very small percentage of the naive human T cell repertoire, it is possible that the size of the naive CD1-restricted T cell population is very small compared with that of the MHC-restricted T cells. Alternatively, many CD1-restricted cells may express little or no PLZF, perhaps because they follow distinct developmental rules or because they are primarily selected by MHC ligands and cross-react against CD1.

Recent reports have begun to assess the frequency and phenotype of fresh CD1-restricted T cells based on their ability to exhibit self-reactivity against CD1-transformants in vitro (24, 25), and one report further demonstrated their presence among PBMC with a naive CD45RA+RO- phenotype (25). Although these studies did not attempt to assess the expression of PLZF in fresh cells, studies of T cell lines and clones, however, may help evaluate the frequency of PLZF expression among CD1-restricted T cells, although it is unknown at present whether PLZF expression is a stable marker in long-term cultures. Mouse models transgenically expressing the human CD1 isotypes that are missing in mice may also prove particularly valuable to elucidate this important issue.

In conclusion, this study provides direct physical evidence for the existence of a sizable population of naive-like CD1d-restricted T cells that express a diverse TCR repertoire and recirculate in human peripheral blood. It suggests that at least a fraction of these cells may express PLZF at low or intermediate levels and therefore belong to a lineage distinct from most MHC-restricted T cells but related to NKT cells. The results also reveal the critical importance of a high threshold of PLZF expression for induction of the effector phenotype in T cells and raise the fundamental issue of the mechanisms regulating PLZF expression during T cell development.

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Disclosures
The authors have no financial conflicts of interest.

References