# Self-tolerance to transgenic γδ T cells by intrathymic inactivation

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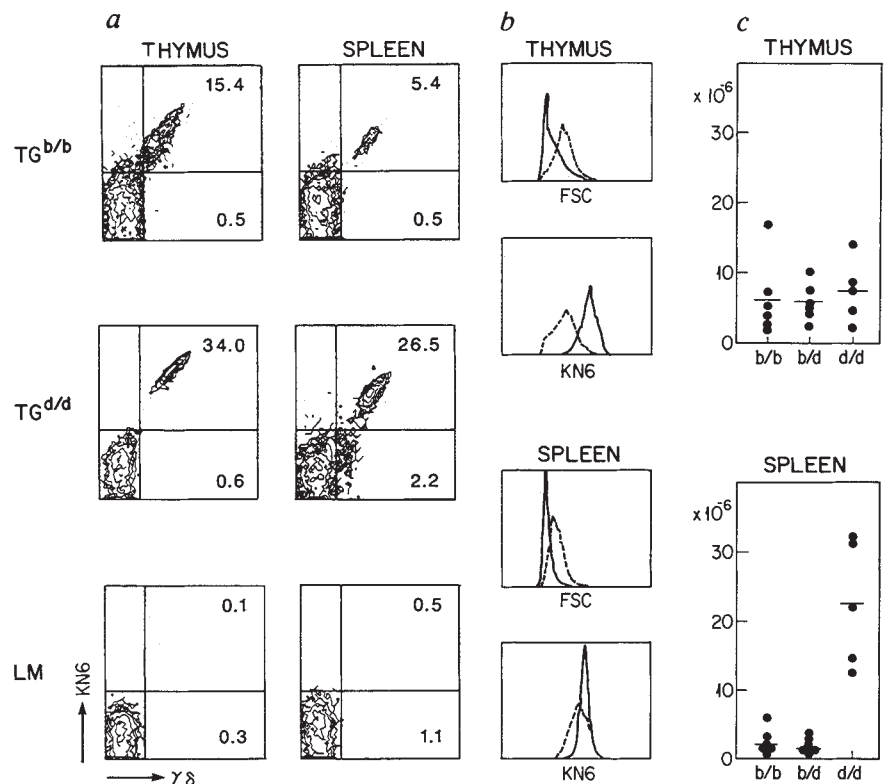§ Hoffman LaRoche AG, Central Research Unit, Basel, Switzerland
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DURING their intrathymic differentiation, T lymphocytes expressing αβ T-cell receptors (TCR) are negatively[1-3] and positively selected[4-8]. This selection contributes to the establishment of self-tolerance and ensures that mature $CD4^+$ and $CD8^+$ cell populations are restricted by the self major histocompatibility complex. Little is known, however, about γδ T-cell development. To investigate whether selection operates in the establishment of the γδ T-cell class, we have generated transgenic mice using γ- and δ-transgenes encoding a TCR that is specific for a product of a gene in the TL-region of the $TL^b$ haplotype. Similar numbers of thymocytes expressing the transgenic TCR were generated in mice of $TL^b$ and $TL^d$ haplotypes. But γδ thymocytes from $TL^b$ and $TL^d$ transgenic mice differed in cell size, TCR density and in their capacity to respond to $TL^b$ stimulator cells or interleukin-2 (IL-2). In contrast to γδ T cells from $TL^d$ transgenic mice, γδ T cells from $TL^b$ transgenic mice did not produce IL-2 and did not proliferate in response to $TL^b$ stimulator cells, but they did proliferate in the presence of exogenous IL-2. These results indicate that functional inactivation of self-antigen-specific T cells could contribute to the establishment of self-tolerance to thymic determinants.

Several γδ TCR-positive T hybridomas were obtained by fusing $CD4^-CD8^-$ thymocytes from adult C57BL/6 (H-2$^b$, $TL^b$) mice with the thymoma BW5147 (ref. 9). Using a hybridoma growth inhibition assay, we identified one hybridoma (KN6) that was specific for syngeneic cells[10]. It recognized a product of the $TL^b$ haplotype, but not a product of the $TL^d$ haplotype, that was expressed not only on peritoneal and spleen cells, but also in the thymus[10]. We were therefore interested in whether the KN6 hybridoma originated from an immature γδ T cell destined to be eliminated in the thymus, but was rescued by the fusion. To investigate this possibility, we generated transgenic mice by introducing into the germ line the rearranged $V4J1C1\gamma$- and $V5DJ1C\delta$-chain genes encoding the KN6 TCR[10-12]. We backcrossed CBA/J × C57BL ($TL^{k/b}$) transgenic mice several times to C57BL/6 ($TL^b$) or BALB/c ($TL^d$) mice, and analysed homozygous $TL^{b/b}$ and $TL^{d/d}$ mice, as well as heterozygous $TL^{b/d}$ transgenic mice. We studied γδ TCR expression by immunofluorescence using monoclonal antibodies that react



FIG. 1 Transgene-encoded γδ TCR are expressed on the surface of γδ T cells from KN6 transgenic mice of $TL^{b/b}$ and $TL^{d/d}$ haplotypes. a, Thymus and spleen cell suspensions were stained with fluorescein isothiocyanate (FITC)-conjugated 3A10 monoclonal antibody (anti-pan γδ)[13] and biotinylated 8D6 monoclonal antibody (anti-Vγ4Vδ5-encoded TCR)[13], followed by streptavidin coupled to phycoerythrin. The dot-plot histograms shown (3A10, horizontal axis; 8D6, vertical axis) were obtained after flow cytometric analysis of thymocytes and splenocytes from KN6 transgenic mice of $TL^b$ ($TG^{b/b}$) and $TL^d$ ($TG^{d/d}$) haplotypes and from nontransgenic littermate is (LM) of $TL^d$ haplotype mice. Fluorescence intensity is expressed in $\log_{10}$ units. The percentage of cells in right quadrants are indicated. b, TCR density and size of γδ T cells from KN6 transgenic mice. Thymus and spleen cell suspensions were stained with 3A10 and 8D6 monoclonal antibodies according to a. The forward scatter (FSC) and the 8D6 fluorescence histograms ($TL^{b/b}$ transgenic, dotted line; $TL^{d/d}$ transgenic, solid line) were obtained from gated γδ T cells. c, Total number of KN6 γδ TCR-positive cells in thymus and spleen from $TL^{b/b}$, $TL^{b/d}$ and $TL^{d/d}$ KN6 transgenic mice. Single-cell suspensions of thymic and splenic cells from KN6 transgenic mice (4–10 week-old) were stained with biotinylated monoclonal antibody 5C10 (KN6 clonotypic) followed by streptavidin coupled to phycoerythrin, and the number of KN6 γδ TCR-positive cells was calculated from the percentage of 5C10$^+$ cells and the total cell number. The obtained mean values (horizontal bars) and s.d.s. were the following (in $10^6$ cells): thymus ($TL^{b/b}$, 6.0 ± 5.1; $TL^{b/d}$, 5.9 ± 2.4; $TL^{d/d}$, 7.5 ± 4.4); spleen ($TL^{b/b}$, 2.2 ± 1.9; $TL^{b/d}$, 1.9 ± 1.0; $TL^{d/d}$, 22.4 ± 9.4).
METHODS. KN6 γδ transgenic mice were generated after injection into fertilized eggs of purified $V4J1C1\gamma$ and $V5DJ1C\delta$ cosmid DNA from the KN6 hybridoma as previously described[11,12]. The CBA/J × C57BL transgenic founders obtained were crossed to C57BL/6.J and BALB/CBy.J mice (Jackson Laboratories) and their progeny backcrossed to their respective strains. Major histocompatibility complex typing was performed on cultured peripheral blood lymphocytes using 28-13-3S (anti-H-2K$^b$), SF1.1.1 (anti-H–2K$^d$) and 16.1.11N (anti-H-2K$^k$) monoclonal antibodies (ATCC No. HB41, HB159 and HB16, Rockville, Maryland). Spleen and thymus cell suspensions from 6-week-old transgenic and nontransgenic mice were prepared according to standard procedures and stained with conjugated 3A10 and 8D6 monoclonal antibodies or with unconjugated 5C10 (anti-KN6 TCR clonotype)[13] monoclonal antibody as described[13]. Flow cytometric analysis was performed on a FACSCAN (Becton Dickinson) using Consort C30 and LYSYS programs. The percentages of 8D6$^+$ and 5C10$^+$ cells were virtually identical (data not shown).

with all $\gamma\delta$ TCR (3A10)[13], with most or all $V\gamma 4V\delta 5$-encoded TCR (8D6)[13], or with the KN6 $\gamma\delta$ TCR only (5C10)[13]. Virtually all thymic and splenic $\gamma\delta$ T cells from $TL^{d/d}$ or $TL^{b/b}$ transgenic mice expressed the transgene-encoded TCR (Fig. 1a), due to blockade of endogenous $\gamma$- and $\delta$-gene rearrangement[10–12]. Anti-CD4 and anti-CD8 monoclonal antibodies did not stain any KN6 TCR-positive cells (data not shown). The total number of peripheral and thymic $\alpha\beta$ T cells varied in different litters, and will not be further considered here. By contrast, the numbers of KN6 TCR-positive thymocytes was rather constant and not significantly different between transgenic mice of the $TL^{b/b}$ and $TL^{d/d}$ haplotypes (Fig. 1c). But the numbers of splenic KN6 TCR-positive cells was on average 10 times lower in $TL^{b/b}$ or $TL^{b/d}$ transgenic mice than in $TL^{d/d}$ transgenic mice. Also $\gamma\delta$ T cells in $TL^{b/b}$ or $TL^{b/d}$ transgenic mice were larger than those in $TL^{d/d}$ transgenic mice, and expressed surface KN6 TCR at a lower density. This difference was more pronounced in thymocytes than in spleen cells (Fig. 1b).

To assess functional properties of $\gamma\delta$ T cells in the various TCR transgenic mice, we purified these cells from thymus and spleen by treatment with anti-CD4 and anti-CD8 monoclonal antibodies and complement. The resulting cell population (<1% $\alpha\beta$ T cells, >90% $\gamma\delta$ T cells) was cultured either in medium alone or in medium with $TL^{b}$ stimulator cells or IL-2, or both. No spontaneous proliferation was observed in medium alone (Table 1 and Fig. 2a). IL-2 receptor $\alpha$-chain expression (Fig. 3) and proliferation (Fig. 2) were induced in thymocytes from $TL^{b/b}$ or $TL^{b/d}$ transgenic mice by IL-2 but not by $TL^{b}$ stimulator cells. $TL^{b}$ stimulator cells also failed to induce IL-2 or IL-4 production, or both, by $TL^{b/b}$ thymocytes (Table 1). Opposite results were obtained with thymocytes from $TL^{d/d}$ transgenic mice: IL-2 or IL-4 production, or both, proliferation and IL-2

receptor $\alpha$-chain expression were induced by $TL^{b}$ stimulator cells but not by IL-2 (Table 1, and Figs 2 and 3). In addition, although thymocytes from both $TL^{b/b}$ and $TL^{d/d}$ transgenic mice proliferated and expressed IL-2 receptor $\alpha$-chain when exposed to IL-2 and $TL^{b}$ stimulator cells (Figs. 2d and 3d, h), the response of the latter was much stronger. Essentially the same results were obtained with spleen cells, except splenic $\gamma\delta$ cells from $TL^{b/b}$ transgenic mice did not respond to IL-2 alone (data not shown). These results indicate that the anergized splenic $\gamma\delta$ T cells are resting.

The results described above show that a self-antigen that is expressed in the thymus does not necessarily delete $\gamma\delta$ T cells expressing TCR for that antigen; we did not see any reduction in the number of KN6 TCR-positive thymocytes in $TL^{b}$ transgenic mice relative to that in $TL^{d}$ transgenic mice. This finding differs from the results of studies with $\alpha\beta$ TCR transgenic mice which demonstrates clearly the deletion of self-antigen-reactive cells[3,6]. In all these previous studies, the TCR genes that were used for the construction of the transgenic mice were obtained from in vitro-stimulated T-cell clones, whereas the KN6 TCR-encoding genes expressed in the transgenic mice that we used were obtained from a $\gamma\delta$ TCR-positive thymocyte. It is possible that the affinity of the transgenic TCR that we were using for a product of the $TL^{b}$ haplotype was below the threshold required for deletion of immature cells as well as for activation of mature cells. This possibility seems unlikely, because the affinity of the KN6 TCR is sufficient to mediate a proliferative response of mature $\gamma\delta$ T cells from $TL^{d}$ mice to $TL^{b}$ stimulator cells. It is more likely that it is the nature of the cell presenting an antigen in the thymus that determines whether T cells reactive with that antigen are deleted or inactivated. It is possible that a minor subset of KN6 TCR-positive thymocytes is in fact deleted in

TABLE 1 Functional properties of $\gamma\delta$ T cells from $TL^{b/b}$ KN6 transgenic mice

| Transgenic responder cells (CD4−CD8−) | $TL^{b}$ stimulator cells | $^{3}$H-labelled thymidine uptake (c.p.m.) | IL-2/4 production (c.p.m.) |
|---|---|---|---|
| $TL^{d/d}$ thymocytes | − | $400 \pm 100$ | $1,600 \pm 500$ |
| | + | $50,500 \pm 4,700$ | $31,100 \pm 1,800$ |
| $TL^{b/b}$ thymocytes | − | $700 \pm 300$ | $3,400 \pm 800$ |
| | + | $1,500 \pm 400$ | $1,200 \pm 300$ |
| $TL^{d/d}$ splenocytes | − | $1,600 \pm 400$ | $3,300 \pm 1,100$ |
| | + | $55,200 \pm 2,600$ | $27,300 \pm 500$ |
| $TL^{b/b}$ splenocytes | − | $1,000 \pm 300$ | $1,100 \pm 300$ |
| | + | $2,100 \pm 600$ | $1,400 \pm 400$ |

When exposed to specific antigen-bearing cells, $\gamma\delta$ T cells from $TL^{b/b}$ KN6 transgenic mice are unable to proliferate or secrete lymphokines. CD4−CD8− thymocytes and splenocytes from $TL^{d/d}$ and $TL^{b/b}$ KN6 transgenic mice were cultured with or without irradiated peritoneal cells of C57BL/6J origin ($TL^{b}$). Proliferative activity of responder cells, assessed by $^{3}$H-labelled thymidine uptake, was assayed at day 2 after the initiation of the culture. Culture supernatants were collected at day 1 and tested for their ability to trigger proliferation of IL-2/4-dependent HT-2 cells. The experiment was performed three times, obtaining similar results. A representative result from one of the three experiments is shown. All measurements were made in triplicate and the data are expressed as mean $\pm$ s.d. Spleen and thymus cell suspensions from 6-week-old $TL^{b/b}$ and $TL^{d/d}$ KN6 transgenic mice were incubated for 1 h at 37 °C with anti-CD4 (RL172.4) and anti-CD8 (3.155.D14) monoclonal antibodies and complement, and live cells were recovered by centrifugation over Ficoll-hypaque. Double-negative responder cells ($5 \times 10^{3}$) were cultured in 96-well plates in 200 $\mu$l complete culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine) with or without $2.5 \times 10^{4}$ irradiated (1,500 rad) peritoneal cells from C57BL/6J mice. Culture supernatant (100 $\mu$l) was collected at day 1. At day 2, cultures were pulsed for 6 h with [$^{3}$H-methyl] thymidine (1 $\mu$Ci per well), collected and counted in a scintillation counter. To determine IL-2/4 production, dilutions of day-1 culture supernatants were added to culture of IL-2/4-dependent HT-2 cells. [$^{3}$H-methyl] thymidine incorporation by these cells was determined on day 2. The results shown are the mean c.p.m. $\pm$ s.d. of triplicates that were obtained with 1/4 final dilutions.
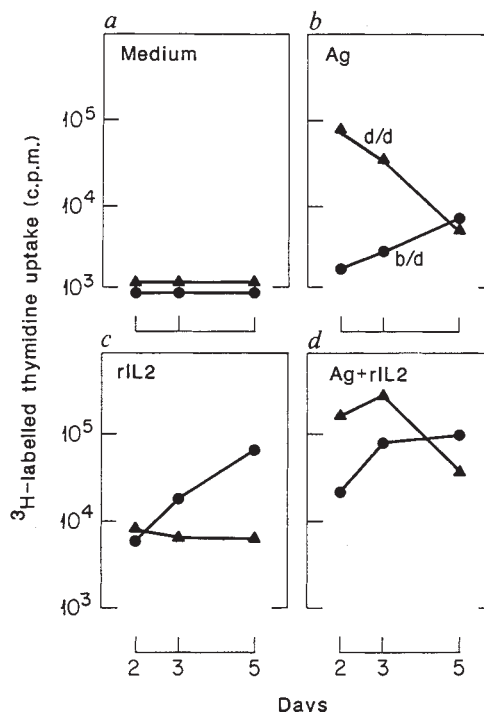


FIG. 2 Addition of recombinant IL-2 (rIL-2) partly restores the proliferation of $\gamma\delta$ T cells from $TL^{b/d}$ KN6 transgenic mice to $TL^{b}$ stimulator cells. CD4−CD8− thymocytes ($TL^{b/d}$ transgenic, $\bullet$; $TL^{d/d}$ transgenic, $\blacktriangle$) were cultured without (a, c) or with (b, d) irradiated $TL^{b}$ peritoneal cells (Ag), in the absence (a and b) or the presence (c and d) of rIL-2. Their proliferative activity, assessed by $^{3}$H-labelled thymidine uptake, was assayed at day 2, 3 and 5 after the initiation of the culture.
METHODS. CD4CD8 double-negative responder cells and irradiated $TL^{b}$ peritoneal cells were prepared and cultured as described in Table 1. IL-2 was added to a final concentration of 100 U ml$^{-1}$. All cultures were pulsed with 1 $\mu$Ci of [$^{3}$H-methyl]thymidine 6 h before collection.

the thymus of $TL^b$ mice. But it is clear that most of these cells are not eliminated even though they interact with products of the $TL^b$ haplotype in the thymus. As a result of this interaction, KN6 TCR-positive cells increase in size, down-modulate their TCR and probably express IL-2-receptor β-chains, which would confer on them the ability to respond to IL-2 alone[14]. This effect of the $TL^b$ product does not reflect an essential positive selection step, because KN6 TCR-positive cells do survive in, and are exported from, the thymus of $TL^d$ transgenic mice. Whereas KN6 TCR-positive cells from $TL^d$ thymi respond to $TL^b$ stimulator cells with IL-2 production and proliferation, KN6 TCR-positive cells from $TL^b$ thymi lose the capacity to produce IL-2 and, as a result, respond to $TL^b$ stimulator cells only in the presence of exogenous IL-2. Therefore the response of KN6 TCR-positive cells from $TL^b$ mice to $TL^b$ stimulator cells is dependent on helper cells that supply IL-2. The lack of such helper cells in $TL^b$ mice could explain the lack of destructive autoimmune responses in $TL^b$ transgenic mice and the lower number of transgenic TCR-positive cells in the periphery of $TL^b$ mice versus $TL^d$ mice. The unresponsive state of the TG-positive cells that is generated in the thymus of the transgenic mice used in the study described here is reminiscent of the state of clonal energy that can be induced in peripheral αβ T cells[15-18], as well as in the thymocytes of P→F$_1$ chimaeras[19].

Our study shows that certain T cells may become dependent on helper cells if they encounter self-antigen in the thymus as well as in the periphery. Like self antigen-specific B cells, self antigen-specific T cells are not necessarily harmful to the host. In fact they could have beneficial functions provided that helper cells are absent, which would facilitate their proliferation and differentiation to potentially harmful effector cells in response to host components. □

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FIG. 3 Blastogenesis and IL-2 receptor α-chain (IL-2Rα) expression of cultured DN thymocytes from KN6 TG mice. DN thymocytes from transgenic mice of $TL^b$ ($TG^{b/b}$) or $TL^d$ ($TG^{d/d}$) haplotypes were cultured in culture medium alone (a, e), with irradiated $TL^b$ peritoneal cells (b, f), with rIL-2 alone (c, g), or with rIL-2 and $TL^b$ stimulator cells (d, h). After 2 days of culture, cells were stained with 8D6 (anti-Vγ4V$\delta$5) and PC61 (anti-IL-2Rα) monoclonal antibodies. Shown are the dot-plot histograms (IL-2Rα fluorescence intensity ($\log_{10}$ scale) on the vertical axis, and forward scatter (FSC; linear scale) on the horizontal axis of gated 8D6$^+$ cells, and the percentage of cells in each quadrant.
METHODS. Cultured cells were incubated with PC61 monoclonal antibody (ATCC No. T1B222) followed by FITC-coupled purified goat anti-rat IgG antiserum (CALTAG, San Francisco). After a third incubation in 10% normal rat serum, cells were stained with biotin-coupled 8D6 monoclonal antibody followed by streptavidin-phycoerythrin. Flow cytometry was performed according to procedures described in Fig. 1 legend.

# A second B cell-specific enhancer 3' of the immunoglobulin heavy-chain locus

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THE expression of immunoglobulin heavy-chain (IgH) genes is generally thought to be regulated by the combination of the $V_H$ promoter with the enhancer element which is located in the $J_H$–$C_H$ intron[1-4]. This is probably an oversimplification: there are cell lines that transcribe IgH genes despite the deletion of the intron-enhancer[5-8]. These findings could imply that other enhancer element(s) exist in the IgH locus[9-11]. Here we show that a strong B-cell-specific enhancer is indeed located at the 3'-end of the rat IgH locus, 25 kilobases downstream of $C\alpha$. This enhancer should be retained downstream of all rearranged IgH genes, regardless of the $V_H$ or $C_H$ segment used. Taken together with analogous findings for the mouse κ locus[12], the results prompt a re-evaluation

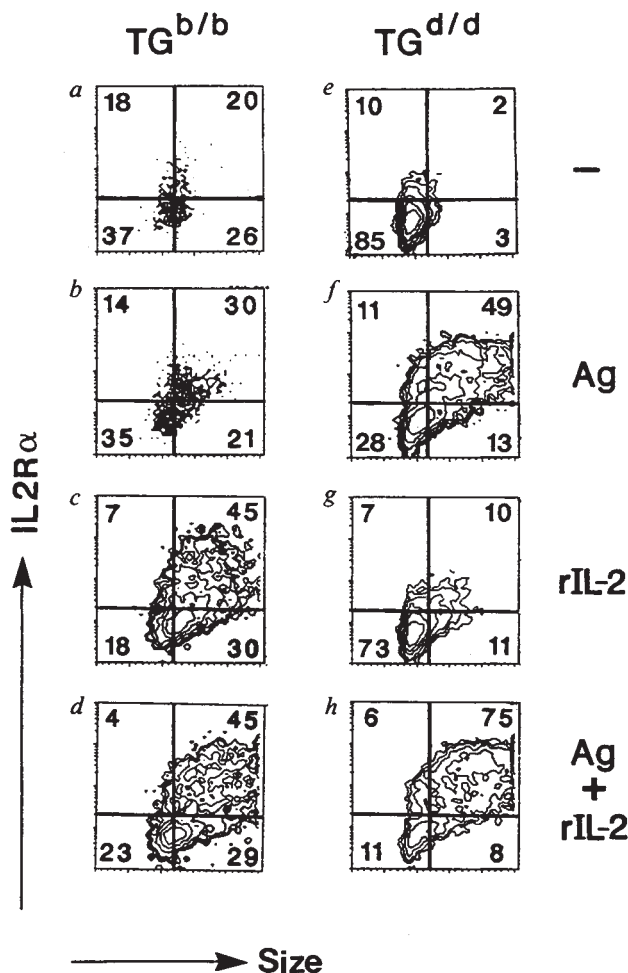* Present addresses: Centre for Biotechnology, Blickagången 6, 14152 Huddinge, Sweden (S.P.); IAPGR, Babraham, Cambridge CB2 4AT, UK (M.B.).