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Original Publication Citation
Wei, D. G., S. A. Curran, P. B. Savage, L. Teyton, and A. Bendelac (26, May). Mechanisms imposing the vbeta bias of valpha14 natural killer t cells and consequences for microbial glycolipid recognition. J. Exp. Med. 23 (5), 1197-127.

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Savage, Paul B.; Wei, Dasten G.; Curran, Shane A.; Teyton, Luc; and Bendelac, Albert, "Mechanisms imposing the VBeta bias of V14a natural killer T cells and consequences for microbial glycolipid recognition" (2006). Faculty Publications. 314.
https://scholarsarchive.byu.edu/facpub/314

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Mechanisms imposing the Vβ bias of Vα14 natural killer T cells and consequences for microbial glycolipid recognition

Datsen G. Wei,1,3 Shane A. Curran,1 Paul B. Savage,4 Luc Teyton,5 and Albert Bendelac1,2

Mouse and human natural killer T (NKT) cells recognize a restricted set of glycosphingolipids presented by CD1d molecules, including self iGb3 and microbial α-glycuronosylceramides. The importance of the canonical Vα14-Jα18 TCR α chain for antigen recognition by NKT cells is well recognized, but the mechanisms underlying the Vβ bias in mouse have not been explored. To study the influences of thymic selection and the constraints of pairing with Vα14-Jα18, we have created a population of mature T cells expressing Vα14-Jα18 TCR α chain in CD1d-deficient mice and studied its recognition properties in vitro and in vivo. Transgenic cells expressed a diverse Vβ repertoire but their recognition of endogenous ligands and synthetic iGb3 was restricted to the same biased Vβ repertoire as expressed in natural NKT cells. In contrast, α-GalCer, a synthetic homologue of microbial α-glycuronosylceramides, was recognized by a broader set of Vβ chains, including the biased NKT set but also Vβ6, Vβ9, Vβ10, and Vβ14. These surprising findings demonstrate that, whereas Vβ8, Vβ7, and Vβ2 represent the optimal solution for recognition of endogenous ligand, many Vβ chains that are potentially useful for the recognition of foreign lipids fail to be selected in the NKT cell repertoire.
TCR α transgene in CD1d-deficient (CD1d<sup>−/−</sup>) mice. Our studies demonstrate that the invariant TCR α chain could pair indiscriminately with the entire Vβ repertoire, ruling out pairing biases as a substantial factor. Second, we found that only Vβ7, Vβ8, and Vβ2 allowed for recognition of endogenous ligand and iGb3, with a Vβ7>Vβ8>Vβ2 hierarchy of responses paralleling the corresponding Vβ enrichment by thymic selection in vivo, demonstrating a predominant role of positive selection over negative selection in shaping the Vβ repertoire. In contrast, many Vβ families that are not represented in the natural NKT cell repertoire were nevertheless capable of recognition of foreign and microbial lipids, revealing a surprisingly inefficient selection of potentially useful TCRs.

RESULTS

**Vα14-Jα18 transgenic (Tg) cells in CD1d<sup>−/−</sup> mice**

Vα14-Jα18 TCR α chain Tg mice have an increased population of canonical CD44<sup>low</sup>/NK1.1<sup>+</sup> NKT cells with the expected Vβ8, Vβ7, Vβ2 bias and CD4 or double negative (DN) phenotype. We previously reported that some Tg lines also harbored in their thymus and spleen a peculiar subset of mature T cells with a CD44<sup>low</sup>/NK1.1<sup>+</sup> DN phenotype and a conspicuous absence of Vβ bias (15). By crossing the Vα14-Jα18 transgene onto a CD1d<sup>−/−</sup> background, we found that the canonical NK1.1<sup>+</sup> NKT cells had disappeared, as expected, whereas the CD44<sup>low</sup>/NK1.1<sup>−</sup> DN T cells persisted (Fig. 1, top and middle). Most of the CD44<sup>low</sup>/NK1.1<sup>−</sup> DN T cells lacked endogenous Vα chains because only 2% were stained by a combination of antibodies against Vα2, Vα3.2, Vα8, and Vα11, which otherwise accounted for 26% of the TCR α chains expressed by other T cells (Fig. 1, bottom). Thus, it is likely that the DN T cells express the invariant Vα14-Jα18 TCR α chain encoded by the transgene, whereas other T cells express endogenous Vαs. Consistent with the expression of Vα14-Jα18, up to 25% of the DN T cells were brightly stained by the α-GalCer-CD1d tetrampo (Fig. 1, bottom). Such a peculiar DN lineage maturing in the absence of thymic ligand has been described previously in many TCR Tg systems and is believed to be induced by the early expression of the Tg TCR α chain at the DN3 stage (as we observed in this Tg line; unpublished data) competing with the pre-TCR α chain for signaling (16–20). Importantly, these Tg cells were reported to respond to their ligand in a dosage-dependent manner despite the absence of thymic selection (21).

**Nonbiased Vβ repertoire of Vα14–Jα18 Tg DN cells in CD1d<sup>−/−</sup> mice**

We next studied the Vβ repertoire associated with the invariant TCR α chain in the Vα14-Jα18 Tg DN T cells of CD1d<sup>−/−</sup> mice. For comparison with another nonselected Vβ repertoire, we used CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) thymocytes from β<sub>m</sub><sup>−/−</sup>-I-A<sup>−/−</sup> “MHC-null” mice that express high levels of surface TCR readily stained by anti-Vβ antibodies (22). For comparison with the CD1d-restricted repertoire, we used CD3<sup>+</sup>NK1.1<sup>+</sup> thymocytes from WT B6 mice thymuses, which contain >95% canonical tetramer<sup>+</sup> NKT cells (23). Fig. 2 shows that, in dramatic contrast with the biased Vβ8, Vβ7, Vβ2 repertoire of natural NKT cells in WT mice, the CD1d<sup>−/−</sup> Tg DN cells expressed a very diverse repertoire. This repertoire appeared relatively close to the nonselected DP thymocytes in MHC-null mice, although significant differences were found for some Vβs suggesting that, in the absence of selection by CD1d, Vα14-Jα18 imparts only modest pairing bias compared with the average Vα.

**Figure 1. Mature DN T cells in Vα14tg,CD1d<sup>−/−</sup> mice.**

(top) The spleen of Vα14tg mice harbors a sizeable population of CD4<sup>+</sup>CD8<sup>+</sup>-CD3<sup>+</sup> DN T cells (boxed in gate b) that is independent of CD1d expression. (Middle) Gated DN T cells have a uniform CD44<sup>low</sup>/NK1.1<sup>−</sup> phenotype in Vα14tg,CD1d<sup>−/−</sup> mice. (Bottom) The DN T cells of Vα14tg, CD1d<sup>−/−</sup> mice do not express endogenous TCR α chains; 29% of them are α-GalCer-CD1d tetramer<sup>+</sup>. Note that, in contrast, the CD4 and CD8 T cells (boxed in gate a) in these mice express endogenous Vαs but are α-GalCer-CD1d tetramer-negative. Data are representative of four independent experiments with two mice per group.
To further evaluate the diversity of the \( V_\beta \) repertoire associated with \( V_\alpha 14-J_\alpha 18 \), we cloned and sequenced multiple CDR3 segments generated by RT-PCR from sorted, CD1d\(-/-\) Tg DN cells. We chose three TCR \( \beta \) chains, \( V_\beta 8.2 \), \( V_\beta 8.1 \), and \( V_\beta 12 \), as representatives of \( V_\beta \)s highly, poorly, or nonenriched in natural NKT lineage cells, respectively. Fig. 3 shows a broad distribution of CDR3 lengths and a broad usage of \( J_\beta \) segments for each of these TCR \( \beta \) chains, with a similar overrepresentation of the \( J_\beta 2 \) over the \( J_\beta 1 \) cluster as previously reported for conventional T cells (24, 25). We found 77–94% of unique sequences in each set of TCR \( \beta \) chains analyzed, similar to previous findings reported for other T cell populations (26, 27). Together, these results establish the broad diversity of the \( V_\beta \) repertoire expressed by the CD1d\(-/-\) Tg DN cells. Thus, pairing of \( V_\alpha 14-J_\alpha 18 \) with TCR \( \beta \) chains is largely promiscuous and unlikely to be a significant factor in the \( V_\beta \) repertoire bias of \( V_\alpha 14 \) NKT cells. Furthermore, comparison of the non-selected \( V_\beta \) repertoire of mature NKT thymocytes allowed a precise calculation of the degree of enrichment associated with thymic selection for each \( V_\beta \) family. Thus, \( V_\beta 2 \) was enriched 2.6-fold, \( V_\beta 8.2 \) was enriched 8-fold, and \( V_\beta 7 \) was enriched 12-fold. A similar percentage of \( V_\beta 7 \) and \( V_\beta 8.1+8.2 \) as in the CD1d\(-/-\) Tg DN cells was also found in DP thymocytes obtained from another CD1d\(-/-\) \( V_\alpha 14-J_\alpha 18 \) TCR strain where the transgene was driven by a CD4 promoter to avoid premature expression at the DN3 stage (unpublished data). Thus, this striking \( V_\beta 7 > V_\beta 8.2 > V_\beta 2 \) hierarchy contrasts with previous estimates derived merely from the relative frequency of \( V_\beta \)s expressed by NKT cells, where \( V_\beta 8.2 > V_\beta 7 \) because it was six times more frequent than \( V_\beta 7 \). It also differs significantly from estimates derived by comparing the NKT lineage repertoire with mainstream DP thymocytes where \( V_\beta 7 \) “enrichment” was similar to that of \( V_\beta 8.2 \) (14).

**V\( \beta \)-specific binding of \( \alpha \)-GalCer-CD1d tetramers**

\( \alpha \)-GalCer-CD1d tetramers stain nearly all mouse NKT cells expressing \( V_\alpha 14-J_\alpha 18 \) combined with \( V_\beta 8 \), \( V_\beta 7 \), and \( V_\beta 2 \) (23, 28). In the nonselected \( V_\alpha 14-J_\alpha 18 \) Tg DN T cells, 69–85% of cells expressing \( V_\beta 8 \), \( V_\beta 7 \), and \( V_\beta 2 \) were tetramer\(^+\) (Fig. 4), indicating that these \( V_\beta \)s are usually, though not always, compatible with \( \alpha \)-GalCer recognition independently of the CDR3 sequence. Surprisingly, however, 36–84% of cells expressing additional \( V_\beta \) families that are not significantly represented in the natural NKT cell repertoire (Fig. 2), including \( V_\beta 3 \), 6, 9, 10, and 14, were also stained by the tetramers.

**Figure 2.** \( V_\beta \) repertoire of \( V_\alpha 14tg.CD1d^{-/-} \) DN T cells. The \( V_\beta \) repertoires of splenic CD3\( ^{\epsilon +} \) DN T cells in \( V_\alpha 14tg.CD1d^{-/-} \) mice, DP thymocytes in MHC-null mice, and CD3\( ^{\epsilon +}NK1.1^{+} \) thymic NKT cells in C57BL/6 mice are compared. Histograms show staining by a panel of anti-\( V_\beta \) mAbs with percentages as indicated. Data are means \( \pm \) SD of three independent experiments with at least two mice per group.

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Published May 1, 2006
Other Vβs, such as Vβ4, 5, 11, 12, and 13 were mostly negative, although they consistently included 3–10% positive cells. Thus, these results reveal many new Vβ families that allow for α-GalCer recognition but had escaped prior recognition because of their absence from the natural NKT cell repertoire. As expected, an extended Vβ repertoire was also found for the microbial lipid α-glucuronylceramide of which α-GalCer is a mimic (unpublished data).

**Vβ-specific induction of CD69 by CD1d/lipid ligands**

Because iGb3-CD1d tetramers could not stain NKT cells (6), we devised T cell stimulation assays to compare the Vβ repertoires specific for iGb3 and α-GalCer. Thymocytes have very poor endocytic properties and are therefore defective in the presentation of exogenous lipid ligands requiring endosomal access for processing or loading onto CD1 molecules (reference 29 and unpublished data). Therefore, we used DCs pulsed with antigen to stimulate DN T cells obtained from the spleen of Vα14tg.CD1d−/− mice. After 4–6 h, DCs were harvested and stained for CD69. Fig. 5 shows that a large fraction of Vβ2, 7, and 8 cells up-regulated CD69 in response to DCs pulsed with either iGb3 (20–46%) or α-GalCer (56–77%) when compared with unpulsed DCs from CD1d−/− mice (3–4%). Consistent with the tetramer staining experiments, many additional Vβs were stimulated by α-GalCer, including Vβ3, 6, 9, 10, 14 (23–63%), whereas others (Vβ4, 5, 11, 12, 13) gave very weak responses (4–12%). Interestingly, when compared with CD1d−/− DCs, unpulsed WT DCs weakly but consistently stimulated Vβ2, 7, and 8 cells (14–33%), suggesting that natural ligand expression was sufficient to induce CD69 on a subset of cells expressing the Vβ families naturally associated with NKT cells. Thus, in contrast with α-GalCer, both the natural ligands expressed by DCs and exogenously added synthetic iGb3 elicited the

![Figure 3. Vα14tg.CD1d−/− DN T cells have diverse CDR3 sequences.](image)

![Figure 4. Vβ-specific binding of α-GalCer-CD1d tetramers to Vα14tg.CD1d−/− DN T cells.](image)
very same Vβ repertoire as expressed by natural NKT cells. Importantly, neither synthetic iGb3 nor naturally expressed ligands stimulated other Vβs, suggesting that the Vβ bias of natural NKT cells must reflect positive selection of these Vβs rather than negative selection of other Vβs.

**Vβ-specific induction of cell division by CD1d/lipid ligands**

Although CD69 can be induced by partial TCR signaling, T cell proliferation requires stronger “agonist” signals (30). Indeed, iGb3 was reported to be an agonist ligand for mouse and human NKT cells and a prime candidate for mediating the positive selection and the expansion of NKT cell precursors in the thymus (6). Therefore, to model the expansion of selective Vβs during NKT development, we used CFSE-labeled DN T cells to compare the mitogenic properties of endogenous and exogenous NKT ligands in a cell culture system. IL-7 was added to prevent the death of unstimulated cells over the 4-d culture period with antigen-pulsed DCs. When DN T cells were incubated with CD1d−/− DCs alone, a background CFSE dilution of ~50%, likely the result of IL-7, was observed for all Vβs in different experiments. Remarkably, CD1d-expressing WT DCs induced a significant and selective expansion of Vβ7 cells (84%) in the absence of added ligands, above the background CFSE dilution observed in Vβ6 and Vβ12 cells (45–48%) (Fig. 6). Vβ2 (57%) and Vβ8 (62%) were slightly higher than Vβ6 and Vβ12. These data suggest that naturally expressed ligands can act as true albeit weak agonists capable of inducing cell proliferation, with the highest affinity for Vβ7, similar to the Vβ7>Vβ8>Vβ2 hierarchy observed in the CD69 induction system.

**Figure 5. Vβ-specific expression of CD69 by antigen-stimulated Vα14tg.CD1d−/− DN T cells.** Enriched DN T cells from Vα14tg.CD1d−/− spleens were cocultured with CD1d−/− DCs or WT DCs pulsed with 100 ng/ml iGb3, 1 ng/ml α-GalCer, or no antigen. T cells were stimulated for 4 h with α-GalCer or for 6 h with iGb3. Percentages of CD69-positive DN T cells gated according to Vβ usage are indicated in top right quadrants as mean ± SD from four independent experiments.
Published May 1, 2006

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Figure 6. Vβ-specific proliferation of antigen-stimulated Vα14tg. CD1d−/− DN T cells. CFSE-labeled Vα14tg.CD1d−/− DN T cells were cocultured with antigen-pulsed DCs in the presence of 10 ng/ml IL-7 for 4 d and CFSE dilution profiles were gated according to Vβ expression. DCs were pulsed with no antigen, iGb3 at 50 ng/ml or α-GalCer at 10 ng/ml. Control stimulation was with a low dose of 50 ng/ml of anti-CD3ε directly added to the culture wells. Data are representative of three independent experiments.

Vβ-specific hierarchy in responses to glycolipid ligands

To extend the aforementioned findings to the larger Vβ repertoire and to more rigorously quantitate the apparent differences in individual Vβ responses, DCs were pulsed with a range of concentrations of α-GalCer and iGb3 before coculture with CFSE-labeled DN T cells. Fig. 7 shows the CFSE dilution profiles according to Vβ expression, with statistics displayed whenever dilution was >50% background level. Percentages of CFSE-negative (>7 divisions) and CFSE-low cells were displayed separately to provide a quantitation of the rate of proliferation in addition to the frequency of cells having proliferated. Furthermore, absolute cell recoveries according to Vβ expression were also recorded (Table I).

| Vβ | α-GalCer (ng/ml) | iGb3 (ng/ml) |
|----|----------------|--------------|
|    | 0.01 0.1 1 10   | 0.5 5 50 500 |
| Vβ2| 0.6 0.9 4.2 9.2 | 0.5 0.7 3.7 4.3 |
| Vβ6| ND ND 1.7 2.7   | ND ND <0.5 <0.5 |
| Vβ7| 2.1 4.0 15.6 27.3 | 1.4 3.0 10.2 14.6 |
| Vβ8.1 + 8.2| 1.2 1.9 8.7 17.3 | 0.6 1.2 5.1 6.8 |
| Vβ8.2| 1.5 2.6 12.0 23.1 | 0.7 1.3 6.0 7.2 |
| Vβ9| ND ND <0.5 4.7 | ND ND <0.5 <0.5 |
| Vβ10| ND ND <0.5 4.1 | ND ND <0.5 <0.5 |
| Vβ14| ND ND 3.2 9.4 | ND ND <0.5 <0.5 |
| non-Vβ2, 7, & 8 | ND ND 0.6 0.7 |

The fold increase in the absolute number of cells expressing indicated Vβs at the end of a 4-d culture period is shown for a range of concentrations of α-GalCer and iGb3. Data are representative of four independent experiments. ND, not determined.
thus faithfully mimicking the natural NKT cell bias. This highly selective effect was further illustrated by the near background CFSE dilution (57%) found for all the non-Vβ2, 7, 8 cells examined together at the highest concentration of iGb3 (Fig. 7, bottom right). Furthermore, iGb3 was 10 times more stimulating for Vβ7 than for Vβ8.2 cells, as judged by the proportion of cells reaching the CFSE-negative stage and by the cell expansion measured in absolute numbers (Table I). For example, 40% of Vβ7 cells had reached the CFSE-negative stage at 0.5 ng/ml of iGb3 compared with only 34% of Vβ8.2 at 5 ng/ml (Fig. 7), and the level of expansion of Vβ7 at 0.5 ng/ml was not reached until 5 ng/ml for Vβ8.2 (Table I). Thus, the data establish a clear hierarchy of Vβ stimulation by iGb3, with Vβ7>Vβ8.2>Vβ2, fully consistent with their respective 12-, 8-, and 2.6-fold enrichments during thymic selection (Fig. 2). This good correlation between the hierarchy measured in vitro and the selection bias in vivo is consistent with iGb3 being a major thymic ligand for NKT precursors and further suggests that positive selection rather than negative selection plays the primary role in shaping the Vβ repertoire of Vα14 NKT cells.

Correlations between Vβ selection and CD1d levels in vivo

Previous reports have shown that changes in Vβ selection occurred in mice expressing lower or higher levels of CD1d.
and were hypothesized to result from changes in positive and negative selection according to VB affinities for naturally expressed ligands. Thus, in mice expressing very high levels of CD1d (~50 times greater than WT) under a MHC class I promoter (13), NKT cells were decreased in absolute numbers to 1/3 of WT, suggesting negative selection, with VB2 NKT cells being relatively overrepresented compared with VB8 and VB7 cells. Conversely, VB7 were overrepresented in BALB/c, CD1d<sup>−/−</sup> mice as compared with WT (14). Although the intricacies of positive and negative selection and clonal competition preclude a definitive interpretation of such results when considered alone, the data are fully consistent with the hierarchy of VB responsiveness to iGb3 and naturally expressed ligands as determined in the present study. Here, we present two additional CD1d expression models to further test this hierarchy in vivo. In pLck/high mice expressing CD1d under the control of the proximal Lck promoter in a NOD, CD1d<sup>−/−</sup> background, the level of CD1d was 12 times higher than that of WT mice (3). These Tg mice exhibited a dramatic decrease in the proportion of VB7<sup>+</sup> NKT cells from 21 to 12%, without significant changes in total cell numbers (Fig. 8). Although this Tg model was studied in the NOD background, which is known to be a low NKT expressor, the results were significant because they could be compared with littermate controls in the very same genetic background. Conversely, in C57BL/6 mice expressing a CD1d null allele and a CD1d low allele (an unintended consequence of a loxP insertion in the 5′UTR of CD1d locus) (CD1d<sup>−/−;Lk</sup> mice), the percentage of VB7 cells reached 43% as compared with 22% in WT controls, with a corresponding decrease of VB8 cells from 57 to 41% (Fig. 8). Overall, NKT cell numbers were drastically reduced, suggesting that in the presence of a limited amount of ligands, positive selection could only rescue the highest affinity cells. Thus, combining these two new models with the other two previously published, the in vivo thymic selection outcomes are remarkably consistent with the VB hierarchy of affinity directly established from our ligand stimulation assays.

**Figure 8. VB repertoire changes in mice expressing high or low levels of CD1d.** (top) Levels of CD1d expression (mean fluorescence intensity indicated above histograms) by thymocytes of indicated mice. CD1d<sup>−/−</sup> are mice with a null (−) allele and a low expression (LE) allele the result of a loxP insertion in the 5′UTR of CD1d1. pLck/high are mice with high level expression of a CD1d transgene driven by the proximal Lck promoter. (bottom) Percentages of VB7 and VB8 cells among α-GalCer-CD1d tetramer<sup>+</sup> cells were determined by FACS analysis of indicated mice. Bars represent mean and SD (white, VB7; gray, VB8) based on indicated "n" number of mice examined. "CD1d =": relative CD1d expression level (mean fluorescence intensity) compared with B6 WT mice; "NKT =": relative numbers of NKT thymocytes compared with B6 WT mice. *, statistical significance (P < 0.05) using unpaired Student’s t test.

**VB-specific induction of CD69 upon intrathymic cell transfer**

To further study the recognition of naturally expressed NKT ligands, we transferred VB nonbiased Vα14-Jα18 Tg DN T cells into the thymus of Ly5.1 congenic recipients. Cells were recovered from the host thymuses after 6 h and subjected to an analysis of VB and CD69 expression. In the absence of exogenous antigen, CD69 was induced on a subset of transferred cells with a selective VB7 (46%) > VB8 (43%) > VB2 (38%) pattern (Fig. 9) reminiscent of iGb3 stimulation assays in vitro. Surprisingly, however, two additional Vβs, VB9 and VB14, exhibited significant induction of CD69, albeit to lower levels than the canonical VB7, VB8, VB2, suggesting weaker signaling. This surprising result was consistently observed in 3/3 independent transfer experiments and may suggest that, in addition to iGb3, other thymic ligands may be able to activate NKT precursors. These putative antigens, however, must be unable to meet the critical signaling requirements for inducing cell expansion and/or survival. Unfortunately, the transferred Vα14-Jα18 Tg DN T cells could not be efficiently tracked over the longer period of time required to evaluate cell proliferation, precluding a more complete assessment of the fate of these VB9 and VB14 cells.

**DISCUSSION**

By transgenically expressing the Vα14-Jα18 transgene in a CD1d-deficient background, we have created a DN T cell population with a TCR repertoire made of the canonical invariant α chain of NKT cells combined with a broad, nonbiased VB repertoire. Several features suggest that these Tg cells display a faithful representation of the preselection repertoire of TCR β chains associated with Vα14-Jα18 α chain. First, they originate in the thymus in the absence of selection by CD1d/ligand. Second, they express an endogenous TCR β...
repertoire of breadth and diversity comparable to that of nonselected DP thymocytes. Third, their ontogeny is related to the premature expression of the Tg TCR α chain that binds to the TCR β chains expressed at the DN3 stage. It is believed that, by modifying the signals that normally emanate from the pTα–TCRβ complexes, the TCR α transgene bypasses the subsequent need for TCR-mediated positive selection (16–20, 32). Finally, similar to a subset of mature NKT cells, these Tg thymocytes do not express CD4 or CD8. These Tg DN cells provide, therefore, a unique tool to explore the structure and the functional properties of the preselection repertoire of NKT precursor thymocytes and to contrast them with those of normal mature NKT cells.

Our findings elucidate some long-standing issues regarding the mechanisms underlying NKT cell repertoire selection. Because the nonselected Vβ repertoire associated with Vα14-Jα18 differed only slightly from the one found in the nonselected DP thymocytes of MHC-null mice, which represents an average of all Vβs, we conclude that the Vβ-pairing bias of Vα14-Jα18 α chain must be modest compared with other Vαs. This finding contrasts with isolated or indirect evidence suggesting an inherent instability of many Vα14-Jα18/Vβ complexes (10, 11).

Second, by comparing the frequency of Vβ families in the unselected and postselection repertoire, it was possible for the first time to measure the degree of enrichment for individual families, with Vβ7 (x12) clearly above Vβ8.2 (x8) and Vβ2 (x2.6) at the bottom. The picture would be significantly different if the baseline for comparison was the DP repertoire because Vβ7 and Vβ8.2 would appear to be equally selected (14). Remarkably, these data matched precisely the hierarchy of Vβ responses observed after stimulating the nonselected Vα14-Jα18 Tg T cells with naturally expressed ligands or with synthetic iGb3. Thus, we conclude that the Vβ repertoire associated with Vα14-Jα18 in NKT lineage cells has undergone predominant positive selection and only modest negative selection.

Importantly, the close resemblance between stimulation by iGb3 and stimulation by the naturally expressed NKT ligands provides new and independent support for the notion that iGb3 is the main NKT ligand involved in these thymic processes. A large proportion ranging from 20 to 50% of the Vβ8-, Vβ7-, and Vβ2-expressing cells responded to iGb3 in the CD69 induction assay, whereas nearly all of them responded in the CFSE dilution assay. Although the different nature and kinetics of these assays may underlie these differences, the results converge to indicate that a substantial proportion of the naturally arising Vβ8, Vβ7, and Vβ2 TCRs may be selected during NKT cell development. Surprisingly, Vβ9- and Vβ14-expressing cells consistently exhibited some level of CD69 induction upon intrathymic transfer, despite the absence of response to iGb3 and despite the absence of corresponding cells in the natural NKT cell pool. This observation could indicate that additional unidentified ligands partially activate NKT precursors during thymic development, yet may not promote their selection or expansion, perhaps as a result of lower affinity.

Another important insight gained from this study relates to the much larger repertoire elicited by synthetic α-GalCer, a mimic of microbial glycuronosylceramides, than by endogenous ligands. Thus, many Vβs that are not stimulated by iGb3 and are not found in the natural NKT repertoire, including Vβ3, 6, 9, 10, 14, could nevertheless be stimulated by nanomolar concentrations of α-GalCer. These unexpected findings demonstrate that the NKT cell repertoire against foreign and microbial antigens is severely limited because thymic exposure to natural ligands failed to select potentially useful TCRs. This stands in sharp contrast with positive selection driven by polymorphic MHC ligands, where the opposite outcome is observed, namely a gain in recognition of foreign peptides after positive selection by self-ligands (33). We propose that these differences may be inherent to the processes of positive selection by partial agonists/antagonists versus full agonists.

Finally, our results directly confirm and extend to the entire nonselected repertoire recent reports (14) suggesting that different Vβ families generically encode different affinities for lipid ligands. Vβ7 responded much better than Vβ8.2 to low doses of natural ligand, whereas both responded comparably to α-GalCer and microbial ligands. Because of the absence of CDR3 restriction in these responses, the CDR1 and 2 regions of the β chain may confer the differences in affinity. Future structural studies of these interactions, including crystallography and mutational analysis, will...
shed light on this intriguing mode of TCR recognition. In the meantime, our quantitative determination of the hierarchy of natural ligand recognition by different Vβ families provides potentially useful knowledge to assess the contribution of affinity to the functional properties of NKT cell subsets and to the selection of NKT cells in mice exhibiting NKT cell defects.

**Materials and Methods**

**Mice.** C57BL/6 and NOD mice were purchased from The Jackson Laboratory. C57BL/6.βM-/-,1-A4/−/− (MHC-null) and C57BL/6. Ly5.1 congenic mice were purchased from Taconic Farm. C57BL/6.Va1h.G and C57BL/6. CD1d−/− mice were described previously (15, 34), and were intercrossed to obtain C57BL/6.Va1h.G CD1d−/− mice. The CD1dβ2 (LE, low expression) allele was the unintended consequence of the insertion of a loxP site in the 5′UTR of the C57BL/6 CD1d gene after homologous recombination in ES cell and excision of a neomycin gene and a STOP sequence cassette. This insertion resulted in the modification of the genomic sequence upstream of the ATG site, 5′-TGAAGCCTCGCCATGTCATG-3′, which included a deletion of the underlined GCC and an insertion at the position marked ^ of the sequence 5′-CATACCTCTAGATGACTTTACGAA-GTTATATATTGGTATGATATGCGAAGATCTCTCCGACCG-3′. The C57BL/6 CD1d−/− mouse carrying a null allele and a LE allele expressed very reduced levels of CD1d on the surface of thymocytes. The LE allele was screened using PCR (forward primer: 5′-TGTTGAG-AACCTGTCAATCCG-3′, reverse primer: 5′-GGGACGCGTATCAGCGACCCG-3′). All mice were housed in a 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, CD44, CD69, Ly5.2, NK1.1, CD4, and CD8 were purchased from BD Biosciences. Streptavidin conjugated to allophycocyanin (APC) or R-PE was purchased from BD Biosciences. The LE allele was screened using PCR (forward primer: 5′-CATACCTCTAGATGACTTTACGAA-GTTATATATTGGTATGATATGCGAAGATCTCTCCGACCG-3′, reverse primer: 5′-GGGACGCGTATCAGCGACCCG-3′). All mice were housed in a pathogen-free facility at the University of Chicago according to the guidelines of the institutional animal care and use committee.

Cell stimulation assays. Single cell suspensions prepared from four to five adult C57BL/6. Va1h.G CD1d−/− spleens were first enriched for DN T cells by depleting B, CD4, and NK cells with B220, CD4, CD8α, and DX5 mAb-conjugated magnetic microbeads and AutoMACS (Miltenyi Biotech) according to manufacturer’s instructions. DX5 is not expressed by the DN T cells. For the CFSE expansion assay, this enriched population (>50% were DN CD3ε+ cells) was labeled with CFSE before stimulation with lipid-pulsed BM-DCs. The DCs were prepared from cultures of BM cells in a 1:1 mixture of EHAA and RPMI 1640 (Biofluids) supplemented with 10% heat-inactivated fetal calf serum, glutamine, antibiotics, 5 × 10^-3 M of 2-mercaptoethanol and 2 ng/ml of mouse GM-CSF (Biosource). After 6 d of culture, BM-DCs were pulsed with different concentrations of α-GalCer or iGb3 overnight, washed, and plated at 10^6 per well in six-well plates (Corning Costar). 10 × 10^6 enriched DN T cells were added per well and cultured in the presence of mouse recombinant IL-7 at 10 ng/ml (Bio-source) for 4 d before FACS analysis. For the CD69 induction assay, the lipid-pulsed DCs were allowed to adhere to culture wells for 16–24 h before adding the enriched DN T cells. These cocultures were spun down briefly (1,200 revolutions/min) to favor cell-to-cell contact. After 4–6 h, the DN T cells were harvested and processed for FACS analysis, using anti-CD69, Vβ, CD3ε, and CD4 and CD8α antibodies.

**Intrathymic cell transfers.** DN T cells enriched from C57BL/6. Va1h.G CD1d−/− spleens (Ly5.2) by depleting B, CD4, and CD8, and NK cells with B220, CD4, CD8α, and DX5 mAb-conjugated magnetic microbeads and AutoMACS were injected intrathymically into C57BL/6.Ly5.1 congenic hosts as described previously (37). After 6 h, thymocytes were harvested and depleted of CD4− and CD8α-expressing cells using magnetic microbeads and AutoMACS (Miltenyi Biotech) before FACS analysis. Transferred DN T cells were identified using anti-Ly5.2, CD69, Vβ, and CD4 and CD8α antibodies.

We thank B. Jabri for expert advice in TCR repertoire analysis, C. Borowski and K. Grewalk for valuable discussions, and all members of the Bendelac Lab for help and support. This work was supported by National Institutes of Health grants to A. Bendelac (no. AI038339) and A. Bendelac, P.B. Savage, and L. Teyton (no. P01 AI053725). The authors have no conflicting financial interests.

Submitted: 21 February 2006

Accepted: 27 March 2006

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