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18. In our investigations of the N_2 cleavage reaction, we are attempting to isolate and investigate the intermediates depicted in Fig. 2.
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CD1 Recognition by Mouse NK1$^+$ T Lymphocytes

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Rare major histocompatibility complex (MHC) class I–like CD1-specific T cells have been isolated from human blood, but it has not been determined whether these clones are part of a defined subset of CD1-specific T cells selected during T cell development, or whether their recognition of CD1 is a fortuitous cross-reaction. In mice, an entire subset of αβ thymocytes with a unique phenotype was found to be CD1-specific. This particular subset, and its human counterpart, provide evidence that CD1 has a general role in selecting and interacting with specialized αβ T cells.

Mouse NK1$^+$ T cells constitute up to 20% of the mature compartment of the thymus and are also found in most peripheral tissues, with particular frequency in bone marrow and liver (*1–7*). They consist of CD4$^+$ and CD4$^-$8$^-$ double-negative (DN) cells. Unlike other T cells, NK1$^+$ T cells express the surface receptors normally associated with natural killer (NK) cells, including NKR-P1 and Ly-49, and they can lyse NK-sensitive target cells (*8*). In addition, they have the unique ability to secrete large amounts of cytokines, especially interleukin-4 (IL-4), upon primary stimulation through their αβ T cell receptors (TCRs) in vitro (*1*, *5*, *7*) and in vivo (*9*). Because this secretion of IL-4 is rapid, NK1$^+$ T cells are likely to promote the differentiation and recruitment of T helper 2 (T_H2) cells over T_H1 cells in immune responses in which they are engaged (*10*). Elucidating the contribution of these specialized T cells thus depends on the identification of their TCR ligands.

Several findings have suggested that the ligand is a nonpolymorphic MHC class I molecule that is conserved in different species. First, thymic selection of NK1$^+$ T cells depends on the expression of β_2-microglobulin (β_2M) (*2*, *4*) and thus, by implication, on the expression of class I molecules. Second, the TCR repertoire of both CD4$^+$ and DN NK1$^+$ T cells is largely restricted to TCRs comprising a single, invariant TCRα chain, $V_\alpha14$-$J_\alpha281$, paired with $V_\beta8$, $V_\beta7$, or $V_\beta2$ TCRβ chains (*11*). Third, the same TCR repertoire exists in different strains of mice, regardless of MHC haplotype (*11*). Fourth, a human counterpart to this subset exists that uses TCRs comprising an invariant TCRα chain, $V_\alpha24$-$J_\alpha Q$ (the homolog of mouse $V_\alpha14$-$J_\alpha281$), paired with $V_\beta11$ TCRβ chains (homologs of mouse $V_\beta8$) (*11*, *12*).

A clue to the identity of the NK1$^+$ T cell ligand is the ligand's tissue distribution. Expression of β_2M on bone marrow–derived cells, but not on thymic epithelium, enables thymic selection of NK1$^+$ T cells (*2*, *4*). The relevant bone marrow–derived cell is probably a thymocyte rather than a dendritic cell or macrophage, because mice with severe combined immunodeficiency disease that were inoculated with β_2M-deficient fetal liver cells did not generate NK1$^+$ T cells (although they developed mainstream T cells), even though their thymic dendritic cells were largely β_2M-positive (*13*). In addition, NK1$^+$ T cells induce cytolysis of cortical thymocytes (*14*). These findings suggest that immature thymocytes express the NK1$^+$ T cell ligand, whereas thymic epithelial cells and professional antigen-presenting cells (APCs) do not. This pattern fits that of the *TL* and *CD1* gene families, which, in contrast to classical MHC class I molecules, are mainly expressed by immature CD4$^+$8$^+$ thymocytes (*15*, *16*).

Table 1. Recognition of an MHC class I ligand by DN32.D3, a NK1$^+$ T cell–derived hybridoma. Duplicate samples of DN32.D3 hybridoma cells (3×10^4) were cultured with 5×10^5 cells from the thymus, spleen, or bone marrow of the indicated mouse strains in 96-well flat-bottom microplates for a period of 20 to 24 hours. IL-2 released in the supernatant was measured in units per milliliter, where 1 U/ml corresponds to 3 pM recombinant human IL-2 as measured by CTLL indicator cells (*1*). Mean values for duplicate samples are given. Standard errors were less than 20% of this value. Purified F23.2 mAb to $V_\beta8.2$ was used at 1 μg/ml, versus the same concentration of 28.8.6S, an isotype-matched mAb to H-2K^{b}D^b. These results were confirmed in two to five separate experiments.

Tissue source	Stimulator cell strains and IL-2 production (U/ml)				
	B6	B10.A	BALB/c	129	B6.β_2M^{-*}
		Experiment 1			
Thymus	27	20	44	32	<1
		Experiment 2			
Thymus + anti-H-2K^{b}D^b mAb	32				
Thymus + anti-$V_\beta8.2$ mAb	<2				
		Experiment 3			
Thymus	29				
Spleen	0.5				
Bone marrow	0.2				

*B6.β_2M$^-$ mice bear a homozygous inactivation of their gene encoding β_2M and were backcrossed eight times to C57BL/6.

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Unlike *TL*, the *CD1* family of genes is conserved in various species, including humans (*17*). The mouse *CD1* family consists of two genes, *CD1.1* and *CD1.2*, that are 95% identical. *CD1.1* and *CD1.2* are homologs of human *CD1d*, one of five members of the human *CD1* family, and sequence analysis suggests that the *CD1d* and *CD1a*, *b*, *c*, and *e* types arose from distinct ancestral genes that predated speciation (*17*). Although no function has yet been ascribed to the *CD1* family members, there is evidence in humans that they can be recognized by some γδ T cells and by unusual αβ T cells that lack CD4 and CD8 expression (*18, 19*).

To search for the NK1$^+$ T cell ligand, we derived T cell hybridomas from NK1$^+$ T cells (*11*). Thymocytes that express different MHC and non-MHC genes, from various mouse strains [including the TL-expressing strains B10.A, BALB/c, and 129 as well as the non–TL-expressing strain C57BL/6 (B6)], stimulated DN32.D3, a hybridoma bearing a canonical V_α14-J_α281–V_β8.2 TCR (Table 1). Thymocytes that express neither classical MHC class I nor CD1 molecules (*13*), from $\beta_2M^{-/-}$ mice, did not stimulate this hybridoma. Recognition of the ligand depended on TCR expression because soluble monoclonal antibodies (mAbs) directed at the TCRβ chain completely blocked IL-2 production, whereas an isotype-matched mAb to the B6 H-2K^{b}D^b MHC class I molecules had no effect. In addition, the ligand had a restricted tissue distribution; it was expressed on thymocytes but was expressed very poorly, or not at all, on spleen or bone marrow cells. These observations imply that the NK1$^+$ T cell ligand is not a classical MHC class I molecule, but rather a nonpolymorphic ligand with an expression pattern similar to that of CD1 (*15*).

To determine whether recognition of a ligand expressed by thymocytes was a general feature of NK1$^+$ T cells, we tested two additional NK1$^+$ T cell–derived hybridomas that were randomly chosen from a panel (*11*) derived from C57BL/6 thymic CD4 or DN NK1$^+$ T cells. In addition, to test directly whether CD1 was the ligand for the hybridomas, we repeated the stimulation in the presence of 3C11, a rat immunoglobulin M (IgM) mAb to mouse CD1.1 (*15*). All three hybridomas responded to thymocytes (Table 2, experiment 1). The mAb to CD1.1 specifically blocked the response of hybridomas DN32.D3 and 432.B9. Stimulation of the third hybridoma, 431.A11, was not blocked by the mAb to CD1.1.

To further explore the recognition of CD1 by the NK1$^+$ T cell–derived hybridomas, we infected a mouse fibroblast cell line with a recombinant vaccinia virus (VV) that expressed the mouse *CD1.1* gene under the control of an early-late viral promoter (VV-CD1). VV-CD1.1–infected cells stimulated each of the three hybridomas, including 431.A11, whose recognition of thymocytes was not blocked by the mAb to CD1.1 (Table 2, experiments 2 and 3). In contrast, mock-infected fibroblasts did not stimulate the hybridomas, nor did fibroblasts that were infected with wild-type VV (VV-wt) or with a recombinant VV that expressed H-2K^b (VV-K^b). Stimulation by VV-CD1.1–infected fibroblasts was consistently blocked by the mAb to CD1.1 for all T cell hybridomas. Thus, all three hybridomas recognized CD1.1, which suggests an explanation for the failure of the mAb to CD1.1 to block thymocyte stimulation of 431.A11: 431.A11 might cross-react to CD1.2, which is also expressed on thymocytes [as indicated by mRNA expression (*20*)] and may not be recognized by the CD1.1 mAb. This interpretation is strengthened by the demonstration that the thymocyte ligand of 431.A11 is also an MHC class I molecule, because it is not expressed on B6.$\beta_2M^{-/-}$ thymocytes (Table 2, experiment 3).

To determine whether the recognition pattern of the panel of hybridomas was representative of the NK1$^+$ T cell population, we investigated whether freshly isolated NK1$^+$ T cells could also respond in vitro to CD1. Fresh NK1$^+$ T cells produce more IL-4 than IL-2 upon stimulation through their αβ TCRs (*1, 5, 7*); therefore, IL-4 was used to measure TCR-mediated stimulation. In preliminary experiments, freshly isolated NK1$^+$ T cells were not significantly stimulated by syngeneic thymocytes. However, for optimal lymphokine secretion, fresh cells are more dependent on costimulatory signals expressed by professional APCs than are hybridomas (*21*), and CD1-expressing thymocytes—mainly cortical CD4$^+$8$^+$ thymocytes—may be poor costimulators. Because IL-4 secretion by NK1$^+$ T cells is increased by IL-2 and CTLA-4 ligands (*9, 13, 22*), we used a fibroblast cell line, P13.9, that had been transfected with genes encoding the ICAM-1 and B7.1 molecules and added low doses of IL-2. The P13.9 cells infected with VV-CD1.1, but not those infected with VV-K^b, stimulated freshly isolated NK1$^+$ T cells to secrete IL-4 in amounts up to 600 times the background, and this stimulation was blocked by the CD1.1 mAb (Table 3). Although high, this amount of IL-4 production may nevertheless be an underestimate because fresh cells need an incubation time with their APCs that is substan-

Table 2. Recognition of CD1 by NK1$^+$ T cell–derived hybridomas. Duplicate samples of 3 × 10^4 T hybridoma cells were cultured with 5 × 10^5 C57BL/6 thymocytes or 5 × 10^4 P13.9 fibroblasts for 20 to 24 hours, and IL-2 production was measured (*1*). Mean values for duplicate samples are given; standard errors were less than 20% of this value. P13.9 cells were preincubated for 1.5 hours with 5 × 10^5 plaque-forming units (PFU) of wild-type (WR strain), CD1.1, or K^b recombinant VV (VV-wt, VV-CD1.1, and VV-K^b, respectively) or were mock-infected (mock) (*28*). The cells were washed twice before addition of the hybridomas. Expression of the K^b or CD1 recombinant proteins was detected by flow cytometry on >80% of the P13.9 fibroblasts at the end of the assay. The mAb to CD1.1, 3C11, was used at a 1:100 dilution of a dialyzed ammonium sulfate precipitate of hybridoma culture supernatant. This concentration was six times the concentration necessary to reach maximum blocking. The control mAb (J11d2, a rat IgM isotype-matched antibody to the heat-stable antigen) was prepared and used in the same way as the mAb to CD1.1; both mAbs are known to bind cortical thymocytes. Similar results were obtained in separate experiments that used VV-CD1.1 to infect a variety of cell lines. DN32.D3 hybridoma cells (V_α14-J_α281–V_β8.2-J_β2.4) were derived from DN NK1$^+$ T cells, whereas 432.B9 cells (V_α14-J_α281–V_β8.2-J_β2.5) and 431.A11 cells (V_α3.2-J_α8–V_β8.2-J_β1.6) were derived from CD4$^+$ NK1$^+$ T cells (*11*).

Stimulator cells	Hybridomas, mAbs, and IL-2 production (U/ml)					
	DN32.D3		432.B9		431.A11	
	Control	Anti-CD1.1	Control	Anti-CD1.1	Control	Anti-CD1.1
			Experiment 1			
Thymus (B6)	32	3	0.3	0.05	30	26
			Experiment 2			
Thymus (B6)	105	18				
P13.9 VV-CD1.1	109	<2				
P13.9 mock	<2	<2				
P13.9 VV-K^b	<2	<2				
P13.9 VV-wt	<2	<2				
			Experiment 3			
Thymus (B6)	3	0.2	0.35	0.07	10	10
Thymus (B6.β_2M^-)	0.1		<0.05		1	
P13.9 VV-CD1.1	8	1	0.4	0.06	9	1
P13.9 VV-K^b	1		<0.05		1	

tially longer than that required by hybridomas (48 versus 24 hours) to achieve optimal cytokine secretion, and VV infection results in substantial cytolysis after the first 24 hours.

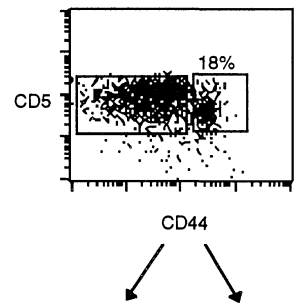
These results establish that NK1$^+$ T cells, which use a restricted repertoire of structurally related TCRs, recognize CD1. We do not yet know whether the CD1 molecules are recognized in isolation or whether they present a peptide or other antigen to the NK1$^+$ T cells. Indeed, human CD1b presents, to a human T cell clone, a lipid derived from *Mycobacterium tuberculosis* cell walls (*23*).

Regardless of whether NK1$^+$ T cells recognize CD1 alone or in conjunction with associated self molecules, it would be rather unexpected to find that the same ligand that induces positive selection of thymocytes also induces full cellular activation of mature T cells, yet this appears to be the case for NK1$^+$ T cells. Although T cell hybridomas may provide a sensitive assay—that is, be able to respond to the putative selecting ligand (*24*)—fresh cells should be nonresponsive, otherwise they would be self-reactive. It is possible that the very large amount of CD1 expression that is induced upon CD1–recombinant vaccinia infection (*25*) exceeds the tolerance threshold established in vivo.

If NK1$^+$ T cells can respond to their selecting self ligand in vivo, then they could be reactivated in the periphery when this ligand is expressed. Mouse CD1 is normally expressed very poorly on resting peripheral cells, but little is yet known about the regulation of its expression in various inflammatory conditions. Human CD1b is highly inducible by lymphokines such as IL-4 and granulocyte-macrophage colony-stimulating factor (*19*). It is possible that the induction of CD1 expression could recruit NK1$^+$ T cells and activate them to release large amounts of lymphokines. Because the NK1$^+$ T cells are a potent and rapid source of IL-4, a cytokine that plays a key role in the T_H1-T_H2 differentiation of CD4 T cells, their activation might promote the T_H2 phenotype of immune responses in which they were recruited. The identification of CD1 as the ligand of NK1$^+$ T cells allows this hypothesis to be tested.

The role of mouse CD1 in positively selecting NK1$^+$ T cells in the thymus may also shed some light on the long-standing enigma of the constitutive expression in the thymic cortex of nonpolymorphic MHC class I genes of the *CD1* and *TL* families. It is likely that these genes participate in the positive selection of T cell subsets that are later capable of recognizing them in the periphery, particularly in conditions where the molecules are acutely induced (*19*, *26*). Our results support the interpretation that the rare CD1-specific T cell clones originally obtained in humans (*18*, *19*) indeed represent a selected, specialized population rather than occasional cross-reactive αβ T cells. Mouse $V_\alpha14$-$J_\alpha281^+$ CD1-specific T cells and their human counterpart ($V_\alpha24$-$J_\alpha Q^+$ T cells, which are likely to be CD1d-specific) may specialize in recognizing lipid antigens; this phenomenon has been observed with a human αβ T cell clone specific for the mycolic acid of *M. tuberculosis* presented by human CD1b (*25*). Alternatively, these cells may be less concerned with the recognition and removal of foreign antigens than with the internal signals required to regulate immune responses (*11*, *27*).

Table 3. Response of freshly isolated mature NK1.1$^+$ thymocytes to CD1. Mature NK1.1$^+$ and NK1.1$^-$ thymocytes were obtained by treating C57BL/6 thymocytes with 3.155 mAb to CD8 and J11d2 mAb to HSA in the presence of rabbit complement (*1*, *11*), staining with mAbs to CD5–fluorescein isothiocyanate and CD44-phycoerythrin, and sorting for CD5$^+$CD44$^-$ or CD5$^+$CD44$^+$ cells (left and right gates of the flow cytometry dot plot). The sorted subsets correspond to αβ TCR$^+$ NK1.1$^-$ mainstream T cells and αβ TCR$^+$ NK1.1$^+$ T cells, respectively (*2*, *11*), and were 99 and 96% pure, respectively. Duplicate samples of 1.5×10^5 cells were cultured for 48 hours, in the presence of low doses of recombinant human IL-2 (10 U/ml), with 5×10^4 P13.9 cells preincubated for 1.5 hours with VV-CD1.1 or VV-K^b. Supernatants were assayed for IL-4 production with the use of the CT4.S indicator line, as described (*1*). In this assay, supernatants were irradiated with ultraviolet light to inactivate VV that accumulated during the longer incubation time (48 hours) required for stimulation of fresh cells. Results are expressed in units per milliliter, where 1 U/ml is equivalent to 0.03 pM mouse recombinant IL-4. Standard errors were less than 20% of the mean values. Similar results were obtained in a separate experiment.



Stimulator cells	Thymocytes, mAbs, and IL-4 production (U/ml)			
	CD44$^-$ (NK1$^-$)		CD44$^+$ (NK1$^+$)	
	No mAb	Anti-CD1.1	No mAb	Anti-CD1.1
P13.9 VV-CD1.1	6	2	282	4
P13.9 VV-K^b	<0.5	<0.5	<0.5	<0.5

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