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Targeted Expression of Human CD1d in Transgenic Mice Reveals Independent Roles for Thymocytes and Thymic APCs in Positive and Negative Selection of Vα14i NKT Cells

Jens Schümann, Paola Pittoni, Elena Tonti, H. Robson MacDonald, Paolo Dellabona, and Giulia Casorati

CD1d-dependent invariant Vα14 (Vα14i) NKT cells are innate T lymphocytes expressing a conserved semi-invariant TCR, consisting, in mice, of the invariant Vα14-Jα18 TCR α-chain paired mostly with Vβ8.2 and Vβ7. The cellular requirements for thymic positive and negative selection of Vα14i NKT cells are only partially understood. Therefore, we generated transgenic mice expressing human CD1d (hCD1d) either on thymocytes, mainly CD4+ CD8+ double-positive, or on APCs, the cells implicated in the selection of Vα14i NKT cells. In the absence of the endogenous mouse CD1d (mCD1d), the expression of hCD1d on thymocytes, but not on APCs, was sufficient to select Vα14i NKT cells that proved functional when activated ex vivo with the Ag α-galactosylceramide. Vα14i NKT cells selected by hCD1d on thymocytes, however, attained lower numbers than in control mice and expressed essentially Vβ8.2. The low number of Vβ8.2+ Vα14i NKT cells selected by hCD1d on thymocytes was not reversed by the concomitant expression of mCD1d, which, instead, restored the development of Vβ7+ Vα14i NKT cells. Vβ8.2+, but not Vβ7+, NKT cell development was impaired in mice expressing both hCD1d on APCs and mCD1d. Taken together, our data reveal that selective CD1d expression by thymocytes is sufficient for positive selection of functional Vα14i NKT cells and that both thymocytes and APCs may independently mediate negative selection.

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4 Abbreviations used in this paper: m, mouse; DN, double negative; ABS, Ab binding site; BM, bone marrow; DC, dendritic cell; DN, double negative; DP, double positive; α-GalCer, α-galactosylceramide; h, human; HSA, heat-stable Ag; M6, macrophage; MNC, mononuclear cell; pLck, proximal Lck; tg, transgenic.

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hCD11c promoter was chosen to drive the expression of the transgene mainly on mDCs (21). The hCD11d expressed by mAPCs is recognized in vitro by mVα14I NKt cell hybridomas (22) without the addition of α-GalCer, indicating that hCD11d can present endogenous lipid Ags recognized by mNKt cells. Furthermore, its use in this model would allow us to discriminate between the expression of the transgene and that of the endogenous mCD11d molecule.

Materials and Methods

Generation of hCD11d-tg mice

Full-length hCD11d cDNA was PCR amplified from cDNA obtained from hCD11d-expressing cells using the up (5'-CGAGGTGGTCCAGCCAGGCGG GATAT-3') and down (5'-CGAGGTGGTCCAGCCAGGACACA-3') oligonucleotides, sequenced, and cloned into the vectors for transgene generation.

The pLck-hCD11d construct was made by cloning the full-length hCD11d cDNA into the EcoRI site of the pBS-CD11c-prom plasmid, containing the murine pLck promoter and the human growth hormone minigene (20). A 6.6-kb NotI fragment containing the murine pLck promoter, the hCD11d cDNA, and the human growth hormone minigene (Fig. 1A) was microinjected into FVB-fertilized eggs.

The CD11c-hCD11d construct was made by subcloning the full-length hCD11d cDNA into the EcoRI site of the pBS-CD11c-prom plasmid, containing the murine CD11c promoter and a rabbit β-globin cDNA expression cassette (21). An 8.9-kb SpeI-XhoI fragment containing the murine CD11c promoter, the hCD11d cDNA, and the rabbit β-globin cassette (Fig. 1A), was microinjected into FVB-fertilized eggs.

Tg founders and offspring were screened by PCR on tail DNA with the up (5'-GACACCTGGAGACGCCCTAA-3') and down (5'-GCGGAGGCCGACT-3') hCD11d-specific primers. Founders were backcrossed three times with C57BL/6 mice in a specific pathogen-free environment. The hCD11d-tg mice were further crossed with CD11d-/- mice (referred to hereafter as mCD11d-/- mice; provided by A. Bendelac, University of Chicago, Chicago, IL) (23). Animal studies were approved by the institutional animal care and use committee, San Raffaele.

Cell preparations

Single-cell suspensions of thymocytes and hepatic lymphocytes were prepared as described previously (24). Mature thymocytes were obtained by depleting heat-stable Ag (HSA)-1+ cells with B2A2 mAb plus rabbit complement, followed by a Lympholyte M gradient (Cedarlane Laboratories). For APC enrichment, thymic fragments were digested with collagenase D (0.5 mg/ml; Roche) and DNase I (40 μg/ml; Roche) in RPMI 1640 medium plus 5% FCS for 10 min at 37°C, followed by cold iso-osmotic Optiprep separation (density, 1.062 g/ml; containing 5 mM EDTA (pH 7.2); Nycoprep; Diagnostics) at 1700 g for 10 min. The low-density fraction, ~1% of the starting population, was harvested and washed twice.

Flow cytometry

Vα14I NKt cells were specifically identified by α-GalCer-loaded mouse CD11c-IgG1 dimers, as described previously (25). The following mAb conjugates were used for cell surface stainings, anti-TCR-β-FITC (H57-597), CD69-FITC (53-6.7), anti-CD11c-PE (HL3), anti-hCD11d-PE (CD11d2), anti-CD4-Pacific Blue (RM4-5), anti-CD8-PE-Cy5 (53-5), anti-F4/80-PE-Cy7 (PK136), anti-TIA-1-PE-Cy5 (F23-2), anti-CD45R/B220-allophycocyanin (RA3-6B2), anti-VEGF-biotin (TR310), anti-mCD1d-biotin (1B1), and F4/80-biotin (F4/80). The biotinylated mAbs were revealed with streptavidin-PE-Cy5.5 (eBioscience; in the case of Vβ7), streptavidin-allophycocyanin (Molecular Probes; in the case of mCD1d), or streptavidin-PerCP (BD Pharmingen; in the case of F4/80). mAbs were either purified and conjugated at our institute or purchased from BD Pharmingen (1B1, H57, CD11d2, PK136, RA3-6B2, and 1B1) or e-Bioscience (RM4-5). Samples were passed on a FACSCanto flow cytometer (BD Biosciences), gated to exclude nonviable cells on the basis of light scatter. Data were analyzed using FACSDiva and CellQuest software (BD Biosciences).

Ab binding sites (ABS) for mouse and human CD11d

The numbers of mouse and human CD11d molecules expressed by various thymic cell types were indirectly determined by counting the ABS for rat anti-mouse CD11d and mouse anti-human CD11d mAbs. Anti-hCD11d-PE (CD11d2) and anti-mCD11d-PE (1B1) mAb containing a one PE molecule/one Ab molecule ratio were obtained by gel filtration. Thymocytes and DCs were purified from wild-type and tg animals as described above and

FIGURE 1. Generation of hCD11d-tg mice and transgene expression. A, Schematic representation of the microinjected tg constructs. B, Total thymocytes from non-tg, pLck-hCD11d-tg, and CD11c-hCD11d-tg mice on the CD11d-/- background were stained with anti-CD4, CD8ε, and hCD11d mAbs. Histograms show hCD11d expression on thymocyte subsets. C, APC-enriched thymic cells from the same set of mice were stained with anti-CD1c, CD45R/B220, F4/80, and hCD11d mAbs. Histograms show hCD11d expression on thymocyte subsets. D, APC-enriched thymic cells from the same set of mice were stained with anti-CD1c, CD45R/B220, F4/80, and hCD11d mAbs. Histograms show hCD11d expression on thymocyte subsets.
stained for either CD4 and CD8 or CD1d-tg together with a saturating amount of anti-mCD1d-PE or anti-hCD1d-PE mAbs. Human thymocytes were purified from two thymi obtained from 21-wk-old fetuses after therapeutic abortion, under the approval of the local ethical committee, and stained with anti-CD4-allophycocyanin, anti-CD8-FITC (BD Pharmingen) and anti-hCD1d-PE. Quantification of the ABS for anti-hCD1d mAb was determined using either the Quantum Simply Cellular (Sero tec) or the QuantiBrite PE (BD Biosciences) method, which gave comparable results. The ABS for the anti-mCD1d mAb were determined using QuantiBrite PE method. Both methods determine ABS independently of the relative affinities of the mAb used (26).

**In vitro Va141 NKT cell activation**

Human CD1d-IgG1 dimers (BD Pharmingen) were loaded with α-GaICer as described above and attached in duplicate wells of flat-bottom, 96-well plates starting from 1 μg/well with 3-fold dilutions. After washing, purified T cells were added to wells in 200 μl of RPMI 1640 medium supplemented with 10% FCS and 2 μg/ml anti-CD28 mAb (BD Pharmingen). T cells were purified from hepatic mononuclear cells (MNC) using either anti-CD5-bio mAb (BD Pharmingen), followed by avidin-coated magnetic beads (Miltenyi Biotec), or anti-Thy-1.2 mAb coated magnetic beads (Miltenyi Biotec). The final number of NKT cells added to each well was normalized in different mice by determining the percentage of lymphocytes stained with α-GaICer-hCD1d dimers present among the purified T cells. That corresponded to a final number of either 10^4 or 2.5 × 10^4 α-GaICer-hCD1d dimer-reactive Va141 NKT cells/well depending on the experiment. Staining with α-GaICer-hCD1d dimers was performed as described for the mouse dimers. IFN-γ and IL-4 production was measured after 48 h by ELISA (BD Pharmingen).

**Statistical analysis**

The results were analyzed by one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison test. A value of p ≤ 0.05 was considered significant.

**Results**

**Generation of hCD1d-tg mice**

The hCD1d-tg mice were generated using a pLck cassette for thymocyte-specific expression (20) or a CD1c cassette for DC-specific expression (21) (Fig. 1A). Six founder mice for pLck-hCD1d-tg and one founder mouse for CD1c-hCD1d-tg were identified by genomic analysis of tail DNA (data not shown) and crossed onto C57BL/6 mice to generate tg lines.

Two lines of pLck-hCD1d-tg mice did not express hCD1d on thymocytes, two lines showed only partial expression of hCD1d on thymocytes, and one line expressed the transgene on both thymocytes and peripheral T cells (data not shown). The number of tg hCD1d molecules expressed by DP thymocytes in this tg line was in a range similar to that of mCD1d molecules expressed by the same subset in wild-type mice as well as of hCD1d molecules expressed by DP thymocytes from human fetal thymi (Table I), suggesting that the hCD1d transgene was expressed at physiologic levels. This line was selected for additional analysis. Thymic lymphoid DCs (defined as CD11c^+CD45R/B220^-F4/80^+), plasmacytoid DCs (defined as CD11c^+CD45R/B220^-F4/80^-), B cells (defined as CD11c^+CD45R/B220^-F4/80^-), or Mϕ (defined as CD11c^+CD45R/B220^-F4/80^-) did not express hCD1d in the selected pLck-hCD1d-tg line (Fig. 1C).

The CD11c-hCD1d-tg line clearly expressed high levels of hCD1d on lymphoid DCs, but not on plasmacytoid DCs (Fig. 1C), and the number of transgenic hCD1d molecules expressed on thymic DCs was much higher than that of endogenous mCD1d (Table I). Lymphoid DCs accounted for >80% of the hCD1d^+ cells in the thymus of CD11c-hCD1d-tg mice. The hCD1d transgene expression was also detected on a small subset of B cells and on Mϕ (Fig. 1C), accounting, however, for a minority of hCD1d^+ cells in these mice (12 and 6%, respectively). DP, CD4^+CD8^+, and DN thymocytes (Fig. 1B) or peripheral T cells (not shown) were, by contrast, completely negative for hCD1d in the CD11c-hCD1d-tg line. Overall, even though the majority of cells expressing hCD1d are lymphoid DCs, we refer to transgene expression in CD11c-hCD1d-tg mice as APC specific.

In both pLck-hCD1d-tg and CD11c-hCD1d-tg mice, transgene expression was not influenced by the presence of endogenous mCD1d, nor was the expression of endogenous mCD1d altered by the transgene (not shown). Furthermore, the frequencies and numbers of all thymic cell populations analyzed, i.e., DP, CD4^+, CD8^+, DN, B cells, DCs, and Mϕ, were not significantly influenced by the expression of the transgene (data not shown).

The selected tg mouse lines were crossed with mCD1d^-/- mice to obtain mice expressing hCD1d in a tissue-specific manner in the presence or the absence of endogenous mCD1d (hCD1d-tg mCD1d^-/- and hCD1d-tg mCD1d^+/-, respectively).

**Expression of hCD1d on thymocytes is sufficient for positive selection of Va141 NKT cells**

We first determined whether Va141 NKT cells developed in mice expressing hCD1d mainly on DP thymocytes, with or without the influence of endogenous mCD1d. Total thymocytes and liver MNCs from pLck-hCD1d-tg mCD1d^-/-, pLck-hCD1d-tg mCD1d^+/-, or non-tg mCD1d^+/- mice were stained with α-GaICer-loaded mCD1d-IgG1 dimers (hereafter referred to as dimer) and mAbs against TCR-β and NK1.1 (representative stainings shown in Fig. 2). Mature Va141 NKT cells were defined as TCR-βhighdimer “NK1.1+”, and conventional T cells as TCR-βhighdimer “NK1.1-”.

<table>
<thead>
<tr>
<th>Thymus</th>
<th>Species of CD1d</th>
<th>ABS^+ Per Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>mCD1d</td>
<td>2350 ± 200^a</td>
</tr>
<tr>
<td>Non-tg mCD1d^+/-</td>
<td>mCD1d</td>
<td>1500 ± 4</td>
</tr>
<tr>
<td>pLck-hCD1d tg mCD1d^+/-</td>
<td>hCD1d</td>
<td>2050 ± 300</td>
</tr>
<tr>
<td>pCD11c-hCD1d tg mCD1d^+/-</td>
<td>hCD1d</td>
<td>220,000 ± 127,000</td>
</tr>
<tr>
<td>Human fetal</td>
<td>hCD1d</td>
<td>1700–2700^d</td>
</tr>
</tbody>
</table>

^a The quantification of ABS, reflecting the number of hCD1d or mCD1d molecules expressed on various thymic cell types, is independent of the affinities of the mAbs used, and has been performed at saturating concentration of either mAb as described in Materials and Methods.

^b Indicated are the mean ± SD of values obtained from four to six mice.

^c nd, not done.

^d Values obtained from the two independent samples are indicated.
The number of total thymocytes or liver MNC between tg and non-tg mCD1d<sup>+</sup>/<sup>-</sup> and mCD1d<sup>−/−</sup> mice did not significantly differ (data not shown). Vα14i NKT cells developed in tg mice expressing hCD1d mainly on DP thymocytes in the absence of endogenous mCD1d, although numbers of mature Vα14i-NKT cells in thymus and liver were lower in pLck-hCD1d-tg mCD1d<sup>-/−</sup> mice than in non-tg mCD1d<sup>-/−</sup> mice (Fig. 3A). Similar results were obtained with splenic Vα14i-NKT cells (3.1 ± 0.2 and 5.2 ± 0.6 mature Vα14i-NKT cells in pLck-hCD1d-tg mCD1d<sup>-/−</sup> and non-tg mCD1d<sup>-/−</sup> mice, respectively) and also when hepatic Vα14i NKT cells were analyzed using α-GalCer-loaded mCD1d tetramers (data not shown). In contrast, conventional T cell numbers were not significantly influenced by the absence of mCD1d or the expression of the hCD1d transgene on thymocytes (Fig. 3A). Previous

**FIGURE 2.** Phenotype and Vβ usage of Vα14i NKT cells in control and pLck-hCD1d-tg mCD1d<sup>-/−</sup> mice. Total thymocytes, HSA-depleted mature thymocytes, or liver MNCs from control non-tg mCD1d<sup>+</sup>/<sup>-</sup> mice (A) or pLck-hCD1d-tg mCD1d<sup>-/−</sup> mice (B) were stained with dimer, anti-TCR-β, NK1.1, and, in the case of HSA-depleted thymocytes and liver MNC, Vβ8.2 and Vβ7 mAbs. Vα14i NKT cells are identified as TCR-β<sup>dim</sup>dimer<sup>+</sup>, and T cells as TCR-β<sup>high</sup>dimer<sup>+</sup>. Shown are the regions defining T or Vα14i NKT cells and the expression of NK1.1, Vβ8.2, and Vβ7 among Vα14i NKT cells. Similar data were obtained from five mice.

**FIGURE 3.** Positive and negative selection of Vα14i NKT cells by DP thymocytes expressing hCD1d. A, Total numbers of mature Vα14i NKT and conventional T cells (±SD). B, Mean percentages of Vβ8.2<sup>+</sup> or Vβ7<sup>+</sup> cells (±SD) among mature Vα14i NKT cells. C, Total numbers of Vβ8.2<sup>+</sup> or Vβ7<sup>+</sup> mature Vα14i NKT cells (±SD) in thymus and liver of pLck-hCD1d-tg mice on the mCD1d<sup>+/−</sup> or mCD1d<sup>-/−</sup> background (n = 5). Data from non-tg mCD1d<sup>-/−</sup> mice are included as controls. Horizontal lines in B indicate the mean percentages of Vβ8.2<sup>+</sup> or Vβ7<sup>+</sup> cells among mature Vα14i NKT cells from non-tg mCD1d<sup>-/−</sup> mice. *, p < 0.05. In A and B: **, values statistically different from non-tg control mice.
reports have shown the necessity for DP thymocytes in the positive selection of Vα14i NKT cells. These studies, however, relied on cell transfer or exclusion experiments (6, 11, 16) in which other CD1d-expressing cell types were present. Our data, therefore, formally prove that CD1d expressed by thymocytes, mainly DP, is sufficient to mediate positive selection of Vα14i NKT cells.

**Thymocytes may also contribute to negative selection of Vα14i NKT cells**

The reduced number of Vα14i NKT cells found in pLck-hCD1d-tg mCD1d<sup>+</sup> mice could be attributed to inefficient positive selection of Vα14i NKT cells by hCD1d. If this were the case, concomitant expression of mCD1d should restore normal numbers of Vα14i NKT cells. Alternatively, thymocytes expressing hCD1d could negatively select, to some extent, developing Vα14i NKT cells. In this scenario, the coexpression of mCD1d would not restore normal numbers of Vα14i NKT cells. As shown in Fig. 3A, the coexpression of hCD1d and mCD1d on thymocytes in pLck-hCD1d-tg mCD1d<sup>+</sup> mice was not able to restore normal numbers of Vα14i NKT cells, favoring the hypothesis of negative selection of Vα14i NKT cells by hCD1d. Compelling evidence in favor of negative selection of Vα14i NKT cells driven mainly by DP thymocytes came from analysis of the TCR Vβ usage in Vα14i NKT cells. The frequencies of Vβ8.2<sup>+</sup> and Vβ7<sup>+</sup> cells were determined by five-color flow cytometry in thymic and liver Vα14i NKT cells from pLck-hCD1d-tg mCD1d<sup>+</sup>, pLck-hCD1d-tg mCD1d<sup>−/−</sup>, or non-tg mCD1d<sup>−/−</sup> mice (representative stainings shown in Fig. 2). As shown in Fig. 3B, thymocytes expressing hCD1d, in the absence of endogenous mCD1d, selected Vα14i NKT cells exhibiting a TCR repertoire heavily skewed toward Vβ8.2. Coexpression of mCD1d with hCD1d on thymocytes increased the frequency and absolute number of Vβ7<sup>+</sup> Vα14i NKT cells, but did not modify the low number of Vβ8.2<sup>+</sup> Vα14i NKT cells selected by hCD1d on thymocytes (Fig. 3B and C).

Collectively, these data indicate that hCD1d interacts selectively with mouse Vβ8.2, but apparently ignores Vβ7. Taking into account the high avidity binding of α-GalCer-hCD1d by Vβ8.2<sup>+</sup> Vα14i NKT cells (22, 25) and the comparable expression levels of endogenous mCD1d and tg hCD1d on thymocytes, our data suggest that the reduced numbers of Vβ8.2<sup>+</sup> Vα14i NKT cells selected by hCD1d-expressing thymocytes, mainly DP, are due to negative selection.

**Thymic APCs mediate negative, but not positive, selection of Vα14i NKT cells**

In contrast to hCD1d expressed on thymocytes, hCD1d expressed on APCs was unable to select Vα14i NKT cells, as shown by the complete absence of Vα14i NKT cells in CD11c-hCD1d-tg mCD1d<sup>−/−</sup> mice (Fig. 4A). Similar results were obtained when Vα14i NKT cells were detected using α-GalCer-loaded mCD1d tetramers (not shown). It is likely that APCs cannot mediate positive selection of Vα14i NKT cells in line with other data showing that mice expressing only tg mCD1d driven by the MHC class II promoter (with close to normal expression levels of mCD1d on thymic DCs, epithelial cells, and Mcf) also completely lacked Vα14i NKT cells (11). However, it cannot be formally ruled out that the absence of Vα14i NKT cells in CD11c-hCD1d-tg mCD1d<sup>−/−</sup> mice is due to APC-driven negative selection dominating over positive selection.

In the presence of endogenous mCD1d, hCD1d expressed on APCs dramatically reduced the development of Vα14i NKT cells (Fig. 4A), suggesting a role for APCs in the negative selection of Vα14i NKT cells. These studies, however, relied on cell transfer or exclusion experiments (6, 11, 16) in which other CD1d-expressing cell types were present. Our data, therefore, formally prove that CD1d expressed by thymocytes, mainly DP, is sufficient to mediate positive selection of Vα14i NKT cells.

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these cells. In contrast, conventional T cell numbers were not significantly influenced by the presence of hCD1d on APCs (Fig. 4A). The TCR Vβ repertoire analysis of Vα14i NKT cells developing in mice coexpressing hCD1d on APCs and mCD1d revealed a profound depletion of Vβ8.2+ Vα14i NKT cells in both thymus and liver (Fig. 4, B and C). Hence, these findings definitively confirm a role for APCs in the negative selection of Vα14i NKT cells, independently of thymocytes, and emphasize the selective and high affinity interaction between hCD1d and mouse Vβ8.2. Furthermore, APCs appear to be more efficient in negatively selecting Vα14i NKT cells than thymocytes, as shown by the more dramatic decrease in the Vβ8.2/Vβ7 ratio among Vα14i NKT cells in CD11c-hCD1d-tg mCD1d+/+ mice (Fig. 4, B and C) than in pLck-hCD1d-tg mCD1d+/+ mice (Fig. 3, B and C) compared with that in non-tg mice. Given that lymphoid DCs constitute the vast majority of cells expressing hCD1d in CD11c-hCD1d-tg mice, and that thymic B cells do not seem to affect Vα14i NKT cell development (16), it is tempting to conclude that DCs are the critical thymic cell type involved in the negative selection of Vβ8.2+ Vα14i NKT cells observed in this transgenic model.

Peripheral Vα14i NKT cells selected in tg mice expressing CD1d only on thymocytes are functional

It has been previously shown that transgenic expression of mCD1d can result in altered Vβ repertoire and hyporesponsiveness of murine NKT cells (17). Moreover, even though the peripheral homeostasis of Vα14i NKT cells seems independent of the interaction with CD1d (29, 30), it is not known whether peripheral NKT cells present in an environment devoid of CD1d might be functionally compromised. In the light of these considerations, we determined the in vitro reactivity to α-GalCer-hCD1d of hepatic Vα14i NKT cells obtained from pLck-hCD1d-tg mCD1d+/+ or pLck-hCD1d-tg mCD1d+/− mice, representing a similar situation in terms of thymic selection of Vβ8.2+ Vα14i NKT cells, but differing with respect to peripheral CD1d expression. To directly determine the IFN-γ production potential of Vα14i NKT cells, without the amplification produced by bystander NK cells, T cells were purified from the total hepatic MNC before the in vitro activation. Care was taken to normalize the number of α-GalCer-hCD1d-reacting Vα14i NKT cells from the different mice added to each experimental well. The absolute numbers of peripheral α-GalCer-hCD1d-reacting Vα14i NKT cells in the different tg

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**FIGURE 5.** Peripheral Vα14i NKT cells from pLck-hCD1d-tg mCD1d+/+ mice are functional in vitro. A, Purified hepatic T cells from either pLck-hCD1d-tg mCD1d+/+ or pLck-hCD1d-tg mCD1d+/- mice were stained with α-GalCer-loaded hCD1d dimers and anti-TCRβ mAb. The percentages of gated human dimer− NKT cells are indicated. B, Purified hepatic T cells were added to wells in the presence of increasing doses of plastic-bound hCD1d-α-GalCer dimers and anti-CD28. To plate 2.5 × 10⁵ human dimer+ NKT cells, 2.1 × 10⁵ or 1.9 × 10⁵ purified T cells from pLck-hCD1d-tg mCD1d+/+ and pLck-hCD1d-tg mCD1d+/- mice were seeded, respectively. IFN-γ and IL-4 production was measured after 48 h by ELISA. Purification of hepatic T cells performed with either anti-CD5 (shown) or anti-Thy1 mAb gave similar results. One representative experiment of three performed with anti-CD5-purified hepatic T cells is shown.
mice was inferred from the percentage of hepatic T cells stained by α-GalCer-hCD1d dimers (Fig. 5A). Equal numbers of α-GalCer-hCD1d-reacting Vα14i NKT cells were plated in the presence of increasing doses of plastic-bound α-GalCer-hCD1d dimers and co-stimulatory anti-CD28 mAb. We chose to activate Vα14i NKT cells with α-GalCer-hCD1d dimers to restrict our focus mainly on the activation of Vβ8.2+ cells. As shown in Fig. 5B, purified hepatic Vα14i NKT cells from the pLck-hCD1d-tg mCD1+− produced more IFN-γ and IL-4 than equivalent cells from pLck-hCD1d-tg-mCD1+− mice, although only the production of IFN-γ was significantly different in the several experiments performed. Together, these findings indicate that NKT cells from pLck-hCD1d-tg mice devoid of CD1d in the periphery are functional and are more responsive to the antigenic stimulus than NKT cells from mice expressing CD1d in periphery, at least for IFN-γ production.

Discussion

Due to the specific interaction of hCD1d bound with endogenous mouse lipid(s) to Vβ8.2+ Vα14i NKT cells, we have been able to dissect the roles of thymocytes and APCs in positive and negative selection of Vα14i NKT cells by tracking the fate of the Vβ8.2− subset. Our data show that CD1d expression by thymocytes, mainly DP, is sufficient for positive selection of functional Vα14i NKT cells, whereas both thymocytes and APCs may independently contribute to negative selection. In line with the skewed mouse TCR repertoire selected by hCD1d in Vα14i NKT cells, we have recently reported that α-GalCer-loaded hCD1d dimers selectively bind with high avidity to mouse Vβ8.2+, but not Vβ7+, Vα14i NKT cells (25). Furthermore, we have shown that a mouse Vβ8.2+ T cell hybridoma responded in the absence of α-GalCer much more strongly (~12-fold) to hCD1d than mCD1d transfectants of mouse A20 B lymphoma cells (22), again suggesting a possibly stronger interaction between hCD1d, presenting mouse endogenous ligands, and Vβ8.2− NKT cells. Similar to α-GalCer-loaded hCD1d dimers, therefore, hCD1d-presenting endogenous mouse lipid ligand(s) interacts selectively with Vβ8.2+ Vα14i NKT cells during their development. The preference for Vβ8.2 appears not to depend on the source of the endogenous or exogenous lipid ligand(s) presented by hCD1d, thus suggesting that residues on the mouse Vβ8.2 domain mainly interact with hCD1d.

APCs appear to be more efficient in negatively selecting Vα14i NKT cells than thymocytes. This might be due to two, not mutually exclusive, reasons: 1) hCD1d transgene expression is higher on APCs than on thymocytes (Fig. 1, B and C, and Table I); or 2) avidity thresholds are lower for APC-driven negative selection of Vα14i NKT cells because of the presence of T cell costimulatory molecules such as CD80 and CD86, which are missing on DP thymocytes. Previous reports showing thymic negative selection of Vα14i NKT cells in vitro and in vivo relied on the use of the strong agonist ligand α-GalCer to activate developing NKT cells (17, 19). Other data consistent with the negative selection of Vα14i NKT cells by endogenous lipid ligands were obtained in vivo by injecting CD11c− BM-derived DCs from K b-mCD1d-tg mice into irradiated RAG−/− mice reconstituted with wild-type BM cells (17). Our data confirm and extend those findings using endogenous thymic APCs, largely represented by hCD1d-expressing DCs, which can export negative selection specifically on Vβ8.2+ Vα14i NKT cells.

It has been shown that Vα14i NKT cells undergo limited homeostatic proliferation and survive independently of the presence of CD1d in both spleen and liver, demonstrating that the homeostatic requirements of CD1d-restricted NKT cells resemble those of CD8+ memory T cells (29, 30). In light of these observations, we infer that the lower number of Vα14i NKT cells found in the periphery of pLck-hCD1d-tg mCD1+− mice is probably due to reduced NKT cell output from the thymus, rather than to the absence of CD1d in the periphery. Moreover, peripheral mature Vα14i NKT cells from tg mice devoid of CD1d expression in the peripheral compartment are functional in vitro, suggesting that the interaction between CD1d plus endogenous ligands and the invariant TCR in the periphery is dispensable to maintain the functional integrity of mature NKT cells. It appears, therefore, that the expression of CD1d mainly on DP thymocytes suffices to generate functional peripheral Vα14i NKT cells. Interestingly, hepatic Vα14i NKT cells from pLck-hCD1d-tg mice that coexpress endogenous mCD1d produce lower levels of cytokines than the NKT cells that develop in mice expressing only hCD1d on thymocytes, suggesting that the interaction between the Vα14i TCR and mCD1d expressed on other cell types may tune their functional status. While this manuscript was under revision, Wei et al. (31) reported similar data on the generation of functional peripheral NKT cells in mice selectively expressing tg mCD1d on cortical thymocytes.

The ability of thymocytes to negatively select Vα14i NKT cells has not been demonstrated previously. This points to a very delicate balance between positive and negative selection driven by CD1d on DP thymocytes and implies that strict regulation of CD1d levels is required for proper development of Vα14i NKT cells. Considering that mature Vα14i NKT cells are autoreactive by design (4) and exert their effector functions by possibly recognizing stress-induced self-lipid ligands presented by CD1d molecules (1), it is thus possible that a double check by DP thymocytes and APCs during negative selection of CD1d-dependent Vα14i NKT cells might be essential to finely tune the avidity of their mature TCR repertoire for self ligands.

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Disclosures

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