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The Influence of CD1d in Postselection NKT Cell Maturation and Homeostasis

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After being positively selected on CD1d-expressing thymocytes, NKT cells undergo a series of developmental changes that can take place inside or outside the thymus. We asked whether CD1d continues to play a role in late-stage NKT cell development and, in particular, during the functionally significant acquisition of NK1.1 that is indicative of NKT cell maturity. We report that CD1d is indeed crucial for this step, because immature NK1.1− NKT cells fail to fully mature when transferred to a CD1d-deficient environment. Surprisingly, however, the lack of CD1d did not greatly affect the long-term survival of NKT cells, and they continued to express CD69 and slowly proliferate. This directly contradicts the currently held view that these phenomena are caused by autoreactivity directed against CD1d/TCR-restricted self-Ags. Our findings demonstrate an ongoing role for TCR-mediated signaling throughout NKT cell development, but the characteristic semiautomated basal state of NKT cells is controlled by CD1d-independent factors or is intrinsic to the cells themselves. The Journal of Immunology, 2005, 175: 3762–3768.

Positive selection of immature thymocytes in the thymus usually involves an interaction between the TCR and MHC-peptide complexes on thymic stromal cells. NKT cells share the earlier stages of thymic development with their mainstream counterparts, but they diverge at positive selection when the semi-invariant NKT cell TCR (Vβ14-Jα18 in mice and Vβ24-Jα18 in humans) binds instead to glycolipids presented in the context of CD1d by surrounding thymocytes (1–3). Unlike most developing T cells, newly selected, CD1d-restricted NKT cells are almost immediately capable of responding to stimulation by releasing cytokines such as IL-4 and IFN-γ (4–6). The role for cytokines produced by NKT cells in the thymus has not been defined, but activated NKT cells in the periphery use cytokines to selectively enhance or suppress the responsiveness of other immune cells (7, 8). In fact, cytokines mediate many of the immunoregulatory activities of NKT cells, activities best illustrated by their ability to modulate different immune responses and, through the increased predisposition toward autoimmune disease, some types of cancer and other immune system-related diseases/disorders conferred by NKT cell deficiency in humans and mice (7, 9, 10).

Despite their capacity for cytokine production, newly selected NKT cells are not fully mature. They are yet to acquire many of the NK-related markers that characterize the lineage (e.g., NK1.1, Ly49, and NKG2 family members), and they respond differently to mature NKT cells when activated (11, 12). NKT cell maturation is best defined by up-regulation of the NK1.1 Ag (4–6). Its expression coincides with critical changes in cytokine production that signal a shift toward the higher IFN-γ and lower IL-4 production normally attributed to mature NKT cells (5, 6). The change is thought to enhance the ability of mature NK1.1+ NKT cells to promote inflammatory activities such as IFN-γ-dependent antitumor responses (13), although they also have the capacity to suppress some other immune responses (such as autoreactivity) through mechanisms that are most often linked to IL-4 (8, 9). Interestingly, the NK1.1− to NK1.1+ transition is not restricted to the thymus, and most NKT cells are actually exported to the periphery as immature NK1.1− cells that up-regulate NK1.1 over the following days to weeks.

The NK1.1− to NK1.1+ transition is also significant because developmental failures at this stage have been linked to peripheral NKT cell deficiencies. A number of experimental models of peripheral NKT cell deficiency have an increased proportion of thymic and peripheral NK1.1− NKT cells that stem from a specific failure of positively selected NKT cells, but not mainstream T cells, to mature fully (14–16). Some of these models have involved inhibition of different NF-κB elements, but the large number of different stimuli that can potentially activate the NF-κB signaling pathway has greatly complicated the task of finding the initiating trigger.

One prominent example of how important this peripheral step appears to be is found in diabetes-prone NOD mice, which have a systemic NKT cell deficiency that predisposes the strain to a breakdown of immune self-tolerance and the onset of autoimmune disease (17–19). The deficiency appears to be caused by a failure of NK1.1− NKT cells to mature. Using NK1.1 congenic NOD mice, we showed that the NKT cell deficiency was primarily restricted to the NK1.1− fraction, whereas NK1.1+ NKT cells were largely normal in number and frequency (20). It is not clear whether the deficiency represents a breakdown of thymic or peripheral NKT cell maturation, but it again highlights the importance of understanding the factors that control the NK1.1− to NK1.1+ maturational stage.
It is not known whether the signals that promote NK1.1 expression in the thymus are identical with those in the periphery, and it remains possible that peripheral NK1.1 expression simply reflects a delayed response to an earlier thymic stimulus. Hence, two important issues remain to be resolved. Firstly, what signal(s) is required for NK1.1 expression by positively selected NKT cells, and secondly, are separate signaling events required for thymus and peripheral up-regulation of NK1.1? Strong candidate triggers for NK1.1 expression in both compartments are signals initiated through TCR engagement of CD1d. CD1d is clearly responsible for NKT cell selection in the thymus, and there is strong circumstantial evidence of continued TCR engagement in the periphery, meaning that CD1d is available to NKT cells throughout their ontogeny (7, 10, 21–24). NKT cells also display a partially activated/memory phenotype reflected by the expression of molecules such as CD69 and CD44, even in germfree mice and human cord blood, suggesting that these cells have an inherent autoreactivity toward ubiquitous self-glycolipids presented by CD1d (7, 10, 21, 25–28).

This study investigates the importance of CD1d-mediated signaling in the maturation of NK1.1+NKT cells in both the thymus and the periphery of wild-type (WT)4 and CD1d-deficient (CD1d−/−) mice and defines how this signaling affects the survival, proliferation, and activation status of resting peripheral NKT cells. The results provide important clues about how NKT cell deficiencies might arise and challenges the widely held view that NKT cells are inherently autoreactive.

Materials and Methods

Mice

Inbred C57BL/6 WT and CD1d−/− mice were bred in-house at the Department of Microbiology and Immunology Animal House, Melbourne University. All experiments were performed according to animal experimental committee guidelines.

Isolation of NKT cells

Lymphocytes were isolated from perfused liver tissue by being gently pressed through 200-μm pore size mesh sieves into PBS containing 2% FCS (Commonwealth Serum Laboratories; FCS.PBS). Lymphocytes were separated from hepatocytes and cellular debris via a 33% isotonic Percoll density gradient (Pharmacia Biotech) conducted at room temperature. Exposed DNA was removed using DNase (35 μg/ml; Roche). Lymphocytes were isolated from the thymus and spleen by gentle grinding between two frosted glass slides into 2% FCS.PBS. Blood was collected through heparinized capillary tubes to prevent clotting. Liver, spleen, and blood were depleted of RBC using red cell lysis buffer (Sigma-Aldrich) conducted at room temperature. Cells were washed before being surface labeled for flow cytometric sorting.

NKT cell enrichment

For thymus-derived NKT cell enrichment, thymocytes were labeled with anti-CD8 (clone 3.155; grown in-house) and anti-CD24 (clone J11D; grown in-house). Ab-bound cells were then depleted using rabbit complement-mediated depletion of CD8+ and HSA+ cells from the enriched thymocytes that were either DN (cells, but not CD8+ and HSA− that were later identified and excluded from analysis. Instruments were washed between removal of each organ, and in the case of FITC injections, the injected thymus was always removed last to avoid cross-contamination of samples.

Results

The transition of NKT cells from NK1.1− to NK1.1+ can occur in the thymus or periphery. To determine the importance of CD1d for NK1.1 up-regulation in each environment, NK1.1− NKT cells were introduced to a CD1d-deficient thymus or peripheral pool, and their subsequent expression of NK1.1 was measured at different times. NKT cells were definitively identified using the αGC-loaded CD1d tetramer reagent (hereafter called CD1d tetramer), which binds to the semi-invariant TCR of NKT cells (29, 32). In the mouse thymus, positively selected NKT cells that are not fully mature are CD1d tetramer− and NK1.1−, whereas mature NKT cells are CD1d tetramer+ and NK1.1+. Both subsets are αβTCR+ and HSA− and contain CD4+CD8− (CD4+) and CD4−CD8− (DN) cells, but not CD8+ cells (4).

Development of NK1.1−/− NKT cells in thymus

NKT cells usually comprise ~0.5% of total thymocytes, but complement-mediated depletion of CD8+ and HSA+ cells enriches the frequency to 15–30%, which enables more efficient flow cytometric sorting. The sorting strategy produced two populations of CD4+ HSA− cells from the enriched thymocytes that were either NK1.1− or NK1.1+. CD4 was used as a sorting parameter to exclude non-T cell lineages, such as NK cells or B cells, and to select the most immature NKT cells (previously identified as CD4+NK1.1− (4)). TCR-specific reagents such as CD1d tetramer were not used in the sorting process to avoid inadvertent signaling through the TCR that could potentially trigger phenotypic changes or interfere with physiological receptor-ligand interactions. This strategy meant that the sorted fractions included some non-NKT cells (primarily CD4+ HSA−/− mainstream T cells within the NK1.1− fraction) that were later identified and excluded from analysis by CD1d tetramer staining. At the time of transfer, each fraction was injected directly into the thymus of congenitally distinct WT or CD1d−/− mice. Recipient mice were killed 7 days after injection and the thymus was removed to assess the purity of these populations.

Adaptive transfer of CFSE-labeled NKT cells

NKT cell-enriched thymocytes were washed once in 0.1% BSA.PBS before being labeled in 1 ml of 0.1% BSA.PBS with 4 μl of 1 μM CFSE (Molecular Probes) for 10 min at 37°C in the dark. The reaction was quenched with 20% FCS.PBS before cells were washed twice in RPMI 1640 and transferred into recipient mice via i.v. injection in PBS.

Thymus transplantation

Thymic lobes were removed from newborn pups and engrafted beneath the kidney capsule of 5–6-wk-old mice in accordance with a previously described technique (30).

Intrathymic injection of FITC dye or cells

Details of this technique were described previously (30, 31). Briefly, animals were anesthetized, and the chest was opened (or kidney was exposed, in the case of grafted mice) to reveal the thymic lobes. Each lobe was injected with ~10 μl of 350 μg/ml FITC (in PBS), which typically resulted in random labeling of 40–60% of the thymocyte population (70–80% for grafted thymuses). For intrathymic injection of sorted lymphocytes, cells were injected in a suspension of 20 μl of PBS. The wound was closed with a surgical stapler, and the mouse was warmed until fully recovered from anesthesia. Mice were killed at different time points after injection (detailed in Results), and graft thymus and lymphoid organs were removed for analysis. Instruments were washed between removal of each organ, and in the case of FITC injections, the injected thymus was always removed last to avoid cross-contamination of samples.
after intrathymic injection, whereupon the transferred cells were identified via CD45.1 expression, and CD1d tetramer αβTCR− NK T cells were analyzed for the expression of NK1.1 (Fig. 1).

Our results show a clear reliance on CD1d for NK1.1 up-regulation. In WT recipients of NK1.1− NK T cells, >70% of donor-origin NK T cells had become NK1.1+ 7 days after transfer (Fig. 1B). This is consistent with previous reports from our group and others and reflects the normal pattern of NK T cell maturation in the WT thymic microenvironment (4, 5). In stark contrast, however, ∼75% of NK1.1− NK T cells transferred into the thymus of CD1d−/− mice remained NK1.1− 7 days after transfer, suggesting that their maturation was CD1d/TCR dependent. NK1.1+ NK T cells maintained NK1.1 expression when transferred into either strain, although there was some minor NK1.1 down-regulation in CD1d−/− recipients, which implies that NK1.1 expression is partly maintained, as well as initiated, by interactions with CD1d.

Similar experiments using NK T cells derived from the liver were conducted, and it is noteworthy to report that the recovery of liver-derived NK T cells from injected thymuses was always vastly lower (typically >90% reduced) than that of thymus-origin cells (data not shown). This was not unexpected, because previous studies had shown that mature conventional T cells are not retained in the thymus after adoptive transfer (33, 34). Nevertheless, the trend among recovered liver NK T cells was similar to that of thymic NK T cells, with NK1.1+ NK T cells becoming mostly NK1.1− in WT mice, but remaining NK1.1− in CD1d−/− recipients (data not shown).

Development of NK1.1+ NK T cells in the periphery

NK T cells can become NK1.1− in the thymus, but they are more often exported to the periphery bearing a NK1.1− phenotype and only become mature NK1.1+ cells over the following days or

FIGURE 1. A, The NK1.1− to NK1.1+ NK T cell transition in the thymus requires CD1d. Thymocyte suspensions from CD45.1+ WT mice were enriched for NK T cells by depleting CD8+ and HSA+ cells (far left dot plot). NK1.1− and NK1.1+ populations obtained by FACS sorting were intrathymically (I.T.) injected into CD45.2 WT or CD1d−/− recipient mice (middle left dot plots). Recipient thymuses were harvested 1 wk later, and donor-derived cells were identified based on CD45.1 expression (middle right dot plots). After exclusion of autofluorescent or nonspecifically bound cells with an unloaded-CD1d tetramer control, αGC-loaded CD1d tetramer αβTCR− CD45.1− NK T cells were examined for NK1.1 vs CD4 expression (far right dot plots) Data shown are representative results from four separate experiments. B, Data from all intrathymic transfer experiments were pooled to determine the extent of NK1.1 expression by NK T cells previously transferred into either WT (□) or CD1d−/− (■) mice. Results are from four separate experiments involving a total of six WT and six CD1d−/− recipients of NK1.1− cells and four WT and five CD1d−/− recipients of NK1.1+ cells. Statistical significance was determined using the Mann-Whitney rank-sum U test. *, p < 0.001.
weeks (4, 5). Whether this transition is simply a delayed response to the CD1d-dependent thymic signal or requires a distinct peripheral trigger (that may or may not be CD1d dependent) is not known.

To directly test the importance of peripheral CD1d expression for NKT cell development, we used a congenic thymus transplantation strategy that allowed new NKT cells to be tracked from the time they were exported from the thymus. This was a more stringent approach than adoptively transferring sorted NKT cells, because NKT cells from the grafted thymus are true thymic emigrants, whereas the maturational status of sorted NKT cells from the thymus (or other organs) would not properly reflect that of normal recent thymic emigrants. Many different groups have previously shown thymus grafts to be physiologically normal in their production and export of mainstream T cells, and we confirmed that this was also the case for NKT cells. (Fig. 2 and data not shown) (30). The export of NKT cells was checked by directly injecting FITC into the thymus grafts so that FITC+ emigrant cells could be easily identified in the periphery. Consistent with previous studies of export from the endogenous thymus, recent thymic emigrant T cells from the graft were mature αβTCR+ T cells (30, 31), and ~2% of these were NKT cells, which were predominantly NK1.1+ (Fig. 2) (4, 5). These results confirm the stringency of NKT and mainstream T cell export from the grafted thymus.

Consistent with previous reports that NK1.1− emigrant NKT cells start to progressively become NK1.1+ after export from the thymus (5), >70% of graft-derived NKT cells were NK1.1− 6 wk after initial engraftment in WT recipients (Fig. 3). In contrast, graft-derived NKT cells in CD1d−/− recipients persisted, but did not significantly up-regulate NK1.1 from the levels in recent thymic emigrants. It should be made clear that most congenic cells are exported from the grafted thymus over a period beginning 2–3 wk after transplantation, so when the recipient mice were harvested 6 wk after transplantation, most congenic cells in the periphery were likely exported 3–4 wk earlier (30).

To exclude the possibility that the differences we observed in peripheral NKT cell development between WT and CD1d−/− mice were due to a selective loss or expansion of NKT cells, we CFSE-labeled NKT cell-enriched thymocyte suspensions from WT mice. These cells contained ~20% NKT cells and were adoptively transferred into WT and CD1d−/− mice that were harvested 2, 4, or 6 wk later. The presence of mainstream T cells alongside the NKT cells served as an important control to measure the persistence of NKT cells in CD1d-deficient mice.

The proportion of NKT cells among all donor cells showed the normal variation between organs, with NKT cell frequency being far higher in the liver than in other organs (20, 35). More importantly, however, the relative proportions of donor T and NKT cells among donor cells in the spleen, liver, and blood were consistently similar in WT and CD1d−/− recipient mice, suggesting that the lack of CD1d had little impact on the survival (or loss) of NKT cells or on the pattern of NKT cell homing to particular organs (Fig. 4A).

This strategy also enabled us to directly measure the impact of CD1d on the basal activation status of NKT cells. NKT cells have long been regarded as being constitutively activated, because they express markers such as CD69 that reportedly reflect an inherent autoreactivity toward self-Ags (21, 25, 26, 35, 36). Autoreactivity of this nature would clearly depend on CD1d-mediated presentation of a glycolipid self-Ag, because CD1d is required for presentation of all known NKT cell Ags. Importantly, despite the long duration of these cells in the CD1d−/− environment, there was no indication of reduced survival, proliferation, or CD69 expression by transferred NKT cells (Fig. 4B). In fact, if anything, the basal proliferation levels appeared to be slightly increased in the absence of CD1d. The transferred NKT cells slowly divided in the spleen, liver, and blood of both WT and CD1d−/− mice, suggesting that CD1d was not required in this process. This was also supported by the consistent levels of CD69 expression on NKT cells in CD1d−/− and WT mice, which indicated that their activated phenotype was not being maintained through TCR recognition of glycolipid Ags presented by CD1d.

**Discussion**

The process by which positively selected NKT cells become mature is not yet fully characterized, but the acquisition of NK1.1 clearly defines an important checkpoint at which the cytokine response of NKT cells in normal mice becomes mature (4–6). This is also the stage at which NKT cell development has failed in some models of NKT cell deficiency (14–16). We specifically tested the role of CD1d in this process, because interactions between the semi-invariant NKT cell TCR and CD1d are implicated in many different aspects of NKT cell biology, including the selection of immature NKT cells in the thymus and activation of mature NKT cells in the periphery. Our data suggest that this role extends to the acquisition of NK1.1 by NKT cells in the thymus and periphery, because in the absence of CD1d, most NK1.1− NKT cells fail to become NK1.1+. The small proportion of NKT cells that did up-regulate NK1.1 suggests that the requirement for CD1d may not be absolute, but it is perhaps more likely that these were cells that had received the trigger for up-regulation before isolation from the donor tissue, but had not yet fully expressed NK1.1.

Our findings provide two especially significant insights into the role played by CD1d in NKT cell homeostasis. First, even after positive selection, NKT cells require ongoing contact with CD1d to complete their development regardless of whether it occurs in the thymus or periphery. This is particularly relevant to clinical studies, because a breakdown in the later stages of NKT cell development can lead to the peripheral NKT cell deficiencies that have been increasingly linked to compromised immunity in humans and mice (7, 13, 37, 38). Of course, one important question that remains unanswered is the nature of the Ag(s) presented to NKT cells at each CD1d-dependent stage of development and, in

**FIGURE 2.** Export from transplanted thymic lobes is normal for mainstream and NKT cells. Recent thymic emigrants were identified as FITC+ cells in the peripheral lymphoid organs of mice intrathymically injected 24 h previously with FITC. Consistent with previous studies and results from the endogenous thymus (not shown), emigrant cells were almost exclusively αβTCR+ and contained ~2% NKT cells, the majority of which were NK1.1+. This contrasted with longer-lived resident NKT cells of endogenous (CD45.2+) and graft thymus (CD45.1+) origin, which were mainly NK1.1+. Consistent with the random nature of FITC labeling in the thymus, both FITC− and FITC+ NKT cells contained similar levels of NKT cells and showed similar patterns of NK1.1 expression. The results shown are representative of five separate mice.
particular, whether different Ags are required at each stage. Although the identities of these ligands are not absolutely clear, one very good candidate is the iGb3 glycolipid that was recently reported to be essential for normal NKT cell selection and can also promote NKT cell activation in the periphery (39–41). Whether the role of iGb3 extends to the later stages of NKT cell development remains to be seen, but because it is capable of selecting and activating NKT cells and is constitutively expressed in mammals, it is entirely possible that it may also drive the NKT cell transition from NK1.1<sup>+</sup> to NK1.1<sup>+</sup>. Therefore, examining whether the late-stage maturation of NKT cells is compromised to the same extent in iGb3-deficient HexB mice as in CD1d<sup>−/−</sup> recipients is likely to prove informative.

The second important finding is that the long held opinion of NKT cells being maintained in a quasi-activated state because of an inherent autoreactivity is probably incorrect (reviewed in Refs. 7 and 10). In the absence of CD1d, NKT cells continued to display an activated phenotype and proliferated to a similar extent as NKT cells in a normal CD1d-intact environment. Hence, these traits are either stimulated by a CD1d-independent factor or are inherent to the cell itself, neither of which is indicative of autoreactivity. Cell survival was not an obvious factor in the comparison between NKT cells in the periphery of CD1d<sup>−/−</sup> and WT mice because the proportion of NKT cells among all donor cells was similar for both recipient groups even when cells were harvested 6 wk after adoptive transfer. One potential caveat was that CD1d expressed by the transferred cells was sufficient to promote NKT cell activation in CD1d<sup>−/−</sup> mice. This is very unlikely because of the extent to which donor cells were diluted within the periphery of CD1d<sup>−/−</sup> recipient mice and because those mice remained completely devoid of the CD1d<sup>+</sup> dendritic cells and B cells that are more strongly linked to NKT cell activation. Given these circumstances, if the basal activation of NKT cells was a CD1d-dependent phenomenon, it is difficult to conceive how CD69 expression and proliferation by NKT cells could be as completely maintained in CD1d<sup>−/−</sup> mice as they were in this study. Moreover, our data are

![FIGURE 3](http://www.jimmunol.org/)

**A**. The NK1.1<sup>−</sup> to NK1.1<sup>+</sup> transition in the periphery requires CD1d. Four to six thymic lobes from neonatal CD45.1<sup>+</sup> C57BL/6 mice were grafted under the kidney capsule of CD45.2<sup>−</sup> recipient mice that were either WT or CD1d<sup>−/−</sup> (far left illustrations). Spleen and liver lymphocytes were harvested from recipient mice 6 wk after engraftment, and graft-origin cells were identified based on CD45.1 expression (histograms). NKT cells endogenous to the recipient (CD1d tetramer<sup>+</sup> CD45.1<sup>+</sup> cells) and NKT cells of graft origin (CD1d tetramer<sup>+</sup> CD45.1<sup>−</sup> cells) were examined for NK1.1 vs CD4 expression (dot plots). Unloaded CD1d tetramer reagent was used to exclude cells that were autofluorescent or had nonspecifically bound reagents. The results shown are representative of two separate experiments involving a total of four mice per group. **B**, Pooled data from grafted thymus experiments were graphed, and the mean proportions (±SE) of endogenous (□) and graft-origin (■) NKT cells that expressed NK1.1 were plotted. Results are from two separate experiments, with two WT and CD1d<sup>−/−</sup> mice per group (total n = 4/group). Statistical significance was determined using the Mann-Whitney rank-sum U test. *p* < 0.05.
consistent with those from an earlier study in which Ab-mediated blocking of CD1d on NKT cells had no effect on their homeostatic proliferation, leading to a similar finding that CD1d expressed by NKT cells was inconsequential to their rate of division (16).

Hence, although CD1d is clearly essential for the selection of NKT cells in the thymus, the subsequent acquisition of NK1.1, and activation by known agonists such as iGb3 and αGc, CD1d is, somewhat paradoxically, not essential for the survival, basal proliferation, or constitutively activated phenotype of NKT cells. This challenges the widely held assumption that distinctive characteristics, such as their constitutively high levels of CD69 expression, could be attributed to autoreactive NKT cells continually encountering one or more agonist glycolipid Ags. Our evidence that the lack of CD1d does not impact upon CD69 expression or on basal turnover levels removes the need to identify ubiquitously expressed self-Ags that might cause these phenomena and instead suggests that NKT cells are more akin to traditional memory T cells that respond rapidly to agonist Ags (self or non-self), but do not require their continual presence for peripheral longevity.

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**Disclosures**

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