

Expansion of Natural (NK1⁺) T Cells that Express $\alpha\beta$ T Cell Receptors in Transporters Associated with Antigen Presentation-1 Null and Thymus Leukemia Antigen Positive Mice

By Sebastian Joyce,* Izumi Negishi,[§] Alina Boesteanu,*
A. Dharshan DeSilva,* Padmanee Sharma,* Michael J. Chorney,*[‡]
Dennis Y. Loh,[§] and Luc Van Kaer^{||}

From the *Departments of Microbiology & Immunology, and [‡]Pediatrics, the Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033; [§]Departments of Medicine, Genetics and Molecular Microbiology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110; and ^{||}Department of Microbiology & Immunology, Howard Hughes Medical Institute, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Summary

Thymic selection of natural killer-1⁺ natural T cells that express $\alpha\beta$ T cell receptors requires a conserved $\beta 2$ -microglobulin-associated molecule, presumably CD1d, displayed by CD4⁺8⁺ thymocytes. Here we demonstrate that positive selection of natural T cells occurs independent of transporters associated with antigen presentation-1 (TAP-1) function. Moreover, natural T cells in TAP-1^{0/0} mice are numerically expanded. Several H-2 class Ib molecules function in a TAP-independent manner, suggesting that if expressed in TAP-1^{0/0} thymocytes, they could play a role in natural T cell development. Of these class Ib molecules, H-2TL is expressed by TAP-1^{0/0} thymocytes. Moreover, we find that thymi of TL⁺ mice congenic or transgenic for *H-2T18* also have a numerically expanded natural T cell repertoire compared with TL⁻ mice. This expansion, as in TAP-1^{0/0} thymi, is evident in each of the limited T cell receptor V β chains expressed by natural T cells, suggesting that TL and CD1d impact similar repertoires. Thus TL, in addition to CD1d, plays a role in natural T cell development.

Natural T (NT) cells are an unusual population of thymus-derived cells, constituting up to 20% of mature thymocytes in adults, that coexpress natural killer cell receptor, NKRP-1 (NK1), intermediate levels of $\alpha\beta$ or $\gamma\delta$ TCR and activated/memory T cell markers (reviewed in reference 1 and references therein). Their function remains elusive; however, several lines of evidence suggest an immunoregulatory role. NT $\alpha\beta$ cells can promote immune responses attributed to Th2 lymphocytes and are among cells that secrete the largest amount of IL-4 on activation through their TCR complex (2–5).

NT $\alpha\beta$ cells are of two types, CD4⁺8⁻ and CD4⁻8⁻, whose development is controlled by a conserved $\beta 2$ -microglobulin ($\beta 2$ -m)-associated molecule(s) (6–8). Unlike the mainstream T cells that require thymic epithelial MHC

class I and class II for maturation, NT $\alpha\beta$ cell development depends on ligand(s) displayed by CD4⁺8⁺ thymocytes (9). CD1d is a highly conserved $\beta 2$ -m-associated molecule expressed by CD4⁺8⁺ thymocytes that resembles the classical antigen-presenting MHC molecules. Mouse CD1d is encoded by two duplicated non-*H-2* genes, *CD1d1* and *CD1d2*. The control of positive selection of NT $\alpha\beta$ cells by CD1d would be predicted from the recent *in vitro* studies (10). TL is another conserved, $\beta 2$ -m-associated molecule closely related to H-2 class Ia that is expressed by CD4⁺8⁺ thymocytes in certain mouse strains carrying the *H-2T18* gene (11, 12). Because TL⁺ and TL⁻ thymocytes stimulate C57BL/6 (B6)-derived NT $\alpha\beta$ cell clones equally well *in vitro*, it is argued that TL antigen is not the restriction element for NT $\alpha\beta$ cells (10). Since the clones were derived from B6, a TL⁻ strain, and because CD1d⁻TL⁺ stimulator cells were not used in this study, TL's role in NT $\alpha\beta$ cell maturation and function remains unknown.

CD1d1 assembles independent of transporters associated with antigen presentation (TAP) that, like those assembled in TAP⁺ cells, stimulate a V α 14⁺V β 8.2⁺ NT $\alpha\beta$ cell clone

I. Negishi, A. Boesteanu, and A. D. DeSilva contributed equally to this work.

I. Negishi's and D. Y. Loh's present address is Nippon Roche Research Center, 200 Kajiwara, Kamakura, Japan.

to secrete IL-4 in vitro (13). Further, the expression of V α 14J α 281 mRNA (presumably of NK1⁺ T cell origin) is maintained in TAP-1^{0/0} mice (14). Because NT $\alpha\beta$ cells recognize CD1d1 with a highly skewed TCR repertoire (V α 14J α 281 paired with V β 8.2, V β 7, or V β 2), CD1d1 must present itself empty or display an invariant TAP-independent ligand to these T cells. Although these studies convincingly demonstrate that CD1d1 controls NT $\alpha\beta$ cell function(s), several questions remain. Do NT $\alpha\beta$ cells develop normally in vivo, in terms of its TCR repertoire selection and its absolute numbers, in TAP-1^{0/0} mice? Can H-2 class Ib molecules influence the development of NT $\alpha\beta$ cells given that certain inbred strains develop greater numbers of these T cells? Here we demonstrate that the development of NT $\alpha\beta$ cells is TAP independent and is not only influenced by CD1d but also by H-2TL.

Materials and Methods

Mice. C57BL/6 (B6) mice were from the Jackson Laboratory (Bar Harbor, ME); B6.A-Tla^a and B6. β 2-m^{0/0} mice were generous gifts from Dr. D.C. Roopenian (the Jackson Laboratory). B6.TAP-1^{0/0} were generated by backcrossing the stock TAP-1^{0/0} mice (15) for six generations to B6 animals at Vanderbilt University. B6.T18-tg⁺ and B6.T18-tg⁻ (16) were used directly from M.J. Chorney's mouse colony.

Generation of TAP-1^{0/0} Mice. TAP-1^{0/0} mice were generated (at Washington University, St. Louis, MO) by inserting PGK-*neo*-(poly[A]) gene between two EcoRI sites of genomic *TAP-1* isolated from 129/Sv library (Stratagene Inc., La Jolla, CA) deleting ~25% of *TAP-1*. The targeting vector, pTNT1, contains PGK-HSV 1 *tk* gene linked to *neo* that is flanked by 1.4 kb and 4.5 kb of *TAP-1*. Linearized pTNT1 was electroporated into E14 embryonic stem (ES) cells and selected with 0.3 mg/ml G418/2 μ M gancyclovir. Homologous recombination events in the drug-resistant ES cells were screened by PCR (forward: 5'TGTAGC-TTTGGCTCTTCTGGAA^{3'} and reverse: 5'GGGCCAGCTCAT-TCCTCCACTC^{3'} primers) and verified by Southern analysis with a 750-bp SmaI probe. Four independent mutant ES lines were injected into B6 blastocysts; all four generated chimeras that transmitted the disrupted allele to their offsprings. Breeding of chimeric males with B6 females resulted in TAP-1^{+/-} heterozygotes. TAP-1^{+/-} were intercrossed to obtain TAP-1^{0/0} mice. PCR, Southern, and flow cytometric analyses confirmed TAP-1 dysfunction (not shown).

Flow Cytometry. Tail blood leukocytes (not shown) or 10⁶ thymocytes were reacted with anti-K^b (Y3), anti-TL (HD168 [17]) or anti-CD1d (3C11 [18]), stained with anti-mouse Ig-FITC (Y3) or anti-rat Ig-FITC (HD168, 3C11) and analyzed using a FACScan[®] (Becton Dickinson and Co., Mountain View, CA). Y3, HD168, and 3C11 were generous gifts from Drs. S.G. Nathenson (Albert Einstein College of Medicine), M. Kronenberg (UCLA), and S.P. Balk (Harvard Medical School), respectively. For three-color analysis, 10⁶ thymocytes from individual mice were stained with PharMingen (San Diego, CA) antibodies: anti-HSA-PE (M1/69), anti-CD8 α -PE (53-6.7), anti-CD44-FITC (IM7) and anti-NK1.1 (NKRP-1)-biotin (PK136), anti-CD4-biotin (H129.19), anti- $\alpha\beta$ TCR-biotin (H57-597), anti-V β 8.1, 8.2-biotin (MR5-2), anti-V β 7-biotin (TR310), or anti-V β 2-biotin (B20.6); biotinylated antibodies were stained with streptavidin-RED670 (GIBCO BRL, Gaithersburg, MD). HSA^{low}CD8^{low}

population was electronically gated and CD44⁺NK1.1⁺, CD44⁺CD4⁺, CD44⁺ $\alpha\beta$ TCR⁺, CD44⁺V β 8⁺, CD44⁺V β 7⁺, and CD44⁺V β 2⁺ cells were analyzed with a FACScan[®] flow cytometer. Absolute numbers were calculated from the percentages of HSA^{low}CD8^{low} and the double-positive NT $\alpha\beta$ cells.

Results

TAP-1^{0/0} Thymocytes Express CD1d and H-2TL. Thymocytes of TAP-1^{0/0} mice express CD1d as well as TL molecules (Fig. 1 A) as predicted from their expression in TAP-2-deficient cells (13, 19). Thus they are distinct from the MHC class Ia molecules. The prototypic H-2^b mice, B6 and C57BL/10 (B10), are TL⁻ because of a natural deletion in the *H-2T18* gene; the 129 strain (also H-2^b) is TL⁺. Thus the TAP-1^{0/0} mice obtain their *T18* gene from the 129 background (Fig. 1 B) because *TAP-1* and *T18* are genetically linked. In contrast to TAP-1^{0/0} thymocytes, those of β 2-m^{0/0} mice do not express CD1d (Fig. 1 A). This is consistent with the finding that β 2-m-deficient cells do not express recombinant CD1d1 at the cell surface (13). Further, β 2-m^{0/0} mice do not express any of the identifiable highly conserved class Ib molecules, e.g., Qa-2 and TL, akin to class Ia molecules (not shown).

NT $\alpha\beta$ Cells Develop in TAP-1^{0/0} Mice. The fact that a select NT $\alpha\beta$ cell clone can recognize CD1d1 assembled in TAP-deficient cells (13), the expression of CD1d in TAP-1^{0/0} mice suggests that some NT $\alpha\beta$ cells would develop normally. NT $\alpha\beta$ cells are identified by double staining of HSA^{low}CD8^{low} thymocytes with an antibody against CD44 along with antibodies to other characteristic markers such as NKRP-1 (NK1.1), CD4, and $\alpha\beta$ TCR. TAP-1^{0/0} mice (15) backcrossed to B6 for six generations (B6.TAP-1^{0/0}) were used to facilitate detection of NT $\alpha\beta$ cells using NK1.1 mAb that recognize B6's but not 129's NK1 allotype (20). HSA^{low}CD8^{low} thymocytes of >6-wk-old B6.TAP-1^{0/0} mice stained for CD44 and NKRP-1 revealed that they develop NT $\alpha\beta$ cells (Fig. 2 A). B6. β 2-m^{0/0} mice, as shown earlier (6–8), do not develop NT $\alpha\beta$ cells (Fig. 2 A). The B6.TAP-1^{0/0} NT $\alpha\beta$ cells have all the phenotypic characteristics of those of B6, including the expression of Ly6C, IL-2 receptor β (not shown), CD4, and intermediate levels (not shown) of $\alpha\beta$ TCR (Fig. 2 B). In addition, the skewed $\alpha\beta$ TCR repertoires consisting of V β 8 and V β 7 are present in proportions similar to B6 NT $\alpha\beta$ cells (Fig. 2 B); V β 2, however, was disproportionate, probably a reflection of the small sample size ($n = 2$). Interestingly, B6.TAP-1^{0/0} NT $\alpha\beta$ cells were numerically expanded; their absolute numbers were severalfold higher compared with B6 (Fig. 2 B). This difference is not due to higher numbers of B6.TAP-1^{0/0} HSA^{low}CD8^{low} thymocytes (Fig. 2 B). Thus NT $\alpha\beta$ cell development is TAP independent.

Positive Selection of Natural T Cells by H-2TL. Three possibilities can explain the numerical expansion of NT $\alpha\beta$ cells in TAP-1^{0/0} thymus: (a) TAP-1^{0/0} mice are deficient in CD4⁺8⁺ T cells, hence NT $\alpha\beta$ cell expansion could be compensatory to maintain thymic cellularity. (b) Some of the TCR of NT $\alpha\beta$ cells may interact with MHC class Ia

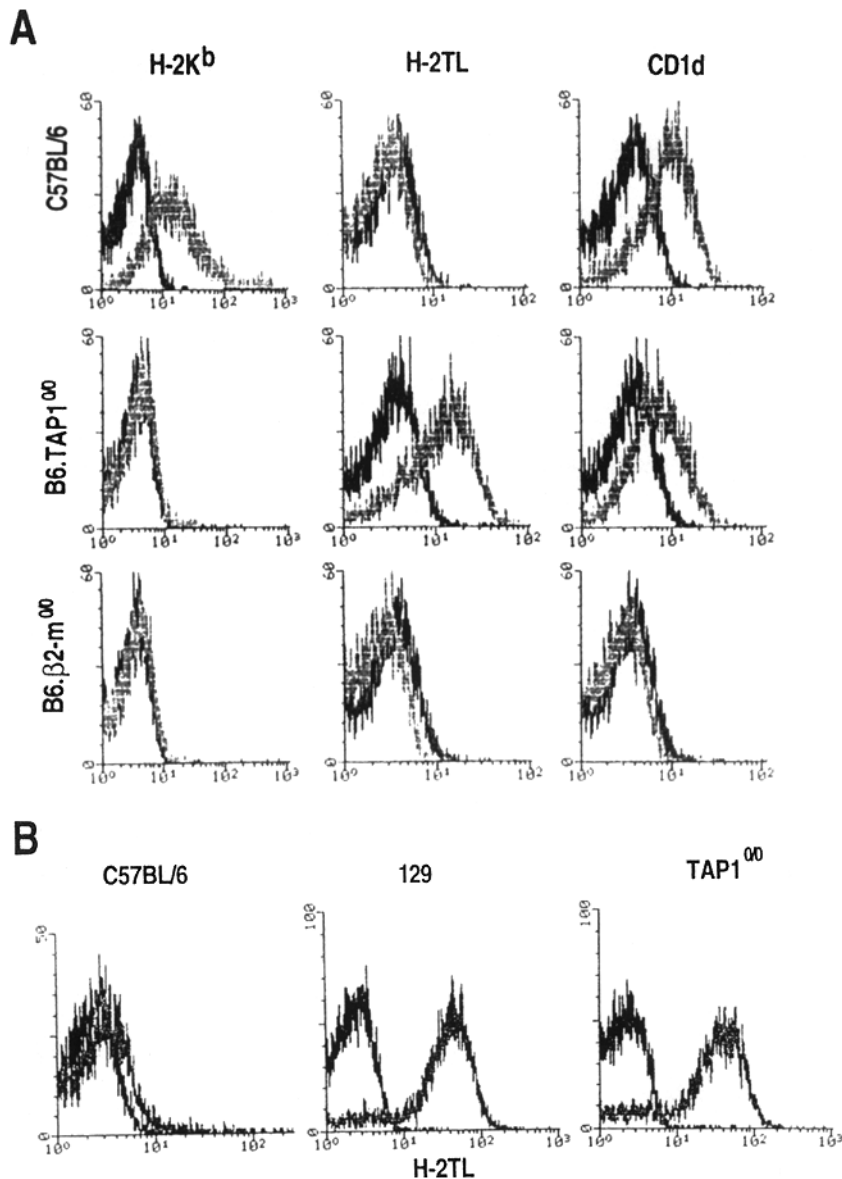


Figure 1. TAP-1-independent expression of TL and CD1d. (A) Expression pattern of K^b, TL, and CD1d in B6, B6.TAP-1^{0/0}, and B6.β2-m^{0/0} thymocytes. The specific antibody-binding profile is superimposed over secondary antibody alone control profile. (B) H-2TL expression by B6, 129, and TAP-1^{0/0} thymocytes. TAP-1^{0/0} mice derives its TL gene from strain 129 because it is linked to TAP-1.

molecules and hence deleted. Thus TAP-1^{0/0} mice protects the class Ia-reactive NTαβ cells from negative selection. (c) Another β2-m-associated class Ib molecule could be involved in the expansion of NTαβ cells in certain mouse strains. Whereas the first two possibilities are difficult to test, the third hypothesis is testable in genetically defined strains that express one or more of the class Ib molecules.

Of all the H-2 class Ib molecules, TL is probably the only molecule known to be expressed by CD4⁺8⁺ thymocytes (11, 12). Since TAP-1^{0/0} thymocytes express TL, it could be the likely other ligand that expands NTαβ cells. Because most mice express CD1d (not shown) it is difficult to assess TL's role in isolation. Therefore, to elucidate TL's role in NTαβ cell development, a quantitative approach was taken using B6 congenic and transgenic mice into which the A strain's H-2T and M region (B6.A-Tla^d) (21, 22) or the BALB/c's H-2T18^d genes (B6.T18-tg⁺) (16), re-

spectively, were introduced. If TL's influence is positive or negative, then the absolute numbers of thymic NTαβ cells would increase or decrease, respectively, in TL⁺ compared with TL⁻ mice. Analysis of HSA^{low}CD8^{low} B6.A-Tla^d thymocytes revealed an expanded repertoire of NTαβ cells that have all the phenotypic characteristics of B6 NTαβ cells including the skewed αβ TCR repertoire (not shown). Thus, the H-2T and M region gene products do influence NTαβ cell development.

Recently, we reported that T18^d transgenic (B6.T18-tg⁺) mice have numerically expanded CD4⁺8⁻ thymocytes (16). Since a large proportion of NTαβ cells is CD4⁺8⁻ (6), NK1⁺ T cells were quantitated in B6.T18-tg⁺ mice. Although our preliminary studies did not show any difference (16), a more thorough analysis revealed that like TAP-1^{0/0} and B6.A-Tla^d mice, the TL⁺ transgenic animals have an expanded NTαβ cell repertoire compared with TL⁻ litter-

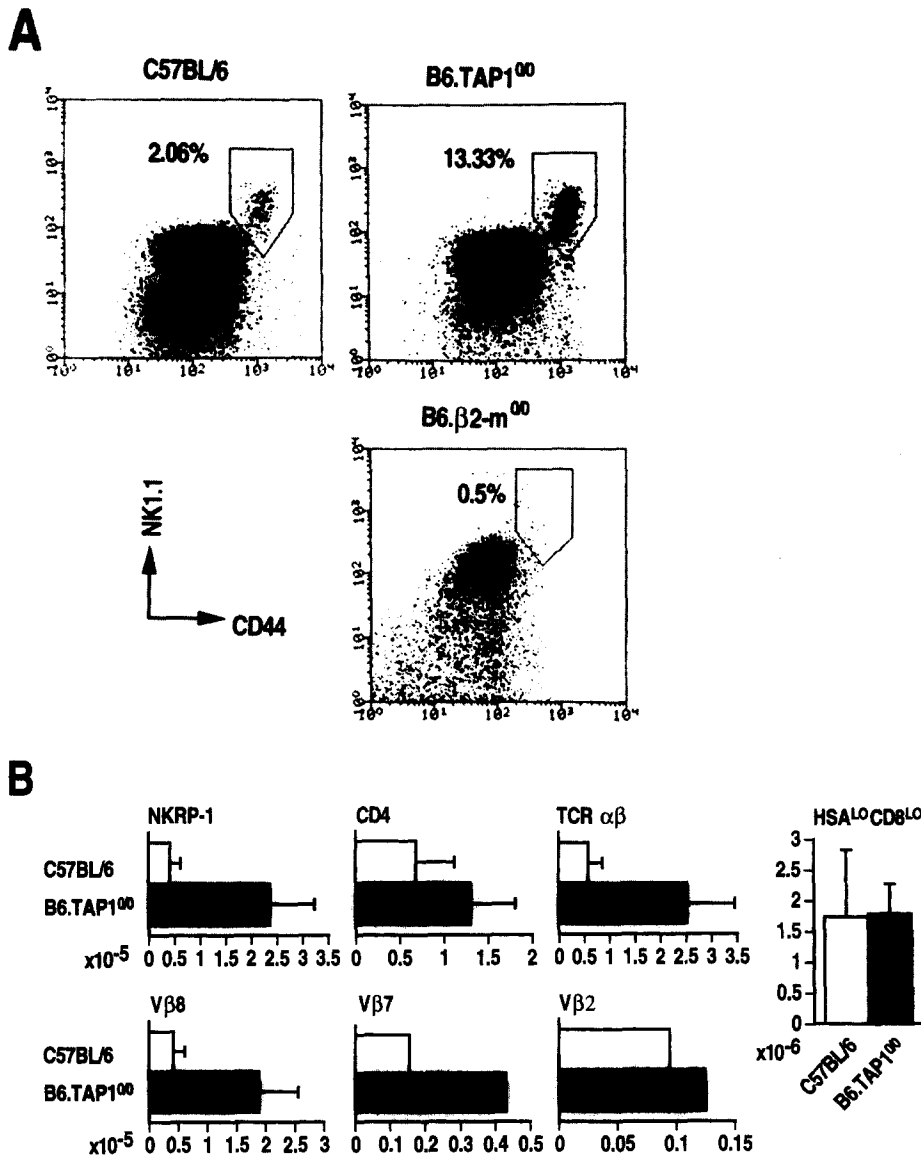


Figure 2. Natural T cells develop in B6.TAP-1^{0/0} mice. (A) Dot plots displaying CD44⁺NK1⁺ T cells among HSA^{low}CD8^{low} thymocytes of B6, B6.TAP-1^{0/0}, and B6.β2-m^{0/0} mice. (B) Absolute numbers of NTαβ cells in B6 (*n* = 3) and B6.TAP1^{0/0} (*n* = 5) mice. Thymocytes from individual animals were stained with four different fluorescent antibodies and quantitated as CD44⁺ and NK1, CD4, αβ TCR, Vβ8.1, 8.2, Vβ7, or Vβ2 within electronically gated HSA^{low}CD8^{low} cells. Note that the numbers of HSA^{low}CD8^{low} cells do not differ significantly between B6 and B6.TAP-1^{0/0} mice. The differences in NTαβ cell numbers were ≥4 SD, except for CD4, Vβ7, and Vβ2.

mates (Fig. 3). Moreover, Vβ8, Vβ7, and Vβ2 are proportionately expanded in ratios similar to B6 NTαβ cells (Fig. 3 B). Thus TL may be significantly involved in NTαβ cell selection.

Discussion

These results suggest that the development, and probably the function, of NTαβ cells is also controlled by TL molecules. Further, this function of TL is TAP independent. It remains to be established whether TL alone can promote the development of NTαβ cells in the absence of CD1d molecules. Such studies will have to await the generation of CD1d null mice. The finding that TL can numerically expand NTαβ cells may explain why certain strains of mice (TL⁺ BALB/c) have greater numbers of NTαβ cells com-

pared with others (TL⁻ B6) (3). It is noteworthy that the limited NT cell αβ receptors interact with two class I-like molecules, CD1d1 (10) and TL. The two molecules may be selecting distinct NTαβ cell subsets that differ in the Vα usage and/or in the Vα and Vβ CDRs not detectable by the TCR-specific mAb. It is less likely that TL-selected and CD1d-selected NTαβ cells differ in their function because the cytokines secreted by B6 and B6.A-*Tla*⁺ splenocytes activated in vivo by TCR crosslinking, a measure of NTαβ cell function (4), were similar (Joyce, S., unpublished data).

MHC class II^{0/0} mice develop a diverse population of CD4⁺8⁻ T cells that recognize CD1d1 and Qa-1 but not TL (23). This is consistent with our finding that NTαβ cells are not expanded in B6.A-*Tla*⁺ and B6.TAP-1^{0/0} spleens (not shown) probably because spleen and lymph node cells do not express TL (12, 24). Another plausible

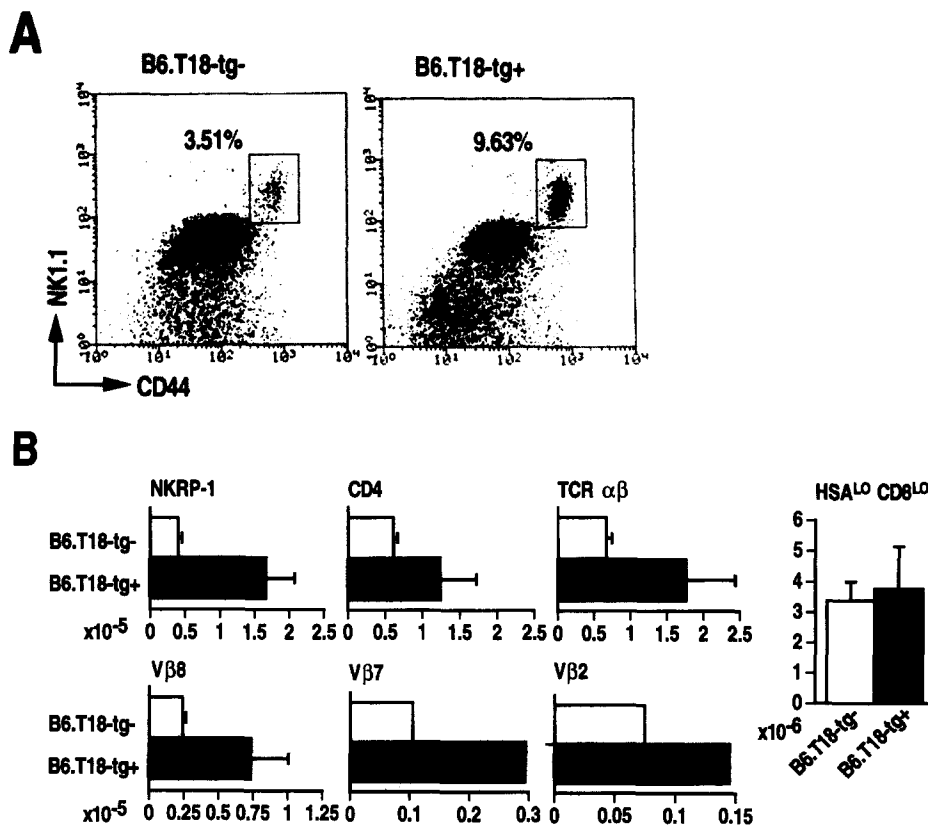


Figure 3. Expanded numbers of NT cells in *H-2T18^t* transgenic mice. (A) Dot plots displaying CD44⁺NK1⁺ T cells in B6.T18-tg⁻ and B6.T18-tg⁺ thymus. (B) Enumeration of NTαβ cells in B6.T18-tg⁻ (*n* = 3) and B6.T18-tg⁺ (*n* = 4) mice as in Fig. 2 B. Note that the numbers of HSA^{low}CD8^{low} cells do not differ significantly between TL⁻ and TL⁺ mice. The differences between NTαβ cells of TL⁻ and TL⁺ strains were ≥ 2 SD, except for Vβ2 (*n* = 2).

explanation could be that, once positively selected by TL, it functions as an antagonist in the periphery.

Our finding that TL may be involved in NTαβ cell selection contradicts those based on studies in Japanese wild mice (*Mus musculus molossinus*). *M m molossinus* (from The Jackson Laboratory) thymocytes express TL and CD1d (DeSilva, A.D., and S. Joyce, unpublished data) but do not express the Vα14Jα281 mRNA (14). Further, the expression of Vα14Jα281 TCR in (B10 × *M m molossinus*)F₁ × *M m molossinus* did not segregate with the *H-2* haplotype of B10 (14). This is probably because B10 is a TL⁻ strain. Further, one or more dominant genes outside the *H-2* region may contribute to NTαβ cell deficiency in *M m molossinus* as seen in SJL (TL⁺CD1d⁺; Boesteanu, A., and S. Joyce, unpublished data) mice (25). Thus the use of genetically defined mice allowed us to determine the role of *H-2* genes in NTαβ cell development.

How can the same TCR of NTαβ cells interact with TL and CD1d? TL and CD1d differ by >90% in their primary structure, therefore, the αβ TCR probably recognizes a ligand(s) presented by the two molecules. TL restricted selection of NTαβ cells might be peptide dependent because they can display NH₂-terminally blocked self peptides (16). CD1d1 can also display peptides recognized by T cells (26). Thus the ligand displayed by TL probably makes it structurally resemble CD1d + ligand and hence capable of interfacing the same αβ TCR of NT cells. A similar situation is seen with antigen-specific T cells that cross-react with allo-MHC molecules (27). Alternatively, CD1d could be presenting TL-derived peptides and/or TL-associated ligands, therefore, TL has an epistatic effect on NTαβ cell development. The elucidation of the molecular nature of the ligands displayed by TL and CD1d should resolve the two models.

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