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The NKT cell pool in the thymus contains immature (NK1.1−) and mature (NK1.1+) subsets that represent distinct linear stages of a linear developmental pathway. An unexplained paradox is why immature NK1.1− NKT cells are mainly exported to the periphery instead of the more mature and more abundant NK1.1+ NKT cells. In this study we have determined that mature NK1.1+ NKT cells are retained by the thymus to form an extremely long-lived resident population capable of rapid and prolonged production of IFN-γ and IL-4. The retention of mature NKT cells provides an explanation for why the periphery is mainly seeded by immature NK1.1− cells despite mature NK1.1+ NKT cells being more abundant in the thymus. This is the first study to identify a mature T cell subset retained within the thymus and is additional evidence of the distinct developmental pathways of mainstream T cells and NKT cells. The Journal of Immunology, 2006, 176: 4059–4065.

The NKT cells are a regulatory T cell subset capable of enhancing or suppressing immune responses through potent cytokine release (1, 2). They are regarded as a potential therapeutic target because numerous studies in humans and mice report a strong association between NKT cell defects and an increased susceptibility to many autoimmune diseases and cancers (1, 3, 4). NKT cells use a semi-invariant, CD1d-restricted TCR that enables recognition of the glycolipid Ags responsible for selection in the thymus and activation in the periphery (5, 6). Although NKT cells were initially identified through their dual expression of a TCR and NK receptors, they are now more definitively identified using CD1d tetramers loaded with the pan-NKT cell agonist α-galactosylceramide (αGC) that targets the distinctive TCR (7, 8).

The earliest tetramer-reactive NKT cells in the thymus are devoid of most NK-associated markers, including the NK1.1 molecule (CD161 in humans) most commonly associated with this lineage (9–11). The CD1d-dependent up-regulation of NK1.1 is a crucial checkpoint in NKT cell maturation and is often the point at which a developmental block occurs in models of NKT cell deficiency (12–15). NK1.1 up-regulation also coincides with the expression of functionally significant cell surface molecules, including inhibitory Ly49 receptors and stimulatory receptors such as NKG2D (10, 16–18). Most importantly, NK1.1 expression signals a maturing of the cytokine response toward high IFN-γ production (9–11).

In sharp contrast to mainstream T cells, most thymic NKT cells carry a mature NK1.1+ phenotype. Paradoxically though, it is mainly immature NK1.1− NKT cells that are exported to the periphery, where they again complete their maturation in a CD1d-dependent manner (9, 10, 12, 19). This developmental pathway is in stark contrast to mainstream T cells where thymic export is tightly restricted to ensure that only mature cells are released to the periphery (20–22). It is not clear why NKT cells are the exception to this rule, but the same phenomenon takes place in human NKT cell development (23, 24).

One obvious question is what happens to the mature NK1.1+ cells that make up >75% of NKT cells in the young adult thymus. In this study we use various assays to track these cells long term and resolve fundamental inconsistencies in the current understanding of NKT cell development. Most strikingly, we identify a population of NK1.1+ NKT cells that are resident in the thymus and capable of potent cytokine production. They appear to be the only T cell subset in the thymus that rapidly turns over, strongly suggesting a functional role for these cells within the confines of the thymus microenvironment.

Materials and Methods

Mice

Inbred CD45.2+ and CD45.1+ C57BL/6 mice were bred at the Department of Microbiology and Immunology House, Melbourne University. Chimeras were generated by irradiating CD45.2+ mice with two doses of 550 cGy and immediately reconstituting them i.v. injection of 5 x 10^6 CD45.1+ bone marrow cells. All experiments were conducted under the approval of the University of Melbourne animal experimental committee.

Thymus transplantation

Thymic lobes were removed from newborn pups and engrafted beneath the kidney capsule of 5- to 6-wk-old mice in accordance with a previously described technique (22, 25).

Intrathymic injection of FITC dye or cells

The details of this technique have been described previously (20, 22). 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 500 μg/ml FITC (in PBS), which typically resulted in random labeling of 40–60% of the thymocyte population (70–80% for grafted thymus). The wound was closed with a surgical staple, and the mouse was warmed until it recovered from anesthesia. Mice were
killed at different time points after injection, with graft thymus and lymphoid organs taken for analysis. Instruments were washed between the removal of each organ, and in the case of FITC injections, the injected thymus was always removed last to avoid cross-contamination of samples.

Flow cytometry

Unfractionated or NKT cell-enriched cell suspensions were labeled with combinations of Abs including: FITC-conjugated anti-αβTCR (clone H57-597); FITC-conjugated anti-CD4 (clone RM4-5); FITC- or PerCP-conjugated anti-CD45.1; FITC- or PerCP-conjugated anti-CD45.2; PE-, PerCy7, or allophycocyanin-conjugated anti-NK1.1 (clone PK-136); PE-conjugated anti-heat-stable Ag; biotinylated anti-CD4 (clone RM4-5); and allophycocyanin-, allophycocyanin-Cy7-, or Pe-Cy7-conjugated anti-αβTCR (clone H57-597). Fluorochrome-labeled, αGC-loaded or unloaded, CD1d tetramer was produced in-house using a construct provided by M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA). The generation and use of this reagent have been described in detail previously (7). To avoid nonspecific binding of Abs to FcR-γ cells were routinely incubated with anti-mouse CD16/32 (clone 2.4G2; grown in-house). All flow cytometry reagents were purchased from BD Pharmingen unless otherwise indicated. Data were collected on an LSR2 flow cytometer (BD Biosciences).

Isolation of liver lymphocytes

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). Lymphocytes were separated from hepatocytes and cellular debris via a 33% isotonic Percoll density gradient (Amersham Biosciences) conducted at room temperature, and red cells were lysed with ammonium chloride.

Results

Mature NKT cells are retained in thymus

We were intrigued by the consistent findings of multiple groups (including our own) that immature NK1.1+ NKT cells were preferentially exported from the thymus of the adult mouse despite the greater abundance of mature NK1.1+ thymic NKT cells. To directly test whether mature NKT cells were retained in the thymus, we transplanted congenic CD45.2+ thymic lobes beneath the kidney capsule of CD45.1+ recipients. Previous studies had shown a highly predictable rate of reconstitution of transplanted thymus lobes in which donor thymocytes are lost from the thymus as they fail selection or are exported, whereas recipient progenitor cells enter the lobes and eventually repopulate all thymocyte compartments (22). The result is that by 4–6 wk after engraftment, <1% of thymocytes are of donor origin. These kinetics are in harmony with estimates from studies of the endogenous thymus, and similar kinetics were observed throughout this study (data not shown) (26–28). We also confirmed that transplanted thymic lobes (as well as the endogenous thymus) in recipient mice contained normal numbers of major thymocyte subsets (including NKT cells), and that NKT cells and mature T cells were exported with normal frequency, again consistent with earlier studies (22) (data not shown).

We harvested thymic lobes at various times after engraftment and, as expected, found cells of donor origin to be rare in all
thymuses transplanted >6 wk earlier. Nevertheless, there were always low, but consistent, numbers of mature (i.e., TCR\^+) residual, donor-derived thymocytes. Strikingly, NKT cells made up the majority of this residual population despite representing <1% of thymocytes in the overall thymus (Fig. 1a). In donor thymuses transplanted 6 wk previously, NKT cells accounted for more than half of all residual cells, and similar results were observed 8 and 12 wk after grafting (Fig. 1b). In each instance, NKT cells represented at least half of the residual donor-derived cells in the donor thymus. The non-NKT cell residents were primarily mature (αβ-TCR\(^{high}\)) T cells, with CD4\(^{+}\)CD8\(^{-}\) T cells most abundant, but small numbers of CD4\(^{-}\)CD8\(^{+}\) and CD4\(^{+}\)CD8\(^{-}\) T cells also present (data not shown).

To exclude any possibility that the persistence of NKT cells was an artifact of the engraftment process, we next examined CD45.1\(^{+}\) mice rendered chimeric by irradiation and subsequent reconstitution with bone marrow from CD45.2\(^{+}\) mice. In such instances, host-derived T cell precursors and progenitors are largely ablated, but some cells persist to produce a limited chimerism between host- and donor-derived, hemopoietically derived cells (26, 27). In the endogenous thymus, this creates a circumstance similar to that in the transplanted thymus, where surviving endogenous thymocytes are exported or otherwise lost as donor-derived progenitors enter and repopulate the thymus microenvironment. We reasoned that this would allow us to confirm that NKT cells were being preferentially retained, this time in the endogenous thymus.

Mice were examined at 3 mo or 1 yr after reconstitution; once again, NKT cell levels were greatly over-represented among residual thymocytes of both groups (Fig. 1c). Despite reports that the mainstream population of T cells is essentially turned over every 4–6 wk, some host-derived cells had persisted in the chimeric thymus, even after 1 yr. NKT cells made up >20% of these host-derived cells, again consistent with their preferential retention. It should be noted that the irradiation/reconstitution model provides a more variable starting point than the consistent reconstitution kinetics of the transplanted thymus, because the relative susceptibilities of the various NKT cell and mainstream T cell subsets to irradiation are not entirely predictable. Nevertheless, the large proportion of NKT cells among residual cells, even 1 yr after reconstitution, confirmed that mature NKT cells were retained in the endogenous thymus.

Most thymic NKT cells are long-term residents

We used the mice analyzed in Fig. 1b to also examine whether long-term resident NKT cells represented a significant proportion of total NKT cells in the thymus. As expected, donor-origin mainstream T cells made up <0.5% of the total T cells in the transplanted thymus 6, 8, and 12 wk after engraftment. In sharp contrast, nearly half of all thymic NKT cells at these time points were donor derived (Fig. 2). This is extremely significant, because it suggests that most mature NKT cells in the thymus are long-lived residents, particularly when residents that are not congenically distinct are taken into account. Again, this is in sharp contrast to mainstream T thymocytes, which are essentially all replaced within 6 wk.

It is important to note that we can exclude the possibility that donor-derived NKT cells had recirculated back to thymic tissue from the periphery after export. If this were the case, a similar phenomenon would have been observed in the endogenous thymus of grafted recipients. Instead, the number of donor NKT cells in the donor thymus was >10-fold higher than that in the endogenous thymus (Fig. 3). Moreover, although donor NKT cells represented >50% of all NKT cells in the donor thymus, they represented <1% of NKT cells in the endogenous thymus (Fig. 2). We have also previously reported that peripheral NKT cells injected into the thymus are very poorly retained compared with thymic NKT cells (12), consistent with earlier evidence that nonactivated T cells do not re-enter the thymus after export (29, 30).

Retained NKT cells are all NK1.1\(^{+}\)

Long-lived thymic NKT cells generally displayed a similar distribution of NK receptors as surrounding NKT cells (Fig. 4). This is not overly surprising, because our data suggest that long-lived NKT cells represent the bulk of the overall population, making it unlikely that the two groups would be greatly dissimilar. One profound difference, however, was in the expression of NK1.1 (Fig. 5). As mentioned previously, NK1.1 expression is a hallmark of NKT cell maturation, yet it is NK1.1\(^{-}\) cells that are preferentially exported to the periphery. As the site of NKT cell development, it is not surprising that the thymus contains a significant population of NK1.1\(^{-}\) cells, and this was again the case in our study. Strikingly, however, the retained NKT cells were overwhelmingly NK1.1\(^{+}\). A difference was also observed for NKG2D, with resident NKT cells expressing higher levels of this NK receptor than surrounding NKT cells. The magnitude of the change was very reminiscent of the increased NKG2D expression that follows chronic Ag stimulation of NKT cells, but other changes observed...
in that report, such as the up-regulation of Ly49 molecules, were not observed in long-term resident NKT cells (31).

**Retained NKT cells are potent cytokine producers**

We next assessed the capacity for retained NKT cells to produce cytokines, because the potent production of cytokines is crucial to the immunoregulatory function of NKT cells. In general terms, mature NK1.1+ NKT cells produced higher levels of IFN-γ than their less mature NK1.1− counterparts, but there was an even greater bias when comparing long- and short-term NKT cell residents. IFN-γ production by the long-term resident cells was considerably higher than that of the younger NKT cells of host origin despite the vast majority of those cells also being NK1.1− (Fig. 6). IL-4 levels were similar in the two groups. The conditions that might lead to intrathymic NKT cell activation are unknown, but resident NKT cells in the thymus clearly possess the capacity for potent cytokine release.

**Age-related changes within NKT cell compartments**

Two implications of our study are, firstly, that overall NKT cell levels might rise in the thymus with age as NK1.1+ cells accumulate and, secondly, that the population might become increasingly skewed toward a NK1.1+ phenotype as a result. We therefore compared the NKT cell compartments in mice of different ages. The analysis of NKT numbers revealed a reduction of overall NKT cell numbers with age in the thymus that was not observed in long-term resident NKT cells (31).

![FIGURE 4.](image)

Expression of NK receptors by resident thymic NKT cells. Recipient CD45.1+ C57BL/6 mice engrafted with newborn CD45.2+ thymic lobes were harvested 8 wk later, and the donor and endogenous thymus were separately examined for donor-derived (CD45.2+) cells. This figure shows the expression of various NK cell-associated markers on NKT cells of donor thymus (○) or host (○) origin. Each symbol represents an individual mouse.

No spontaneous thymic defect in NKT cell-deficient mice

The very specific retention of NK1.1+ NKT cells by both the endogenous and the transplanted thymus suggested that these cells might play a role in the normal functioning of the thymus, particularly since NKT cells are regarded as an immunoregulatory cell. To test this, we compared the frequencies of all major T cell subsets in the thymus of wild-type and the NKT cell-deficient CD1d−/− or Ja18−/− strains; we found no significant differences, other than the lack of NKT cells (data not shown). In addition to the normal frequencies of thymocyte subsets defined by CD4 and CD8, similar proportions of subsets defined by markers, such as heat-stable Ag, CD25, and CD69, were seen in NKT cell-deficient mice. Deletion of immature thymocytes reactive to endogenous mouse mammary tumor virus superantigens was also normal in NKT cell-deficient BALB/c.CD1d−/− and C57BL/6.CD1d−/− mice, showing that there was no gross defect in negative selection (data not shown).
Discussion

This study revealed that many NK1.1+ NKT cells are preferentially retained in the thymus. This was a most unexpected finding, because there is compelling evidence that the vast majority of mainstream thymocytes are usually resident for <4 wk (22, 28, 34), the exception being one early study that described a rare resident population that could not be definitively identified due to the technical limitations of the day (35). Our identification of a resident thymic NK1.1+ NKT cell extends that previous report, but also resolves the paradox of why the thymus exports a high proportion of immature NK1.1+ NKT cells to the periphery when most thymic NKT cells are mature NK1.1+ cells. Our findings complement earlier studies (including our own) that show that NKT cell development can involve the periphery being seeded with immature NKT cells (9, 10), but it now appears that many NKT cells are not exported and, instead, develop into extremely long-term thymic residents. The retention of mature NKT cells is consistent with the increased proportion of NK1.1+ NKT cells in the aged thymus and among recent thymic emigrants of older mice. Significantly, neither of these events was evident in previous studies of NKT cell export, because they were conducted on young adult mice <8 wk of age (9, 10).

The mechanism of NKT cell retention probably involves the differential expression of trafficking molecules, because these are known to influence the export of mainstream T cells (36, 37). There are many reported differences between the chemokine receptors expressed by NKT cells in different organs, but of particular significance might be the observation that immature NK1.1+ NKT cells, and not mature NK1.1+ NKT cells, traffic in response to the CCR7 ligand SLC/CCL21, because this receptor/ligand pairing is known to be partly responsible for thymic export (36, 38). The resident numbers are also likely to be supported by the low basal proliferation described for peripheral NKT cells (10, 14, 39), although NK1.1+ thymocytes in adult mice proliferate far less than their immature NK1.1+ counterparts (10). It is interesting to note, however, that most thymic NK1.1+ NKT cells are proliferating in mice younger than 4 wk (40), suggesting that the proliferation is a function of cellular age and is not directly controlled by the expression of NK1.1.

There are indications that the mature thymic NKT pool may be a functionally distinct subset from its peripheral counterparts. Thymic and peripheral NKT cells differ in their expression of Ly49 receptors (41–43) and respond differently to trafficking molecules, such as the aforementioned SLC/CCL21 (38). More importantly, however, are the very clear functional differences between thymic and peripheral NKT cells when they are used in models of NKT cell-dependent immunity. The functional competence of thymic NKT cells has been demonstrated by their effective suppression of diabetes in NOD mice after adoptive transfer and in vitro assays (44), yet thymic NKT cells cannot provide the same antitumor protection afforded by liver NKT cells (45).

What then is the possible functional significance of long-term thymic resident NKT cells? Given the potent protection against autoimmunity provided by thymic NKT cells in transfer models, it is tempting to speculate that they could suppress potentially damaging immune responses within the thymus. This is consistent with the suppressive role for thymus NKT-like cells in anterior chamber-associated immune deviation (46) and with a similar role proposed for peripheral NKT cells (4, 47). Alternatively, there is some evidence that thymic NKT cells can participate in mainstream selection events through Fas-mediated deletion of immature CD4+CD8+ thymocytes (48), although our analysis of mouse mammary tumor virus superantigen-mediated deletion in NKT cell-deficient mice would appear to exclude a fundamental role in negative selection.
Resident NKT cells are unlikely to play a day-to-day role in T cell development because they do not constitutively release cytokines, and T cell development is normal in NKT cell-deficient mice and in mice before the appearance of NK1.1 + NKT cells on day 8 of ontogeny. However, given that the importance of peripheral NKT cells is only revealed in NKT cell-deficient mice when they are challenged with NKT-dependent pathogens, a more likely scenario is that resident NKT cell activity is only triggered under very particular circumstances. Theoretically, the most obvious means to assess the function of resident thymic NKT cells is to identify RTEs. The method used to identify RTEs. The upper middle and upper left panels are typical staining patterns of the thymus and liver, respectively, after intrathymic injection of FITC 24 h previously. The lower left panels are representative FACS plots showing CD4 + (x-axis) and NK1.1 + (x-axis) expression of resident NKT cells (left) and RTE NKT cells (right). The far right graphs summarize the NK1.1 expression data from one representative experiment (of four total experiments) involving six to eight mice per group.

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Disclosures
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References


