

# Characterization of the early stages of thymic NKT cell development

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Upon reaching the mature heat stable antigen (HSA)<sup>low</sup> thymic developmental stage, CD1d-restricted V $\alpha$ 14-J $\alpha$ 18 thymocytes undergo a well-characterized sequence of expansion and differentiation steps that lead to the peripheral interleukin-4/interferon- $\gamma$ -producing NKT phenotype. However, their more immature HSA<sup>high</sup> precursors have remained elusive, and it has been difficult to determine unambiguously whether NKT cells originate from a CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) stage, and when the CD4<sup>+</sup> and CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN) NKT subsets are formed. Here, we have used a CD1d tetramer-based enrichment strategy to physically identify HSA<sup>high</sup> precursors in thymuses of newborn mice, including an elusive DP<sup>low</sup> stage and a CD4<sup>+</sup> stage, which were present at a frequency of  $\sim 10^{-6}$ . These HSA<sup>high</sup> DP and CD4<sup>+</sup> stages appeared to be nondividing, and already exhibited the same V $\beta$ 8 bias that characterizes mature NKT cells. This implied that the massive expansion of NKT cells is separated temporally from positive selection, but faithfully amplifies the selected TCR repertoire. Furthermore, we found that, unlike the DN  $\gamma\delta$  T cells, the DN NKT cells did not originate from a pT $\alpha$ -independent pathway bypassing the DP stage, but instead were produced during a short window of time from the conversion of a fraction of HSA<sup>low</sup> NK1.1<sup>neg</sup> CD4 cells. These findings identify the HSA<sup>high</sup> CD4<sup>+</sup> stage as a potential branchpoint between NKT and conventional T lineages and between the CD4 and DN NKT sublineages.

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Abbreviations used: CD1d- $\alpha$ GalCer; CD1d- $\alpha$ -galactosylceramide; DP, double-positive; DN, double-negative; HSA, heat stable antigen; MACS, magnetic-activated cell sorting; SAP, SLAM-associated protein; SLAM, signalling lymphocyte activation molecule.

Mouse V $\alpha$ 14-J $\alpha$ 18/V $\beta$ 8,V $\beta$ 7,V $\beta$ 2 and human V $\alpha$ 24-J $\alpha$ 18/V $\beta$ 11 NKT cells are a conserved CD1d-restricted innate-like lymphocyte lineage that is involved in various infectious, allergic, autoimmune, and tumor conditions through the recognition of conserved endogenous and exogenous glycolipids (1–5). These glycolipids include the self-antigen, iGb3, generated in lysosomal compartments (4); microbial  $\alpha$ -glycuronosylceramides found in the cell wall of Gram<sup>-</sup> LPS<sup>-</sup> bacteria (3, 5); and mycobacterial phosphatidylinositolmannosides (6).

The development of this unusual lineage has remained largely elusive. Two main models have been proposed: a precommitment model (7), which suggests that NKT cells originate from a committed precursor before TCR expression, and an instructive model (8) whereby mainstream thymocytes expressing the rare canonical CD1d-reactive TCR are instructed—by virtue of their interaction with CD1d-expressing cells—to differentiate into NKT cells. In strong support of the instructive model, transgenic expression of the canonical NKT cell receptor

was sufficient to induce NKT cell differentiation (9). In addition, staining with CD1d tetramers identified developmental intermediates after positive selection, but preceding the acquisition of NK differentiation (10, 11). The earliest, well-characterized precursor identified was a rare, mature heat stable antigen (HSA)<sup>low</sup> CD44<sup>low</sup> thymocyte with a CD4<sup>+</sup> or double-negative (DN) phenotype. Unlike other mature thymocytes, this cell seemed to be engaged in cell cycle, and gave rise to the CD44<sup>high</sup> memory-type stage. Cycling CD44<sup>high</sup> cells migrated to the periphery where they induced an “NK program”—including the expression of NK1.1, Ly49, and CD94/NKG2A—over the course of several weeks. In parallel with these phenotypic events, NKT cells acquired the ability to produce IL-4, then also IFN- $\gamma$  upon TCR stimulation.

The conspicuous activation and expansion undergone by mature HSA<sup>low</sup> NKT thymocytes suggest that they must have received unusual signals. Unlike mainstream T cells, NKT development depends on interaction

with antigen expressed on the surface of bone marrow-derived cells, including double-positive (DP) thymocytes, rather than epithelial cells (12–16). Furthermore, recognition of the endogenous glycosphingolipid iGb3, an agonist antigen for mouse and human NKT cells, also is likely to be involved in the unusual signaling that leads to NKT cell expansion and differentiation (4). Consistent with the hypothesis of differential signaling, NKT development is unaffected by dominant negative forms of Ras or Mek-1 (17), but is abrogated completely in Fyn null mice (18, 19). The recent finding that signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) connects Fyn to SLAM family members expressed on the cell surface (20–22) suggests that, in conjunction with TCR signaling, key signals may emanate from homotypic interaction between SLAM family members expressed by thymocytes (i.e., between the developing NKT precursors and neighboring CD1d-expressing DP thymocytes; references 23–26).

Integration of these recent advances in the general scheme of NKT cell development requires precise characterization of the immature HSA<sup>high</sup> stages; however, their very low frequency (estimated at less than 1/10<sup>6</sup>) has precluded physical identification. For example, it is unclear whether NKT cells transit through a DP stage. The existence of very rare tetramer-positive DP thymocytes is controversial (11, 27, 28). PCR-based studies have revealed rare V $\alpha$ 14-J $\alpha$ 18 joints among DP thymocytes; however, it has not been determined whether the corresponding cells are enriched in or deprived of NKT cell precursors because the coexpression of V $\beta$  chains (V $\beta$ 8, V $\beta$ 7, V $\beta$ 2) allowing endogenous ligand recognition has not been determined (27). In one study, purified DP thymocytes generated rare mature NKT cells upon intrathymic transfer; however, the inordinately high number of cells injected (10<sup>7</sup>) may have included enough immature DN cell contaminants to account for NKT cell generation independent of the DP stage (27).

The origin of the DN sublineage of NKT cells, which is found in mouse and human, also is elusive. Because DN and CD4 cells are represented at the first developmental stage identified, the HSA<sup>low</sup> CD44<sup>low</sup> cell, it remains unclear whether DN cells originate early from precursors that bypass the DP stage (e.g., as is the case for DN  $\gamma\delta$  T cells), or later from DP or even from CD4 T cells. Although the CD4 and CD8 coreceptors are dispensable for the development of the CD4 and DN NKT sublineages, the absence of CD8 has a measurable impact on the V $\beta$  repertoire of CD4 and DN cells (12). This suggests that earlier expression of CD8 influences positive or negative selection, perhaps through interaction with CD1d. Transgenic expression of CD8 $\alpha\beta$ —under a CD2 promoter—drastically reduced the thymic NKT cell population; this also supports the hypothesis that CD8 may interact with CD1d (12). Finally, in mice transgenically expressing the V $\alpha$ 14-J $\alpha$ 18 TCR  $\alpha$  chain, CD8 cells were depleted of the V $\beta$ 8 and V $\beta$ 7 chains that confer reactivity to CD1d (9). These findings suggested that the conspicuous ab-

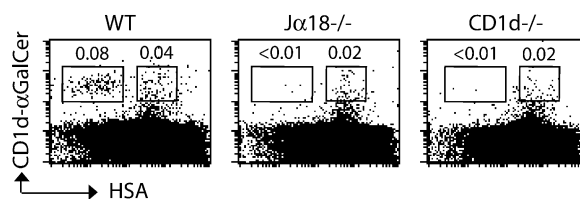
sence of CD8<sup>+</sup> NKT cells in WT mice may reflect negative selection or, as previously observed in a TCR transgenic system with agonist ligand signaling (29), the direct transformation of DP into DN or CD8 $\alpha\alpha$  cells.

In this study, we used a CD1d tetramer-based strategy to enrich NKT cell precursors from the thymus of WT newborn mice. Comparison with J $\alpha$ 18<sup>-/-</sup> and CD1d<sup>-/-</sup> NKT deficient controls allowed the identification of rare, noncycling HSA<sup>high</sup> DP<sup>low</sup> and HSA<sup>high</sup> CD4<sup>+</sup> precursors; intrathymic cell transfers demonstrated that the DN subset originated later from cycling HSA<sup>low</sup> CD4 cells. These findings that identify the early stages of NKT cells in the thymus provide significant additions to the developmental map of NKT cells at the cellular level, and have implications on the molecular mechanisms that underlie this enigmatic pathway of T cell development.

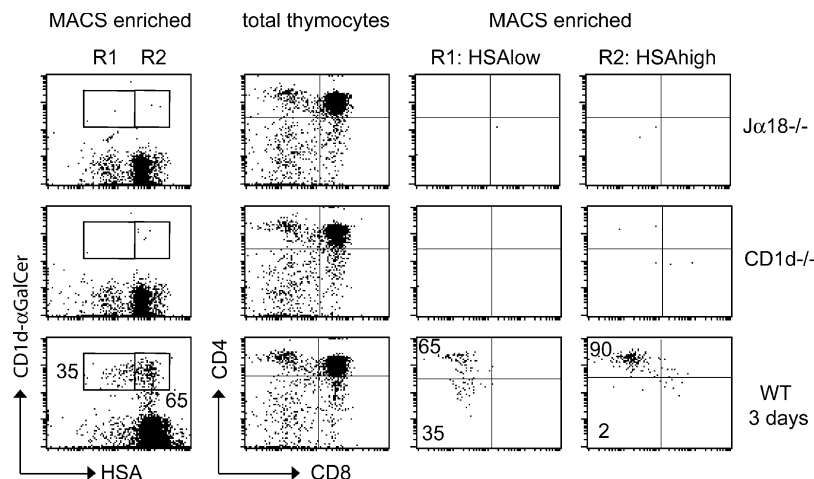
## RESULTS

### Identification of HSA<sup>high</sup> V $\alpha$ 14 NKT cells in newborn thymus

Because of their massive intrathymic expansion, NKT cells are readily detectable among adult mature HSA<sup>low</sup> thymocytes by specific tetramer staining. In contrast, as shown in Fig. 1, their frequency among the immature HSA<sup>high</sup> population is very low and comparable to that of J $\alpha$ 18-deficient thymuses where the canonical NKT TCR is absent or of CD1d-deficient thymus where NKT cells are not selected. This indicates that HSA<sup>high</sup> NKT lineage cells are too rare to be detected over a “nonspecific” staining background of 0.02%. However, upon magnetic-activated cell sorting (MACS)-enrichment with the same tetramers, bona fide, predominantly HSA<sup>high</sup> tetramer-positive cells were recovered from WT 3-d-old thymuses, but not J $\alpha$ 18-deficient or CD1d-deficient controls (Fig. 2). This result indicates that the cells that were stained “nonspecifically” by FACS were not recovered after MACS enrichment, perhaps because of their lower affinity interactions with the tetramers. Using this strategy, we were able to enrich the frequency of NKT lineage cells >1,000 fold; however, we estimate that under MACS conditions, only one third of the NKT lineage cells



**Figure 1. Detection of HSA<sup>high</sup> NKT lineage cells in total thymocyte preparations.** Total thymocytes from 2-wk-old WT, J $\alpha$ 18<sup>-/-</sup>, and CD1d<sup>-/-</sup> mice were stained with HSA and CD1d-tetramers. Gates identifying the tet<sup>+</sup>HSA<sup>low</sup> and tet<sup>+</sup>HSA<sup>high</sup> cells are shown with corresponding frequencies. Note that the level of background staining in the HSA<sup>high</sup> gates (as measured in J $\alpha$ 18<sup>-/-</sup> and CD1d<sup>-/-</sup> mice) precludes the identification of HSA<sup>high</sup> NKT lineage cells in wild type mice. Data representative of >10 individual WT or mutant mice.



**Figure 2. Improved detection of HSA<sup>high</sup> NKT lineage cells in MACS-enriched thymocyte preparations.** Thymocytes from 3-d-old WT, J $\alpha$ 18<sup>-/-</sup>, and CD1d<sup>-/-</sup> mice were MACS-enriched for tet<sup>+</sup> cells and stained for HSA, CD4, and CD8. The left column shows CD1d-tetramer/HSA staining of MACS-enriched preparations, the other columns show CD4/CD8 staining of whole thymocytes, or MACS-enriched thymocytes in the R1 (HSA<sup>low</sup>) and R2 (HSA<sup>high</sup>) gates, as indicated. The numbers on the HSA/CD1d-tetramers dot plots represent the percentages of HSA<sup>low</sup> and

HSA<sup>high</sup> cells among the tet<sup>+</sup> population. The numbers on the R1 and R2 gated CD4/CD8 FACS dot plots represent the percentage of CD4<sup>+</sup> and DN populations among HSA<sup>low</sup> and HSA<sup>high</sup> NKT lineage cells. Note that in the tet<sup>+</sup> MACS-enriched panels, each dot represents a cell: there were 3 and 5 tet<sup>+</sup> dots in the MACS-enriched preparations of J $\alpha$ 18<sup>-/-</sup> and CD1d<sup>-/-</sup> thymuses, respectively, and 224 tet<sup>+</sup> dots in the WT. Data are representative of at least three individual experiments with pooled thymuses from 40 to 60 3-d-old newborns.

were recovered from these early thymuses. Therefore, the rarity of these NKT lineage cells in newborns required large-scale experiments; typically, we pooled 40–60 newborn thymuses to enrich and examine  $\sim$ 200–300 “authentic” tet<sup>+</sup> cells, because the procedure recovered approximately one NKT cell per 10<sup>6</sup> thymocytes at this age (Table I). In these 3-d-old mice,  $\sim$ 70% of the tet<sup>+</sup> cells, on average, were composed of HSA<sup>high</sup> cells (Fig. 2 and Table I), whereas the average proportion decreased to 57% at day 4, 13% at day 5, and 2% at day 8 (Fig. 3 and Table I). Nearly all of the tet<sup>+</sup>HSA<sup>high</sup> cells found in 3-d-old mice were CD4<sup>+</sup> (Fig. 2). Rare other cells included DP<sup>low</sup> cells that could not be distinguished readily from the background staining of control J $\alpha$ 18<sup>-/-</sup> thymuses. In older newborn mice, the HSA<sup>high</sup> cells were largely of the CD4<sup>+</sup> phenotype, although minor proportions of DN cells could be detected (Fig. 3). In contrast, in the same tetramer-enriched population, the tet<sup>+</sup>HSA<sup>low</sup> cells were composed of nearly equal proportions of CD4<sup>+</sup> and DN cells as early as day 3 of age (Figs. 2 and 3). As shown in Figs. 2 and 3, HSA<sup>high</sup> CD4<sup>+</sup> cells were absent from the thymus of CD1d-deficient mice, which supports the notion that they are the product of positive selection.

Given the extreme rarity of the HSA<sup>high</sup> CD4<sup>+</sup> cell, it is not surprising that we could not detect earlier stages, such as putative DP precursors. The lower TCR level and lower frequency of these precursors likely renders them undetectable. A population of DP<sup>low</sup> cells belonging to the NKT lineage became visible at 2 wk of age (Fig. 3). A similar phenotype had been reported previously (27, 28), but was not confirmed in a subsequent study (11). Therefore, it was not clear whether these rare, unusual cells were bona fide NKT

lineage cells or a staining artifact. The absence of DP<sup>low</sup> cells in J $\alpha$ 18<sup>-/-</sup> mice (Fig. 3) indicates that they represent an authentic NKT lineage cell type, and their appearance at 2 weeks may help to explain seemingly contradictory reports. Their similar frequency to HSA<sup>high</sup> CD4<sup>+</sup> cells suggests that they are a product of positive selection, as confirmed by their absence in the CD1d<sup>-/-</sup> thymus. In addition, the DP<sup>low</sup> phenotype was identical to that of CD69<sup>+</sup> DP thymocytes previously associated with positive selection (30; see the position of the pink gate in Figs. 3 and 4). The absence of this phenotype in younger mice may reflect different kinetics in the down-regulation of the CD4 and CD8 coreceptors and regulation of TCR following positive selection.

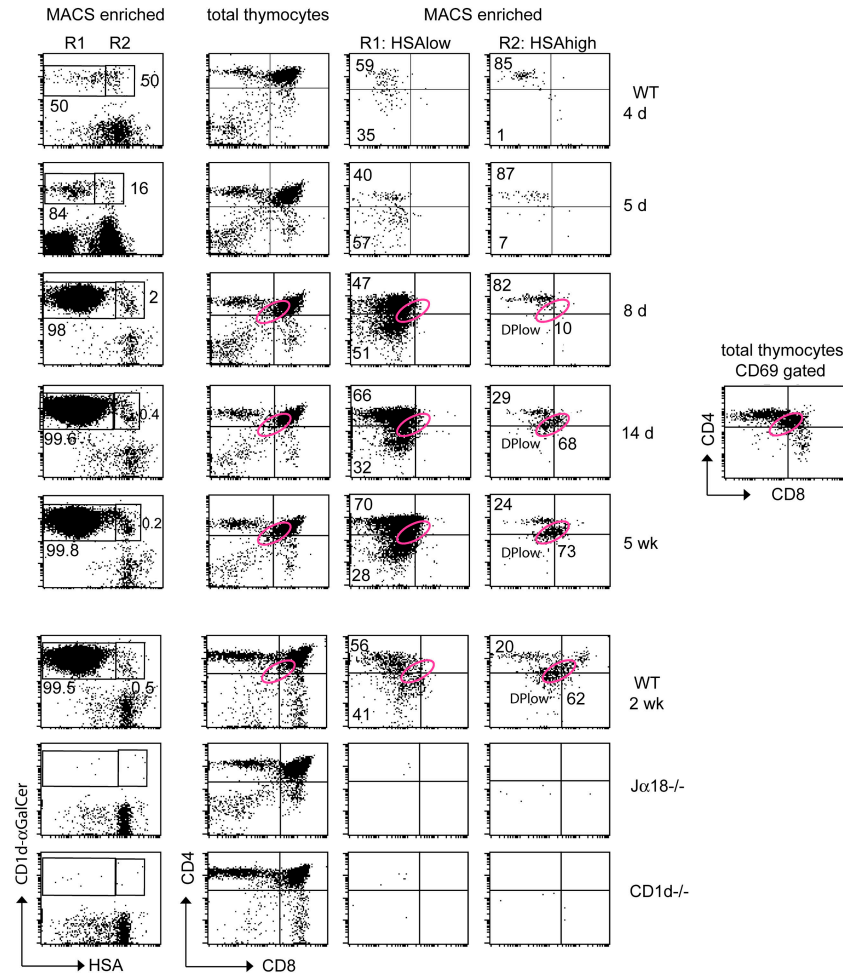
**Table I.** Ontogeny of NKT lineage thymocytes

Day	Total thymocyte no. <sup>a</sup> $\times 10^6$	tet <sup>+</sup> cells recovered/thymus <sup>b</sup>	Frequency among total thymocytes <sup>c</sup>		Frequency among tet <sup>+</sup> cells
			HSA <sup>high</sup>	HSA <sup>low</sup>	HSA <sup>high</sup>
3	12 $\pm$ 3	8 $\pm$ 3	3.5 $\pm$ 1.5	1.5 $\pm$ 1	70.5 $\pm$ 4.5
4	18 $\pm$ 2	10 $\pm$ 1	2.5 $\pm$ 0.5	2 $\pm$ 0.5	57.5 $\pm$ 7.5
5	25 $\pm$ 4	38 $\pm$ 5	2.8 $\pm$ 1	16 $\pm$ 2.5	13 $\pm$ 3
8	37 $\pm$ 2	3,729 $\pm$ 70	16 $\pm$ 4	982 $\pm$ 52	1.5 $\pm$ 0.5

<sup>a</sup>Means  $\pm$  SD are calculated from at least three separate experiments using pools of up to 40–60 newborn thymuses.

<sup>b</sup>tet<sup>+</sup> cells were counted after MACS enrichment. Only approximately one third of the tet<sup>+</sup> cells were recovered by this method in 8-d-old thymuses.

<sup>c</sup>Calculated based on tet<sup>+</sup> cells recovered after MACS.



**Figure 3. Ontogeny of HSA<sup>high</sup> and HSA<sup>low</sup> NKT lineage thymocytes.** Thymocytes of WT mice at different ages were enriched and analyzed as in Fig. 2. The total numbers of MACS-enriched tet<sup>+</sup> cells at 4 and 5 d was 208 and 265, respectively (note that all dots are displayed and each dot represents a cell). Tet<sup>+</sup> DP<sup>low</sup> cells are in the pink elliptical gate and have the same DP<sup>low</sup> phenotype as the CD69<sup>+</sup> DP cells in the total thymocyte population shown in the rightmost panel of fourth row. Note that for consistency of

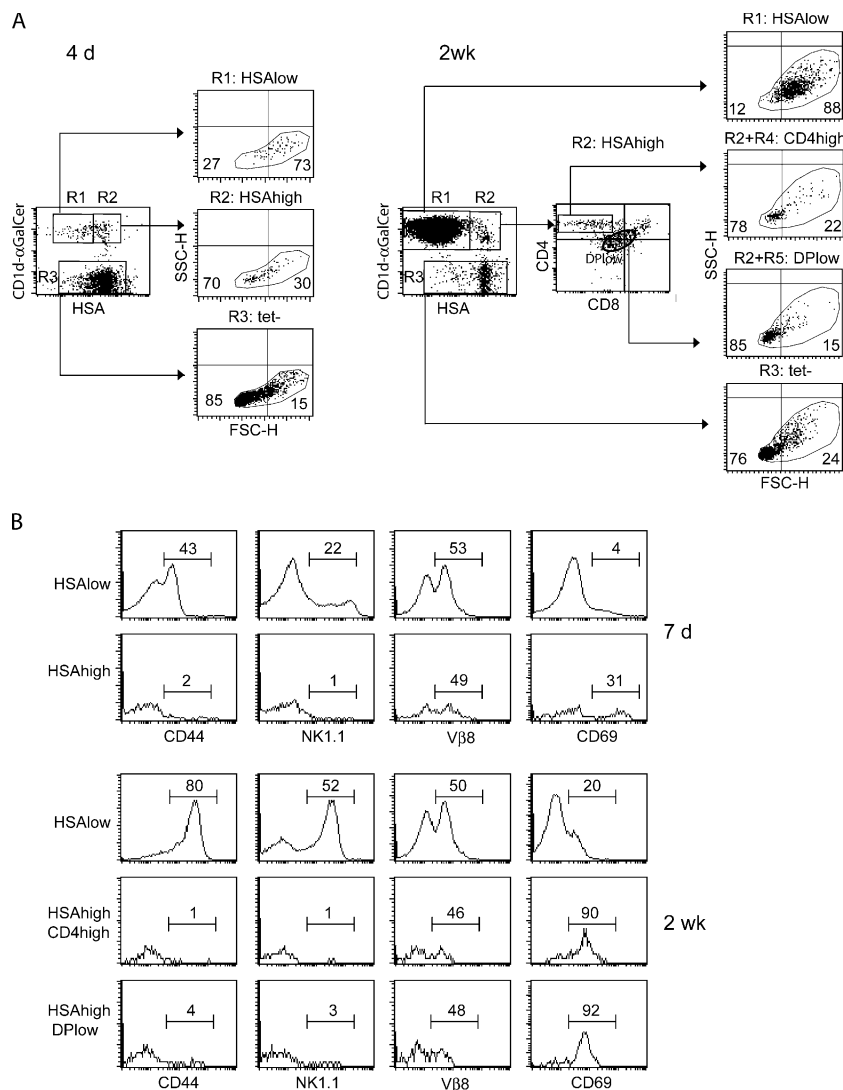
CD4/CD8 staining, thymuses at 8 d, 14 d, and 5 weeks were analyzed side by side in the same experiment (rows 3–5), with doublet and propidium iodide uptake exclusion. The 2-wk-old thymocytes also were analyzed side by side with Jα18<sup>-/-</sup> and CD1d<sup>-/-</sup> thymuses in the same experiment (bottom three rows). Data are representative of at least three individual experiments where 5 to 60 thymuses were pooled.

**HSA<sup>high</sup> Vα14 NKT cells are not cycling and express a biased Vβ8 usage**

Mature HSA<sup>low</sup> NKT cells are large, cycling cells that characteristically undergo multiple rounds of cell division that lead to massive intrathymic expansion (10). We found that the size of the HSA<sup>high</sup> cells, both DP<sup>low</sup> and CD4, was comparable to that of the majority of thymocytes, and much smaller than the cycling HSA<sup>low</sup> cell (Fig. 4 A). Although the rarity of these cell types precludes BrdU incorporation studies, their small size likely reflects the lack of cell cycling. This is consistent with the lack of expansion of this population after birth, compared with the explosive growth of HSA<sup>low</sup> cells between days 5 and 8 (Table I). Thus, cell division is a late event that can be separated from the stages of development. The HSA<sup>high</sup> DP and HSA<sup>high</sup> CD4 cells expressed a CD44<sup>low</sup> NK1.1<sup>-</sup> phenotype similar to that of the following

HSA<sup>low</sup> stage (Fig. 4 B). In addition, they expressed CD69, as reported for cells having recently undergone positive selection (30).

Mature HSA<sup>low</sup> NKT cells express a highly biased repertoire overusing Vβ8, Vβ7, and Vβ2; however, it is unclear whether this Vβ hierarchy results from preferential expansion at mature stages or from preferential selection at immature stages. Although the CD1d-α-galactosylceramide (CD1d-α-GalCer) tetramers may bias the analysis to cells expressing these three Vβ chains, Fig. 4 B shows that the HSA<sup>high</sup> cells exhibited the same frequency of Vβ8 chain usage as found in the HSA<sup>low</sup> stages. This result suggests that the hierarchy of Vβ chain usage, at least among the three Vβ families, is already established after positive selection and is not modified substantially later by preferential cellular expansion.



**Figure 4. Phenotypic characterization of HSA<sup>high</sup> NKT cells.** Thymocytes were MACS-enriched for tet<sup>+</sup> cells and (A) analyzed for size (forward scatter, FSC) and granularity (side scatter, SSC) or (B) stained for HSA, CD44, NK1.1, Vβ8, or CD69. Line graphs display the frequency of

CD44<sup>+</sup>, NK1.1<sup>+</sup>, Vβ8<sup>+</sup>, and CD69<sup>+</sup> cells among tet<sup>+</sup>HSA<sup>low</sup> or tet<sup>+</sup>HSA<sup>high</sup> cells gated as indicated. Data are representative of three individual experiments using pools of 7-d-old or 2-wk-old mice as indicated.

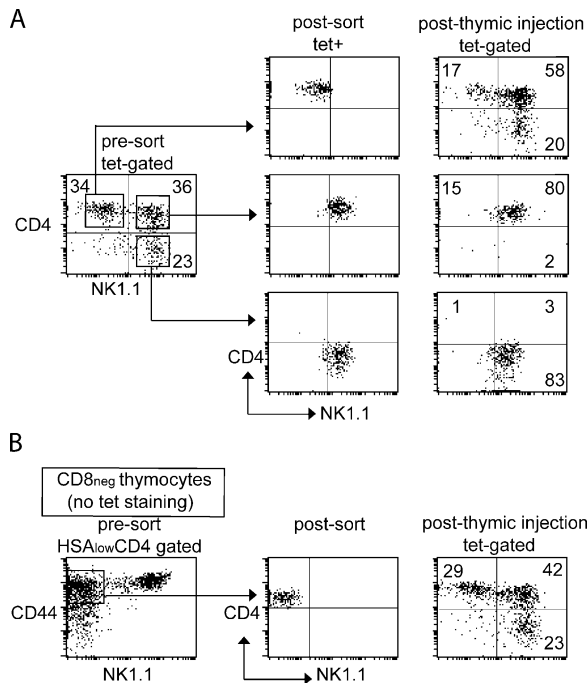
#### DN cells arise from CD4 cells at the HSA<sup>low</sup> NK1.1<sup>-</sup> stage

The exclusive CD4<sup>+</sup> phenotype of the HSA<sup>high</sup> stage and the appearance of DN cells at the cycling HSA<sup>low</sup> CD44<sup>low</sup> stage suggested that DN cells might arise at this latter stage from CD4<sup>+</sup> precursors. We sorted the CD4<sup>+</sup> subset of thymic HSA<sup>low</sup> NK1.1<sup>-</sup> tet<sup>+</sup> cells from WT mice and injected it into the thymus of Jα18<sup>-/-</sup> (NKT-deficient) recipients. In this transfer assay, the transferred NK1.1<sup>-</sup> cells matured to NK1.1<sup>+</sup> within 5–7 d, as previously shown. In addition, the progeny of the CD4 subset included CD4<sup>+</sup> and DN cells (Fig. 5 A), which demonstrated a precursor product relationship between CD4 and DN cells. A similar relationship was suggested previously (11). In contrast, the mature NK1.1<sup>+</sup> cells conserved their CD4 or DN phenotype in the same intrathymic transfer conditions (Fig. 5 A). To rule out poten-

tial artifacts caused by tetramer staining, we sorted CD4<sup>+</sup> CD44<sup>high</sup>NK1.1<sup>-</sup> cells from a preparation of HSA<sup>low</sup>CD8<sup>neg</sup> thymocytes, and transferred them intrathymically. The progeny of the CD4<sup>+</sup> cells contained CD4<sup>+</sup> and DN tet<sup>+</sup> NKT cells (Fig. 5 B). Together, these results demonstrate that DN cells arise from the conversion of CD4<sup>+</sup> cells during a short developmental window.

#### DN NKT cells do not arise through a pTα-independent “γδ-like” pathway

It was hypothesized that a proportion of DN NKT cells might originate through a distinct pTα-independent “γδ-like” pathway that is characterized by the absence of induction of CD4 or CD8 coreceptor. Although pTα-deficient mice have normal γδ T cell compartments, they were re-

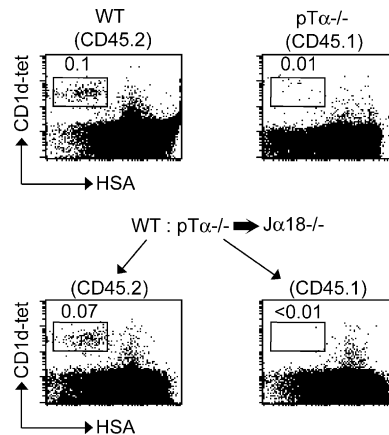


**Figure 5. DN NKT cells arise from CD4<sup>+</sup> precursors.** NKT thymocyte subsets were isolated and FACS-sorted from 2–4-wk-old mice for intrathymic injection into NKT-deficient  $\text{J}\alpha 18^{-/-}$  recipients. The subsets injected included (A)  $\text{tet}^+\text{NK1.1}^- \text{CD4}^+$ ,  $\text{tet}^+\text{NK1.1}^+ \text{CD4}^+$ , and  $\text{tet}^+\text{NK1.1}^+ \text{CD4}^-$  (as gated) or (B)  $\text{CD4}^+ \text{CD44}^{\text{high}} \text{NK1.1}^-$  cells (as gated) obtained from a preparation of  $\text{HSA}^{\text{low}} \text{CD8}^- \text{I}\alpha\beta^{-/-}$  thymocytes without tetramer staining. CD4/NK1.1 profiles of sorted cells and their progeny 7 d after intrathymic injection are shown, as indicated. Numbers represent the percentages of each subpopulation in indicated gate or quadrant. Data are representative of three separate experiments.

ported to lack NKT cells, a finding that apparently is inconsistent with the hypothesis (31). However, it is recognized that the NKT cell defect in these mice might not be cell autonomous, but could be secondary to the profound depletion of thymocytes acting as CD1d-presenting cells for NKT cell selection. Therefore, we generated mixed, congenically marked  $\text{pT}\alpha^{-/-}:\text{WT}$  into irradiated  $\text{J}\alpha 18^{-/-}$  recipient bone marrow radiation chimeras. Fig. 6 demonstrates that, in contrast with WT NKT cells, which developed normally, there was a profound, cell autonomous NKT cell defect in the CD45.1<sup>+</sup>  $\text{pT}\alpha^{-/-}$  compartment of these mixed chimeras.

**DISCUSSION**

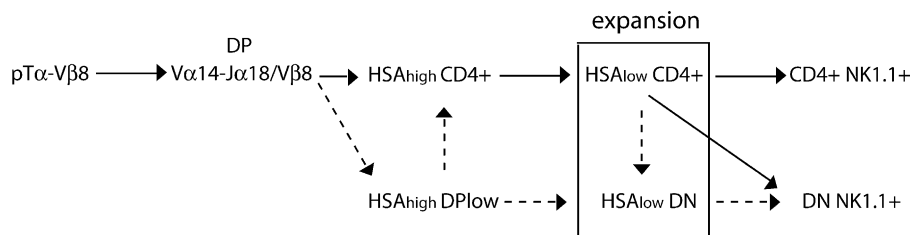
By enriching NKT precursors from pools of up to 60 newborn thymuses with CD1d- $\alpha$ -GalCer tetramers and MACS sorting, we have been able to identify and characterize very rare ( $10^{-6}$ )  $\text{HSA}^{\text{high}}$  cells that represent the earliest reported stages of NKT cell development (10, 11, 27, 28). The physical identification of the  $\text{HSA}^{\text{high}}$   $\text{DP}^{\text{low}}$  and  $\text{HSA}^{\text{high}}$   $\text{CD4}^+$  stages essentially completes the map of this enigmatic developmental pathway (Fig. 7). The nearly exclusive  $\text{CD4}^+$  phe-



**Figure 6. Cell-autonomous requirement for  $\text{pT}\alpha$  in NKT thymocyte development.** Mature NKT cells in WT,  $\text{pT}\alpha^{-/-}$ , and  $\text{pT}\alpha^{-/-}:\text{WT}$  bone marrow  $\rightarrow \text{J}\alpha 18^{-/-}$  radiation chimeras. In the mixed chimera, the WT compartment is identified by CD45.2 gating and the  $\text{pT}\alpha^{-/-}$  compartment by CD45.1 as indicated. Data are representative of at least four mice in each group.

notype of the rare  $\text{HSA}^{\text{high}}$  precursor in 3-d-old mice, and the demonstration by cell transfer experiments that the  $\text{CD4}^+$  cells could give rise to a substantial fraction of DN cells clearly support a model whereby all DN cells arise late in development, after down-regulation of CD4 by a fraction of  $\text{CD4}^+$  cells. However, the presence of rare DN cells among the  $\text{HSA}^{\text{high}}$  subset of older mice and the appearance of a population of  $\text{DP}^{\text{low}}$  cells in 2-wk-old mice suggest that the respective kinetics of CD4/CD8 coreceptor down-modulation may vary with age, and that commitment to the CD4 versus DN sublineage already may be imparted at a DP stage. The existence of  $\text{DP}^{\text{low}}$  cells has been controversial (11, 27, 28). Our results differ markedly from those of Gapin et al. (27) and Gadue et al. (28) who found relatively high frequencies of  $\text{DP}^{\text{low}}$  cells among total  $\text{tet}^+$  thymocytes. Instead, we found that authentic  $\text{DP}^{\text{low}}$  cells were very rare, and were restricted to the previously unrecognized  $\text{HSA}^{\text{high}}$  stage of development.

This study could not detect  $\text{tet}^+$  DP thymocytes preceding positive selection in  $\text{CD1d}^{-/-}$  mice. However, the unambiguous presence of  $\text{DP}^{\text{low}}$  cells after positive selection is important because it provides critical and direct evidence for the existence of such a DP precursor stage. Other experimental results support this conclusion. For example, the cell-autonomous requirement for  $\text{pT}\alpha$ , as demonstrated in the present study, is highly suggestive of such a stage because  $\text{pT}\alpha$  signaling induces CD4 and CD8 expression. The genetic role of  $\text{CD8}$  in shaping the  $\text{V}\beta$  repertoire of CD4 and DN NKT cells also suggests their previous expression of CD8, and its interaction with CD1d (12). Finally, it was shown recently in a TCR transgenic model of agonist-mediated selection that immature DP cells could give rise to mature DN or  $\text{CD8}\alpha\alpha$  cells, a direct demonstration of the down-regulation of CD4 and  $\text{CD8}\beta$  (29). This example is particularly relevant to the development of NKT cells, be-



**Figure 7. New model outlining the cellular developmental stages leading to mature CD4 and DN NKT cells.** The dashed arrows indicate hypothetical precursor-product relationship.

cause their thymic ligand, iGb3, also is an agonist that is capable of full activation of mature cells.

Given the rarity of the HSA<sup>high</sup> DP<sup>low</sup> and HSA<sup>high</sup> CD4<sup>+</sup> cell, it may not be surprising that, for technical reasons, preselection DP precursors would not be detected. The rarity of these precursors also supports the notion that canonical Vα14-Jα18 rearrangements occur stochastically at very low frequency.

It remains unclear what determines the CD4 versus DN fate of NKT cells. Their near 1:1 ratio might suggest a stochastic decision; however, an alternative model is that CD8 could be forcibly down-regulated from DP cells undergoing high levels of signaling. The CD4 versus DN choice also might be instructed by different TCR affinities for ligand.

The newly identified HSA<sup>high</sup> DP<sup>low</sup> and HSA<sup>high</sup> CD4<sup>+</sup> cells have a small size and express CD69. These stages, which precede the previously identified HSA<sup>low</sup> CD4<sup>+</sup> at which cells blast and undergo intense cell division, are analogous to the HSA<sup>high</sup> CD69<sup>+</sup> stage following positive selection of mainstream MHC-restricted T cells (30). These positively selected MHC-restricted T cells will not engage in cell cycle. Thus, the HSA<sup>high</sup> CD4<sup>+</sup> cell represents the likely branchpoint in the development of mainstream T versus NKT cells. An unresolved issue is whether commitment to cell division already is imparted at this stage, or whether it occurs later at the HSA<sup>low</sup> stage (e.g., upon second antigenic stimulation by medullary dendritic cells). Although there is no example, to our knowledge, of mitogenic stimuli imparted at the immature HSA<sup>high</sup> stage, some arguments suggest that this may be the case. First, entry into the cell cycle is delayed by 1 or 2 d following TCR stimulation, corresponding to the lag time between HSA<sup>high</sup> and HSA<sup>low</sup> CD4 T cells. Second, recent experiments have demonstrated that restricted expression of CD1d on DP thymocytes was sufficient to drive the major expansion observed at the medullary HSA<sup>low</sup> stage (32).

These different lines of evidence raise the surprising possibility that a single set of signaling interactions between HSA<sup>high</sup> NKT cell precursors and neighboring cortical thymocytes—involving TCR and perhaps SLAM family members as coreceptors—may represent the defining event of the NKT lineage imparting the subsequent NKT expansion and differentiation program.

## MATERIALS AND METHODS

**Mice.** C57BL/6, C57BL/6.CD45.2, and C57BL/6.1-Aβ<sup>b-/-</sup> mice were obtained from Taconic Farms. CD1d<sup>-/-</sup> mice were used after 12 back-

crosses to C57BL/6 (33), Jα18<sup>-/-</sup> (34) and pTα<sup>-/-</sup> (35) mice on C57BL/6 backgrounds were gifts from M. Taniguchi (RIKEN, Yokohama, Japan) and I. Aifantis (University of Chicago, Chicago, IL), respectively. All mice were maintained under specific pathogen-free conditions at the University of Chicago, and experimental studies were in accordance with the Institutional Animal Care and Use Guidelines.

**Flow cytometry.** FITC-conjugated mAbs against CD8, Vβ8.1/8.2, CD45.2, CD4, CD24 (HSA), CD44, and CD69; PE-conjugated mAbs against CD69, HSA, CD44, and NK1.1; PE-Cy7-conjugated mAb against CD4; cytochrome-conjugated mAbs against CD24, CD4, and CD8; and allophycocyanin-conjugated mAbs against CD4, CD44, CD8, and NK1.1 were obtained from BD Biosciences. Propidium iodide was from Sigma-Aldrich. CD1d-αGalCer tetramers were produced with streptavidin-PE or -allophycocyanin and used for staining as described previously (36). Samples were analyzed using a four-color FACSort equipped with argon and 635-nm diode lasers (Becton Dickinson) and CELLQuest software. For five-color staining, samples were analyzed using BD LSR II equipped with 488 sapphire and 633 HeNe lasers (Becton Dickinson) and FACSDiva software. In some experiments, doublet and propidium iodide exclusion was applied to remove irrelevant dots.

**Enrichment of CD1d-αGalCer tetramer<sup>+</sup> cells with paramagnetic microbeads.** Thymuses were pooled from batches of 40 to 60 newborns (day of birth considered as day 1), and CD1d tetramer<sup>+</sup> thymocytes were enriched using anti-PE or -allophycocyanin microbeads and the AutoMACs system (Miltenyi Biotec) at ice cold temperature following the manufacturer's instructions.

**FACS sorting of NKT cells.** Pooled thymuses from batches of 25 to 50 C57BL/6.IAβ<sup>b-/-</sup> or C57BL/6 mice (2–4-wk-old) were depleted of CD8<sup>+</sup> cells using anti-CD8 microbeads and the AutoMACs system (Miltenyi Biotec); stained with CD1d-αGalCer streptavidin-PE along with anti-CD4 and -NK1.1; and the NKT cells were sorted into CD4<sup>+</sup>NK1.1<sup>-</sup>, CD4<sup>+</sup>NK1.1<sup>+</sup>, and CD4<sup>-</sup>NK1.1<sup>+</sup> subsets. Alternatively, to avoid TCR cross-linking by tetramer staining, CD8-negative thymocyte preparations were stained with HSA, CD4, CD44, and NK1.1 for sorting of HSA<sup>low</sup>CD4<sup>+</sup>CD44<sup>high</sup>NK1.1<sup>-</sup> cells.

**Intrathymic injection.** Sorted tetramer-positive CD4<sup>+</sup>NK1.1<sup>-</sup>, CD4<sup>+</sup>NK1.1<sup>+</sup>, and CD4<sup>-</sup>NK1.1<sup>+</sup> subsets or sorted CD4<sup>+</sup>CD44<sup>high</sup>NK1.1<sup>-</sup> cells (4–8 × 10<sup>6</sup>) were injected intrathymically as described, and their progeny were analyzed 5 to 7 d later after MACS enrichment of CD1d-tetramer positive cells.

**Bone marrow radiation chimeras.** C57BL/6.Jα18<sup>-/-</sup> mice received whole body γ-irradiation (1,000 Rad) with a cesium source (Gammacell 40), and were reconstituted 6 h later with one i.v. injection of 5–10 × 10<sup>6</sup> bone marrow cells from various adult donors.

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