Brief Definitive Report

Positive Selection of Mouse NK1$^+$ T Cells by CD1-expressing Cortical Thymocytes

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Summary

Mouse NK1$^+$ T cells constitute a subset of α/β TCR$^+$ T cells that specialize in the rapid production of cytokines, in particular IL-4, and may promote the differentiation of Th2-type CD4 T cells. Their TCRs, like those of a homologous subset of human T cells, use an invariant TCR α chain and were recently shown to be specific for the β2-microglobulin–associated, MHC class I-like CD1 molecules, which are encoded outside the MHC. In contrast to mainstream thymocytes, which recognize their positively selecting MHC ligand on thymic epithelial cells, positive selection of NK1$^+$ T cells requires their CD1 ligand to be expressed on bone marrow-derived cells. To investigate the nature of the bone marrow-derived cell involved, chimeric mice were constructed with tissues from normal, SCID, and MHC-deficient mice, so that CD1 could be selectively expressed by different subsets of bone marrow-derived cells in the thymus. CD1 expression was also directly assessed using an anti-CD1 mAb, and a CD1-specific T cell hybridoma. The results suggest that immature (CD4$^+$8$^+$ double-positive) cortical thymocytes are the source of CD1 presentation for positive selection of NK1$^+$ T cells.

NK1$^+$ T cells constitute a subset of mouse T cells that bear natural killer (NK) surface receptors, and have T cell and NK-like functions (reviewed in reference 1). They include CD4$^+$ (2–8) as well as CD4$^-$8$^-$ double-negative (DN) (2–3, 6, 9–11) T cells but no CD8$^+$ T cells (because CD8 imparts negative selection [6]) and are found in the thymus, where they account for up to 20% of the adult mature (HSA^{low}) thymocyte compartment, as well as in the periphery, particularly in the bone marrow and the liver (8). Their TCR repertoire is very restricted, using a single, invariant TCR α chain, Vα14-Jα281, paired with Vβ8$^+$, Vβ7$^+$, or Vβ2$^+$ TCR β chains (12). In humans, a similar TCR repertoire using Vα24-JαQ paired with Vβ11, the homologues to mouse Vα14-Jα281 and Vβ8, respectively, defines an equivalent subset of T cells (12–15).

NK1$^+$ T cells were recently shown to be specific for the non-polymorphic, β2-microglobulin (β2M)–associated MHC class I-like CD1 molecules encoded outside the MHC region (16). Mouse CD1 is encoded by two genes, CD1.1 and CD1.2 that are 95% identical, and are homologous to CD1d, one of five CD1-family members in humans (17). Since the most striking property of NK1$^+$ T cells is the ability to secrete large amounts of IL-4 upon primary stimulation (3, 5, 18–20), it was suggested that the CD1/NK1$^+$ T cell pathway may direct the Th2 differentiation of some immune responses and that induction of CD1 during immune responses may recruit and activate NK1$^+$ T cells (16, 21). Indeed, animals that are deficient in CD1 expression and/or NK1$^+$ T cell function, such as β2-microglobulin–deficient mice and the SJL mouse strain, have lost the potential to produce the early burst of IL-4 that follows T cell activation by anti-CD3 antibody in vivo, and cannot secrete polyclonal IgE in response to the intravenous injection of polyclonal anti-IgD antibodies (21a, 21b).

The development of such CD1-specific, IL-4 producer NK1$^+$ T cells, occurs mainly in the thymus because NK1$^+$ T cells can be generated in fetal thymic organ culture (6), and they are rare or absent in athymic mice (1, 22–23). Like mainstream MHC class I-specific T cells, the NK1$^+$ T cells depend on β2-microglobulin expression for positive selection in the thymus (6–8, 11). However, in contrast to mainstream T cells, which depend for positive selection on MHC expression by radioresistant thymic epithelial cells, the positively selecting ligand of NK1$^+$ T cells is exclusively presented by bone marrow-derived cells, because lethally irradiated β2M$^{+/+}$ mice reconstituted with β2M$^{-/-}$ fetal liver cells do not generate NK1$^+$ T cells whereas the reciprocal chimeras (β2M$^{-/-}$ mice reconstituted with β2M$^{+/+}$ fetal liver cells) do (6, 8, 11). Since CD1 is normally expressed constitutively in the bone marrow-derived compartment, particularly on immature cortical thymocytes (24–26), the possibility exists that immature thymocytes present CD1 to each other for positive selection. Alternatively, other cell-types, such as dendritic cells, macrophages, or B cells, could be involved in this process.

To begin to investigate this peculiar pathway of positive

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selection of CD1-specific T cells, in vivo and in vitro experiments were designed to identify the CD1-expressing cell that mediates this process. The results suggest that immature ($CD4^+8^+$ double-positive) cortical thymocytes themselves are the source of CD1 presentation for positive selection of developing thymocytes.

Materials and Methods

Mice. C57BL/6, C57BL/6.MHCII$^-$ (I-Aβ^b knock-out [27] backcrossed six times to B6), C57BL/6.β2M$^{-/-}$ (β2-microglobulin knock-out [28], backcrossed eight to nine times to B6), C57BL/6.MHCII$^{-/-}$ β2M$^{-/-}$ (double knock-outs, backcrossed four times to B6), C57BL/6.C$\mu^{-/-}$ (μ heavy chain knock-out [29], backcrossed eight times to B6) and B10.SCID mice were raised under specific pathogen-free conditions at Bioqual (Frederick, MD), at the National Institutes of Allergy and Infectious Diseases (National Institutes of Health, Bethesda, MD), or at Princeton University (Princeton, NJ). TCR $\alpha^{-/-}$ (30), TCR $\beta^{-/-}$ (30), and RAG-$2^{-/-}$ (31) mice backcrossed four to eight times to B6 were purchased from the Jackson Laboratories (Bar Harbor, ME).

Fetal Liver Chimeras. Recipient mice were treated with two i.p. injections of 0.5 mg purified PK136 anti-NK1.1 antibody at day 1 and day 0 of whole body γ-irradiation (1,000 Rads) with a cesium source (Gammacell 40, Ontario, Canada). 6 h after irradiation, they were reconstituted with an i.v. injection of 5–10 $\times$ 10^6 day 14 fetal liver cells from various donors.

Cell Preparations and FACS Analysis/Sorting. Thymocyte suspensions were treated with anti-heat stable antigen (HSA) mAb (J11d2) and rabbit complement (Cedarlane, Hornby, Canada) or anti-HSA + anti-CD8 (3.155) and rabbit complement in a one-step 45-mn 37°C incubation, and viable cells were collected after centrifugation over a density gradient (Lympholite, Cedarlane, Hornby, Canada). Cells were stained for three-color FACS analysis with directly conjugated antibodies obtained from Pharmingen (San Diego, CA): 53.7.3 anti-CD5, PK1.36 anti-NK1.1, IM-7.8 anti-CD44, H57 anti-pan-TCRβ, RM4-5 anti-CD4, 53.6.7 anti-CD8 or made in the laboratory: Y3P anti-I-A^b, R1.21.2 anti-$K^{b,k}$, F23.1 anti-Vβ8. CD1.1 was stained with the rat 3C11 antibody (24), followed by PE-conjugated goat anti-rat Ig (Southern Biotech), washes and then saturation of the rat Ig binding sites with unlabeled rat Ig, before staining with FITC anti-pan-TCRβ. FACS analyses were performed on a FACSCAN (Becton Dickinson, Mountain View, CA) with conventional settings, except in one set of experiments where the argon laser was used at 20 mW instead of 5 mW, to increase the resolution of the dimly staining 3C11 anti-CD1 antibody. FACS-sorting was performed using a FACS-STAR^{PLUS} (Becton Dickinson) or an EPICS 753 (Coulter, Hialeah, FL) equipped with dual (argon and dye) lasers.

Antigen Stimulation of T Cell Hybridomas. Unirradiated whole thymocytes or thymocyte fractions (5×10^5 cells) were incubated in flat-bottom microwells in the presence of 3×10^4 hybridoma T cells, in a final volume of 0.2 ml of a 1:1 mixture of Click's medium and RPMI (Biofluids, Rockville, MD) enriched with 10% heat-inactivated FCS, glutamine, antibiotics, and 5×10^{-5} M 2-ME. After overnight incubation, supernatants were harvested and IL-2 measured using the CTLL bioassay as previously described (3).

Thymic Dendritic Cells Enrichment. Thymocyte suspensions (20×10^6 cells/ml in culture medium as above) were allowed to adhere on plastic culture dishes (Falcon 3025; Becton Dickinson Labware, Lincoln Park, NJ) for 2 h at 37°C in a 5% CO_2 incubator. Nonadherent cells were then removed with several washes and vigorous pipetting, and adherent cells reincubated overnight. Cells that detached during the second culture period contained an enriched proportion (1–5% vs. less than 0.1% before enrichment) of dendritic cells, identified as large cells expressing very high levels of MHC class II molecules by flow cytometry analysis.

Results and Discussion

The Bone Marrow-derived, CD1-presenting Cell Required for Positive Selection of NK1$^+$ T Cells Is Not a Professional Antigen Presenting Cell. CD1-specific NK1$^+$ T cells can be identified in the mature (HSA^{low}) compartment of the thymus, by their expression of NK1.1 or CD44 (in the C57BL/6 background), and by their bias in Vβ8 usage (usually above 45–50%, vs. 18–25% in mainstream T cells) (6). Previous experiments have established that the positively selecting CD1 ligand of NK1$^+$ T cells is exclusively presented by bone marrow-derived cells, because NK1$^+$ T cells do not develop in lethally irradiated β2M$^{+/+}$ mice reconstituted with β2M$^{-/-}$ fetal liver cells whereas they develop normally in the reciprocal chimeras (β2M$^{-/-}$ mice reconstituted with β2M$^{+/+}$ fetal liver cells) (6, 8, 11). To dissociate the responding thymocyte from the CD1-presenting, positively selecting cell in the bone marrow-derived compartment of the thymus, we reconstituted unirradiated SCID mice (β2M$^{+/+}$) with fetal liver cells from β2M$^{-/-}$ donors. In these chimeras, T cells can only come from β2M$^{-/-}$ cells whereas other bone marrow-derived components of the thymus, including dendritic cells and macrophages, can originate from both β2M$^{+/+}$ (SCID) cells and β2M$^{-/-}$ stem cells. Within a month, the thymus of the recipients was reconstituted, and β2M$^{-/-}$ mature T cells had seeded the periphery. When dendritic cells in these thymuses were partially enriched by an in vitro adherence–deadherence procedure, and identified by their high level of expression of MHC class II molecules, they were found to express the same levels of classical MHC class I molecules as dendritic cells in control β2M$^{+/+}$ into SCID chimeras (Fig. 1, *e* and *f*, compare upper and lower right quadrants of the dot plots). Thus, the majority of thymic dendritic cells in the β2M$^{-/-}$ into SCID chimeras are of SCID (host) origin. However, though the control β2M$^{+/+}$ reconstituted SCID mice generated normal populations of NK1$^+$ T cells with the Vβ8-skewed canonical repertoire, the β2M$^{-/-}$ reconstituted SCID mice failed to generate such cells. Their populations of CD44$^+$ $\alpha\beta$TCR$^+$ cells (which in the thymus almost completely overlap with NK1.1$^+$ $\alpha\beta$TCR$^+$ cells (6)) were reduced in number (4% vs. 11%, Fig. 1, *a* and *c*) and contained only 18% Vβ8$^+$ cells (0.7 out of 4, Fig. 1, *c* and *d*), vs. 45% Vβ8$^+$ cells in control chimeras (5 out of 11, Fig. 1, *a* and *b*). These results indicate that the CD1-expressing cell that positively selects NK1$^+$ T cells is not of myeloid origin (i.e., not a dendritic cell or a macrophage).

The CD1-presenting, Positively Selecting Cell Is Not a B Cell. Rare B cells are occasionally found in the thymus, and β2M$^{+/+}$ B cells would be missing in the SCID chimeras described in the previous section. Fig. 2 shows that C$\mu^{-/-}$ mice, which do not generate mature B cells because of a

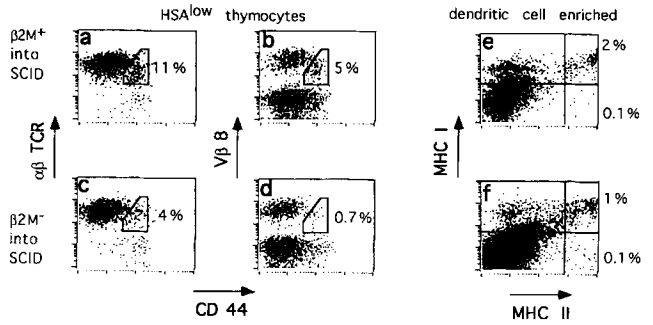

Figure 1. SCID mice reconstituted with $\beta 2M^{-/-}$ fetal liver cells are deficient in generating canonical $NK1^+$ T cells. Adult B10.SCID mice were injected i.v. with 5×10^6 fetal liver cells from 14 d B6 or B6.$\beta 2M^{-/-}$ embryos. To prevent NK-mediated rejection of the $\beta 2M^{-/-}$ inoculum, SCID mice in both groups were previously treated with two injections of PK1.36 anti-NK1.1 antibody. Eight weeks after injection, B6 or B6.$\beta 2M^{-/-}$ fetal cell-injected SCID mice were analyzed. Their thymuses were of similar size (1.5×10^8 vs. 1.6×10^8 cells on average). However, mature (HSA^{low}) thymocytes of B6.$\beta 2M^{-/-}$ fetal cell-injected SCID mice had a similar proportion of α/β TCR^+ $CD44^+$ cells (*c*) than those of B6 fetal cell-injected SCID mice (*a*). Moreover, the residual $CD44^+$ cells did not show the canonical bias in TCR-Vβ8 usage seen in control B6-reconstituted mice (*b* and *d*; the frequency of $V\beta 8^+$ cells is calculated to be 18% (0.7 out of 4) vs. 45% (5 out of 11) of the $CD44^+$ ($NK1.1^+$) population. Right panels (*e* and *f*) however show that most dendritic cells, i.e., the rare MHC class II bright cells enriched after adherence–deadherence treatment of the thymocyte preparations, were in the upper right quadrant (H-2 K^{b+}, i.e., $\beta 2M^{+/+}$) and not in the lower right quadrant (H-2 K^{b-}, $\beta 2M^{-/-}$). Similar results were observed in two separate batches of chimeras, including a total of four chimeras of each kind.

homozygous inactivation of their Cμ gene (29), nevertheless generate normal amounts of thymic $NK1^+$ T cells with the typical bias in Vβ8 usage, ruling out the possibility that B cells are a major source of CD1 presentation for the positive selection of $NK1^+$ T cells in the thymus. In addition, the frequency of $NK1^+$ T cells in the bone marrow was found to be normal in these mice, suggesting that B cells also do not contribute to any significant expansion of $NK1^+$ T cells in this peripheral tissue (data not shown).

The CD1-presenting, Positively Selecting Cell Is Not a Mature T Cell. Altogether, the previous results point to the T cell compartment itself as the source of CD1-presentation for positive selection of $NK1^+$ T cells. To test the possibility that mature thymocytes were the selecting cells, despite the fact that they are located in the medulla, we generated chimeric mice by injecting MHC $II^{-/-}$ fetal liver cells into lethally irradiated, $\beta 2M^{-/-}$MHC $II^{-/-}$ recipients. In these animals, the radioresistant host tissues express neither MHC class I nor class II, while the bone marrow-derived cells express only class I. Because of the lack of MHC molecules on thymic epithelium, the development of mainstream T cells is arrested at the $CD4^+8^+$ immature state and, as a consequence the chimeras produce virtually no mature mainstream T cells. They do, however, produce normal numbers of $NK1^+$ T cells (with the bias in Vβ8 usage), which represent most of the mature thymic pool in these animals (Fig. 3).

These results indicate that mature mainstream T cells are not necessary for the positive selection of the $NK1^+$ T cell pool and, taken together with the evidence against a role for dendritic cells, B cells, and thymic epithelium, indicate that the CD1-presenting cells that positively select Vα14-Jα281$^+$ thymocytes are the immature thymocytes themselves.

CD1 Expression in the Thymus. Previous studies based on in situ hybridization and immunostaining of tissue sections have suggested that cortical thymocytes are the main CD1-expressing population in the thymus (24–26). Using flow cytometry analysis with an anti-CD1.1 mAb, we showed that CD1.1 is indeed expressed by the majority of immature, $\alpha\beta TCR^{low}$ thymocytes, and is down-modulated at the $\alpha\beta TCR^{high}$ state (Fig. 4).

To confirm that the $NK1^+$ T cell ligand is expressed by double-positive thymocytes, thymocyte fractions were FACS-sorted and used as CD1-presenting cells for DN32.D3, a T cell hybridoma derived from $NK1^+$ T cells that uses a canonical Vα14$^+$/Vβ8.2$^+$ TCR (12) and was previously shown to be CD1.1-specific (16). Indeed, double-positive thymocytes induced a strong IL-2 secretion by the hybridoma, accounting for most of the CD1 presentation by the whole thymocyte presentation, whereas single-positive

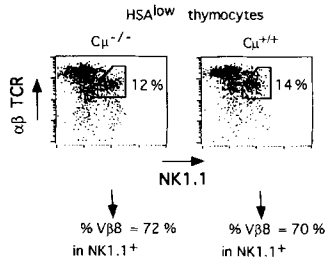

Figure 2. Normal generation of $NK1^+$ T cells in $C\mu^{-/-}$ mice. Mature (HSA^{low}) thymocytes from B6 or B6.$C\mu^{-/-}$ mice generate comparable proportions of α/β TCR^+ $NK1.1^+$ cells with the canonical bias in TCR Vβ8 usage (70 and 72% of the NK1.1 T cells, respectively).

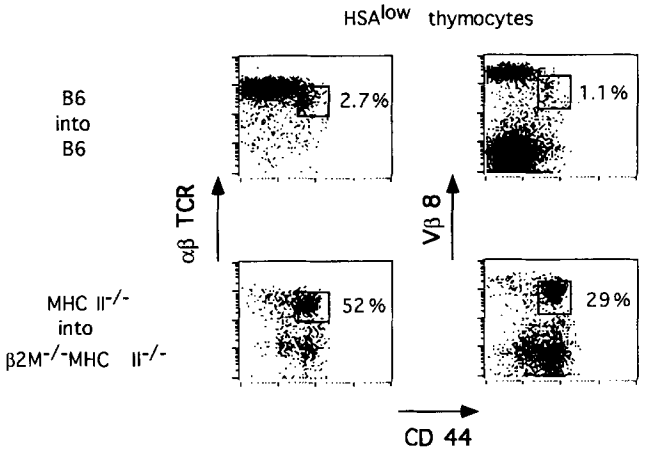

Figure 3. Normal generation of $NK1^+$ T cells in the absence of mainstream single-positive thymocytes. Mature (HSA^{low}) α/β TCR^+ thymocytes from MHC $II^{-/-}$ into $\beta 2M^{-/-}$MHC $II^{-/-}$ fetal liver radiation chimeras are almost exclusively $CD44^+$ ($NK1.1^+$) T cells, and display the canonical bias in TCR Vβ8 usage (56% $V\beta 8^+$, i.e., 29 [*bottom right*] out of 52 [*bottom left*]). They lack the mainstream T cell component ($CD44^-$) that is present in B6 into B6 pseudo-chimeras. Average recoveries of HSA^{low} cells after anti-HSA + complement kill were 1.4×10^6 in the control chimeras and 0.15×10^6 in the MHC $II^{-/-}$ into $\beta 2M^{-/-}$MHC $II^{-/-}$ chimeras. Similar results were observed in two separate experiments including five B6 into B6 control chimeras and four MHC $II^{-/-}$ into $\beta 2M^{-/-}$ MHC $II^{-/-}$ chimeras.

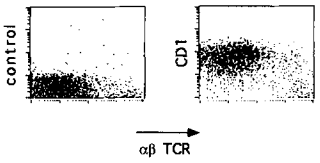

Figure 4. Expression of CD1.1 in the mouse thymus. Thymocytes from B10.A mice were stained with anti-CD1.1, or a control isotype-matched antibody, and anti-αβ TCR.

thymocytes were only weakly (30–50 times less) stimulatory (Table 1).

To more precisely identify the stage of thymocyte development at which CD1 is first being expressed in a form that is recognizable by $NK1^+$ T cells, we used thymocytes from RAG-, TCRβ- and TCRα-deficient mice that are arrested at the double-negative, late double-negative/early double-positive, and double-positive stages, respectively (30–31). Maximal stimulation of DN32.D3 was induced by thymocytes from TCRα-deficient mice, whereas those of TCRβ-deficient mice induced only partial stimulation and RAG-deficient thymocytes were not at all stimulatory (Table 1). These results suggest that induction of the $NK1^+$ T cell ligand is initiated at the double-positive or possibly the late double-negative stages.

Models for Positive Selection of CD1-specific, Vα14-Jα281$^+$ Thymocytes. Altogether, these results indicate that CD1-expressing, double-positive thymocytes are the main cell type that mediates the positive selection of $V\alpha14\text{-}J\alpha281^+$, CD1-specific thymocytes. Because there are a number of indirect suggestions that $NK1^+$ T cells go themselves through a double-positive stage (1, 2, 6), the most likely scenario is that $V\alpha4\text{-}J\alpha281^+$ double-positive thymocytes are presented with CD1 for positive selection by their CD1-expressing double-positive clustermates.

There have been previous reports suggesting that cell types other than epithelial cells could also mediate positive selection (34–36). Bone marrow-derived cells can present for some level of positive selection of CD8 cells in $\beta2M^{+/+}$ into $\beta2M^{-/-}$ fetal liver chimeras, although this process is inefficient (36). Our results suggest that the inefficiency of this process might be due to the fact that cortical thymocytes express very little of the classical MHC class I molecules necessary for positive selection of mainstream T cells. On the other hand, the predominant role of double-positive thymocytes in selecting Vα14-Jα281 cells could be related to their higher constitutive expression of CD1.

A major remaining question is why Vα14-Jα281–specific cells have a particular functional differentiation that includes features of activated/memory T cells as well as of NK cells. For example, in contrast to mainstream T cells, they display activation/memory surface receptors and secrete a large set of cytokines of both the Th1 and Th2 types upon primary stimulation, they express NK surface receptors and they can kill NK targets such as YAC cells (reviewed in reference 1). It is possible that this differentiation is genetically programmed before TCR engagement by CD1 during the positive selection process. This is rather unlikely however, because the Vα14-Jα281 rearrangements themselves do not appear to be directed (12). Alternatively, the differentiation could be the consequence of a particular type of TCR engagement and/or interactions occuring during the positive selection events. Indeed, the avidity of Vα14-Jα281 TCRs for CD1 appears to be higher than average, because $CD8^+$ $V\alpha14\text{-}J\alpha281^+$ cells are negatively selected (6, 12). In addition, as reported here, the antigen presenting cell mediating positive selection is not an epithelial cell, but a cortical thymocyte.

Further studies should now aim at understanding how this unusual phenotype is imparted to $NK1^+$ T cells. $NK1^+$ T cells play an important functional role in regulating the Th1/Th2 differentiation in some immune responses, and mice that do not select functionally competent $NK1^+$ T cells have been identified (21a, 21b). Because a similar pathway is likely to operate in humans, it may be of high clinical relevance to identify the various steps that can be affected during the thymic development of $NK1^+$ T cells.

Table 1. *Stimulation of a CD1-specific T Cell Hybridoma by Thymocytes*

	$RAG^{-/-}$	$TCR\beta^{-/-}$	$TCR\alpha^{-/-}$	Normal
		IL-2* *(u/ml)*		
Whole thymoctes	0.1	5	18	16
$CD4^+8^+$				20
$CD4^+8^-$				0.6
$CD8^+4^-$				0.4

*IL-2 released in the supernatant of microcultures of 3×10^4 DN32.D3, CD1-specific T hybridoma cells after 20 h of culture with 5×10^5 whole thymocytes or cells from different thymocyte subsets. Background IL-2 release (hybridoma alone) is <0.1 unit/ml.

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