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A Thymic Precursor to the NK T Cell Lineage

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CD1d-restricted autoreactive natural killer (NK1.1$^+$) T cells function as regulatory cells in various disease conditions. Using improved tetramer tracking methodology, we identified a NK1.1$^-$ thymic precursor and followed its differentiation and emigration to tissues by direct cell transfer and in situ cell labeling studies. A major lineage expansion occurred within the thymus after positive selection and before NK receptor expression. Surprisingly, cytokine analysis of the developmental intermediates between NK$^-$ and NK$^+$ stages showed a T helper cell T_H2 to T_H1 conversion, suggesting that the regulatory functions of NK T cells may be developmentally controlled. These findings characterize novel thymic and postthymic developmental pathways that expand autoreactive cells and differentiate them into regulatory cells.

In addition to mainstream CD4 and CD8 αβ T cells, the thymus produces specialized subsets of autoreactive regulatory cells, including CD1d-restricted CD4$^+$ and CD4$^-$8$^-$ (DN) NK T cells and major histocompatibility complex (MHC) class II–restricted CD4$^+$CD25$^+$ cells. These cells exist at relatively high frequencies in peripheral tissues, where they regulate conventional T cell responses through the secretion of cytokines such as interleukin-4 (IL-4), IL-10, transforming growth factor–β, or interferon-γ (IFN-γ) [reviewed in (*1, 2*)]. Although little is known about the steps that govern the development of these cells, the study of T cell receptor (TCR) transgenic models has shown that the autoreactive nature of their TCRs is essential for their differentiation into regulatory cells (*3, 4*). However, direct study of the development of natural, nontransgenic regulatory cells has not been feasible because of their rarity and the lack of specific markers.

We focused on the developmental steps of the major population of regulatory NK T cells, which express the conserved Vα14-Jα18/Vβ8 TCR (Vα24-Jα18/Vβ11 in humans), and which regulate a broad range of disease conditions such as type I diabetes, cancer, and infections [reviewed in (*5, 6*)]. The development of these cells is particularly intriguing because they are a hybrid of the T and NK lineages, expressing both the αβ TCR and the inhibitory MHC-specific NK receptors (*5, 6*). Previous studies have shown that, unlike other autoreactive T cells, autoreactive NK T cells are not deleted in the thymus but are positively selected upon recognition of CD1d (presumably with an endogenous ligand) expressed by CD4$^+$8$^+$ cortical thymocytes (*7, 8*). The inherent autoreactivity of NK T cells seems to be controlled by the inhibitory NK receptors, because it is revealed in hybridomas lacking these NK receptors and in fresh cells exposed to dendritic cells (DCs) lacking the classical MHC class I ligands of these NK receptors (*9, 10*).

To study the developmental branchpoints of NK T cells relative to the T and NK lineages, the acquisition of T_H1 or T_H2 profiles, and the mechanisms underlying their high frequency in tissues, we identified these cells using tetramers of CD1d loaded with the synthetic lipid α-galactosylceramide (αGC). CD1d-αGC tetramers uniformly stain all NK T cells expressing the Vα14-Jα18/Vβ8 TCR (*11, 12*). Because NK T cells are rare, nonspecific background staining is a problem; this can be partly overcome by simultaneous staining with empty CD1d tetramers (referred to as CD1d) conjugated with a different fluorochrome. After gating on CD1d-αGC$^+$ CD1d$^-$ thymocytes of 2-week-old B6 mice, the proportions of cells found were 0.08% in wild-type mice versus <0.01% in control Jα18$^{-/-}$ mice (*13*) lacking NK T cells (Fig. 1A) (*14*). Nearly all of the cells were found among CD8$^-$ thymocytes expressing low levels of the early cell surface marker HSA, whereas the few CD8$^+$ cells were HSA^{high} and indistinguishable from background (as measured in Jα18$^{-/-}$). By focusing on these HSA^{low} cells in 2-week-old mice, we observed a predominance of NK1.1$^-$ cells, as reported by others (*15*), in contrast to the predominantly NK.1.1$^+$ phenotype of their adult counterparts (Fig. 1B). Furthermore, nearly all of the NK1.1$^+$ cells were CD44^{high}, whereas the NK1.1$^-$ population could be separated into CD44^{low} and CD44^{high} subsets. Analysis of 3- and 6-week-old mice suggested a developmental sequence from CD44^{low}NK1.1$^-$ to CD44^{high}NK1.1$^-$ to CD44^{high}NK1.1$^+$. Other NK lineage receptors (such as Ly49A, Ly49G/I, Ly49C, and CD94/NKG2) were also expressed late, along with or after NK1.1 (*15, 16*).

In situ labeling of thymocytes with fluorescein isothiocyanate (FITC) allowed the identification of recent emigrants in the spleen and liver (*17*). At 24 hours, most of these emigrants failed to express NK lineage markers and had the same CD44^{high}NK1.1$^-$ phenotype as the thymic pre-NK1.1 stage (Fig. 2A). After 24 hours, a progressive expression of NK1.1 was observed (Fig. 2, A and B), suggesting that the thymic emigrants acquired the NK phenotype after entry into peripheral sites (*18*).

The frequency of Vα14 T cells among recent thymic emigrants was conspicuously high, at 4% in Fig. 2A and on average (*16*), and we estimate that as many as 1×10^5 Vα14 T cells could be exported to the spleen every day, a relatively large number compared with the size of the resident NK T population (5×10^5 to 10×10^5) (*19*).

To directly test the precursor-product relation between NK1.1$^-$ and NK1.1$^+$ cells, we sorted the NK1.1$^-$ cells and transferred them intrathymically into Jα18$^{-/-}$ hosts, which lack NK T cell precursors. We mixed into the inoculum 3% congenically marked, sorted NK1.1$^+$ thymocytes (*20*) to rule out the possibility that a minor population of NK1.1$^+$ contaminants could expand and account for the NK1.1$^+$ cells found later. We found that most of the NK1.1$^-$ cells expressed NK1.1 within a week, both in the thymus (Fig. 2C) and in the spleen and liver (*16*), whereas the congenically marked contaminants were not detectable. These results show that NK1.1$^-$ cells represent precursors to those expressing NK1.1.

Because the thymic emigration experiments suggested a major expansion of Vα14 thymocytes before emigration, we examined the cell cycle status and turnover rate of NK T cell developmental intermediates (*21*). Indeed, we found that a large fraction of both CD44^{low}NK1.1$^-$ and CD44^{high}NK1.1$^-$ cells were actively dividing, as shown by the 16 to 18% bromodeoxyuridine-positive (BrdU$^+$) cells at 12 hours and the 2.5 to 4.5% cells in S/M

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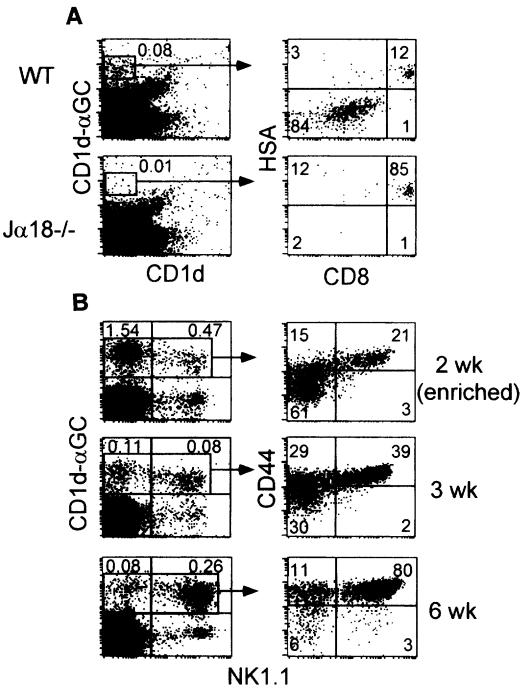

Fig. 1. Ontogeny of thymic NK T cells. (**A**) Thymocytes of 2-week-old wild-type or control Jα18$^{-/-}$ mice were labeled as indicated (four-color). Cells stained by αGC-loaded CD1d (CD1d-αGC) but not by unloaded CD1d (CD1d) tetramers (left panels) were gated for analysis of HSA and CD8 expression (right panels). Comparison between WT and Jα18$^{-/-}$ shows that only the HSA^{low}CD8^{-} subset contains Jα18^{+} cells. (**B**) CD1d-αGC^{+} thymocytes of normal mice of different ages were gated for analysis of CD44 and NK1.1 expression. To reduce background staining, all panels were pre-gated to exclude dots staining with unloaded CD1d tetramers. Two-week-old thymocytes were pre-enriched by treatment with antibodies to HSA and with complement (*34*). Numbers in the plots denote percentages and are representative of >16 experiments.

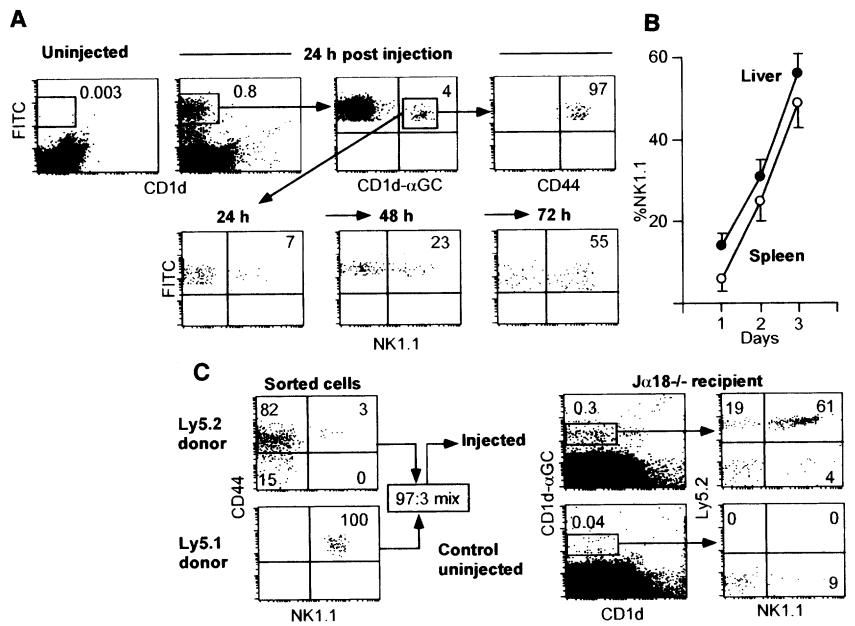

Fig. 2. Precursor-product relation between NK1.1^{-} and NK1.1^{+} cells. (**A**) Recent thymic emigrants in the spleen of 4- to 6-week-old mice were labeled by in situ injection of 10 μl of FITC into one thymic lobe (*34*). For increased specificity of CD1d-αGC tetramers, cells were preincubated with unloaded CD1d tetramers and gated CD1d^{-}FITC^{+} cells were analyzed for CD1d-αGC staining. CD1d-αGC^{+}CD1d^{-}FITC^{+} cells representing the recent thymic Vα14 emigrants (boxed) were further analyzed for expression of CD44 (upper right panel) or NK1.1 (lower three panels) at different time points after FITC injection, as indicated. Uninjected mice (upper left panel) served as controls for background staining. (**B**) Time course analysis of NK1.1 expression among recent thymic CD1d-αGC^{+} emigrants in the spleen and liver, compiled from at least three different mice per data point and representative of four experiments. (**C**) Sorted CD1d-αGC^{+} NK1.1^{-} thymocytes from 2- to 4-week-old Ly5.2^{+} donors (upper left panel) were mixed 97:3 with sorted CD1d-αGC^{+} NK1.1^{+} thymocytes from Ly5.1^{+} donors (lower left panel, to control for NK1.1^{+} contaminants) and injected intrathymically into NK T–deficient Jα18$^{-/-}$ (Ly5.1^{+}) recipients. Seven days later, recipient thymocytes were recovered and enriched by treatment with antibodies to HSA and CD8 and with complement, and CD1d-αGC^{+} cells were analyzed for Ly5.2 and NK1.1 expression. Similar expression of NK1.1 was observed among Ly5.2^{+} donor cells recovered from the spleen and liver of thymus-injected recipients. Data are representative of five experiments.

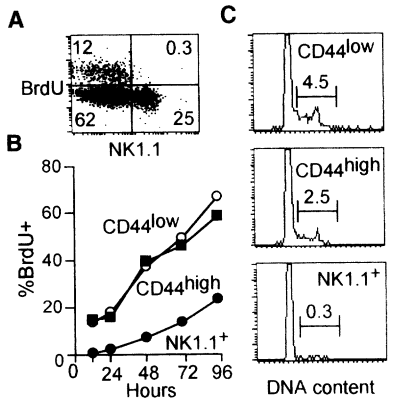

Fig. 3. Turnover and division rate of NK T cell subsets. (**A**) Mice injected intraperitoneally with BrdU showed rapid incorporation in the NK1.1^{-} but not the NK1.1^{+} subsets of gated CD1d-αGC^{+} thymocytes at 12 hours, indicating cell division of NK1.1^{-} cells. (**B**) Kinetics of BrdU incorporation in the different thymic NK T cell subsets upon repeated intraperitoneal injections (twice daily) of BrdU, representative of five experiments in 2- to 3-week-old mice. The NK1.1^{-} (CD44^{low} and CD44^{high}) subsets show rapid turnover, whereas the NK1.1^{+} subset is a sessile population. (**C**) DNA content of CD1d-αGC^{+} subsets enriched from 2-week-old thymocytes, measured by Hoechst staining. Note the high frequency of cells in S/M phase in the NK1.1^{-} but not the NK1.1^{+} subset.

phase (Fig. 3, A to C). In addition, the NK1.1^{-} populations were rapidly turning over, with a 50% turnover rate in 3 days; in contrast, the NK1.1^{+} thymocytes were nondividing cells with a slow turnover, as reported in (*15*). Similarly, the peripheral CD44^{high}NK1.1^{-} cells (which contain the thymic emigrants) were actively dividing and rapidly turning over, whereas the CD44^{high}NK1.1^{+} cells were a nondividing and sessile population (*16*). Together, these results indicate that a lineage expansion occurs at the pre-NK1.1 stage, both in thymocytes and in recent thymic emigrants, and they suggest that NK1.1 expression occurs independently in the thymus (for the cells that will stay resident in the thymus) and in peripheral sites (for the cells that have emigrated). Thus, the CD44^{high}NK1.1^{+} cells represent end-stage sessile populations in different tissues, which do not appear to communicate, as also suggested by their different pattern of NK receptor expression (*22*).

Next, we investigated the functional properties of NK T cell developmental intermediates. Sorted CD44^{low}NK1.1^{-}, CD44^{high}NK1.1^{-}, and CD44^{high}NK1.1^{+} subsets were stimulated in vitro with αGC-pulsed DCs, and cytokines released in the supernatants were measured (*23*). Surprisingly, we found that these fresh thymocyte subsets expressed strikingly different cytokine potentials. Thus, as they progressed in vivo through the CD44^{low}NK1.1^{-}, CD44^{high}NK1.1^{-}, and CD44^{high}NK1.1^{+} stages, the cells expressed IL-4, IL-4 and IFN-γ, and IFN-γ, respectively, with the IL-4:IFN-γ ratio changing from 130:1 to 15:1 to 1:4 (Fig. 4A). The

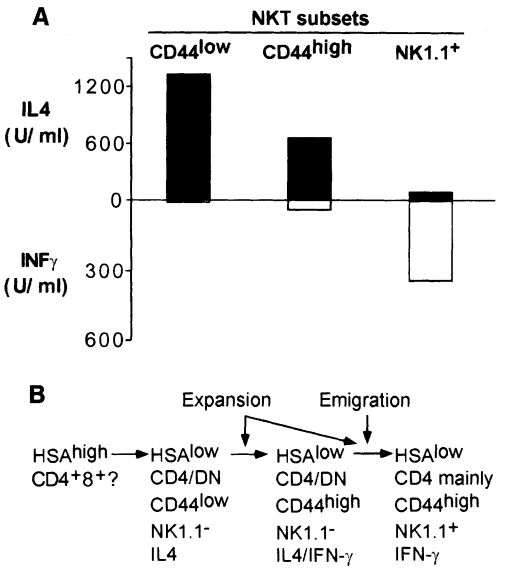

Fig. 4. T_H2 to T_H1 reversal during NK T cell development. (**A**) Fresh $CD44^{low}NK1.1^-$, $CD44^{high}NK1.1^-$, and $CD44^{high}NK1.1^+$ NK T thymic subsets were sorted and stimulated with αGC-pulsed splenic DCs. Cytokines released in the supernatant were measured at 48 hours. Similar results were obtained in six experiments, for purified $CD4^+$ as well as for whole ($CD4^+$ and DN) NK T subsets. T_H1 polarization was also observed for splenic and liver $NK1.1^+$ NK T cells (*16*). CD1d-deficient DCs pulsed with αGC were used as negative controls to establish that cytokines were induced by TCR engagement (*16*). (**B**) NK T cell lineage thymic and postthymic development scheme. Note that, although peripheral NK T cells express NK1.1 after emigration into tissues, a fraction of NK T cells acquire NK1.1 in the thymus and do not emigrate.

precursor-product relation between these intermediates suggests that the developing NK T cell lineage reverses its cytokine profile from T_H2 to T_H1 during the expansion phase.

The development of NK T cells has been controversial, with conflicting but indirect evidence for thymic (*24, 25*) versus extrathymic (*26–28*) origin, early (*27, 29*) versus late (*3*) (i.e., pre- or post-TCR) fate specification, and directed (*27, 29*) versus random (*30*) TCR gene rearrangement. Our results identify a mature $HSA^{low}CD44^{low}NK1.1^-$ Vα14 T cell, found only in the thymus, as a precursor to the NK T cell lineage, consistent with a thymic origin and a late fate specification of NK T cells. This developmental intermediate is itself the product of positive selection at the HSA^{high} stage because it is absent from CD1d-deficient mice, but the rare HSA^{high} precursors remain to be directly identified, although they are likely to include DP thymocytes (*15, 24, 31*). The rarity of these HSA^{high} precursors, below our current detection levels, suggests that Vα14-Jα18 rearrangements occur at a very low frequency, consistent with random rather than directed V-J joining.

Our study reveals a massive expansion at the mature HSA^{low} stage, which is unprecedented among mainstream T cell lineages. This expansion mimics an antigen-driven proliferative response, establishing an abundant pool of peripheral NK T cells. Expression of NK lineage receptors is a late event, correlating with cessation of cell division and establishment of long-term residence either in the thymus or after emigration into peripheral tissues (Fig. 4B). Because inhibitory NK receptors regulate TCR autoreactivity (*10*) and differ in expression pattern according to tissues (*22*), this finding implies that local environmental influences shape the final pattern of reactivity of NK T cells and might explain the tissue-specific autorecognition of CD1d reported for some NK T cells (*32*). Autoreactivity is ultimately controlled by expression of inhibitory NK receptors in peripheral tissues, but it is tempting to speculate that cell division of HSA^{low} thymocytes is a consequence of CD1d autoreactivity against cortical thymocytes or medullary DCs, both of which express CD1d.

The physical identification and functional characterization of developmental NK T intermediates identifies a novel developmental pathway that expands rather than deletes autoreactive cells and turns them into cytokine-producing regulatory cells. Other autoreactive lymphocytes belonging to the γδ T, B-1, and NK cell lineages may share some aspects of their development with NK T cells (*2*). Further, a T_H2 to T_H1 progression similar to the one we described for NK T cells has also been recently documented for human NK cells (*33*), suggesting common pathways and mechanisms of development. Future studies will shed light on the molecular and cellular regulation of these developmental stages and their potential defects in autoimmune disease.

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14. B6 and B6.Jα18$^{-/-}$ (*13*) thymuses were stained (four-color) and analyzed as described (*11*), except that cells were preincubated with 2.4G2 antibody to FcγRII/III and with unlabeled streptavidin, then with unloaded CD1d tetramers labeled with Cychrome, before exposure to a mixture of APC-labeled CD1d-αGC tetramers and antibodies to HSA, CD8, CD44, or NK1.1 (BD Pharmingen).
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17. In some experiments, spleens of FITC-injected 4- to 6-week-old B6 mice were treated with CD8 monoclonal antibody 3.155, panned on plates coated with antibody to mouse immunoglobulin to remove CD8 cells and B cells, and centrifuged over Lympholyte (Cederlane Laboratories, Hornby, Ontario, Canada).
18. While recent thymic emigrants progressively acquired NK1.1, they maintained their $CD44^{high}$ phenotype at all times, indicating that they do not give rise to $CD44^{low}$ cells. Moreover, only CD4 cells could be observed among thymic emigrants in the spleen and liver, consistent with the rarity of the $CD4^-8^-$ subset of NK T cells in mice (*16*).
19. Given that 4% of recent thymic emigrants belong to the Vα14 NK T lineage, and taking into account the fraction of thymocytes labeled by FITC (10 to 30% in different experiments), the frequency of $FITC^+$ splenocytes (0.2 to 0.8%), and the number of splenocytes ($\sim 10^8$) in these mice, we calculate that as many as 1×10^5 Vα14 T cells are exported to the spleen every day.
20. $CD1d\text{-}\alpha GC^+$ $NK1.1^-$ cells were enriched and sorted from 40 2- to 4-week-old B6.Ly5.2.MHCII$^{-/-}$ mice (Jackson Labs, Bar Harbor, Maine), whereas the $NK1.1^+$ subset was obtained from B6 mice (Ly5.1). Cells (1×10^5 to 3×10^5) were injected intrathymically and their progeny were analyzed 7 days later, after thymocyte enrichment by treatment with antibodies to HSA and CD8 and with complement.
21. B6 mice (2 to 3 weeks old) were injected twice daily with BrdU (*8*). Enriched thymocytes (recovered after treatment with antibodies to HSA and CD8 and with complement) were stained with CD1d-αGC tetramers and antibodies to BrdU, CD44, and NK1.1 (BD Pharmingen) or with Hoechst DNA dye (Sigma).
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