T Cell Development in Mice Expressing CD1d Directed by a Classical MHC Class II Promoter

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CD1d and nonclassical MHC molecules differ markedly from classical MHC ligands in their ability to promote the selection and differentiation of developing T cells. Whereas classical MHC-restricted T cells have a predominantly naive phenotype and a broad TCR repertoire, most other T cells have a memory and/or NKT phenotype with a restricted repertoire. Because the nonclassical ligands selecting these memory-type cells are expressed by bone marrow-derived cells, it has been suggested that the development of large repertoires of naive-type cells was dependent on the classical MHC expression pattern in the thymus cortex, high on epithelial cells and low on cortical thymocytes. We redirected CD1d expression using the classical MHC II Eα promoter. pEα-CD1d mice lacked memory-type NKT cells, but, surprisingly, they did not acquire the reciprocal ability to select a diverse population of naive CD1d-restricted cells. These findings suggest that, whereas the development of NKT cells is dependent on the pattern of CD1d expression, the absence of a broad, naive CD1d-restricted T cell repertoire may reflect intrinsic limitations of the pool of TCR genes or lipid Ags. The Journal of Immunology, 2003, 171: 4096–4104.

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4 Abbreviations used in this paper: DN, double negative; αGalCer, α-galactosylceramide; KO, knockout.

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Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice deficient in H2-KbD (KbD knockout (KO)), I-Aβ (I-Aβ KO), CD1d1/CD1d2 (CD1d KO) were used after 9–14 backcrosses onto C57BL/6 (2).

pEα/βCD1d-transgenic mice were obtained by crossing the transgenic mice expressing CD1d1 under the MHC class II Eα/β-promoter (described below) with CD1d KO mice. These mice were further crossed to KbDβ/CD1d KO and I-Aβ/CD1d KO to obtain KbDβ KO/pEα/βCD1d mice and I-Aβ/CD1d KO/pEα/βCD1d mice, respectively, where the pEα/βCD1d transgene was expressed in the absence of classical MHC class I or class II and in the absence of endogenous CD1d genes.

All mice were raised in a specific pathogen-free barrier environment at Princeton University according to the institutional animal care and use committee guidelines. Results reported in this study were derived from comparative analysis of littermates of relevant genotypes.

Generation of pEα-CD1d-transgenic mice

Full-length C57BL/6 CD1d1 cDNA generated by PCR with forward primer 5'-AAGCGCAGAAGTCGGAGCCG-3' (5') and reverse primer 5'-GCAGGTACGCACATTTGCAGTTGTG-3' (5') was cloned into pCR3.1 (Invitrogen, Carlsbad, CA). The 1.2-kb EcoRI fragment containing mCD1d1 cDNA was inserted at the EcoRI site in the Eα/β-globin exon of the pDOI-5 vector (18), which drives gene expression under the control of mouse MHC class II (I-Eα/β) promoter. After BglII digestion, a 5-kb fragment containing the Eα/β-promoter, CD1d1 cDNA, and poly(A) signal as described in Fig. 1 was inserted at the EcoRI site in the β-globin exon of the pDOI-5 vector (18), which drives gene expression under the control of mouse MHC class II (I-Eα) promoter. After BglII digestion, a 5-kb fragment containing the Eα promoter, CD1d1 cDNA, and poly(A) signal as described in Fig. 1

![FIGURE 1. Schematic of the 5-kb pEα-CD1d transgene (not to scale) constructed using the vector described by Kouskoff et al. (18) as detailed in Materials and Methods.](image1)

![FIGURE 2. Surface expression of transgenic CD1d in pEα-CD1d mice. Whole thymocytes, splenic TCRβ T cells, B220 B cells, bone marrow-derived mature CD11c+ DCs, splenic or peritoneal CD11b+ macrophages were stained for CD1d and I-Aβ. Cells from B6.pEα-CD1d mice expressing the transgene but lacking endogenous CD1d molecules were compared with wild-type (WT) and CD1d KO B6 mice. Mean fluorescence intensity of CD1d is reported in the relevant (arbitrary) gates delineating MHC IIhigh vs MHC IIlow cells. Results are representative of three separate experiments. Mo, macrophage.](image2)

![FIGURE 3. pEα-CD1d transgene-encoded molecules are functionally active. A, Fresh splenocytes or bone marrow-derived TNF-α-matured DCs from wild-type (WT) or pEα-CD1d B6 mice were pulsed with graded doses of αGalCer before stimulation of the Vα14-Jα18/Vβ8 DN32.D3 hybridoma, measured by IL-2 release. B, Bone marrow-derived TNF-α-matured DCs from wild-type or pEα-CD1d mice were used to stimulate IL-2 release by the CD1d-autoreactive hybridomas DN32.D3 (Vα14-Jα18/ Vβ8) or TCB11 (Vα8/Vβ8). Results are representative of three experiments.](image3)
was purified and injected into fertilized C57BL/6 eggs. Four lines of transgene-positive mice were crossed with C57BL/6.CD1d−/− mice to obtain pEa-CD1d-transgenic mice.

**Abs and CD1d tetramers**

Conjugated mAbs anti-CD3, CD4, CD8, CD24 (HSA), CD45, NK1.1, B220, panTCRβ, and streptavidin were purchased from BD Pharmingen (San Diego, CA). Y3-P anti-I-Ab and 19G11 anti-CD1d were prepared and conjugated to FITC or biotin in our laboratory. Anti-Ly51 (clone 6C3), mAb 29 (of the same rat origin as the anti-CD1d mAb, we used a biotinylated 19G11 anti-CD1d mAb followed by fluorescent streptavidin (αGalCer) tetramers were produced and used for staining as described previously (20).

Flow cytometry analysis was performed with a four-color FACSort equipped with argon and 635-nm diode lasers (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences).

The following secondary reagents, purchased from Molecular Probes (Eugene, OR) were used for immunohistochemical studies: Alexa Fluor 488 or 568 goat anti-rat IgG, Alexa Fluor 488 or 568 goat anti-hamster IgG, Alexa Fluor 488 goat anti-mouse Ig, and Alexa Fluor 488 or 568-streptavidin.

**Frozen section and immunohistochemistry**

Thymi were removed and immediately soaked in ice-cold PBS. Unfixed tissues were embedded in OCT freezing medium (Tissue-Tek, Sakura Finetek, CA) and frozen at −80°C until use. Tissue sections (8- to 10-μm thick) were generated with a cryostat and mounted on glass slides (Fisher Scientific, Pittsburgh, PA), air dried, and fixed by immersion into −20°C acetone for 5 min.

For immunohistochemical staining, sections were incubated in PBS with 10% goat serum containing avidin/streptavidin block (Vector Laboratories, Burlingame, CA) for 30 min before incubation with primary Ab for 1 h in humid chamber. Sections were washed with PBS and incubated with the appropriate secondary Ab. For double staining, we used secondary anti-Ig reagents monospecific for rat and hamster. When the other primary mAb (CD11b, 29, or 6C3) was of the same rat origin as the anti-CD1d mAb, we used a biotinylated 19G11 anti-CD1d mAb followed by fluorescent streptavidin in the last steps of the staining processing. Y3P anti-I-Ab was used directly conjugated to FITC. Sections were mounted in Mowiol and staining was examined with a confocal microscope Axiovert 100M Zeiss LSM510. Images were processed with Adobe Photoshop 6.0 software (Adobe Systems, Mountain View, CA).

**Purification of thymic epithelial cells**

Thymic epithelial cells were purified from 6- to 8-wk-old mice that had been irradiated (990 rad) 24–48 h previously. Thymi were cut into small pieces, washed with PBS, and incubated with a mixture of 0.3% trypsin (Sigma-Aldrich, St. Louis, MO) and 0.1% DNase I (Sigma-Aldrich) in PBS at 4°C for 4 h on a rotator. Cells present in suspension were harvested and the remaining fragments were reincubated with fresh trypsin-DNase for 30 min at 37°C and then stirred with fresh trypsin and DNase at room temperature to achieve complete digestion. Cells recovered in suspension were pooled, collected by centrifugation, and resuspended in PBS-1% BSA for flow cytometry analysis.

**Generation of liver lymphocytes, thymocytes, splenocytes, DCs, and peritoneal macrophages**

Lymphocytes were recovered from thymus, spleen, and liver as previously described (2). Peritoneal macrophages were recovered from peritoneal cavity washes with PBS-10% sucrose. Spleen-derived DCs were obtained by plastic adherence (2 h at 37°C) followed by deadherence of splenocytes and culture for 12–24 h in the presence of GM-CSF (10 ng/ml). Bone marrow-derived DCs were generated by culturing fresh bone marrow cells at 1 × 10⁶ cells/ml in 24-well plates for 7 days in medium supplemented with GM-CSF (5 ng/ml) and IL-4 (5 ng/ml; BioSource International, Camarillo, CA). To induce DC maturation, TNF-α (20 ng/ml; BioSource International) was added for the last 18 h of culture.

**Ag presentation assay**

Briefly, 5 × 10⁵ thymocytes, splenocytes, peritoneal macrophages, or 2 × 10⁵ splenic DCs, immature, or mature bone marrow-derived DCs were plated in 96-well-bottom microwell plates pulsed with various concentrations of αGalCer or medium alone for 6 h, washed, and incubated with 5 × 10⁴ NKT hybridoma DN32.D3 (Vα14-Jα18/Vβ8) or TCB11 (Vα8/Vβ8) for 18–24 h in RPMI 1640 enriched with 10% heat-inactivated FCS, glutamine, antibiotics, and 5 × 10⁻⁷ M 2-ME. The IL-2 content of supernatants was measured using the CTLL bioassay (2).

**Results**

**CD1d expression and Ag-presenting function in pEa-CD1d mice**

We constructed the pEa-CD1d transgene by inserting the B6 mouse CD1d1 cDNA into a transgenic expression cassette (Fig. 1). Two of four independent founder lines created in the B6 background had a faithful MHC class II-type pattern of expression, with levels of CD1d in the physiological range, as determined after crossing to B6.CD1d KO mice lacking endogenous CD1d1 and CD1d2. These lines, thereafter referred to as pEa-CD1d mice, were selected for further studies. In addition, one pEa-CD1d-transgenic line was further crossed with I-Abβ/CD1d and Kdβ/
CD1d doubly and triply deficient mice, respectively, to evaluate transgenic pEα-CD1d-restricted selection in the absence of both endogenous CD1d and MHC molecules (see below). Fig. 2 depicts the level of expression of transgenic CD1d molecules in pEα-CD1d mice, as quantitated by flow cytometry on various relevant cell types. As expected, CD1d was redistributed among the I-Aβ-positive cell types, whereas it was no longer expressed on I-Aβ-negative cells. Thus, thymocytes, splenic T cells, and the CD11b+/I-Ab-negative splenic or peritoneal macrophages, which in wild-type mice express CD1d but are MHC class II negative, lost CD1d expression. Conversely, pEα-CD1d was expressed on all I-Aβ-expressing cells, including B cells, bone-marrow derived DCs, and splenic CD11b+/I-Ab+ macrophages, and the expression levels achieved with the pEα promoter (expressed as mean fluorescence intensity in the relevant quadrants of the dot plots in Fig. 2) were generally very close to, or within a 0.5- to 1.5-fold range of, those observed in wild-type B6 mice. Furthermore, the subset of splenic marginal zone B cells lost the distinctively very high levels of CD1d expression characteristically seen in wild-type mice (21).

We next examined the ability of the pEα-CD1d transgene to present exogenous as well as endogenous Ags. Splenocytes and bone-marrow derived mature DCs from wild-type and pEα-CD1d mice were pulsed with a range of concentrations of αGalCer, the exogenous glycolipid ligand recognized by NKT cells expressing the canonical Vα14-Jα18/Vβ8 TCR, and then cultured with the Vα14-Jα18/Vβ8 DN32D3 hybridoma. The pEα-CD1d APCs elicited strong IL-2 production at levels identical to or slightly above those of wild type (Fig. 3A), closely reflecting the respective surface levels of CD1d measured in Fig. 2. Furthermore, pEα-CD1d DCs elicited strong autoreactive responses from both the Vα14-Jα18/Vβ8 DN32D3 and the Vα8/Vβ8 TCB11 hybridomas which recognize distinct endogenous ligands loaded in the endosomal and secretory pathway, respectively (Ref. 22 and Fig. 3B).

Thus, the hemopoietic elements in pEα-CD1d mice presented the appropriate pattern of CD1d expression expected from a MHC class II promoter and supported normal Ag-presenting functions. Expression of pEα-CD1d in thymic DCs and epithelium

To quantify surface expression of pEα-CD1d in thymic epithelial and DCs, we isolated these cell types from the thymus and analyzed them by flow cytometry in comparison with wild-type and CD1d KO mice examined in the same experiments (Fig. 4). Approximately 11% of thymic cells recovered after in vivo irradiation and in vitro collagenase digestion of the thymus were epithelial cells positive for the MHC class II molecule I-Aβ and negative for the hemopoietic marker CD45. Thymic DCs present at low frequency (1.3%) were recognized by their high expression of I-Aβ and CD45 (Fig. 4A). Importantly, both thymic epithelial cells and DCs in pEα-CD1d mice expressed CD1d on their surface and their level of expression were in the same range (compare Fig. 4, B and C). Surprisingly, we also found that wild-type thymic epithelial cells expressed significant levels of CD1d on their surface, a finding which, to our knowledge, had not been previously reported. In fact, the levels of CD1d in wild-type DCs and epithelial cells were approximately twice those found in pEα-CD1d. Because B6 mice heterozygous for the CD1d null mutation (+/−) express half the surface CD1d levels of wild type (+/+), and have normal NKT cells (data not shown), the levels achieved in pEα-CD1d mice are considered to be within physiological range. This was further supported by the in situ analysis of CD1d expression in thymic frozen sections showing strong expression by both cortical and medullary epithelial cells as shown below.

![Image of CD1d and I-Aβ expression in the thymus](image-url)
Immunohistochemical analysis of CD1d expression in the thymus

To further compare the pattern of expression of CD1d and MHC class II molecules in the thymus, we examined frozen tissue sections. In the wild-type thymus, CD1d was broadly expressed on all thymic cells in both the cortical and medullary zones (Fig. 5, upper panels). In contrast, I-Ab expression, which is restricted to epithelial cells, DCs, and macrophages, was scattered in the cortex and abundantly expressed in the medulla. In pEα-CD1d mice, CD1d faithfully acquired the MHC class II pattern (Fig. 5, lower panels), as evidenced by the near perfect colocalization of CD1d and I-A^b in the merged picture.

To more precisely examine the CD1d-expressing cells in the thymus, we analyzed macrophages and DCs by double staining for.

**FIGURE 6.** Immunohistofluorescence analysis of CD1d expression by macrophages and DCs in the thymus of wild-type (WT) and pEα-CD1d mice. A, CD11b^+ macrophages. B, CD11c^+ DCs. Arrows point to the macrophages that did not express CD1d. Bar, 100 μm.
lineage markers and CD1d. In wild-type mice, nearly all CD11b+ macrophages, scattered throughout all thymic areas, constitutively expressed CD1d (Fig. 6A, upper panels). In contrast, in pEa-CD1d mice, a significant proportion of macrophages failed to express CD1d (arrows in Fig. 6A, lower panels). This was expected from the MHC II pattern because a proportion of thymic CD11b+ macrophages failed to express I-A^b as well (data not shown). Most CD11c+ DCs expressed CD1d in wild-type as in pEa-CD1d mice (Fig. 6B).

We next analyzed thymic epithelial cells using the mAb 29 and Ly51 specific for the medullary and the cortical subsets, respectively. CD1d was expressed by both epithelial cell subsets in wild-type mice (Fig. 7, upper panels) as well as in pEa-CD1d mice (Fig. 7, lower panels). In particular, the strong expression of CD1d in cortical and medullary epithelial cells of pEa-CD1d mice mirrored the natural pattern of expression of I-A^b (data not shown) and confirmed the flow cytometry data shown in Fig. 4.

pEa-CD1d mice lacked NKT cells

Having established that CD1d was faithfully expressed according to the MHC class II pattern, that its expression levels were within the range of those found in wild-type mice, and that its Ag-presenting functions were conserved, we next examined the selection of CD1d-restricted T cell subsets. Using CD1d-aGalCer tetramers, which specifically stain cells expressing the canonical Vex14-Jo18/VJ8 TCRs, we examined the presence both in nonlymphoid and nonlymphoid tissues. Strikingly, we found that CD1d-aGalCer+ cells were undetectable in the thymus, spleen, lymph node, and liver of pEa-CD1d mice (Fig. 8). Their frequency, which ranged from 0.1 to 8% in these different tissues in wild-type mice, was <0.01%, the background level in CD1d KO mice. The absence of the Vex14 T cell lineage was not due to negative selection by the transgenically expressed molecules because mice coexpressing endogenous and transgenic CD1d genes had normal NKT cell frequencies in all tissues examined, including the thymus. Indeed, B6 mice (bearing two copies of the endogenous CD1d genes) that were heterozygous for the pEa-CD1d transgene had TCR^NK1.1+ frequencies (percent) of 0.6, 0.6, and 21 in the thymus, spleen, and liver, respectively, over background (in CD1d KO), whereas B6.CD1d^+/− littermates that did not express the transgene, and thus had a single copy of endogenous CD1d, had similar frequencies of 0.3, 0.4, and 14. Altogether, these results suggested that positive selection of Vex14-Jo18/VJ8 T cells was entirely abrogated in pEa-CD1d mice.

pEa-CD1d failed to select conventional CD4 or CD8 T cells

We next investigated whether reverting the pattern of CD1d expression to that of classical MHC class II might “rescue” the selection of a diverse, naive population of T cells. By crossing pEa-CD1d mice expressing the transgene but lacking the endogenous CD1d genes with mice lacking both classical MHC class I and CD1d (KbD^b/CD1d KO) or classical MHC class II and CD1d (I-A^b/CD1d KO), we eliminated conventional MHC-restricted T cells and directly measured the frequency of residual CD8 or CD4 T cells, respectively, which would include any CD1d-restricted cell selected by pEa-CD1d. For comparison, we examined KbD^b/CD1d KO or I-A^b/CD1d KO littermates which did not express the transgene. As expected, the control KbD^b/CD1d KO and I-A^b/CD1d KO expressed very few residual CD8 or CD4 T cells, respectively, in the thymus (Fig. 9) or in peripheral tissues (Fig. 10). Remarkably, expression of the pEa-CD1d transgene failed to rescue a detectable population of either CD8 or CD4 T cells above background. pEa-CD1d transgene expression also failed to increase the size of the rare population of CD4^-CD8^- T cells (data not shown). Thus, we conclude that re-expression of CD1d according to the classical MHC class II pattern did not reveal a significant CD1d-restricted repertoire of T cells.

Discussion

By transgenically redirecting CD1d expression with the MHC class II Ee promoter in CD1d KO mice, we have tested the hypothesis that the marked differences in thymic expression patterns between CD1d and MHC molecules were responsible for the corresponding differences in T cell repertoires.

Using flow cytometry, immunohistochemistry, and functional Ag presentation assays, we identified transgenic lines expressing functional CD1d molecules both in the thymus and in peripheral tissues. The relative levels of transgenic CD1d in various cell types tightly reflected those observed for MHC class II, indicating faithful expression of CD1d according to the MHC class II pattern. The levels of CD1d expressed on the surface of thymic epithelial cells, macrophages, DCs, and B cells were within close range (0.5- to 1.5-fold) of those of wild-type mice. Such levels normally support the selection of NKT cells by CD1d when it is driven by its own promoter, because both CD1d^+/− mice (where the level of CD1d is 0.5-fold that of +/-) and a CD1d-transgenic line where CD1d expression was driven by its own promoter at levels twice above wild-type exhibited normal NKT cell frequencies (S.-H.P., D.W., and A.B., unpublished data).

In this context, it is remarkable therefore that pEa-CD1d mice completely failed to develop CD1d-aGalCer tetramer+ Vex14 T cells. This result seems consistent with previous experiments with bone marrow chimeras, indicating that NKT cell selection required
CD1d expression in bone marrow-derived cells, most likely cortical thymocytes (5–9), as cortical thymocyte expression of CD1d was abolished in our pEα-CD1d mice. However, it had not been formerly recognized that CD1d was also expressed by the thymic cortical epithelial cells of normal B6 mice as we have now shown by both immunohistochemistry and FACS analysis. In fact, surface expression of CD1d in B6 mice was as intense in cortical epithelial cells as in cortical thymocytes. In retrospect, this surprising finding indicates that the previously reported lack of NKT cell selection in the CD1d/H11002 into CD1d/H11001 bone marrow radiation chimeras must be reinterpreted, as it could not simply be explained by an absence of CD1d expression in the cortical area of the thymus where developing thymocytes undergo positive selection. It is clear that CD1d expression by thymic epithelial cells in these chimeras was not sufficient for selection of NKT cells. One possibility therefore might be that the natural development of NKT cells required a sequence of interactions, first, in the cortex with either cortical thymocytes or epithelial cells and, second, in the medulla or at the corticomedullary junction with bone marrow-derived DCs where the marked expansion and acquisition of the memory phenotype by NKT cells might be induced (2). Such a scenario would be interrupted in the CD1d− into CD1d+ bone marrow radiation chimeras. This scenario was precisely tested and ruled out by the pEα-CD1d mice because they expressed CD1d in both cortical epithelial cells and in DCs, yet CD1d-αGalCer tetramer+ Vα14 T cells were absent. Taken together, our results therefore point to some unique nonredundant property of CD1d-expressing cortical thymocytes that is absolutely required for NKT cell development. One possibility is that the level of surface expression of the selecting CD1d-associated ligands, presumably of glycolipid or lipid nature, might be limiting in thymic epithelial cells, perhaps because these ligands are insufficiently generated or processed. Another nonexclusive possibility is that cortical thymocytes might provide additional signals that are specifically required for NKT cell development. For example, NKT cells, which express intrinsically autoreactive TCRs, might normally be deleted unless a specific program, perhaps including the expression of inhibitory NK-like receptors, was activated to down-regulate TCR signaling.

Another major result of our study is that, in the absence of MHC molecules, pEα-CD1d mice failed to select naive CD4, CD8, or DN T cells. The genetic approach used in our study allowed for CD1d expression according to the global MHC II pattern in the diverse hemopoietic and nonhemopoietic elements of the thymus, a pattern well known to support the selection, differentiation, and maintenance of naive T cells. Yet, such naive and diverse T cells were conspicuously absent. Much genetic, biochemical, and structural evidence suggests that CD8 acts as a coreceptor for various CD1 family members both in mouse and humans (6, 17, 23–26), yet no CD8 T cells could be observed above background in KdDα−deficient pEα-CD1d mice. A concurrent report of T cell development in a transgenic mouse model where CD1d was expressed under a classical MHC class I promoter and at higher levels than in our pEα-CD1d mice confirmed the absence of a naive repertoire of CD8 T cells (30). Given the present demonstration that CD1d is normally expressed by thymic cortical and medullary epithelial cells, previous reports that KdDα KO or TAP KO mice lacked a diverse population of naive CD8 T cells (2) are consistent with the results observed with these new transgenic models. However, the high constitutive levels of CD1d on cortical thymocytes in these KdDα KO or TAP KO mice, which are never

FIGURE 8. Lack of CD1d-αGalCer+ Vα14-3α18 T cells in pEα-CD1d mice. FACS dot plots show CD1d-αGalCer tetramer staining on the y-axis and unloaded CD1d tetramer+ B220 staining in the x-axis. Percentage of CD1d-αGalCer+ T cells in indicated tissues of wild-type (WT), pEα-CD1d, and CD1d KO mice are shown in the upper left quadrants of FACS dot plots. Results are representative of three independent experiments examining at least five mice per group.
observed for classical MHC molecules, might have prevented the selection of naive CD8 T cells (e.g., by inducing negative selection). Therefore, by faithfully reconstituting the MHC pattern of expression, these transgenic mice provided a more accurate model to test whether the differences between the MHC and CD1 pattern of expression were directly responsible for the lack of selection of naive diverse CD8 T cells. We could not detect other non-CD8 T cell subsets either, as shown by the fact that, in I-A<sup>b</sup>/H<sub>9252</sub>b-deficient pE<sub>a</sub>-CD1d mice, CD4 T cells remained at the residual background level observed in I-A<sup>b</sup>/H<sub>9252</sub>b/CD1d doubly deficient mice. Finally, there was no increase in the rare DN T cell subset either upon pE<sub>a</sub>-CD1d expression. Altogether, these results demonstrate that expression according to a classical MHC pattern does not confer to CD1d the property to support the development of a diverse and naive T cell repertoire. These surprising findings suggest a fundamental difference between the CD1d and MHC systems, i.e., that, unlike MHC molecules, CD1d molecules may be intrinsically unable to support the development of a diverse naive T cell population. We envision two nonexclusive explanations for this intriguing observation. One possibility is that the lipid ligand repertoire displayed by CD1d in the thymus is highly restricted and cannot support the development of a diverse TCR repertoire. However, although knowledge of the lipid repertoire naturally bound to CD1d is still limited, several reports suggest that a diverse repertoire of endogenous and exogenous lipids is likely to be found associated with CD1d on the cell surface (reviewed in Refs. 27 and 28). The other possibility is that the TCR gene repertoire is intrinsically deficient in CD1d-restricted combinations. We favor this possibility because it seems logical, from an evolutionary point of view, that the majority of the TCR gene pool evolved to recognize MHC-peptide rather than CD1d-lipid complexes, since microbial peptides can mutate at a high rate while lipid changes are rare and often deleterious for bacteria. In addition, we have previously shown that the MHC-restricted repertoire is intrinsically poorly cross-reactive to CD1d (2). Together, these observations

![FIGURE 9. pEa-CD1d transgene expression does not allow for CD4 or CD8 thymocyte selection in MHC/CD1d-deficient mice. Thymic cell suspensions of indicated mice were four-color stained to measure the frequencies of CD4 and CD8 T cells (percent shown in right panels) among mature HSA(CD24<sup>hi</sup>T<sub>CR<sup>lo</sup></sub>hight) thymocytes gated as shown in left panels. Note that pEa-CD1d indicates pEa-driven (transgenic only) expression of CD1d (endogenous CD1d genes are homozygous KO). Results representative of three experiments examining a total of three to six mice per group.](image-url)

![FIGURE 10. pEa-CD1d transgene expression does not increase the frequency of residual CD4 or CD8 T cells in peripheral tissues of MHC/CD1d-deficient mice. A, CD3<sup>hi</sup>CD4<sup>hi</sup>T cells (frequency indicated in the upper right quadrants of each dot plots) were stained in the spleen, peripheral lymph node, and liver of wild type (WT), I-A<sup>b</sup>/CD1d KO, and I-A<sup>b</sup> KO/pEa-CD1d mice. Note that pEa-CD1d indicates pEa-driven (transgenic only) expression of CD1d (endogenous CD1d genes are homozygous KO). B, CD3<sup>hi</sup>CD4<sup>-</sup>T cells in KbD<sup>b</sup> CD1d KO and KbD<sup>b</sup> KO/pEa-CD1d mice. Results representative of three experiments examining a total of three to six mice per group.](image-url)
might suggest that the MHC- and CD1d-restricted repertoires have evolved under radically different selection pressures and do not overlap. The CD1d-restricted repertoire might be limited and display memory-like features ensuring rapid and forceful innate-type responses, whereas the MHC-restricted repertoire is broad and adaptive.

Our findings should be considered in the broader context of T cell development mediated by the various classical and nonclassical MHC and MHC-like molecules. The emerging picture is that many more T cell developmental pathways exist than previously thought based on the early studies of classical CD4 and CD8 T cells. Several nonclassical MHC or MHC-like molecules such as Qa1, H2-M3, and MR1 are also associated with unusual T cell subsets that are dominated by a memory rather than naïve phenotype (13–16). In all cases, the development of such memory-type cells is mainly supported by bone marrow-derived thymic cells. It is possible that selection on bone marrow-derived cells might influence thymocytes engaged in low avidity or nonagonist interactions and only rescue those recognizing ligands with higher avidity or agonist properties. The memory phenotype might therefore be imparted by the specificity (i.e., autoreactivity) of the TCR rather than by the restricting element. Bone marrow-derived cells might also support the development of naive type cells, as reported in the case of one MHC-restricted TCR (29). However, because classical MHC molecules are absent or very poorly expressed in cortical thymocytes, the main bone marrow-derived cell type in the cortex, it is difficult at present to assess quantitatively or qualitatively the developmental pathways that they might support if they were abundantly expressed in the bone marrow-derived compartment, e.g., with a CD1d-like pattern. Interestingly, recent studies indicate that epithelial cells could also support the development of memory-type cells, the so-called CD25 T regulatory cells (11). Future studies aiming at unraveling the molecular and cellular basis of these intriguing new developmental pathways should shed light not only on fundamental aspects of T cell development, but also on the functions and evolutionary roles of the various MHC and MHC-like pathways of Ag presentation and their corresponding TCR repertoires.

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