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

Datsen G. Wei,[1,2] Hyunji Lee,[3] Se-Ho Park,[3] Lucie Beaudoin,[4] Luc Teyton,[5] Agnès Lehuen,[4] and Albert Bendelac[1]

[1]Committee on Immunology, University of Chicago, Chicago, IL 60637
[2]Department of Molecular Biology, Princeton University, Princeton, NJ 08544
[3]School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, South Korea
[4]Institut National de la Santé et de la Recherche Médicale U561, Hospital Cochin-Saint Vincent de Paul, 75014, Paris, France
[5]Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037

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.

CORRESPONDENCE
Albert Bendelac:
abendela@bsd.uchicago.edu

Abbreviations used: α-GalCer, α-galactosylceramide; BrdU, bromodeoxyuridine; DN, double-negative; DP, double-positive; HSA, heat stable antigen; NOD, nonobese diabetic; SAP, SLAM-associated protein; SLAM, signaling lymphocyte activation molecule.

CD1d-restricted mouse Vα14-Jα18/Vβ8 and human Vα24-Jα18/Vβ11 NKT cells are innate-like 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 $CD44^{high}$ 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

The Journal of Experimental Medicine

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 $HSA^{low}CD44^{low}$ 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_2 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 $V\alpha14^+$ 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, 28). 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.

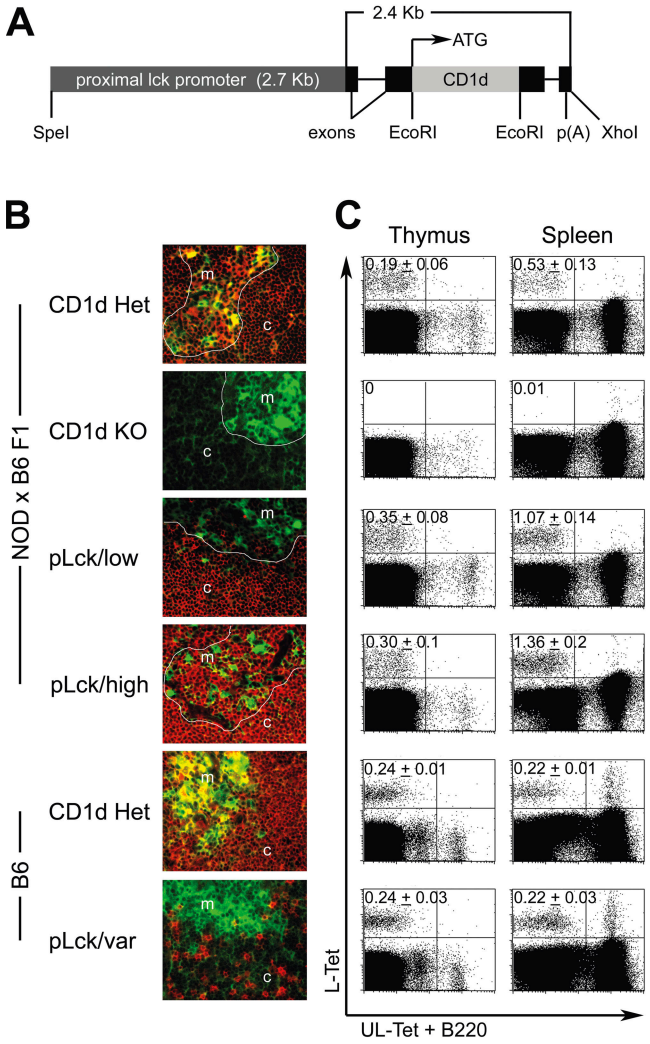

Figure 1. CD1d expression and NKT cells in *pLck-CD1d* mice. (A) Transgenic construct for expression of CD1d cDNA under the control of the proximal *Lck* promoter. CD1d cDNA was inserted via the EcoRI site located in the second exon of the β-globin expression cassette. Black boxes, exons; thin lines, introns; gray box, promoter. The construct was excised via SpeI and XhoI sites from the bacterial vector, purified, and injected into fertilized eggs. (B) CD1d (red) versus $I\text{-}A^b$ (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* ($CD1d^{+/-}$) and *CD1d* KO ($CD1d^{-/-}$) 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.

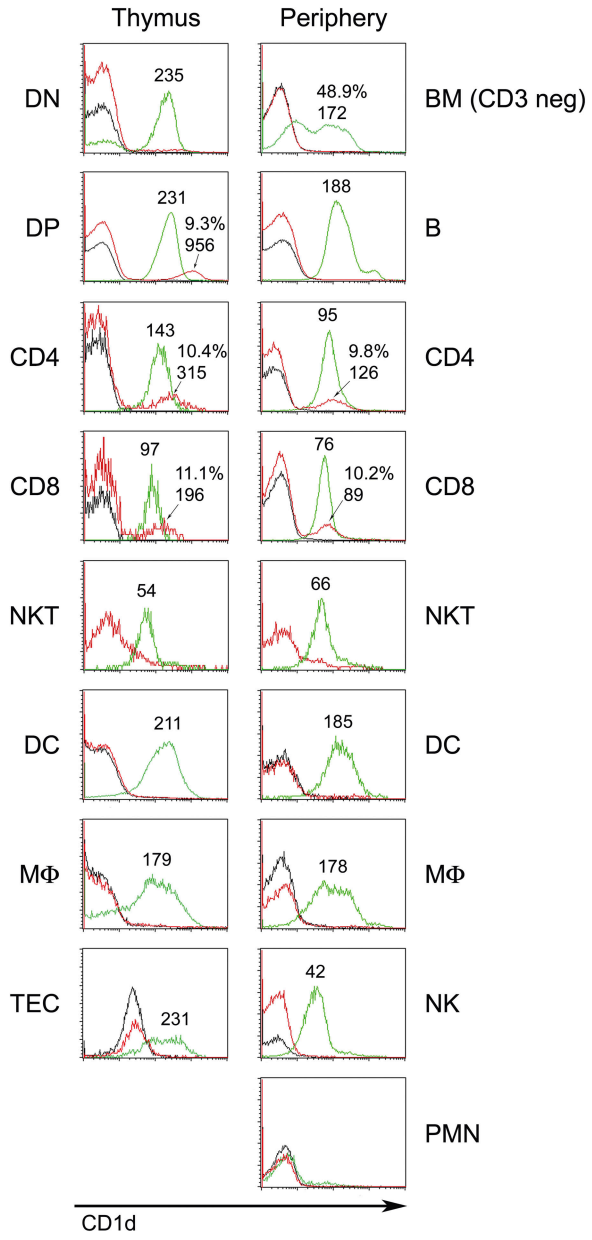

Figure 2. Pattern of CD1d expression in *pLck/var* mice. CD1d expression on gated thymic (left column) and peripheral (right column) subsets shown in green for WT, red for *pLck/var*, and black for *CD1d* KO. Percentages and mean fluorescence intensity of $CD1d^+$ cells are indicated above fluorescence peaks. Results are representative of three independent experiments, and more than three mice are analyzed for each cell type. (left) DN, $CD4^-8^-$ DN and $CD3\varepsilon^-$; DP, $CD4^+8^+$. CD4 and CD8 are mature HSA^{low} single-positive cells. NKT are $NK1.1^+CD3\varepsilon^+$ cells. Thymic dendritic cells (DC) were enriched with anti-CD11c magnetic microbeads and further gated on $CD11c^+I\text{-}A^{b+}$ for analysis. Thymic macrophages (MΦ) were enriched and gated similarly with CD11b; thymic epithelial cells (TEC) were enriched by depleting hematopoietic cells with anti-CD45 magnetic microbeads, and gating on $CD45^-I\text{-}A^{b+}$ cells. (right) BM, $CD3\varepsilon^-$ BM cells. All other subsets are gated from splenic preparations, except PMN, $Gr\text{-}1^+CD3\varepsilon^-$ polymorphonuclears from peripheral blood.

RESULTS

pLck-CD1d mice

To restrict CD1d expression on the T cell lineage, we constructed transgenic vector using the proximal *Lck* promoter (29) to drive CD1d cDNA in a β-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 *pLck-CD1d* mice. For in-depth analysis of CD1d expression, we selected a *pLck/low* and a *pLck/high* as low and high expressors, respectively, as well as a *pLck/var* exhibiting a variegated pattern of expression on ~10–20% of thymocytes. All *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 *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 $CD1d^{+/-}$ (*CD1d Het*) littermate for optimal comparison purposes.

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

A more quantitative assessment of CD1d expression by different cell types was performed using flow cytometry (summarized in Table I for *pLck/low* and *pLck/high* and shown in Fig. 2 for *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 $CD1d^{+/-}$ heterozygous controls, i.e., 2.5 to 10 times the levels of WT $CD1d^{+/+}$. The *pLck-CD1d* transgenic mice exhibited a marked drop in CD1d expres-

Table I. CD1d expression levels in splenic and thymic subsets of *pLck-CD1d* mice

	Thymus				Spleen				
Mice	DN	DP	CD4	CD8	CD4	CD8	B	DC	MΦ
CD1d Het	182	125	109	82	97	74	178	129	79
CD1d KO	12	17	15	13	19	8	8	5	5
pLck/low	437	563	87	57	78	60	5	4	7
pLck/high	3,476	3,038	1,384	1,197	1,154	918	8	5	8

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

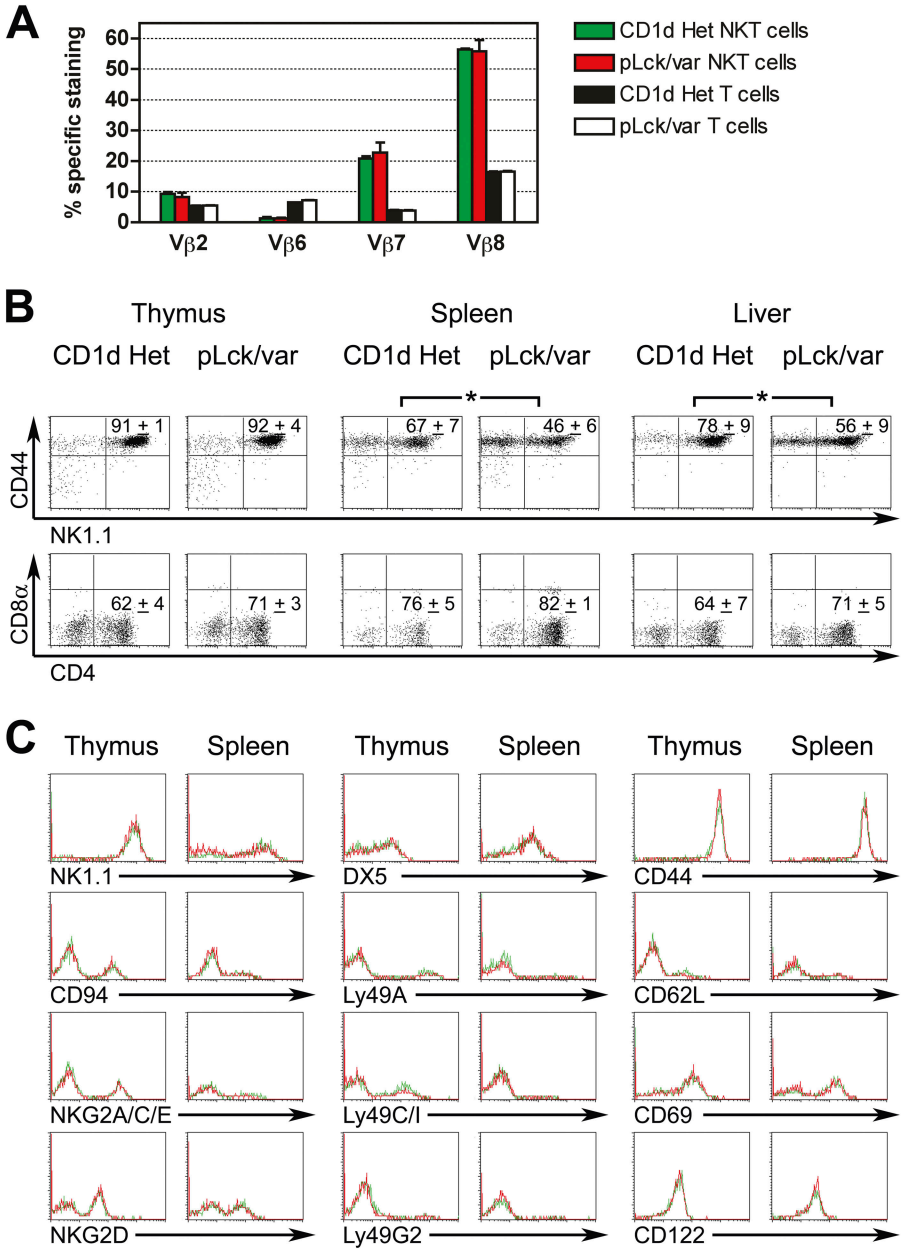

Figure 3. NKT cell phenotype in *pLck/var* mice. (A) TCR Vβ repertoire analysis on gated CD3ε$^+$CD1d-α-GalCer tetramer$^+$ NKT cells and mainstream CD3ε$^+$ T cells in the spleen of *pLck/var* and *CD1d Het* littermate controls, as indicated. Results shown as mean + SD of data collected from nine mice in two independent experiments. (B) CD44/NK1.1 and CD4/CD8α 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 CD44^{low}, CD44^{high}, 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^{low}NK1.1^{-} 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 HSA^{low}CD44^{low} NKT cells (40.1%) was two times higher than expected from the frequency among DP thymocytes (18.6%) (Fig. 5 A). This increased frequency was particularly evident at the early HSA^{low}CD44^{low} NKT cell stage after positive selection from the HSA^{high} 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 evaluate further 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 mainstream 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-

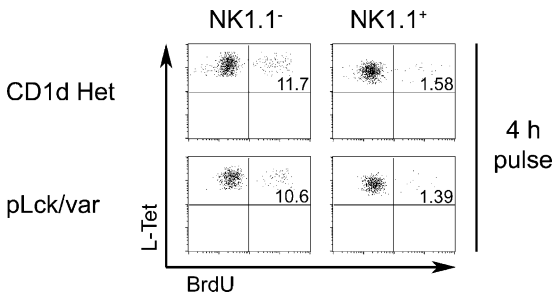

Figure 4. BrdU incorporation by NKT cell subsets in vivo. 4 h after i.v. injection of BrdU, *pLck/var* and control *CD1d Het* showed similar frequency of BrdU^{+} cells among CD1d-α-GalCer tetramer^{+} NKT cell subsets. Similar results were found in three different experiments. Percentages of BrdU^{+}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

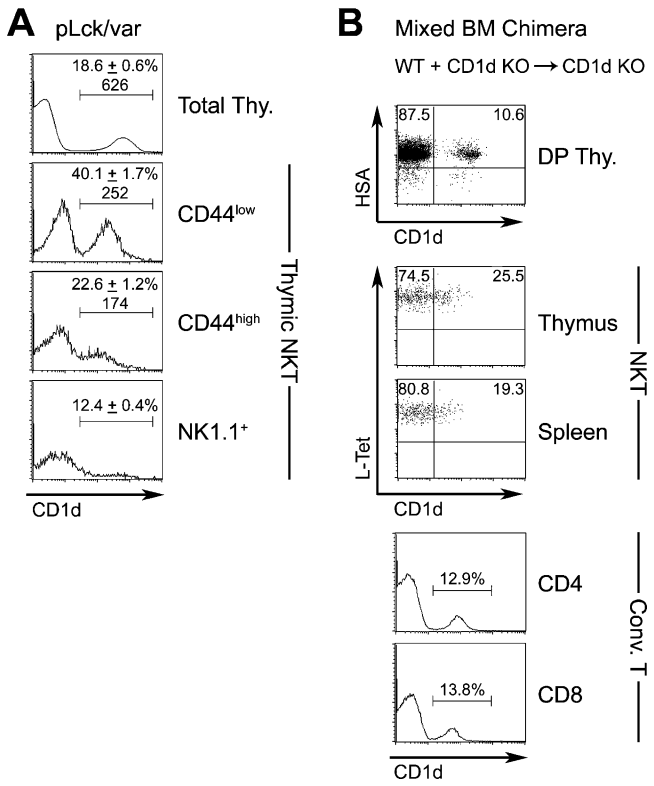

Figure 5. CD1d expression autonomously enhances NKT cell development. (A) Frequency of CD1d expression is higher among NKT cells of *pLck/var* mice than among total thymocytes. The difference is particularly visible at the CD44^{low} NKT stage before extinction of the *pLck* promoter. Frequencies are indicated as mean ± SD of three mice. (B) In mixed WT + *CD1d* KO 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.

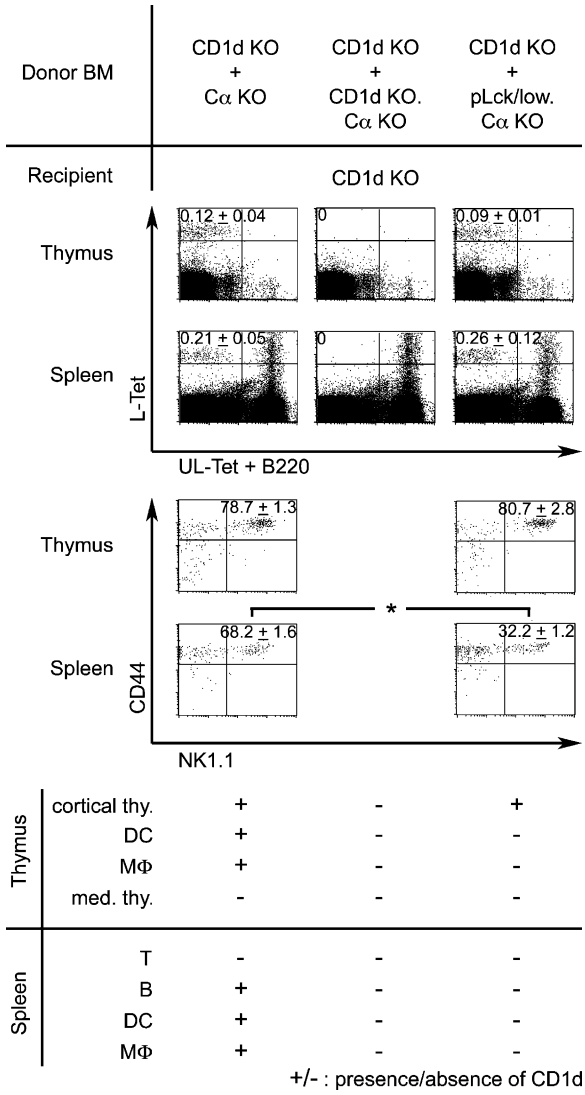

Figure 6. NKT cell development in *CD1d* KO + *pLck/low.Cα* KO mixed BM chimeras. Irradiated (900 rad) C57BL/6.*CD1d* KO recipients of a mixture of BM cells, as indicated, were analyzed for frequency and phenotype of CD1d-α-GalCer tetramer$^+$ NKT cells 6–8 wk after reconstitution. In these experiments, the *pLck/low.Cα* KO BM cells were obtained from a H-2^b donor. Percentages ± SD are calculated from four individual chimeras. *, statistically significant differences ($P < 0.05$, unpaired Student's *t* test). The bottom table recapitulates the thymic and peripheral cell types expressing (+) or not expressing (−) CD1d in these chimeras.

tial contribution by DCs, macrophages, or thymic epithelial cells, it was important to investigate whether transgenic expression of CD1d on mature T cells inside and outside the thymus was contributing to NKT cell development. Indeed, even though the proximal *Lck* promoter is substantially extinguished after the DP stage, residual gene expression combined with the long lifespan of CD1d proteins provides a window of opportunity for mature HSA^{low} NKT thymocytes to interact with CD1d (including CD1d expressed on NKT cells themselves, as described before) after they exit the cortex. Such interaction might account for the rounds of cell division and the effector/memory formation that characterize this developmental stage, as well as terminal NK differentiation in the periphery. To abrogate this residual expression of CD1d in the thymic medulla and in the periphery, we generated mixed BM radiation chimeras 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/var* mice compared with *CD1d Het* 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).

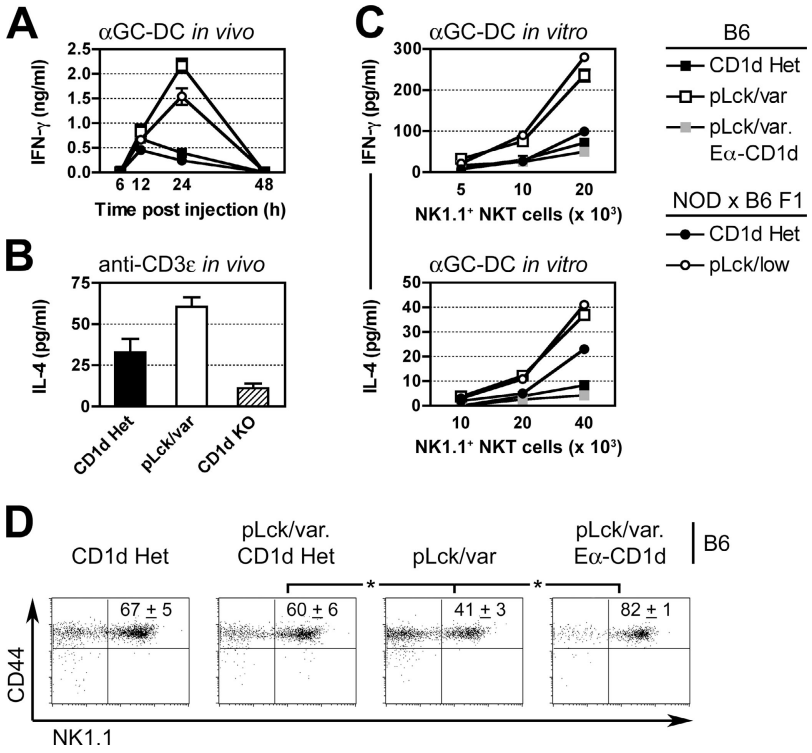

Figure 7. Cytokine secretion by NKT cells in vivo and in vitro. (A) Mice of indicated genotypes were injected i.v. with 5×10^5 α-GalCer-pulsed DCs (αGC-DC), and IFN-γ was measured in sera taken at indicated time points. Results are shown as mean ± SD of two independent experiments, each including four mice per group. (B) Early IL-4 released in vitro by cultured ~1-mm^3 splenic fragments, 90 min after i.v. injection of 1.25 μg anti-CD3ε in vivo. (C) Titrated numbers of sorted CD1d-α-GalCer tetramer$^+$NK1.1$^+$ cells were stimulated with 10^5 α-GalCer-pulsed DCs, before collection of supernatant for cytokine assays 48 h later. Results pooled from at least three independent experiments. Error bars represent 1 SD (not all error bars are visible). (D) CD44/NK1.1 staining of splenic CD1d-α-GalCer tetramer$^+$ NKT cells in age-matched (6 wk) mice of indicated genotypes. Percentages and SD from three mice per group in three independent experiments are indicated. *, statistical significance by unpaired Student's *t* test.

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/var* 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 exhibit 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 CD8αα 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 molecules, 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 Cα* KO mice in the C57BL/6 background were purchased from Jackson ImmunoResearch Laboratories. C57BL/6.*Eα-CD1d* mice expressing a CD1d1 transgene driven by the MHC II Eα promoter in a *CD1d* KO background were previously described (27). To generate *pLck-CD1d* Tg mice, we replaced the *Eα* promoter in the previously described *Eα-CD1d* construct with the proximal *Lck* 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 CD-1 (VAF+) outbred females. Transgenic mice were screened using PCR (forward primer: 5′-TCCTGTGAACTTGGTGCTTGAG-3′; reverse primer: 5′-CCAAAATGATGAGACAGCACAAC-3′) and were bred to C57BL/6.*CD1d* KO or NOD.*CD1d* KO to obtain *pLck-CD1d* mice expressing the *pLck-CD1d* transgene in the C57BL/6.*CD1d* KO ($n = 2$) or to NOD.*CD1d* KO ($n = 3$) background, respectively. Because the NOD strain has a partial, recessive defect in NKT cell development (42), the NOD.*pLck-CD1d* mice were further crossed with C57BL/6.*CD1d* KO mice to generate NODxC57BL/6 F1.*pLck-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, CD8α, CD11c, CD11b, CD24 (HSA), CD44, CD45, CD62L, CD69, CD94, CD122, DX5, Gr-1, Ly49A, Ly49C/I, Ly49G2, NKG2A/C/E, NK1.1, TCR Vβ2, Vβ6, Vβ7, and Vβ8.1+8.2, were purchased from BD Biosciences. Anti-F4/80, B220, NKG2D mAbs, and streptavidin conjugated with allophycocyanin or R-phycoerythrin were purchased from eBioscience. Anti-I-A^b mAb (clone Y3P) was conjugated to FITC in our laboratory. CD1d tetramers loaded with α-GalCer were prepared and used in staining as described previously (43). BrdU staining of cells extracted from mice (after one i.p. injection of 1 mg BrdU) was done according to manufacturer's instructions in BrdU Flow Kits (BD Biosciences). Flow cytometry analysis was performed on a four-color FACSCalibur with CELLQuest software (BD Biosciences).

Frozen section immunohistochemistry. Thymi were harvested, rinsed with PBS, embedded in OCT freezing medium (Tissue-Tek, Sakura Finetek), frozen in a mixture of 2-methylbutane (Sigma-Aldrich) and dry ice, and stored at –20°C. Thymic sections were cut with a cryostat (10 μm), mounted on glass slides (Fisher Scientific International), dried, fixed with acetone (Sigma-Aldrich), and covered with glass coverslips (Fisher Scientific International). Thymic sections were first stained with a ~5 μg/ml of a mixture of three purified rat anti-CD1d mAbs (clones 19G11, 17F5, and 16G9 in 1:1:1 ratio) developed in our laboratory. Biotinylated goat anti–rat IgG, streptavidin-Texas red, and FITC-conjugated Y3P anti–I-A^b antibodies were used in sequential order with washes in between. Fluorescence images were acquired with an Axiovert 200 microscope (Carl Zeiss MicroImaging, Inc.) and processed with OpenLab (Improvision Inc.) and Adobe Photoshop (Adobe Systems Inc.) software.

NKT cell purification and stimulation. Single-cell suspension of splenocytes was first enriched by depleting $B220^+$ cells using B220-conjugated magnetic microbeads with AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. CD1d-α-GalCer $tetramer^+NK1.1^+$ cells were then sorted with FACSAria (BD Biosciences), and cultured with DCs in ER 10% medium, a 1:1 mixture of Eagle's Ham's amino acid and RPMI 1640 (Biofluids) enriched with 10% heat-inactivated FCS, glutamine, antibiotics, and 5×10^{-5} M of 2-mercaptoethanol. The DCs were prepared from cultured BM in ER10% medium supplemented with mouse GM-CSF (Biosource International) at 2 ng/ml for 6 d and pulsed with α-GalCer (100 ng/ml) overnight before washing and culture at 10^5 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 5×10^5 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 10^7 mixed BM cells was injected i.v. Chimeras were analyzed 6–8 wk after injection.

We thank L. Degenstein for oocyte injections, T. Li for help with immunohistochemistry; J. Marvin, R. Duggan, and B. Eisfelder for cell sorting; and all Bendelac laboratory members for expert advice and discussion.

This work is supported by grants from the New Jersey Committee on Cancer Research (to D.G. Wei), the Korea Research Foundation grant no. 2001-015-DS0063 (to S.-H. Park), the French Ministry of Research (to A. Lehuen), and the National Institutes of Health no. RO1 AI38339 (to A. Bendelac).

The authors have no conflicting financial interests.

Submitted: 22 February 2005
Accepted: 1 June 2005

REFERENCES

1. Godfrey, D.I., K.J. Hammond, L.D. Poulton, M.J. Smyth, and A.G. Baxter. 2000. NKT cells: facts, functions and fallacies. *Immunol. Today*. 21:573–583.
2. Brigl, M., and M.B. Brenner. 2004. CD1: antigen presentation and T cell function. *Annu. Rev. Immunol*. 22:817–890.
3. Park, S.H., and A. Bendelac. 2000. CD1-restricted T-cell responses and microbial infection. *Nature*. 406:788–792.
4. Benlagha, K., T. Kyin, A. Beavis, L. Teyton, and A. Bendelac. 2002. A thymic precursor to the NKT cell lineage. *Science*. 296:553–555.
5. Pellicci, D.G., K.J. Hammond, A.P. Uldrich, A.G. Baxter, M.J. Smyth, and D.I. Godfrey. 2002. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *J. Exp. Med*. 195:835–844.
6. Bendelac, A., R.D. Hunziker, and O. Lantz. 1996. Increased interleukin 4 and immunoglobulin E production in transgenic mice overexpressing NK1 T cells. *J. Exp. Med*. 184:1285–1293.
7. Zhou, D., J. Mattner, C. Cantu III, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y.P. Wu, T. Yamashita, et al. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science*. 306:1786–1789.
8. Alberola-Ila, J., K.A. Hogquist, K.A. Swan, M.J. Bevan, and R.M. Perlemutter. 1996. Positive and negative selection invoke distinct signaling pathways. *J. Exp. Med*. 184:9–18.
9. Eberl, G., B. Lowin-Kropf, and H.R. MacDonald. 1999. Cutting edge: NKT cell development is selectively impaired in Fyn-deficient mice. *J. Immunol*. 163:4091–4094.
10. Gadue, P., N. Morton, and P.L. Stein. 1999. The Src family tyrosine kinase Fyn regulates natural killer T cell development. *J. Exp. Med*. 90: 1189–1196.
11. Sivakumar, V., K.J. Hammond, N. Howells, K. Pfeffer, and F. Weih. 2003. Differential requirement for Rel/nuclear factor kappa B family members in natural killer T cell development. *J. Exp. Med*. 197:1613–1621.
12. Stanic, A.K., J.S. Bezbradica, J.J. Park, N. Matsuki, A.L. Mora, L. Van Kaer, M.R. Boothby, and S. Joyce. 2004. NF-kappa B controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. *J. Immunol*. 172:2265–2273.

13. Schmidt-Supprian, M., J. Tian, E.P. Grant, M. Pasparakis, R. Maehr, H. Ovaa, H.L. Ploegh, A.J. Coyle, and K. Rajewsky. 2004. Differential dependence of CD4+CD25+ regulatory and natural killer-like T cells on signals leading to NF-kappaB activation. *Proc. Natl. Acad. Sci. USA*. 101:4566–4571.
14. Coles, M.C., and D.H. Raulet. 1994. Class I dependence of the development of CD4+ CD8− NK1.1+ thymocytes. *J. Exp. Med.* 180: 395–399.
15. Bendelac, A., N. Killeen, D. Littman, and R.H. Schwartz. 1994. A subset of CD4+ thymocytes selected by MHC class I molecules. *Science*. 263:1774–1778.
16. Ohteki, T., and H.R. MacDonald. 1994. Major histocompatibility complex class I related molecules control the development of $CD4^{+}8^{-}$ and $CD4^{-}8^{-}$ subsets of natural killer 1.1^{+} T cell receptor-a/b^{+} cells in the liver of mice. *J. Exp. Med.* 180:699–704.
17. Coles, M.C., and D.H. Raulet. 2000. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J. Immunol.* 164:2412–2418.
18. Hayakawa, Y., S.P. Berzins, N.Y. Crowe, D.I. Godfrey, and M.J. Smyth. 2004. Antigen-induced tolerance by intrathymic modulation of self-recognizing inhibitory receptors. *Nat. Immunol.* 5:590–596.
19. Brawand, P., F.A. Lemonnier, H.R. MacDonald, J.C. Cerottini, and W. Held. 2000. Transgenic expression of Ly49A on T cells impairs a specific antitumor response. *J. Immunol.* 165:1871–1876.
20. Ikarashi, Y., R. Mikami, A. Bendelac, M. Terme, N. Chaput, M. Terada, T. Tursz, E. Angevin, F.A. Lemonnier, H. Wakasugi, and L. Zitvogel. 2001. Dendritic cell maturation overrules H-2D-mediated natural killer T (NKT) cell inhibition. Critical role for b7 in CD1d-dependent NKT cell interferon gamma production. *J. Exp. Med.* 194:1179–1186.
21. Chun, T., M.J. Page, L. Gapin, J.L. Matsuda, H. Xu, H. Nguyen, H.S. Kang, A.K. Stanic, S. Joyce, W.A. Koltun, et al. 2003. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J. Exp. Med.* 197:907–918.
22. Voyle, R.B., F. Beermann, R.K. Lees, J. Schumann, J. Zimmer, W. Held, and H.R. MacDonald. 2003. Ligand-dependent inhibition of CD1d-restricted NKT cell development in mice transgenic for the activating receptor Ly49D. *J. Exp. Med.* 197:919–925.
23. Matsuda, J.L., L. Gapin, S. Sidobre, W.C. Kieper, J.T. Tan, R. Ceredig, C.D. Surh, and M. Kronenberg. 2002. Homeostasis of V alpha 14i NKT cells. *Nat. Immunol.* 3:966–974.
24. Yoshimoto, T., and W.E. Paul. 1994. CD4pos NK1.1pos T cells promptly produced IL-4 in response to in vivo challenge with anti-CD3. *J. Exp. Med.* 179:1285–1295.
25. Stetson, D.B., M. Mohrs, R.L. Reinhardt, J.L. Baron, Z.E. Wang, L. Gapin, M. Kronenberg, and R.M. Locksley. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J. Exp. Med.* 198:1069–1076.
26. Bendelac, A., O. Lantz, M.E. Quimby, J.W. Yewdell, J.R. Bennink, and R.R. Brutkiewicz. 1995. CD1 recognition by mouse NK1+ T lymphocytes. *Science*. 268:863–865.
27. Forestier, C., S.H. Park, D. Wei, K. Benlagha, L. Teyton, and A. Bendelac. 2003. T cell development in mice expressing CD1d directed by a classical MHC class II promoter. *J. Immunol.* 171:4096–4104.
28. Xu, H., T. Chun, A. Colmone, H. Nguyen, and C.R. Wang. 2003. Expression of CD1d under the control of a MHC class Ia promoter skews the development of NKT cells, but not CD8+ T cells. *J. Immunol.* 171:4105–4112.
29. Allen, J.M., K.A. Forbush, and R.M. Perlmutter. 1992. Functional dissection of the Lck proximal promoter. *Mol. Cell. Biol.* 12:2758–2768.
30. Goldrath, A.W., and M.J. Bevan. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature*. 402:255–262.
31. Ashton-Rickardt, P.G., L.V. Kaer, T.N.M. Schumacher, H.L. Ploegh, and S. Tonegawa. 1993. Peptide contributes to the specificity of positive selection of CD8+ T cells in the thymus. *Cell*. 73:1041–1049.
32. Yamagata, T., D. Mathis, and C. Benoist. 2004. Self-reactivity in thymic double-positives commits cells to a CD8aa lineage with characteristics of innate immune cells. *Nat. Immunol.* 5:597–605.
33. Legendre, V., C. Boyer, S. Guerder, B. Arnold, G. Hammerling, and A.M. Schmitt-Verhulst. 1999. Selection of phenotypically distinct NK1.1+ T cells upon antigen expression in the thymus or in the liver. *Eur. J. Immunol.* 29:2330–2343.
34. Schultz, R.J., A. Parkes, E. Mizoguchi, A.K. Bhan, and S. Koyasu. 1996. Development of CD4−CD8− abTCR+ NK1.1+ T lymphocytes. Thymic selection by self antigen. *J. Immunol.* 157:4379–4389.
35. Jordan, M.S., A. Boesteanu, A.J. Reed, A.L. Petrone, A.E. Holenbeck, M.A. Lerman, A. Naji, and A.J. Caton. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2:301–306.
36. von Boehmer, H., J. Kirberg, and B. Rocha. 1991. An unusual lineage of alpha/beta T cells that contains autoreactive cells. *J. Exp. Med.* 174: 1001–1008.
37. Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 3:756–763.
38. Cannons, J.L., L.J. Yu, B. Hill, L.A. Mijares, D. Dombroski, K.E. Nichols, A. Antonellis, G.A. Koretzky, K. Gardner, and P.L. Schwartzberg. 2004. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. *Immunity*. 21:693–706.
39. Davidson, D., X. Shi, S. Zhang, H. Wang, M. Nemer, N. Ono, S. Ohno, Y. Yanagi, and A. Veillette. 2004. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation. *Immunity*. 21:707–717.
40. Engel, P., M.J. Eck, and C. Terhorst. 2003. The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat. Rev. Immunol.* 3:813–821.
41. van Ewijk, W. 1991. T-cell differentiation is influenced by thymic microenvironments. *Annu. Rev. Immunol.* 9:591–615.
42. Esteban, L.M., T. Tsoutsman, M.A. Jordan, D. Roach, L.D. Poulton, A. Brooks, O.V. Naidenko, S. Sidobre, D.I. Godfrey, and A.G. Baxter. 2003. Genetic control of NKT cell numbers maps to major diabetes and lupus loci. *J. Immunol.* 171:2873–2878.
43. Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. In vivo identification of glycolipid antigen specific T cells using fluorescent CD1d tetramers. *J. Exp. Med.* 191:1895–1903.