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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 CD62L<sup>high</sup>CD45RO<sup>+</sup>CD4<sup>+</sup> phenotype and relatively high frequency of ~10<sup>−5</sup> 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.

CD45RO<sup>−</sup>CD45RA<sup>−</sup>CCR7<sup>−</sup>CD161<sup>−</sup>CD4<sup>+</sup> cell-surface phenotype for these Vα24-negative CD1d<sup>+</sup>αGalCer-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>αGalCer 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-y-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>αGalCer tetramers were prepared as described previously (7, 8) using the αGalCer analog PBS-57 (8). Fluorochrome-labeled mAbs (clone indicated in parentheses) against human CD3 (HIT3A or UCHT1), CD4 (RPA-T4 or OKT4), CD8a (GK1.5 or RM4-5), CD8b (RPA-T8), CD45RA (HI100), CD62L (DREG-56), CD61 (DX12 or HP-3G10), CD197 (TG8/CCR7), Vβ2 (MPB2D5), Vβ11 (C21), and Vα24 (C15) were purchased from BD Biosciences, Beckman Coulter, BioLegend, eBioscience, or Pierce Thermo Scientific. Fluorochrome-labeled mAbs against mouse B220 (RA3-6B2), CD4 (GK1.5 or RM4-5), CD8α (53-6.7), CD8β (M1/69), CD44 (IM7), CD62L (MEL-14), and TCRβ (H57-597) were purchased from BD Biosciences, BioLegend, or Invitrogen.

Human PBMC were isolated from the blood of healthy donors after centrifugation over Ficoll-Paque Plus (GE Healthcare). Prior to Ab staining, cells were resuspended in PBS (1% BSA, 10 mM Tris-HCl, pH 7.4) containing 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate (Invitrogen), 1 mM nonessential amino acids (Invitrogen), 55 μM 2-mercaptoethanol (2-ME, Sigma), 8% FCS (Biowest), 2% AB human serum (Atlanta Biologicals), and penicillin-streptomycin (Sigma-Aldrich). A total of 2 × 10<sup>6</sup> freshly isolated PBMC were plated in 24-well plates at a density of 1 × 10<sup>5</sup> cells/ml and cultured in the presence of 100 ng/ml αGalCer 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>αGalCer<sup>−</sup>Vα24<sup>+</sup>, CD3<sup>+</sup>CD1d<sup>+</sup>αGalCer<sup>−</sup>Vα24<sup>−</sup>, and CD4<sup>+</sup>CD3<sup>+</sup>CD1d<sup>+</sup>αGalCer<sup>−</sup> cells were single-cell sorted from PBMC of three healthy donors using a FACSaria (BD Biosciences) or MoFlo (DakoCytomation) 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 CD1d<sup>+</sup>αGalCer 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 sequences were PCR amplified from cDNA using Platinum Taq DNA polymerase (Invitrogen) with the oTCAC4 region primer paired with a V region primer other than oV24, as described previously (10). TRCβ sequences of Vβ11<sup>−</sup> clones were amplified with the CB C region primer and a V region primer other than BV11, whereas the TRCBβ sequences of Vβ11<sup>+</sup> clones were amplified using the C B region primer and BV11 (11). Following amplification, PCR products were gel purified and subcloned using the TOPO TA Cloning Kit (Invitrogen). Single colonies were incubated, DNA-prepared, and sequenced using 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_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 Micro Kit (Qiagen) and reverse-transcribed with random primers using the AffinityScript qPCR cDNA Synthesis Kit (Stratagene). Transcripts for human PLZF were quantified with primers spanning intron 3 (forward primer: 5′-TAGGTGCGGGCAGAAAATGC-3′; reverse primer: 5′-ACCCGACTGACAGACAAAG3′) and normalized to hypoxanthine phosphoribosyltransferase (forward primer: 5′-GGAAGGTTATTTCCACTAGG3′; reverse primer: 5′-CTATTTCTTCCACACCTCTAG3′). Quantitative PCR was performed on an Mx3005p system (Stratagene) using Brilliant SYBR Green qPCR Master Mix (Stratagene, Invitrogen).

**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) and reverse-transcribed with random primers using the AffinityScript qPCR cDNA Synthesis Kit (Stratagene). Transcripts for human PLZF were quantified with primers spanning intron 3 (forward primer: 5′-TAGGTGCGGGCAGAAAATGC-3′; reverse primer: 5′-ACCCGACTGACAGACAAAG3′) and normalized to hypoxanthine phosphoribosyltransferase (forward primer: 5′-GGAAGGTTATTTCCACTAGG3′; reverse primer: 5′-CTATTTCTTCCACACCTCTAG3′). Quantitative PCR was performed on an Mx3005p system (Stratagene) using Brilliant SYBR Green qPCR Master Mix (Stratagene, Invitrogen).

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**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 Vα24<sup>+</sup> CD1d<sup>+</sup>αGalCer<sup>−</sup> PBMC*

Fig. 1 shows that, as previously reported (13, 14), a rare population of Vα24<sup>+</sup> CD1d<sup>+</sup>αGalCer<sup>−</sup> T cells, which is barely seen in whole PBMC, can preferentially expand upon stimulation with αGalCer in vitro and typically express a weaker tetramer staining than canonical Vα24<sup>+</sup> NKT cells. Studies of 18 clones derived...
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 Vβ-chains, the overrepresentation of other components of the canonical NKT TCR, including the Jα18 segment and the Vβ11 chain, and the predominant CD4 phenotype.

**MACS enrichment and characterization of fresh Vα24-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 from PBMC for meaningful analyses. They represented a variable #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 × 10⁻⁶ and 1 × 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 Vα24⁺ CD1d-aGalCer⁺ NKT cells in circulating blood varied widely among these individuals, ranging between 1 × 10⁻⁴ and 5 × 10⁻³, although it remained stable in each individual over time.

In agreement with studies of cultured cells (Table I) (13, 14), the fresh Vα24⁺ CD1d-aGalCer⁺ cells exhibited an overrepresentation of Vβ11, reaching 47–63% on average, unambiguously confirming that they were the bona fide counterparts of the Vα24⁻ negative clones (Fig. 2A, Table II). In marked contrast with the Vα24⁺ CD1d-aGalCer⁺ NKT cells, the majority of Vα24⁺ CD1d-aGalCer⁺ expressed a CD45RA⁺CCR7⁺CD62Lhigh phenotype characteristic of naive lymph node-seeking T cells, and they conversely lacked the CD161 (NK-R-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 Vα24-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 Vα24⁺ CD1d-aGalCer⁺ PBMC**

The expression of the transcription factor PLZF in Vα24⁺ 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 Vα24⁺ CD1d-aGalCer⁺ cells were FACs-sorted after MACS enrichment and mixed with Jα18⁻/- 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 Vα24⁺ positive NKT cells from the same individuals in the same experiments.

**PLZF expression in cultured Vα24⁺ CD1d-aGalCer⁺ PBMC**

PLZF mRNA was quantitated in clones simultaneously derived from Vα24⁺ CD1d-aGalCer⁺ (n = 16), Vα24⁺ CD1d-aGalCer⁺ (n = 12), and control CD4 (n = 15) PBMC from the 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 Vα24⁻ or Vα24⁺, 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 Vα24⁺ CD1d-aGalCer⁺ clones tended to express less PLZF than the Vα24⁺ 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 blastic or 18 d after when they were resting. In these experiments, the differences between the Vα24⁺ CD1d-aGalCer⁺ and the Vα24⁺ 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.

---

**Table I. TCR chain usage and coreceptor expression by CD1d-aGC⁺ Vα24⁺ cells**

<table>
<thead>
<tr>
<th>Clone</th>
<th>CD4</th>
<th>Vα24</th>
<th>Vβ11</th>
<th>TRAV</th>
<th>TRAJ</th>
<th>TRBV</th>
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<tr>
<td>1-2</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>12-3</td>
<td>18</td>
<td>25-1</td>
</tr>
<tr>
<td>1-3</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>1-1</td>
<td>9</td>
<td>27</td>
</tr>
<tr>
<td>1-4</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>27</td>
<td>22</td>
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<tr>
<td>1-5</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>NI</td>
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<td>−</td>
<td>−</td>
<td>1-1</td>
<td>30</td>
<td>20-1</td>
</tr>
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**V and I regions of TCRα-chains and V regions of TCRβ-chains of 18 CD1d-aGC⁺ Vα24⁺ clones obtained from three individuals were determined by sequencing. CD4, Vα24, and Vβ11 expression were assessed by flow cytometry. Note that Vβ11 corresponds to TRBV25-1 in the gene nomenclature. NI, not identified.**

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**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 pre gated 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.
FIGURE 2. Phenotype of CD1d-αGalCer Vo24+ PBMC after MACS enrichment. A. Fresh human PBMC were MACS-enriched with CD1d-αGalCer tetramers prior to FACS analysis. The middle and bottom panels present the phenotype of the CD1d-αGalCer-positive and –negative populations, respectively, with numbers representing frequencies among upper quadrants (Vo24+) and lower quadrants (Vo24–). Summary data of four individuals are presented in Table II. B. Fresh human PBMC were MACS-enriched with CD1d-αGalCer tetramers and then FACS-sorted into tetramer Vo24– and tetramer Vo24+ populations, as indicated, prior to intranuclear staining for PLZF. The CD4 population represents tetramer CD4+ cells from the same individual. The MFI of PLZF is indicated for each population. C. Summary data as in B for three different individuals.

Altogether, these findings establish the existence of a T cell population with a naive-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 T cell phenotype. One fraction expressed 62% of the PLZF levels of the CD4 cells expressed in the heterozygous 1797 line, the CD62L/CD44 phenotype. One fraction expressed 62% of the PLZF levels of the CD4 cells expressed in the heterozygous 1797 line, the CD62L/CD44 phenotype. This was comparable to WT. Interestingly, homozygous expression of PLZF transgene in the same line, which induced downregulation of CD62L, and many also upregulated CD44 compared with wild-type (WT) (Fig. 5A). Thus, <1% of transgenic CD4 cells expressed the naive CD62LhighCD44low 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 1963 line, 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 1979 on average and displayed full conversion to

Table II. Frequency and phenotype of CD1d-αGalCer Vo24+ cells in vivo

<table>
<thead>
<tr>
<th>Individual No.</th>
<th>Population</th>
<th>CD3+ (%)</th>
<th>CD4+ (%)</th>
<th>CD8+ (%)</th>
<th>CD45RA+ (%)</th>
<th>CD62L+ (%)</th>
<th>CCR7+ (%)</th>
<th>CD161+ (%)</th>
<th>Vβ11+ (%)</th>
<th>Vβ2+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tetr Vo24+</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>
<td>97 ± 2</td>
<td>0.093 ± 0.13</td>
</tr>
<tr>
<td>2</td>
<td>Tetr Vo24+</td>
<td>0.0011 ± 0.00051</td>
<td>ND</td>
<td>26</td>
<td>73 ± 20</td>
<td>86 ± 5.2</td>
<td>ND</td>
<td>5.1 ± 2.6</td>
<td>63 ± 18</td>
<td>7.4 ± 3.7</td>
</tr>
<tr>
<td>3</td>
<td>Tetr Vo24+</td>
<td>0.00011 ± 0.000051</td>
<td>ND</td>
<td>12</td>
<td>41.3 ± 4.5</td>
<td>6.8 ± 4.5</td>
<td>ND</td>
<td>9.7 ± 6.6</td>
<td>5.1 ± 5.6</td>
<td>8.3 ± 0.82</td>
</tr>
<tr>
<td>4</td>
<td>Tetr Vo24+</td>
<td>0.00011 ± 0.000051</td>
<td>ND</td>
<td>72</td>
<td>35 ± 17</td>
<td>62 ± 16</td>
<td>ND</td>
<td>61 ± 13</td>
<td>9.7 ± 3.8</td>
<td>0.044 ± 0.068</td>
</tr>
<tr>
<td>5</td>
<td>Tetr Vo24+</td>
<td>0.000011 ± 0.000009</td>
<td>ND</td>
<td>28</td>
<td>59 ± 17</td>
<td>61 ± 16</td>
<td>ND</td>
<td>61 ± 13</td>
<td>9.7 ± 3.8</td>
<td>0.044 ± 0.068</td>
</tr>
</tbody>
</table>

The frequency of CD1d-αGalCer Vo24+ cells within CD3+ PBMC is shown for different individuals. Their phenotype is compared with those of CD1d-αGalCer Vo24+ and CD1d-αGalCer cells. Data shown as mean ± SD of two to four independent blood samples for each individual, except individual 4, who was examined only once. ND, not determined; Tetr, tetramer.
FIGURE 3. CD1d-αGC′Vo24− T cell clones express intermediate amounts of PLZF. PLZF mRNA expression was quantitated by quantitative RT-PCR (A) and microarray (B) experiments. A. Each data point represents the mean of triplicate PCR values of an individual resting clone harvested 18 d after stimulation with PHA and IL-2. Control (tetramer−) CD4+ clones, tetramerVo24− clones, and tetramerVo24+ clones were derived from each of three individuals, and a total of 15, 16 and 12 clones of each respective phenotype are represented in the figure. B. Four to five randomly selected clones of each category were further analyzed by microarray 18 d poststimulation (resting) or 3 d after PHA and IL-2 (stimulated). The normalized expression levels of the 205883_at PLZF probe set from individual Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix) are represented in a two-dimensional plot showing the PLZF level in stimulated (ordinate) and resting (abscissa) clones. The differences in PLZF expression between tetramerVo24− clones and tetramerVo24+ clones were statistically significant (p < 0.05) whether examined at the resting or activated state. Note that most of the dots fell below the diagonal: a linear regression of all data points had a slope of 0.503, significantly different from 1 (p < 0.0001), indicating downregulation of PLZF upon activation.

the CD62Llow/CD44high/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, 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-αGC+ 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 CD45RACCR7CD62Lhigh phenotype that predicted recirculation properties of lymph 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 NK T 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 cells to thymocytes, CD4 thymocytes acquired a CD62LlowCD44high effector phenotype with dual production of IL-4 and IFN-γ, and they expressed PLZF (19, 20). As most MHC-restricted T cells do not express PLZF, the expression of PLZF by the entire population of naive-like CD1d-αGalCer-specific T cells also provides a major argument against the possibility that these CD1d-restricted T cells were primarily selected by MHC-peptide ligands and secondarily cross-reacted onto CD1d-lipid complexes.

The expression of PLZF stands in apparent contradiction with the naive phenotype of these Vo24−CD1d-αGC+ T cells because PLZF is necessary and sufficient for effector differentiation during T cell development. Our study establishes, however, that the expression level of PLZF in these lipid-specific cells was significantly reduced compared with NKT cells in fresh PBMC samples and that, as experimentally assessed in the mouse system, a sharp threshold of expression was required for effector differentiation, as judged by the CD44/CD62L phenotype. Thus, a 2-fold reduction of PLZF protein in PLZF−/− mice was sufficient to drastically impair NKT cell development and prevent their acquisition of effector properties. In addition, transgenic studies
demonstrated that substantial amounts of PLZF protein, estimated between 34 and 64% of the maximal levels observed in NKT cells, were required for effector conversion. Collectively, these findings suggest that, although PLZF may mark T cell lineages selected by ligands expressed on thymocytes, different levels can be induced with substantial consequences on the naive versus effector type of differentiation imparted.

The mechanisms governing the induction of different levels of PLZF remain unclear at present. It is conceivable that the levels may depend on 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).

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-transfectants in vitro (24, 25), and one report further demonstrated their presence among PBMC with a naïve 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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