

# Blockade of transgenic $\gamma\delta$ T cell development in $\beta_2$ -microglobulin deficient mice

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The  $\gamma\delta$  T cell receptor (TCR) of the hybridoma KN6 recognizes the self molecule encoded by a class I gene which maps within the TL region of the major histocompatibility complex (MHC) of H-2<sup>b</sup> mice. Mice transgenic (Tg) for this TCR were crossed with mice genetically deficient in  $\beta_2$ -microglobulin ( $\beta_2m$ ). No mature Tg  $\gamma\delta$  T cells were detected in the thymus or the spleen of the  $\beta_2m^-$   $\gamma\delta$  Tg mice. We conclude that interaction between the Tg  $\gamma\delta$  TCR and a  $\beta_2m$ -associated molecule (probably an MHC class I molecule) is required for the generation of mature Tg  $\gamma\delta$  T cells. **Key words:**  $\gamma\delta$  T cells/ $\beta_2m$  knockout mice/positive selection/thymic differentiation/transgenic mice

## Introduction

In the mouse,  $\gamma\delta$  T cells represent a minor T cell subpopulation in the lymphoid organs (Bluestone *et al.*, 1987; Itohara *et al.*, 1989), but appear in larger proportion in some non-lymphoid tissues such as the skin epidermis (Koning *et al.*, 1987; Kuziel *et al.*, 1987; Stingl *et al.*, 1987), the intestinal epithelia (Bonneville *et al.*, 1988; Goodman and Lefrançois, 1988), and several other epithelial tissues that form the outer and inner surfaces of the body (Augustin *et al.*, 1988; Itohara *et al.*, 1990).  $\gamma\delta$  T cells that reside in different peripheral sites represent distinct subsets that differ in the utilization of  $\gamma$  and  $\delta$  gene segments and in their degree of diversity, and develop in the thymus in a programmed sequence during ontogeny. Thus, the  $\gamma\delta$  T cells associated with the epidermis (referred to as s-IEL) and with the epithelia of vagina, uterus and tongue (referred to as vut-IEL) are the first to appear during fetal life and express distinct and homogeneous TCRs (Asarnow *et al.*, 1988; Itohara *et al.*, 1990). The other  $\gamma\delta$  T cell subsets which are localized in peripheral lymphoid organs and other epithelial tissues are generated later in ontogeny in the thymus or in unknown peripheral sites and express more diverse TCRs (Cron *et al.*, 1988; Lafaille *et al.*, 1989; Takagaki *et al.*, 1989; Happ *et al.*, 1989; Bandeira *et al.*, 1991).

An important issue concerning  $\gamma\delta$  T cells is whether they develop in the thymus in a manner similar to  $\alpha\beta$  T cells. During their intrathymic differentiation,  $\alpha\beta$  T cells undergo positive and negative cellular selection (see Von Boehmer,

1989; Fowlkes and Pardoll, 1989 for reviews). Positive selection ensures that mature  $\alpha\beta$  T cells recognize antigen in the context of self-MHC molecules and negative selection contributes to the establishment of self-tolerance. We have previously presented evidence indicating that the TCR homogeneity of the s-IEL and vut-IEL results from positive cellular selection in the fetal thymus (Lafaille *et al.*, 1989; Itohara and Tonegawa, 1990). However, nothing is known about the putative ligand that mediates such positive selection.

To investigate whether selection also operates in the development of the  $\gamma\delta$  T cell subsets with TCR diversity, we previously generated and analyzed  $\gamma\delta$  TCR transgenic (Tg) mice. These Tg mice were produced by introducing into the germline the rearranged  $\gamma$  and  $\delta$  genes expressed on the surface of a  $\gamma\delta$  T cell hybridoma, KN6. KN6 was derived from a thymocyte of an adult C57BL/6 (H-2<sup>b</sup>) mouse and recognizes the product of the MHC class I gene T22<sup>b</sup> (Bonneville *et al.*, 1989; Ito *et al.*, 1990), which presumably acts as an autologous peptide-presenting molecule. IL-2 production and proliferation of KN6 cells are induced *in vitro* by spleen cells from H-2<sup>b</sup> mice but not by spleen cells from H-2<sup>d</sup> mice, which carry a defective T22 gene (Ito *et al.*, 1990). Analysis of the Tg mice showed that the thymocytes bearing the Tg KN6 TCR are equally numerous in the ligand-negative H-2<sup>d</sup> mice and ligand-positive H-2<sup>b</sup> mice and that spleens from Tg H-2<sup>d</sup> adult mice contained ~10 times more KN6 cells than spleens from Tg H-2<sup>b</sup> adult mice (Bonneville *et al.*, 1990). Moreover, the KN6 cells from Tg H-2<sup>d</sup> mice responded *in vitro* to H-2<sup>b</sup> stimulator cells, while the cells from Tg H-2<sup>b</sup> mice did not (Bonneville *et al.*, 1990). These results indicated that Tg KN6 cells undergo negative selection thereby being inactivated (anergized) in the thymus of H-2<sup>b</sup> mice but develop normally in the thymus of H-2<sup>d</sup> mice.

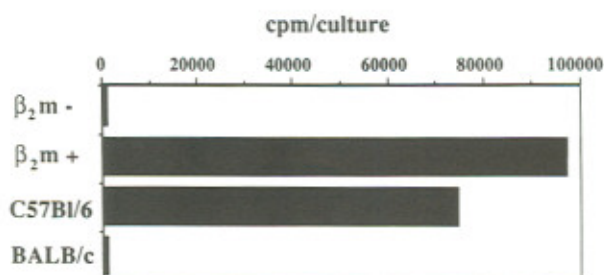
The above results also pose questions regarding the existence of positive selection for Tg KN6 cells in H-2<sup>d</sup> mice and the nature of the ligand involved in the putative positive selection. In order to address these questions we have now crossed the KN6 TCR Tg mice with mice genetically deficient in  $\beta_2m$  (Zijlstra *et al.*, 1989). We reasoned that if maturation of the Tg KN6 cells requires an interaction between the KN6  $\gamma\delta$  TCR and a  $\beta_2m$ -associated molecule (probably an MHC class I molecule), as an obligatory step, we would observe its arrest in the  $\beta_2m^-$  mice regardless of their H-2 haplotype. The experiments described here show that this is indeed the case.

## Results

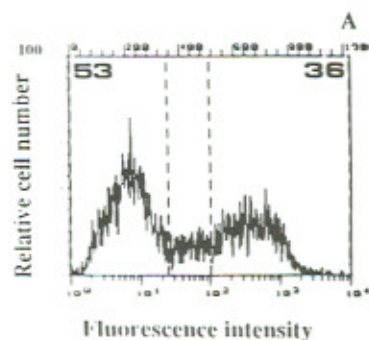
### Expression of T22<sup>b</sup> requires a functional $\beta_2m$ gene

To analyze whether expression of the TL antigen recognized by KN6 cells (T22<sup>b</sup>) depends on expression of  $\beta_2m$ , we studied the ability of spleen cells from  $\beta_2m^-$  mice to stimulate KN6 Tg cells from H-2<sup>d</sup> Tg mice. As shown in

Figure 1, KN6 Tg cells failed to proliferate in response to irradiated splenic cells from H-2<sup>b</sup>  $\beta_2m^-$  mice, while they proliferated when cultured with spleen cells from normal littermates. Figure 1 also shows that, as previously described (Ito et al., 1990), KN6 Tg cells respond to H-2<sup>b</sup>-expressing spleen cells but not to H-2<sup>d</sup>-expressing spleen cells. This observation strongly suggests that a functional  $\beta_2m$  gene is required for the expression of the T22<sup>b</sup> encoded antigen. Therefore, it is likely that  $\beta_2m^-$  mice do not express T22<sup>b</sup>, or the products encoded by the alleles of the T22<sup>b</sup> gene present in other mouse strains.

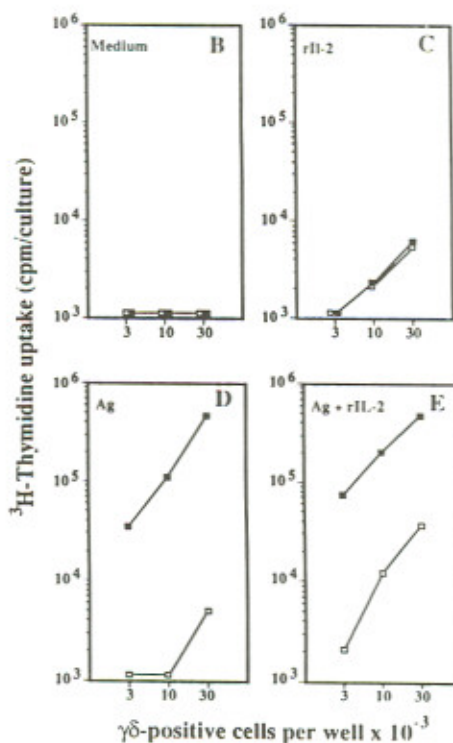


**Fig. 1.** KN6 Tg cells are not induced to proliferate by spleen cells from  $\beta_2m^-$  mice. Blood lymphocytes from an 8 week old BALB/c KN6 transgenic mouse were isolated as described in Materials and methods.  $2 \times 10^4$  CD4<sup>+</sup>CD8<sup>-</sup> responder cells (50%  $\gamma\delta$  cells and <2%  $\alpha\beta$  cells) were cultured with  $2 \times 10^5$  irradiated (1000 rads) spleen cells from the indicated strains. Results shown are the mean c.p.m. of triplicate cultures assayed 3 days after the initiation of the cultures.



### The heat stable antigen is expressed by immature but not by mature Tg KN6 cells

In the analysis of developing  $\alpha\beta$  T cells, CD4 and CD8 antigens have been very useful as differentiation markers. Since  $\gamma\delta$  T cells are largely CD4<sup>-</sup>CD8<sup>-</sup>, these antigens cannot be used for the same purpose in the analysis of those cells. To identify an alternative differentiation marker, we screened KN6 Tg thymocytes for the expression of several T cell specific markers known to be differentially expressed in immature and mature T cells. One such antigen is the heat stable antigen (HSA) detected by the J11d mAb (Bruce et al., 1981; Crispe and Bevan, 1987). As shown in Figure 2A, about half of KN6 Tg thymocytes in H-2<sup>d</sup> mice are stained with the J11d mAb. We examined the relationship between the J11d phenotype and the state of maturation of the  $\gamma\delta$  T cells. The cells in the J11d<sup>-</sup> subpopulation but not the cells in the J11d<sup>+</sup> subpopulation produce IL-2 (data not shown) and proliferate *in vitro* in response to the spleen cells from H-2<sup>b</sup> mice (Figure 2B and D). In the presence of exogenous IL-2, both subpopulations proliferated in response to the spleen cells (Figure 2E). However, extrapolation of the titration curve shows that the fraction of proliferating cells in the cultures containing the J11d<sup>+</sup> cell preparation is 50- to 100-fold lower than that in the cultures containing the J11d<sup>-</sup> cell preparations. Some of the residual proliferation in the former cultures can be ascribed to the response of J11d<sup>+</sup> cells to rIL-2, as suggested by the weak proliferation of these cells in the presence of exogenous IL-2 but in the absence of the stimulator cells (Figure 2C). The rest may be due to contamination of the J11d<sup>+</sup> cell preparation by



**Fig. 2.** The expression of J11d correlates with immaturity in  $\gamma\delta$  Tg thymocytes. (A) J11d profile of  $\gamma\delta$  Tg thymocytes. Thymic cells from two BALB/c KN6 Tg mice were stained with anti- $\delta$  and J11d mAbs and analyzed in a FACSscan. The figure shows the profile of J11d staining of  $\gamma\delta$ -positive cells. The vertical dotted lines show the gates used for the sorting of J11d<sup>+</sup> and J11d<sup>-</sup> subpopulations. The numbers indicate the proportion of cells in each subpopulation. (B-E) The indicated numbers of sorted J11d<sup>+</sup> ( $\square$ ) and J11d<sup>-</sup> ( $\blacksquare$ )  $\gamma\delta$  Tg thymocytes obtained from two BALB/c KN6 Tg mice were cultured either alone (B and C) or with  $2 \times 10^5$  irradiated, T cell depleted C57BL/6 spleen cells (D and E), in the absence (B and D) or in the presence (C and E) of exogenous IL-2. Proliferative responses were measured at day 3 of culture.

J11d<sup>-</sup> cells, or due to *in vitro* maturation of some J11d<sup>+</sup> cells in the assay cultures. Regardless of which interpretation is correct, the results clearly indicate that the bulk of functionally competent and therefore mature  $\gamma\delta$  thymocytes in the H-2<sup>d</sup> KN6 Tg mice are within the J11d<sup>-</sup> subpopulation while the J11d<sup>+</sup> subpopulation is largely composed of immature, functionally incompetent cells.

#### Staining analysis of Tg KN6 cells in $\beta_2m^-$ mice

The staining results of the cells from the mice produced by crossing KN6 TCR Tg mice with  $\beta_2m$  deficient mice as well as the results for control mice are shown in Figures 3 and 4. First, while similar numbers of Tg KN6 cells are present in the thymus from  $\beta_2m^-$  and the control  $\beta_2m^+$  mice, only about half (range: 30–70% in 11 mice) of the KN6 TCR<sup>+</sup> thymocytes from  $\beta_2m^+$  mice were stained with anti-J11d mAb while >95% of these cells from  $\beta_2m^-$  mice were stained with the same mAb. Second, spleens from  $\beta_2m^-$  mice of both H-2<sup>b</sup> and H-2<sup>d</sup> mice contained greatly reduced numbers of KN6 Tg cells compared with spleens of the control  $\beta_2m^+$  mice. As expected, the number of KN6 Tg cells in the spleens from the control  $\beta_2m^+$  mice are mostly of the mature phenotype (i.e. J11d<sup>-</sup>). Note that spleens of the H-2<sup>b</sup>  $\beta_2m^+$  mice contain several-fold less

KN6 Tg cells than the spleens of H-2<sup>d</sup>  $\beta_2m^+$  mice. This is probably related to the inactivation of these cells in the thymus of the former mice (Bonneville *et al.*, 1990). The absence of J11d<sup>-</sup> Tg KN6 thymocytes and the greatly reduced number of Tg KN6 cells in the spleens suggest that KN6 thymocytes fail to mature in  $\beta_2m^-$  mice.

#### Functional analysis of Tg KN6 cells in $\beta_2m^-$ mice

The immaturity of the  $\gamma\delta$  thymocytes from  $\beta_2m^-$  KN6 TCR Tg mice was confirmed by *in vitro* IL-2 production and proliferation assays. As shown in Table I,  $\gamma\delta$  thymocytes from  $\beta_2m^-$  KN6 TCR Tg mice, in contrast to  $\gamma\delta$  thymocytes from  $\beta_2m^+$  H-2<sup>d</sup> KN6 TCR Tg mice, failed to produce IL-2 in response to H-2<sup>b</sup> spleen cells. As we reported previously (Bonneville *et al.*, 1990),  $\gamma\delta$  thymocytes from  $\beta_2m^+$  H-2<sup>b</sup> KN6 TCR Tg mice are anergized and therefore also do not produce IL-2 when cocultured with H-2<sup>b</sup> spleen cells.

The results of the proliferation assay are shown in Figure 5.  $\gamma\delta$  thymocytes from  $\beta_2m^-$  KN6 TCR Tg mice, in contrast to the cells from  $\beta_2m^+$  KN6 TCR Tg mice, failed to proliferate in response to H-2<sup>b</sup> spleen cells in the absence of exogenous IL-2 (Figure 5A). The same cell population proliferated to some extent in the presence of exogenous IL-2

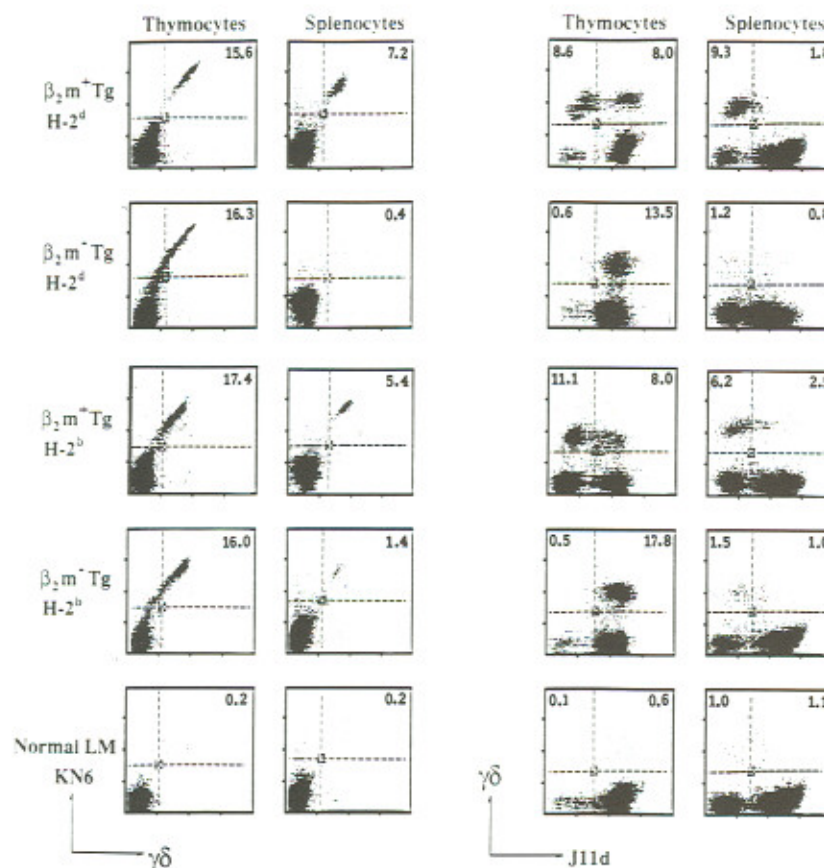
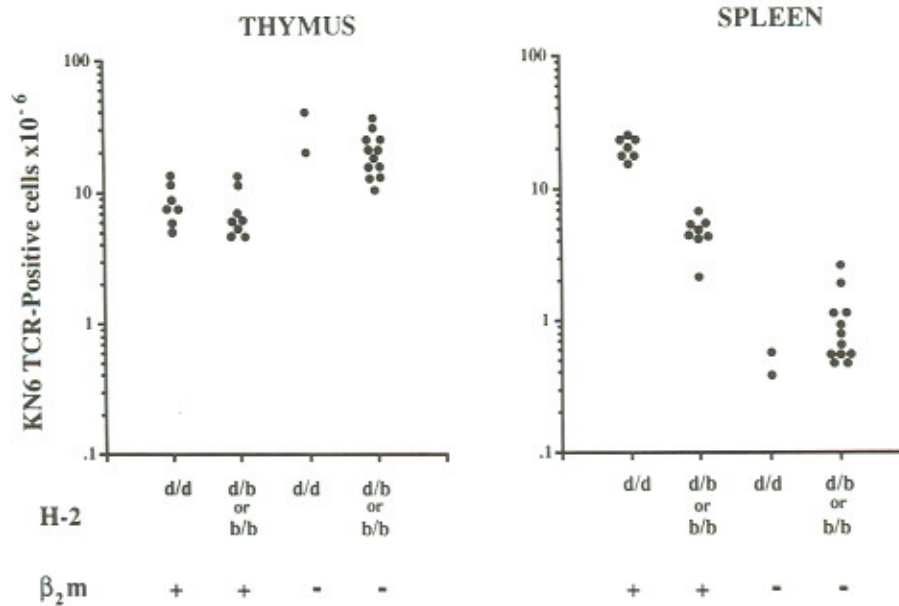


Fig. 3. Expression of KN6 TCR and J11d marker on  $\gamma\delta$  cells in thymus and spleen of different KN6 Tg mice and normal littermates. Thymic and splenic cells from the indicated mice were stained with FITC-labeled 3A10 (anti- $\delta$ ) and biotin-labeled 8D6 (anti-V $\gamma$ 4 V $\delta$ 5) mAbs followed by streptavidin-PE (left panels) or with unlabeled J11d and biotin-labeled anti- $\delta$  mAbs followed by FITC-labeled goat anti-rat Ig and streptavidin-PE (right panels) as described in Materials and methods, and analyzed in a FACScan. In several experiments, cells were also stained with 5C10 mAb (anti-KN6 clonotype). No significant differences were found between the number of cells stained with 8D6 and 5C10 in KN6 Tg mice. Dot plots shown in the figure is lower than that (26.5) we reported in an earlier publication (Bonneville *et al.*, 1990). This difference is due to individual variation. Among the seven mice analyzed here, this proportion ranged between 7 and 31%.



**Fig. 4.** Total number of KN6 Tg cells in the thymus and spleen of different Tg mice. Single cell suspensions of the indicated mice were prepared and stained with 3A10 and 8D6 mAbs as described in Materials and methods. The number of KN6 cells was calculated from the percentage of double positive cells and the total cell numbers. Results were compiled from different experiments. All animals analyzed were littermates within each experiment except five of the H-2<sup>d</sup>  $\beta_2m^+$  mice which were BALB/c KN6 Tg mice.

(Figure 5B), but extrapolation of the titration curves indicates that the fraction of proliferative cells is ~50-fold lower than the  $\gamma\delta$  thymocytes purified from  $\beta_2m^-$  Tg mice than in the  $\gamma\delta$  thymocytes purified from the control  $\beta_2m^+$  H-2<sup>d</sup> Tg mice. The proliferation behavior of the  $\gamma\delta$  thymocytes from the  $\beta_2m^-$  KN6 TCR Tg mice resembles that of the anergized  $\gamma\delta$  thymocytes from H-2<sup>b</sup> KN6 TCR Tg mice (Bonneville *et al.*, 1990 and Figure 5). But the former cells are almost entirely J11d<sup>+</sup> while about half of the latter cells are J11d<sup>-</sup> (Figure 2). Furthermore, the level of the TCR expression of the  $\gamma\delta$  thymocytes from  $\beta_2m^+$  H-2<sup>b</sup> KN6 TCR Tg mice but not from  $\beta_2m^-$  KN6 TCR Tg mice is down-regulated (Figure 6) as a result of the interaction of the former cells with the T22<sup>b</sup> encoded ligand (Bonneville *et al.*, 1990).

We therefore conclude that KN6 cells are subjected to positive selection in the thymus through the interaction of their  $\gamma\delta$  TCR with a  $\beta_2m$ -associated ligand. Cells which are yet to undergo selection express J11d and the TCR at high levels and the positively selected cells lose J11d expression. The latter are functionally competent unless they are subjected to negative selection by virtue of an interaction with the T22<sup>b</sup>-containing ligand, in which case they become anergic and down-regulate their TCR density.

#### **Blockade of Tg KN6 cell development occurs in fetal thymus before mature $\alpha\beta$ T cells appear**

Transgenic KN6 TCR might be expressed in the thymocytes of  $\alpha\beta$  T cell lineage and, if so, the observed blockade of thymocyte development might reflect a developmental program of  $\alpha\beta$  T cells rather than that of  $\gamma\delta$  T cells. Although several arguments make this possibility highly unlikely (see Discussion), we examined the expression of the J11d marker on the  $\gamma\delta$  thymocytes from  $\beta_2m^-$  KN6 TCR Tg mice at day 18 of fetal development, just before the appearance of mature  $\alpha\beta$  T cells (Pardoll *et al.*, 1987). As shown in Figure 6, while virtually all  $\gamma\delta$  thymocytes from

**Table I.** KN6 Tg thymocytes from  $\beta_2m^-$  mice do not produce IL-2 when cocultured with T22-bearing spleen cells

Responder cell <sup>a</sup>	IL-2 production (U/ml)
$\beta_2m^-$ (d/b)	<1
$\beta_2m^-$ (d/d)	<1
$\beta_2m^+$ (d/b)	<1
$\beta_2m^+$ (d/d)	50

<sup>a</sup> $4 \times 10^4$  double negative thymocytes from the indicated mice were cultured with  $2 \times 10^5$  irradiated, T cell depleted C57BL/6 spleen cells. Supernatants were collected 24 h later and the IL-2 was quantified as described in Materials and methods.

$\beta_2m^-$  KN6 Tg mice express the J11d marker, a fraction (range: 12–40% in 11 mice) of the  $\gamma\delta$  thymocytes from the control  $\beta_2m^+$  KN6 TCR Tg mice fail to express this cell surface marker. As these mice contain no mature  $\alpha\beta$  T cells (note that J11d<sup>-</sup>  $\gamma\delta$ TCR<sup>-</sup> T cells are barely detectable in Figure 7), we believe that the conclusion drawn here is relevant to positive selection of at least some  $\gamma\delta$  T cells in normal mice.

#### **Discussion**

The findings described here show that in the absence of expression of  $\beta_2m$ , Tg KN6  $\gamma\delta$  T cells specific for a TL class I antigen fail to differentiate in the thymus, suggesting that interaction of the  $\gamma\delta$  TCR with an MHC class I molecule is required for the maturation of these  $\gamma\delta$  T cells. Lack of generation of mature  $\gamma\delta$  T cells was defined on the basis of three different criteria. First, although similar numbers of Tg KN6 cells are present in the thymi of  $\beta_2m^+$  and  $\beta_2m^-$  mice, >95% of Tg KN6 thymocytes in  $\beta_2m^-$  mice express the differentiation marker J11d, while about half of the Tg KN6 thymocytes in  $\beta_2m^+$  mice do not express this marker. Second, Tg KN6 thymocytes from  $\beta_2m^-$  mice

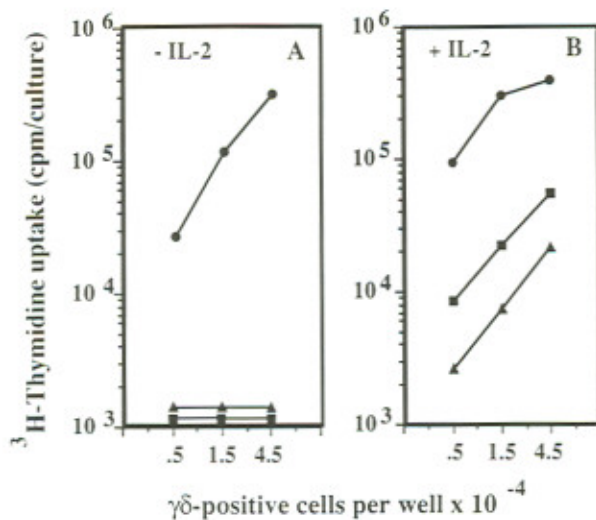


Fig. 5. KN6 thymocytes from  $\beta_2m^-$  thymocytes do not respond to T22<sup>b</sup>-expressing stimulator cells. The indicated numbers of CD4<sup>-</sup>CD8<sup>-</sup> thymocytes from H-2<sup>d</sup>  $\beta_2m^+$  (●), H-2<sup>b</sup> $\beta_2m^+$  (▲) and H-2<sup>b</sup>  $\beta_2m^-$  (■) were cultured with  $2 \times 10^5$  irradiated, T cell depleted, H-2<sup>b</sup> splenic cells in the absence (A) or presence (B) of rIL-2. Their proliferative response was measured 3 days after the initiation of the culture. We also performed a similar experiment with H-2<sup>d</sup>  $\beta_2m^-$  mice and obtained results which are essentially the same as the H-2<sup>b</sup>  $\beta_2m^-$  mouse results.

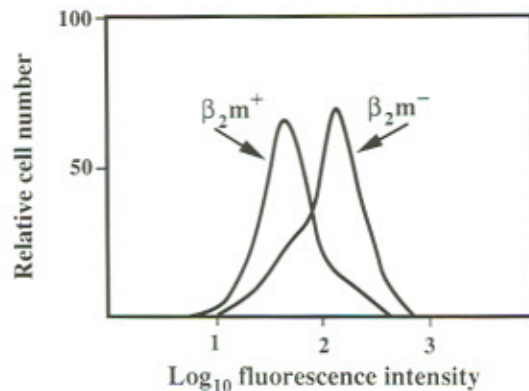


Fig. 6. TCR  $\gamma\delta$  density on KN6 Tg thymocytes from H-2<sup>b</sup>  $\beta_2m^-$  and H-2<sup>b</sup>  $\beta_2m^+$  littermates. Thymocytes from the indicated mice were stained with biotinylated 8D6 mAb followed by streptavidin-PE. The figure shows an overlay histogram of two mice of each type analyzed separately.

were functionally incompetent; these cells, in contrast to the Tg KN6 thymocytes from  $\beta_2m^+$  mice, cannot be induced to produce IL-2 or to proliferate *in vitro* by the ligand-expressing cells from the spleens of H-2<sup>b</sup> mice. Third, spleens of  $\beta_2m^-$  mice of either H-2<sup>b</sup> or H-2<sup>d</sup> contain very few Tg KN6 cells.

The interaction of TCR with self MHC class I or class II molecules has been shown to be an obligatory step for the intrathymic maturation of CD8<sup>+</sup> (Kisielow *et al.*, 1988; Sha *et al.*, 1988; Marusic-Galesic *et al.*, 1989; Pircher *et al.*, 1989; Scott *et al.*, 1989; Koller *et al.*, 1990; Zijlstra *et al.*, 1990) and CD4<sup>+</sup> (Berg *et al.*, 1989; Kaye *et al.*, 1989)  $\alpha\beta$  T cells. Thus, the results presented here would require a different interpretation if the KN6 TCR transgenes were expressed in cells of  $\alpha\beta$  lineage that would then be selected based on their KN6 TCR specificity but following

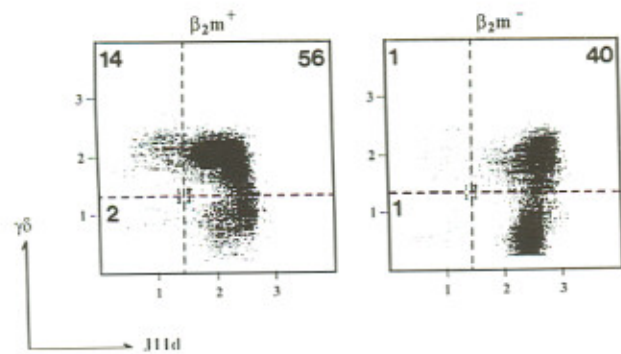


Fig. 7. Expression of KN6 TCR and J11d marker on fetal thymocytes from  $\beta_2m^-$  KN6 Tg and  $\beta_2m^+$  KN6 Tg mice. Thymocytes from a day 18 fetus were stained and analyzed as in Figures 2 and 3. The numbers indicate the percentage of positive cells in each quadrant.

the developmental program governing  $\alpha\beta$  T cells. We believe that this possibility is unlikely because (i) the Tg KN6  $\gamma\delta$  T cells follow the normal  $\gamma\delta$ -type pattern of CD4 and CD8 expression (i.e. double negative in both immature and mature states, unpublished observations) rather than the  $\alpha\beta$ -type pattern (double positive in immature state and single positive in mature state); (ii)  $\alpha\beta$  T cells in the KN6 TCR Tg mice appear to develop normally (Bonneville *et al.*, 1990; Ishida *et al.*, 1990); (iii) since the  $\gamma$  gene utilized to produce KN6 Tg mice contains the transcriptional silencer active in  $\alpha\beta$  lineage T cells (Ishida *et al.*, 1990), it is highly unlikely that the Tg  $\gamma\delta$  TCR is expressed in  $\alpha\beta$  T cells; and (iv) as shown here, the blockade of the maturation of Tg KN6 thymocytes also occurs at day 18 in fetal developmental when mature  $\alpha\beta$  thymocytes are yet to appear.

Mice deficient in  $\beta_2m$  are essentially devoid of mature CD8<sup>+</sup>  $\alpha\beta$  T cells (Koller *et al.*, 1990; Zijlstra *et al.*, 1990). Thus, the arrest of Tg KN6 cell maturation observed in  $\beta_2m^-$  mice could be attributed to the lack of CD8<sup>+</sup> cells rather than to a lack of a positive selection step involving the KN6 TCR. This possibility, however, is unlikely because Tg KN6 cells can develop in SCID mice (Bosma *et al.*, 1983) which lack mature lymphocytes (P. Pereira, D. Spaner, R. Phillips and S. Tonegawa, unpublished observation). We cannot exclude another alternative interpretation of our data, namely that the lack of expression of  $\beta_2m$  in the Tg T cells affects their selection. We consider that this possibility is remote because there is no experimental evidence or theoretical framework which suggests the need of  $\beta_2m$  inside the T cell for the development of any T lymphocytes.

Interactions between MHC class I molecules and CD8 antigens (coreceptors) have also been shown to be required during the development of CD8<sup>+</sup>  $\alpha\beta$  T cells (Ramsdell and Fowlkes, 1989; Zuniga-Pflucker *et al.*, 1990; Fung-Leung *et al.*, 1991). We can exclude the possibility that the maturation of Tg KN6 cells in  $\beta_2m^-$  mice is affected by the lack of ligand for CD8 because Tg KN6 thymocytes do not express CD8 antigens (Bonneville *et al.*, 1990; Ishida *et al.*, 1990; our unpublished observations). It is still possible that  $\gamma\delta$  T cells utilize another type of coreceptor molecule and that interaction of these molecules with MHC class I antigens is required for the maturation of, at least, some  $\gamma\delta$  T cells, but so far no evidence for the existence of such a molecule has been produced.

The precise identity of the  $\beta_2m$ -associated molecule which mediates the positive selection of Tg KN6 cells

remains to be elucidated, but it is probably the T22<sup>b</sup> gene product in H-2<sup>b</sup> mice (see Klein *et al.*, 1990 for TL class I gene nomenclature). In H-2<sup>d</sup> mice, another  $\beta_2m$ -associated molecule must mediate the positive selection because the T22<sup>d</sup> gene is known to be defective (Ito *et al.*, 1990). A probable candidate is the product of the T10<sup>d</sup> gene, which is closely related to the T22 gene. The T10<sup>d</sup> protein differs from the T22<sup>b</sup> protein by four residues in the  $\alpha_1$  and  $\alpha_2$  domains (Ito *et al.*, 1990). Thus, the T10<sup>d</sup> gene might mediate the positive selection of KN6 cells but fail to present the putative autologous peptide that is required for the inactivation of immature KN6 thymocytes and the activation of mature KN6 cells. Previous observations have indicated that mature  $\alpha\beta$  T cells also do not necessarily recognize peptides exclusively in association with those MHC proteins that were involved in their positive selection: due to cross reactivity, T cells positively selected by MHC *a* can recognize a peptide presented by MHC *b* (Hunig and Bevan, 1982). Moreover, the analysis of  $\alpha\beta$  TCR Tg mice has shown that the development of L<sup>d</sup> specific T cells was dependent on positive selection by the K<sup>b</sup> protein (Sha *et al.*, 1990). Thus, positive selection of  $\alpha\beta$  T cells and at least some of the  $\gamma\delta$  T cells with diversified TCR to which KN6 cells belong (Takagaki *et al.*, 1989) appear to follow similar rules.

Recently it has been shown that the number of splenic  $\gamma\delta$  T cells is not significantly reduced in  $\beta_2m^-$  mice as compared with normal mice (Koller *et al.*, 1990; Zijlstra *et al.*, 1990) and these cells can be activated *in vitro* by anti-TCR mAbs (I. Correa and D. Raulet, personal communication). These results suggest that some  $\gamma\delta$  T cells can mature without interacting with  $\beta_2m$ -associated molecules. Indeed it has been shown that at least one  $\gamma\delta$  T cell clone is specific for MHC class II molecules (Matis *et al.*, 1989). On the other hand, besides KN6 cells, at least one  $\gamma\delta$  T cell clone and one  $\gamma\delta$  T cell hybridoma have been shown to be specific for or restricted by MHC class I molecules that map within the TL region (Matis *et al.*, 1987; Vidovic *et al.*, 1989), and our present results demonstrate that one of these T cell clones requires interaction with a  $\beta_2m$ -associated molecule, presumably an MHC class I molecule, for its maturation. Thus, there appear to be two subsets of  $\gamma\delta$  T cells, one which requires interaction with  $\beta_2m$ -associated molecules and another which does not. The total number of  $\gamma\delta$  T cells in a given peripheral organ is affected not only by cellular selection events in the thymus but also by other parameters such as peripheral expansion. It remains to be determined, therefore, what proportion of  $\gamma\delta$  T cells can mature without interaction with  $\beta_2m$ -associated molecules.

## Materials and methods

### Mice

BALB/c and C57BL/6 mice were from our breeding colonies. The KN6 Tg mice have been described previously (Ishida *et al.*, 1990). The mice used here have been backcrossed to BALB/c for five to seven generations. The generation of  $\beta_2m$  deficient mice has also been described (Zijlstra *et al.*, 1990). To produce  $\gamma\delta$  Tg mice lacking expression of MHC class I molecules we crossed (129  $\times$  C57BL/6)F<sub>2</sub> (H-2<sup>b</sup>) mice homozygous for the deficient  $\beta_2m$  gene with H-2<sup>d</sup> homozygous KN6 Tg mice. Transgenic animals of the first generation were further backcrossed to parental  $\beta_2m^-$  mice. All mice obtained this way carried the H-2<sup>b</sup> haplotype in at least one of the two MHC-carrying homologs. KN6 Tg  $\beta_2m^-$  H-2<sup>d</sup> mice were obtained by intercrossing H-2<sup>b/d</sup> heterozygous, KN6 Tg mice obtained in

the first backcrossed generation. All mice used here were between 4 and 6 weeks of age.

### Antibodies

The mAbs 3A10 (anti-pan  $\gamma\delta$ ), 8D9 (anti-V $\gamma$ 4V65) and 5C10 (anti-KN6  $\gamma\delta$  TCR clonotype) have been described previously (Itohara *et al.*, 1989). The mAbs were purified on a Protein A-Sepharose column and labeled with fluorescein isothiocyanate (FITC) or biotin using standard procedures. The mAb J11d was obtained from the American Type Culture Collection (Rockville, MD) and used as a culture supernatant.

### Immunofluorescence staining and cell sorting

Thymus and spleen cell suspensions were prepared according to standard procedures. Cells (10<sup>6</sup>) were incubated in staining buffer (PBS, 3% FCS, 0.1% NaN<sub>3</sub>) with the indicated labeled Abs for 30 min on ice. After three washes the cells were incubated with streptavidin-PE (Southern Biotechnology, Birmingham, AL) or, when unlabeled J11d Ab was used, with FITC-labeled goat anti-rat immunoglobulin (Caltag) together with streptavidin-PE for 15 min on ice. After another three washes, 10 000 viable cells were analyzed using a FACScan flow cytometer (Becton-Dickinson). Dead cells were excluded from the analysis on the basis of their forward and sideways light scattering properties. In some experiments dead cells were gated out by their staining with propidium iodide.

For sorting experiments KN6 thymocytes were stained at a concentration of  $2 \times 10^7$  cells/ml using the procedure described above. Cell sorting was carried out in a FACStar Plus (Becton-Dickinson).

### Cell purification

For the experiment presented in Figure 1, blood lymphocytes were isolated by centrifugation over Ficoll-Hypaque and incubated for 1 h at 37°C with anti-CD4 (RL172.4) and anti-CD8 (3.155) mAbs and complement. Double negative (CD4<sup>-</sup>CD8<sup>-</sup>) thymocytes were prepared by incubating the thymic cell suspensions with the same mAbs as above and complement. T cell depleted spleen cells were obtained by treatment of spleen cells with anti-Thy-1 (J1j, Bruce *et al.*, 1981) plus anti-CD4 and anti-CD8 and complement as described above.

### Cell culture

The indicated numbers of CD4<sup>-</sup>CD8<sup>-</sup> cells or sorted cells in the experiment shown in Figure 2 were cultured in flat bottomed 96-well plates in 200  $\mu$ l of DME medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics. When indicated,  $2 \times 10^5$  irradiated (1000 rads) T cell depleted spleen cells were added as a source of stimulator cells. Exogenous IL-2 was added at a final concentration of 10 U/ml except in the experiment shown in Figure 1, in which IL-2 was added at 25 U/ml. All cultures were pulsed for 4 h 3 days after their initiation with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (sp. act. 20 mCi/mmol), harvested and counted in a scintillation counter. The results shown are the mean c.p.m. of triplicate cultures.

IL-2/IL-4 production was assayed by the growth of HT-2 cells. Supernatants were titered on HT-2 cells with a titration of an IL-2 standard. The growth of HT-2 cells was scored by the MTT assay as originally described (Mosmann, 1983).

### Typing of the $\beta_2m$ genotype, presence or absence of the TCR $\gamma$ transgene and MHC haplotype

The presence of normal or mutated  $\beta_2m$  alleles was detected by Southern blot hybridization of tail DNA as previously described (Zijlstra *et al.*, 1989). Presence of the TCR  $\gamma$  transgene was analyzed on tail DNA by PCR using oligonucleotide primers specific for the V $\gamma$ 4 gene and the junctional sequence of the KN6  $\gamma$  gene. The primers used were 5'-TGTCCTTGCAACCCCTACCC-3' and 5'-ACCTTGTGAAAACCC-CCTTG-3', respectively. MHC typing was also performed by PCR of the same DNA preparation using specific primers located in the first exon and second intron of the I-E $\beta^d$  gene and in the first intron and third exon of the I-A $\beta^b$ . The primers were 5'-CCTAGCAACAGATGTGTCAGTCT-3' and 5'-CTCAACTAAGTCTGAGTCATTTT-3' for the I-E $\beta^d$  gene and 5'-CTTCCCCAGGGGAGTCTCCACATT-3' and 5'-CAGCCCACC-ACCCAGTCTCCAGA-3' for the I-A $\beta^b$  gene. The phenotype of each animal was confirmed at the time of the assay by staining the thymocytes with FITC-labeled anti-K<sup>b</sup> and anti-K<sup>d</sup> mAbs and the spleen cells with FITC-labeled anti-I-A<sup>b</sup> and anti-I-E<sup>d</sup> mAbs (Pharmingen).

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## Note added in proof

Recently, a paper by F.B. Wells *et al.* [*Science*, **253**, 903–905 (1991)] reported similar findings.