Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes

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Unlike conventional major histocompatibility complex–restricted T cells, Vα14–Jα18 NKT cell lineage precursors engage in cognate interactions with CD1d-expressing bone marrow–derived cells that are both necessary and sufficient for their thymic selection and differentiation, but the nature and sequence of these interactions remain partially understood. After positive selection mediated by CD1d-expressing cortical thymocytes, the mature NKT cell lineage undergoes a series of changes suggesting antigen priming by a professional antigen–presenting cell, including extensive cell division, acquisition of a memory phenotype, the ability to produce interleukin-4 and interferon-γ, and the expression of a panoply of NK receptors. By using a combined transgenic and chimeric approach to restrict CD1d expression to cortical thymocytes and to prevent expression on other hematopoietic cell types such as dendritic cells, macrophages, or B cells, we found that, to a large extent, expansion and differentiation events could be imparted by a single-cognate interaction with CD1d-expressing cortical thymocytes. These surprising findings suggest that, unlike thymic epithelial cells, cortical thymocytes can provide unexpected, cell type–specific signals leading to lineage expansion and NKT cell differentiation.

CD1d-restricted mouse Vα14-Jα18/Vβ8 and human Vα24-Jα18/Vβ11 NKT cells are innatelike autoreactive lymphocytes that regulate a number of infectious, autoimmune, and cancerous conditions (1–3). Their development differs strikingly from that of conventional MHC-restricted CD4 and CD8 T cells. Although the thymic precursors to both NKT cells and conventional T cells require interaction with their respective self ligands in the cortex for positive selection, the mature heat stable antigen (HSA)low NKT cells subsequently undergo a complex series of activation and differentiation events. These events include multiple rounds of cell division leading to massive lineage expansion and acquisition of the CD44high memory phenotype before migration to the periphery, sequential activation of the IL-4 and IFN-γ gene loci, and expression of NK lineage markers such as NK1.1, NKG2D, Ly49, and CD94/NKG2 over the course of several weeks (4, 5). Because the NKT cell fate is imparted by the Vα14-Jα18/Vβ8 TCR, (6), it is believed that differential signaling induces the sequential changes leading to full NKT cell differentiation. Indeed, rather than the antagonist/partial agonist ligands that select conventional T cells, an agonist self-glycosphingolipid, iGb3, is recognized by mouse and human NKT cells (7). Expression of dominant negative forms of Ras and Mek1, although abolishing conventional T cell development, does not impair NKT cells (8). Conversely, Fyn (9, 10) and NF-κB (11–13) are specifically required for NKT cells but not for conventional T cells. Finally, BM chimera experiments have demonstrated that, in contrast with MHC for conventional T cells, expression of CD1d on the BM-derived rather than epithelial cell compartments is necessary and sufficient for NKT cell development (14–17). These radical differences have suggested a model whereby agonist ligand signaling in conjunction with uncharacterized accessory signals emanating from BM-derived cell types...
direct NKT cell positive selection and subsequent expansion, differentiation, and survival. Like the NK cell, the final NKT cell product balances TCR autoreactivity with inhibitory signaling by NK receptors (18–20).

The BM-derived cell types involved in NKT cell development have not been directly identified. However, the autoreactivity of NKT cells and the orchestrated series of changes associated with their passage through different compartments of the thymus and periphery have suggested that sequential TCR interactions with a variety of CD1d-expressing cells continue to drive their differentiation. In particular, it is thought that the massive expansion initiated after positive selection, at the HSA<sup>low</sup>/CD44<sup>low</sup> stage, would require interaction with professional APCs in the medulla (4, 21); that the sequential expression of inhibitory NK receptors over several weeks might also require TCR signaling to select appropriate inhibitory receptors that quench TCR autoreactivity (17, 19, 20, 22); and, although CD1d is not required for survival of terminally differentiated NKT cells (23), that the effector status of NKT cells suggested by their explosive cytokine release upon TCR stimulation (24) and their basal low-level transcription of cytokine genes (25) must be maintained by repeated TCR engagement. Mixed BM chimeras using β<sub>2</sub> microglobulin KO BM cells as NKT cell precursors and T cell developmental stage mutant BM cells as CD1d/APCs have revealed a requirement for a cell type present in TCRα- but not TCRβ-deficient thymus, most likely the double-positive (DP) cortical thymocytes (17). Consistent with this finding, DP thymocytes highly express CD1d, and they directly stimulate autoreactive Vα14<sup>+</sup> NKT cell hybridomas (26). However, these chimeras still harbored many other CD1d-expressing cell types, such as DCs, B cells, and macrophages, whose contribution could not be tested. Thus, the issue as to which of these other cell types would control different aspects of NKT cell differentiation, particularly those happening days or weeks after emigration from the cortex into the medulla and into the periphery, has remained unanswered.

CD1d is ubiquitously expressed in the thymus and found in cortical and medullary epithelial cells, macrophages, and DCs, but also is conspicuously expressed at high levels in DP cortical thymocytes and at lower levels on mature T cells (27). Recent experiments have shown that mice expressing CD1d under the control of an MHC class I or MHC class II promoter failed to support NKT cell development, consistent with a requirement of CD1d expression by cortical thymocytes (27). Here, we generated transgenic and chimeric mutant mice expressing CD1d under the proximal Lck promoter to test directly the contribution of the expression of CD1d on cortical thymocytes to NKT cell development. Surprisingly, our results indicate that CD1d expression on cortical thymocytes is not only required but is, in fact, sufficient to induce massive lineage expansion and distinct differentiation and survival events long after emigration from the cortex into the medulla and the periphery. Other peripheral CD1d-expressing cell types, although not essential, seemed to enhance the differentiation process leading up to NK differentiation; this observation suggests some functional influences of prolonged TCR interactions.

Figure 1. CD1d expression and NKT cells in pLck-CD1d mice. (A) Transgenic construct for expression of CD1d cDNA under the control of the proximal Lck promoter. CD1d cDNA was inserted via the EcoRI site located in the second exon of the β-globin expression cassette. Black boxes, exons; thin lines, introns; gray box, promoter. The construct was excised via Spel and Xhol sites from the bacterial vector, purified, and injected into fertilized eggs. (B) CD1d (red) versus I-Ab (green) expression in frozen thymic sections of three different lines of pLck-CD1d mice, pLck/low, pLck/high, and pLck/var, compared with CD1d Het (CD1d<sup>+/−</sup>) and CD1d KO (CD1d<sup>−/−</sup>) littermate controls. Upper four rows are mice in the NODxC57BL/6 F1 background; bottom two rows are in the C57BL/6 background. A thin white line delineates the boundary between cortex (c) and medulla (m). Note the variegated pattern of CD1d expression in pLck/var. (C) Staining of total thymocytes and splenocytes with CD1d tetramers loaded with α-GalCer (L-Tet, y-axis) versus anti-B220 + unloaded CD1d tetramers (UL-Tet + B220, x-axis). Percentages of NKT cells ± SD, compiled from five experiments and five to eight mice per line, are shown in upper left quadrants.
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RESULTS

\textit{pLck-CD1d} mice

To restrict CD1d expression on the T cell lineage, we constructed transgenic vector using the proximal \textit{Lck} promoter \citep{29} to drive CD1d cDNA in a \(\beta\)-globin expression cassette (Fig. 1 A). Five founders were established by injection into fertilized oocytes of inbred B6 (\(n = 2\)) or nonobese diabetic (NOD) strains (\(n = 3\)). The B6 founders subsequently were bred to the B6.CD1d KO background, and the NOD founders were bred to NOD.CD1d KO mice followed by a cross to B6.CD1d KO mice to obtain (NODxB6) F1.CD1d KO mice. The transgenic lines in the CD1d homozygous KO background are thereafter termed \textit{pLck-CD1d} mice. For in-depth analysis of CD1d expression, we selected a \textit{pLck/low} and a \textit{pLck/high} as low and high expressors, respectively, as well as a \textit{pLck/var} exhibiting a variegated pattern of expression on \(\sim 10\% - 20\%\) of thymocytes. All \textit{pLck-CD1d} lines showed faithful expression of CD1d in the T cell lineage, i.e., cortical thymocytes and mature T cells. Examples of expression pattern in \textit{pLck-CD1d} mice are shown in Fig. 1 B and Fig. 2 and are summarized in Table I. Our breeding scheme allowed each transgenic mouse to be analyzed side by side with a nontransgenic \textit{CD1d} het littermate for optimal comparison purposes.

In thymic frozen sections (Fig. 1 B), \textit{CD1d} het controls showed broad expression of CD1d (red) not only on all cortical thymocytes, but also on all MHC class II I-A\(^{b}\) cells (green), including cortical and medullary epithelial cells, CD11c\(^{+}\) DCs, and CD11b\(^{+}\) macrophages, as previously shown \citep{27}. In contrast, \textit{pLck/low}, \textit{pLck/high}, and \textit{pLck/var} expressed CD1d on cortical and, to a lesser extent, medullary thymocytes, but not on any I-A\(^{b}\) cells.

A more quantitative assessment of CD1d expression by different cell types was performed using flow cytometry (summarized in Table I for \textit{pLck/low} and \textit{pLck/high} and shown in Fig. 2 for \textit{pLck/var}). Although CD1d expression was restricted absolutely to the T cell lineage, both in the thymus and periphery, the level of CD1d expression varied in different transgenic lines, ranging from five to 20 times the level expressed by \textit{CD1d} het littermate mice. The \textit{pLck-CD1d} transgenic mice exhibited a marked drop in CD1d expres-

\begin{table}
\centering
\caption{CD1d expression levels in splenic and thymic subsets of \textit{pLck-CD1d} mice}
\begin{tabular}{|l|l|l|l|l|l|l|l|}
\hline
 & \textbf{Thymus} & & & & \textbf{Spleen} & & \\
 & DN & DP & CD4 & CD8 & CD4 & CD8 & B & DC & M\Phi \\
\hline
\textit{CD1d} Het & 182 & 125 & 109 & 82 & 97 & 74 & 178 & 129 & 79 \\
\textit{CD1d} KO & 12 & 17 & 15 & 13 & 19 & 8 & 8 & 5 & 5 \\
\textit{pLck/low} & 437 & 563 & 87 & 57 & 78 & 60 & 5 & 4 & 7 \\
\textit{pLck/high} & 3,476 & 3,038 & 1,384 & 1,197 & 1,154 & 918 & 8 & 5 & 8 \\
\hline
\end{tabular}
\end{table}

Mean fluorescence intensity is shown, averaged from three independent analyses of one or two mice per transgenic line.
sion between the immature cortical DP and the single-positive mature T cell stage, as expected.

**NKT cell selection, expansion, and differentiation in pLck-CD1d mice**

Next, we examined the NKT cell developmental stages in the pLck-CD1d mice using flow cytometry with various stage-specific markers and α-galactosylceramide (α-GalCer)-loaded CD1d tetramers specific for the canonical Vα14-Jα18 TCR. Remarkably, all lines of pLck-CD1d mice expressed substantial percentages of NKT cells (Fig. 1 C). Despite their reduced proportion of DP thymocytes expressing CD1d, the pLck/var mice (C57BL/6 background) expressed the same frequencies and absolute numbers of NKT

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**Figure 3. NKT cell phenotype in pLck/var mice.** (A) TCR Vβ repertoire analysis on gated CD3e⁺CD1d-α-GalCer tetramer⁺ NKT cells and mainstream CD3e⁺ T cells in the spleen of pLck/var and CD1d Het littermate controls, as indicated. Results shown as mean ± SD of data collected from nine mice in two independent experiments. (B) CD44/NK1.1 and CD4/CD8 staining of NKT cells in thymus, spleen, and liver of pLck/var mice and CD1d Het controls. Percentages and SD from 23 mice in seven experiments are indicated. *, P < 0.05 by unpaired Student’s t test. (C) Analysis of NK and activation/memory markers expressed by gated CD1d-α-GalCer tetramer⁺ NKT cells of pLck/var (red) versus littermate CD1d Het control (green). Results representative of six pairs of pLck/var transgenic and WT littermates. Similar results were found for pLck/low mice (data not depicted).
cells in the thymus and spleen as CD1d+/– littermate controls at the adult age (Fig. 1 C) as well at the younger ages of 2 and 4 wk (unpublished data). In the C57BL/6 background, CD1d+/– and CD1d+/+ mice have the same number of NKT cells (unpublished data). The pLck/low and pLck/high mice (NODxC57BL/6 F1) expressed 1.5 to three times more NKT cells than CD1d+/+ controls (Fig. 1 C), or about the same as CD1d+/+ mice in the NODxC57BL/6 background (unpublished data). Similar results were found for liver lymphocyte samples (unpublished data).

NKT cells in pLck/var mice expressed the same Vβ repertoire composed of three families, Vβ8>Vβ7>Vβ2 (Fig. 3 A), and passed through the same CD44low, CD44high, and NK1.1+ stages as WT mice with no difference in their double-negative (DN)/CD4 ratio (Fig. 3 B). However, one consistent defect observed in the three pLck-CD1d transgenic lines examined was an ~30% lower ratio of NK1.1+/NK1.1− cells in peripheral tissues (spleen and liver) (Fig. 3 B and unpublished data). These mature NK1.1+ cells, however, expressed the whole complement of NK lineage receptors examined, with intensity and frequency similar to those seen in WT mice (Fig. 3 C), including NKG2D, Ly49A, C/I, G2, CD94, NKG2A/C/E, and other characteristic markers such as DX5, CD44, CD62L, CD69, and CD122.

We next measured the rate of cell divisions previously reported at the HSA lowNK1.1− stage of NKT cell thymic development. 4 hr after an i.p. injection of 1 mg bromodeoxyuridine (BrdU), 11–12% of NK1.1− thymic NKT cells were labeled in WT, as in pLck/var mice (Fig. 4), demonstrating that the massive lineage expansion that is a hallmark of this NKT cell thymic stage occurred normally.

Cell-intrinsic expression of CD1d enhances positive selection of NKT cell precursors

Further analysis of pLck/var mice with a variegated pattern of transgene expression provided an unexpected clue to the ongoing interactions between the NKT cell precursors and CD1d-expressing cells. We made the surprising observation that the frequency of CD1d expression among positively selected HSAlowCD44low NKT cells (40.1%) was twice higher than expected from the frequency among DP thymocytes (18.6%) (Fig. 5 A). This increased frequency was particularly evident at the early HSAlowCD44low NKT cell stage after positive selection from the HSAhigh stage, when pLck-driven CD1d expression is not yet extinguished. This surprising but consistent result might suggest that developing NKT cells use their own CD1d to enhance positive selection. To further evaluate the general significance of this unexpected result, we generated mixed WT + CD1d KO into CD1d KO radiation BM chimeras in which CD1d expression was confined to a fraction of thymocytes. Here again, the proportion of NKT cells expressing CD1d was approximately twice that of DP or conventional T cells (Fig. 5 B). Thus, the data suggest that, although not essential, intrinsic expression of CD1d significantly enhances the selection and development of NKT cell precursors.

CD1d-expressing DP thymocytes are sufficient for positive selection of NKT cells

Although the quasinormal selection, differentiation, and maturation of NKT cells in pLck-CD1d mice rules out an essen-

Figure 4. BrdU incorporation by NKT cell subsets in vivo. 4 h after i.v. injection of BrdU, pLck/var and control CD1d Het showed similar frequency of BrdU+ cells among CD1d-αGalCer tetramer−α NKT cell subsets. Similar results were found in three different experiments. Percentages of BrdU+ cells are shown in top right quadrants.

Figure 5. CD1d expression autonomously enhances NKT cell development. [A] Frequency of CD1d expression is higher among NKT cells of pLck/var mice than among total thymocytes. The difference is particularly visible at the CD44low NKT stage before extinction of the pLck promoter. Frequencies are indicated as mean ± SD of three mice. [B] In mixed WT + CD1dKO BM radiation chimeras, a higher proportion of NKT cells are CD1d-positive than expected from analysis of the DP and conventional mature CD4 and CD8 T cells. Data are representative of three individual chimeras.
In order to investigate whether transgenic expression of CD1d on mature T cells inside and outside the thymus contributed to NKT cell development, it was important to abrogate this residual expression of CD1d in the thymic medulla and in the periphery. To achieve this, mixed BM radiations chimeras were generated using a mixture of pLck/low.Cα KO BM (as antigen-presenting component, unable to generate T cells) and CD1d KO BM (as responding component, generating the T cell but unable to present antigen). The hosts were irradiated CD1d KO mice. In these chimeras, therefore, CD1d was expressed only on cortical thymocytes that were themselves unable to mature because of the Cα mutation, whereas NKT cells could only arise from cells that were themselves CD1d KO. Surprisingly, NKT cells were quasinormally generated in such chimeras by comparison with control CD1d KO + Cα KO mixed chimeras (Fig. 6).

NKT cells were identical to controls in terms of numbers, Vβ repertoire, and DN/CD4 ratio, as well as pattern of NK receptor expression (unpublished data), although the proportion of NK-lineage receptor-expressing cells was substantially decreased in the periphery (Fig. 6). Similar results were observed using pLck/var.Cα KO cells instead of pLck/low.Cα KO BM cells (unpublished data). Thus, these mixed chimeras firmly establish that, even with CD1d expression strictly confined to the thymic cortex, NKT cell development proceeds through the sequential stages leading to the final NKT cell product weeks after emigration to the periphery. The partial defect in peripheral NK1.1 acquisition previously noted in the pLck-CD1d mice as compared with WT mice was observed again when compared with control chimeras. In fact, in these mixed chimeras, the relative defect tended to be even more severe (50% reduction) than when pLck-CD1d mice were compared with WT littermates (30% reduction), possibly because of the more restricted distribution of CD1d expression.

NKT cell responses in pLck-CD1d mice

To study the functional properties of NKT cells developing in pLck-CD1d mice, in which CD1d expression by peripheral APCs is absent, we first injected DCs loaded with the agonist NKT cell ligand α-GalCer intravenously and measured cytokines released in the sera. Peak IFN-γ secretion not only was preserved but was, in fact, consistently three to four times higher in the two pLck-CD1d lines examined, pLck/var and pLck/low, than in WT cells (Fig. 7 A). IL-4 was not detected in this assay. However, the early IL-4 release induced by anti-CD3ε i.v. injection, a characteristic functional property of effector-stage NKT cells (24), was unimpaired and perhaps was increased slightly in pLck-CD1d mice compared with WT littermate controls (Fig. 7 B). In an in vitro assay combining sorted splenic CD1d-α-GalCer tetramer+NK1.1+ NKT cells and α-GalCer-pulsed DCs, NKT cells from pLck-CD1d mice secreted both IFN-γ and IL-4, again significantly more than WT cells (Fig. 7 C).
We next crossed the pLck/var mouse with the Eα/CD1d transgenic mouse, which expresses CD1d exclusively under the control of the MHC class II Eα promoter. The Eα/CD1d transgenic mouse previously was shown to express CD1d faithfully according to the MHC class II pattern, i.e., in the thymic epithelium as well as in thymus and peripheral APCs, including DCs, macrophages, and B cells (27). Together, the pLck and Eα promoters reconstitute the natural distribution of CD1d (with the exception of hepatocytes). Strikingly, the splenic NKT cell responses in these double-pLck/var.Eα/CD1d transgenic mice were normalized, comparable to WT B6 mice (Fig. 7 C). In addition, the relative defect in the acquisition of NK receptors by peripheral NKT cells was corrected (Fig. 7 D), demonstrating the concomitant influence of peripheral CD1d-expressing APCs in promoting the terminal differentiation. Similar findings were observed when the pLck/var mice were crossed with WT mice (Fig. 7, C and D), further demonstrating that the altered tissue expression pattern of CD1d, restricted to T cells, accounted for the relative decrease in terminal NKT cell differentiation and concomitant increase in cytokine secretion.

DISCUSSION

In an attempt to account for the complex, orchestrated sequence of thymic and peripheral events leading to the natural NKT cell differentiation in response to self-agonist self ligands such as iGb3, this study focused on elucidating the nature of the CD1d-expressing cell types involved at different stages of the process, from positive selection in the thymic cortex to clonal expansion and cytokine gene locus activation in the medulla and to the unique expression of the NK program in the periphery. Previous studies had indicated that the entire sequence of events could be directed by CD1d-expressing BM-derived cells (14–17), that CD1d-expressing DP thymocytes were required for the process (17), and that DCs transgenically overexpressing CD1d could induce negative selection (21). Thus, although the survival of terminally differentiated NKT cells was shown to be independent of CD1d (23), it was logically anticipated that the different stages of NKT cell development and differentiation occurring after positive selection and leading to the terminal NKT cell stage would require additional interactions between the NKT cell, autoreactive TCR, and agonist iGb3 or other ligands in different cell types of the thymus and periphery.
The present findings, however, clearly establish that, to a large extent, a single, temporo-spatially restricted TCR interaction between the immature Vα14+ T cell precursor and CD1d-expressing DP T cells in the thymic cortex is sufficient to induce the whole sequence of distinct events that characterize NKT cell differentiation, even weeks after immigration to the periphery. Careful analysis by flow cytometry and tissue immunohistochemistry of the pattern of CD1d expression in the pLck-CD1d transgenic mice excluded cryptic or aberrant expression of CD1d. Furthermore, because analysis of the pLck−/−ar mouse demonstrated that expression of CD1d by the developing NKT cell itself could have a substantial impact on its differentiation, we generated mixed chimeras in which we could observe the development of CD1d KO thymocytes in response to pLck-CD1d BM cells developmentally arrested at the DP stage. In this experimental set up, in which CD1d could be presented only in trans by cortical thymocytes, NKT cells were selected, amplified, and differentiated largely as in WT. Thus, we conclude that the transient interaction of the immature Vα14+ thymocyte with CD1d expressed by neighboring cortical thymocytes is sufficient to switch on the long-range expansion and differentiation program that characterizes the NKT cell lineage.

This conclusion is surprising for several reasons. First, TCR engagement in the cortex has not been shown previously to induce entry into cell cycle, nor do DP thymocytes express the B7 costimulatory molecules normally required to induce naive T cell expansion. Second, because CD1d expression was largely extinguished in the periphery of pLck-CD1d mice and completely absent in the mixed chimeras, how did NKT cells, unlike other T cells (30), survive and maintain an effector status in the absence of TCR signals? Perhaps the induction of a memory phenotype and expression of the IL−15 receptor CD122 chain in the thymus, before emigration, constitutes the solution to this enigma, because memory cells expressing IL−15 receptor are largely independent of TCR engagement for survival (23). Third, if NK receptors serve the purpose of balancing TCR autoreactivity (18), how could they be selected in the absence of TCR signals? Our findings indicate that the genetic program of NK lineage differentiation, although it unfolds after emigration to the periphery, is activated in the thymus. Although we found no changes in the expression of the individual NK receptors examined, others may be altered, and it is possible that more NKT cells have mismatched NK receptors in pLck-CD1d mice than in WT mice, explaining their greater responses to TCR stimuli. This greater mismatch might explain the tendency of NKT cells from pLck-CD1d mice to have higher TCR reactivity than their WT counterparts, an appealing possibility that could not be tested in this transgenic system. Alternatively, inhibitory receptors may selected based on their ability to antagonize activating NK receptors such as NK1.1 and NKG2D. This plasticity in the developmental scheme may also reflect plasticity in functional activation, because evidence suggests that NKT cells can suppress type I diabetes in a CD1d-independent manner (unpublished data).

Interestingly, although the transition to the NK stage was preserved in the pLck-CD1d mice, it seemed to be somewhat less efficient than in WT mice. This observation was confirmed further in the mixed BM chimeras, an experimental system in which CD1d could be restricted to a subset of developmentally arrested Cα KO thymocytes. Together with the higher reactivity of NKT cells in these mice, these observations suggest that, in WT mice, additional interactions with other types of CD1d-expressing cells may shape further some of their functional properties and terminal differentiation. Although one cannot rule out the possibility that these changes might be related in part to the transgenic system, our results indicated that both the hyperreactivity and the delayed NK receptor expression were corrected after introduction of the Eα-CD1d transgene (driving CD1d with an MHC class II promoter) or the WT CD1d in these pLck-CD1d mice. These findings support the notion that additional interactions with CD1d-expressing APCs fine-tune NKT cell development and function. The mechanisms involved in these tuning events remain to be elucidated.

Agonist-driven thymocyte development has been reported in a number of transgenic systems (31–37), resulting in altered differentiation programs leading to the NKT cell, CD4+CD25+, or CD8αβ lineages with various memory/effector phenotypes. Although both the epithelial and the BM-derived compartments of the thymus could support the differentiation of such autoreactive cells, the specific nature of the signaling events involved has remained elusive. The crucial role of Fyn in Vα14 NKT cell development (9, 10) is particularly intriguing in light of the recent discovery of its upstream connections to signaling lymphocytic activation molecule (SLAM)–associated protein (SAP) and SLAM-type molecules and downstream activation of NF-κB (38, 39). Indeed, NF-κB mutant mice exhibit major NKT cell developmental defects (11–13), and we have found that SAP-deficient mice, like Fyn KO mice, also completely lacked NKT cells (unpublished data). SLAM-type molecules and SAP are prominently expressed by cortical thymocytes (40), suggesting that homophilic interactions between SLAM family members expressed by developing Vα14+ T cells and their fellow CD1d-expressing DP thymocytes might be essential to NKT cell development and differentiation, providing the costimulation needed for expansion and perhaps also for rescue from negative selection. It is striking that, unlike CD1d-expressing DP thymocytes, MHC class I molecules are transiently down-regulated during the DP stage, both in mouse and human (41). It is intriguing, therefore, to speculate that these conspicuous differences underlie fundamentally different pathways of T cell development for the recognition of different categories of antigens, i.e., peptides versus lipids. Future experiments are in progress to test the hypothesis that restricted expression of MHC class I on DP thymocytes may lead to the development of MHC class I–specific NK-type T cells.
MATERIALS AND METHODS

Mice. CD1d KO mice were bred for more than 14 generations onto either NOD or C57BL/6 background in our laboratory. C57BL/6 mice and TCR CX-KO mice in the C57BL/6 background were purchased from Jackson ImmunoResearch Laboratories. C57BL/6 Eta-CD1d mice expressing a CD1d transgene driven by the MHC II Eta promoter in a CD1d KO background were previously described (27). To generate pLlv-CD1d Tg mice, we replaced the Eta promoter in the previously described Eta-CD1d construct with the proximal Lk promoter (29) excised (NotI and BamHI) from the p1017 transgenic expression cassette (provided by Nigel Killeen, University of California, San Francisco), as described in Fig. 1 A. This construct contains exons, introns, and poly-adenylation sequence for optimal expression. The linearized (SpeI and XhoI) construct was injected into fertilized C57BL/6 oocytes and into fertilized NOD oocytes, and the injected oocytes were implanted into pseudopregnant CD1- (VAF+) outbred females. Transgenic mice were screened using PCR (forward primer: 5′-TCTCTGTGAACTTGGTGCTTGAG-3′; reverse primer: 5′-CCAAAT-GATGAGACAGACACAC-3′) and were bred to C57BL/6. CD1d KO or NOD.CD1d KO to obtain pLlv-CD1d mice expressing the pLlv-CD1d transgene in the C57BL/6/CD1d KO (α = 2) or to NOD.CD1d KO (α = 3) background, respectively. Because the NOD strain has a partial, recessive defect in NKT cell development (42), the NOD.pLlv-CD1d mice were further crossed with C57BL/6. CD1d KO mice to generate NODxC57BL/6 F1.pLlv-CD1d mice. All mice were housed in a specific pathogen-free facility at the University of Chicago according to the guidelines of the institutional Animal Care and Use Committee.

Flow cytometry. Fluorochrome-conjugated mAbs anti-CD1d, CD3ε, CD4, CD8a, CD11c, CD11b, CD24 (HSA), CD44, CD14, CD62L, CD69, CD102, DX5, Gr-1, Ly49A, Ly49C/I, Ly49D2, NKG2A/C/E, NK1.1, TCR, VB2, VB6, VB7, and VB8.1+8.2, were purchased from BD Biosciences. Anti-F4/80, B220, NKGD2 mAbs, and streptavidin conjugated with allophycocyanin or R-phycoerythrin were purchased from Biolegend. Anti-F4/80, B220, NKG2D mAbs, and streptavidin conjugated with allophycocyanin or R-phycoerythrin were purchased from eBioscience. Anti-I-Aa mAb (clone Y3P) was conjugated to FITC in our laboratory. CD1d tetramers loaded with α-GalCer (100 ng/ml overnight before washing and culture at 10⁵ per well with titrated numbers of NKT cells in 96 flat-bottom microwell plates (Corning Costar Corporation). Stimulation assays were incubated for 48 h before assaying IL-4 and IFN-γ release with OptEIA ELISA sets (BD Biosciences) according to manufacturer’s instructions. For in vivo experiments, mice were injected i.v. with 5 × 10⁵ α-GalCer-pulsed DCs in PBS. The spleen fragment assay measuring IL-4 release in vitro after 1.25 μg anti-CD3ε injection in vivo was performed as described previously (24).

Radiation BM chimeras. C57BL/6. CD1d KO mice were irradiated (900 rad) 2 h before BM reconstitution. BM cells were harvested from mouse femurs and depleted of T cells with FITC-conjugated anti-CD3ε mAb and anti-FITC magnetic microbeads on AutoMACS (Miltenyi Biotec). BM cells from various donor combinations were mixed in a 1:1 ratio, and a dose of 10⁶ mixed BM cells was injected i.v. Chimeras were analyzed 6–8 wk after injection.

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