

Allelic Exclusion at DNA Rearrangement Level Is Required to Prevent Coexpression of Two Distinct T Cell Receptor β Genes

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Summary

In mice double transgenic for functionally rearranged T cell receptor (TCR) $V\beta 2$ and $V\beta 8.2$ genes we found that most T lymphocytes express both TCR β chains simultaneously. These T cells show no abnormality in thymic selection *in vivo* and their TCRs are capable of transducing activation signals *in vitro*. These results indicate that multispecific T cells may appear in the periphery if allelic exclusion of TCR β genes is not established at the level of gene rearrangement.

Antigen receptors of T lymphocytes are clonally expressed, i.e., all receptors expressed on the surface of a given T cell are identical. Clonal expression of TCR may be important to avoid autoreactivity and to achieve efficient thymic selection of functional T cells. Clonal expression is controlled by a process called allelic exclusion. Using transgenic mice carrying functionally rearranged TCR β genes it has been shown that allelic exclusion is a regulated event (references 1–4; van Meerwijk, J. P. M., A. Iglesias, T. Hansen-Hagge, H. Bluethmann, and M. Steinmetz, manuscript submitted for publication; Iglesias, A., T. Hansen-Hagge, A. von Bonin, and H. U. Weltzien, manuscript submitted for publication). In the presence of a functionally rearranged TCR β transgene, $V\beta$ gene segment rearrangements at the endogenous loci are suppressed and thus only the transgenic β chain is expressed. In contrast, the presence of a functionally rearranged TCR α transgene does not block endogenous $V\alpha$ rearrangement (2, 5). Thus, different mechanisms appear to establish allelic exclusion in TCR α and β genes.

Is allelic exclusion of TCR β genes controlled exclusively at the level of gene rearrangement, or is there a fail-safe mechanism operating *in vivo* to prevent T cells expressing two different receptors to accumulate in the periphery? We have investigated whether T cells with two productively rearranged TCR β genes can express two functional β chains simultaneously *in vivo*.

Materials and Methods

Mice. TCR $V\beta 8.2$ transgenic mice (3), backcrossed with strain C57BL/6 (H-2^b), were crossed with TCR $V\beta 2$ transgenic mice

(Iglesias, A., manuscript in preparation) of H-2^{bd} haplotype. Offspring was analysed at the age of 4 wk. No significant difference in experimental results was observed between H-2^{bb} and H-2^{bd} pups (data not shown).

Cytofluorimetric Analyses. Thymus and lymphnodes were homogenized, cells were recovered and washed twice in PBS/5%FCS/0.1%NaN₃. IgG-positive B lymphocytes were eliminated from the lymphnode cell preparation as described elsewhere (van Meerwijk, J.P.M., A. Iglesias, T. Hansen-Hagge, H. Bluethmann, and M. Steinmetz, manuscript submitted for publication). T cells were stained with the following antibodies: biotinylated anti-mouse $V\beta 2$ (B20.6.5; reference 21) and anti-mouse $V\beta 8$ (F23.1, reference 6) as described previously (7). As secondary reagents FITC-labeled anti-mouse Ig (Silenus Laboratories, LTD., Victoria, Australia) and streptavidin-PE (Southern Biotechnology Associates, Inc., Birmingham, AL) were used. Anti-mouse CD4-PE (L3T4) and CD8-FITC (Lyt-2) are from Becton Dickinson and Co. (Mountain View, CA). List mode data were processed using FACScan research software (Becton Dickinson and Co.) and displayed as dot-plots (log scale).

Immobilized Antibody Assays. Flat-bottom microtiter plates (Nunc immunoplate) were incubated with 10 μ g/ml goat anti-mouse Ig or goat anti-rat Ig (Southern Biotechnology Associates, Inc.) for 2 h at 37°C. Wells were then washed with PBS plus 10% FCS and subsequently coated with dilutions of supernatant of hybridoma culture of B20.6.5, F23.1, or 145-2CII, for 2 h at 37°C. After washing, 5 \times 10⁴ lymphnode cells were plated per well in Iscove medium plus 10% FCS. [³H]thymidine incorporation was measured 40 h later.

In vitro Staphylococcal Enterotoxin B (SEB) Stimulation. 0.5 \times 10⁶ lymphnode cells were cocultured with 10⁶ irradiated syngeneic spleen cells in 0.2 ml complete medium with SEB (100, 10, 1 ng/ml) or without. [³H]thymidine incorporation was measured 48 h later.

Mixed Lymphocyte Reactions. 0.5 \times 10⁶ lymph node cells from

nTg, TgV β 2, TgV β 8.2, and TgV β 2/V β 8.2 mice were cocultured in triplicate with 10^6 irradiated syngeneic or allogeneic H-2^S spleen cells. [³H]thymidine incorporation was measured 48 h later.

Results and Discussion

We crossed mice transgenic for a functionally rearranged V β 8.2 gene (3) with mice transgenic for a functionally rearranged V β 2 gene (van Meerwijk, J. P. M., A. Iglesias, T. Hansen-Hagge, H. Bluethmann, and M. Steinmetz, manuscript submitted for publication; Iglesias, A., T. Hansen-Hagge, A. von Bonin, and H. U. Weltzien, manuscript submitted for publication). Double transgenic mice as well as single- and nontransgenic littermates were analyzed for expression of the V β 8.2 and V β 2 transgenes in thymocytes and peripheral T lymphocytes.

As compared to nontransgenic mice (nTg)¹ and mice single transgenic for the V β 2 (TgV β 2) or V β 8.2 (TgV β 8.2) gene, the thymuses of V β 2/V β 8.2 double transgenic littermates (TgV β 2/V β 8.2) are significantly smaller. The difference in size is most striking at 6 wk-of-age (Table 1).

As shown in Fig. 1 *a* thymocytes from mice single transgenic for the functionally rearranged TCR V β 2 or V β 8.2 genes express the transgene at both low and high levels corresponding to immature and mature thymocytes, respectively. Thymocytes derived from TgV β 2/V β 8.2 double transgenic mice express the products of both transgenes on the cell surface simultaneously (Fig. 1 *a*).

To find out whether thymocytes expressing two different TCRs simultaneously can make their way through thymic selection to the peripheral lymphoid organs, we examined lymphnode T cells for expression of V β 2 and V β 8 transgenes (Fig. 1 *b*). Mice single transgenic for the TCR V β 2 or V β 8.2 gene express only the transgenic β chain at high levels on >95% of peripheral T lymphocytes. Interestingly, peripheral T lymphocytes of TgV β 2/V β 8.2 double transgenic mice express both transgenic β chains simultaneously (Fig. 1 *b*). The V β 2/V β 8 staining pattern shows that not all cells express equivalent densities of V β 2 and V β 8 chains. About one third of the peripheral T lymphocytes carry slightly lower levels of V β 8.2 than V β 2 (population 1, Fig. 1 *b*), one third display equal amounts of V β 2 and V β 8 (population 2) and one third display less V β 2 than V β 8.2 (population 3). The variation in surface expression may be due to different pairing efficiencies of the two transgenic β chains with the different endogenous α chains. Variable surface expression of transfected pairs of TCR α and β chains was observed previously (8).

As far as the CD4/CD8 staining pattern is concerned, thymocytes and peripheral T lymphocytes of TgV β 2/V β 8.2 double transgenic mice are not significantly different from their single transgenic counterparts and the non-transgenic controls (Table 2).

The TCRs on peripheral T lymphocytes of TgV β 2/V β 8.2 double transgenic mice and single- and nontransgenic littermates were examined for their ability to transduce T cell ac-

Table 1. Number of Thymocytes in Nontransgenic and T Cell Receptor V β 2/V β 8.2 Double Transgenic Mice (TgV β 2/V β 8.2) at Different Ages

	2 wk	6 wk	10 wk
nTg	85	200	50
Tg V β 2/V β 8.2	55	20	25

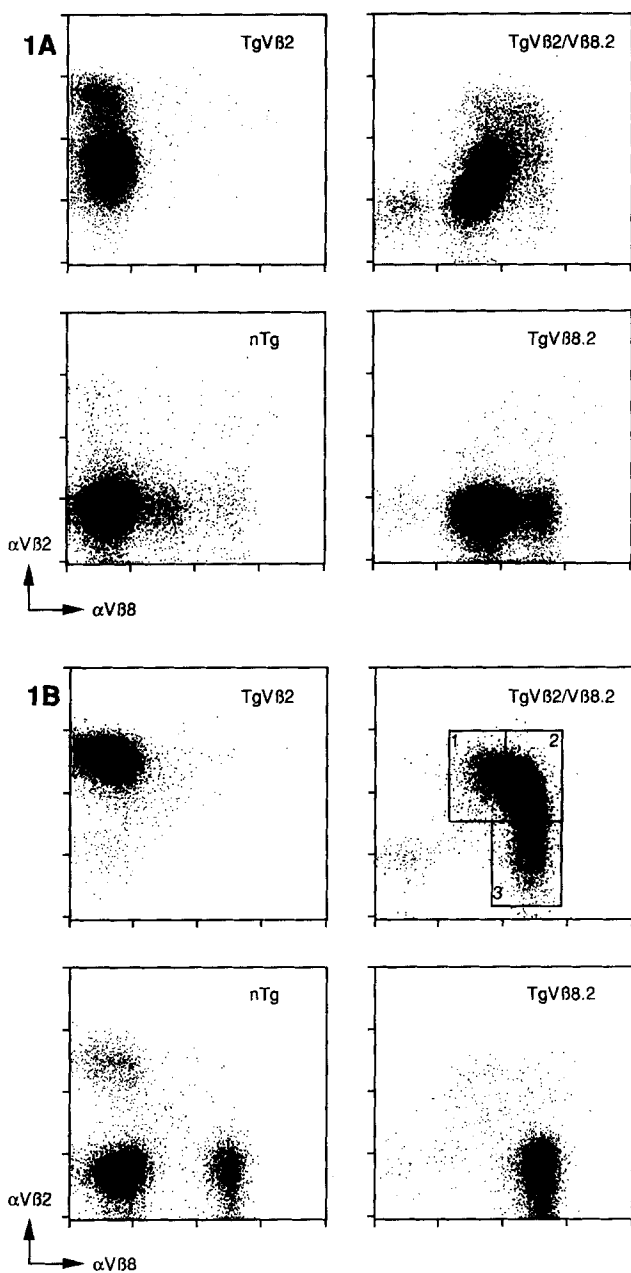


Figure 1. TCR V β 2 and V β 8.2 expression on thymocytes (*A*) and lymphnode T lymphocytes (*B*) from nontransgenic (nTg), V β 2 (TgV β 2), V β 8.2 (TgV β 8.2) and V β 2/V β 8.2 (TgV β 2/V β 8.2) transgenic mice. Boxes in Fig. 1 *b* correspond to populations 1, 2, and 3 as mentioned in the text and legend to Fig. 3 *b*.

¹ Abbreviation used in this paper: nTg, nontransgenic.

Table 2. CD4/CD8 Phenotype of Transgenic and Nontransgenic Thymocytes and Peripheral T Lymphocytes

Genotype	Cells	CD4 ⁻ CD8 ⁻	CD4 ⁺ CD8 ⁻	CD4 ⁻ CD8 ⁺	CD4 ⁺ CD8 ⁺
nTg	Thymocytes	8	9	3	80
TgVβ2		10	12	4	73
TgVβ8.2		15	13	3	68
TgVβ2/Vβ8.2		7	11	6	76
nTg	Lymphnode T cells	3	60	36	1
TgVβ2		4	54	41	1
TgVβ8.2		2	71	26	1
TgVβ2/Vβ8.2		7	54	38	1

Populations are given as percentages of total.

tivation signals in vitro using immobilized antibody assays, mixed lymphocyte reactions and superantigen stimulation.

It was shown previously that T cells can be activated with immobilized antibodies specific for different components of the TCR (9). Using antibodies specific for Vβ8 (F23.1) and Vβ2 (B20.6.5) we could differentially induce proliferation of peripheral T lymphocytes of mice transgenic for the Vβ8 or Vβ2 gene, respectively (Fig. 2 a). Peripheral T lymphocytes of TgVβ2/Vβ8.2 mice were readily stimulated using immobilized antibodies specific for Vβ2, Vβ8 and CD3 and with a combination of anti-Vβ2 and anti-Vβ8 antibodies (Fig. 2 a).

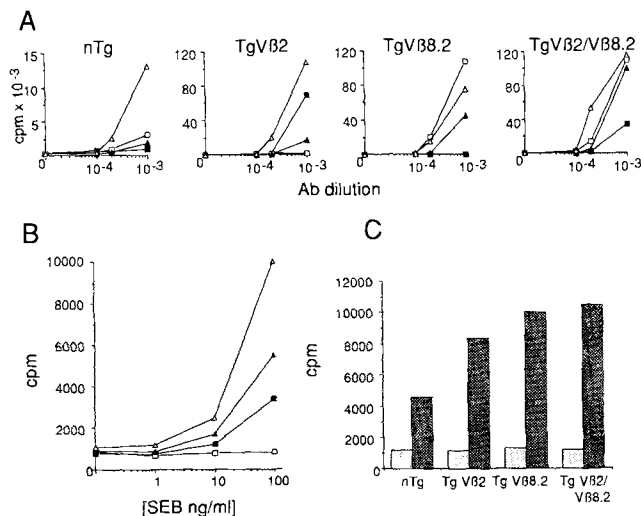


Figure 2. Lymphocytes expressing two different TCRs can be activated in vitro. (A) Responses of lymphnode cells from nTg, TgVβ2, TgVβ8.2 and TgVβ2/Vβ8.2 mice to immobilized antibodies specific for Vβ2 (B20.6.5) (■), Vβ8.2 (F23.1) (□), Vβ2/Vβ8.2 (▲) and CD3ε (145-2CII) (△). (B) Responses of lymphnode cells from nTg (■), TgVβ2 (□), TgVβ8.2 (▲) and TgVβ2/Vβ8.2 (△) mice to Staphylococcal enterotoxin B (SEB). (C) Mixed lymphocyte reaction. Lymphnode cells from nTg, TgVβ2, TgVβ8.2 and TgVβ2/Vβ8.2 mice were co-cultured with irradiated syngeneic (□) or allogeneic (■) spleen cells.

The stimulated cells were analyzed by flow cytometry. The changes in forward (FSC) and side scatter (SSC) patterns were taken as criterion for activation (see legend to Fig. 3 a). Cells carrying both Vβ2 and Vβ8.2 chains at similar levels (population 2) are stimulated in all arrays, as are the cells displaying less Vβ8.2 than Vβ2 (population 1, Fig. 3 b). The popula-

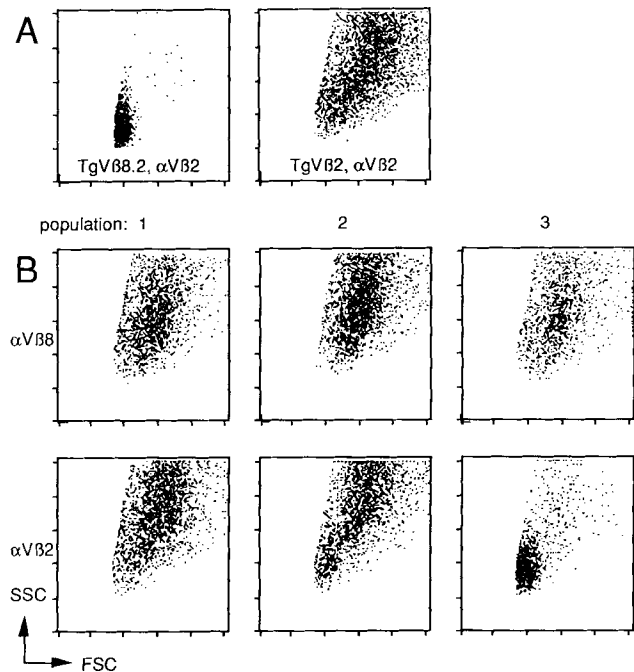


Figure 3. Stimulation of different double transgenic lymphocyte populations. (A) Stimulation by immobilized antibodies leads to increased forward (FSC) and side scatter (SSC) as shown for TgVβ2 cells stimulated by anti-Vβ2 antibody (TgVβ2, anti-Vβ2, see Fig. 2 A for thymidine incorporation data). As negative control the FSC/SSC pattern of TgVβ8.2 cells incubated in the presence of immobilized anti-Vβ2 antibody is shown (TgVβ8.2, anti-Vβ2). FSC is a measure for cell-size and SSC for granularity. (B) FSC/SSC patterns of the three double transgenic (TgVβ2/Vβ8.2) T lymphocyte populations after several stimulations. Populations 1, 2, and 3 are as indicated in Fig. 1 B. Stimulations were as indicated in the figure.

tion displaying less V β 2 than V β 8.2 chains (population 3) is not stimulated by the V β 2 antibody (Fig. 3 b), whereas there is no obvious difference in stimulation of this population by the other antibodies. These results indicate that both the V β 2 and V β 8.2 TCRs are capable of transducing activation signals in vitro. The observation that practically all cells carrying similar levels of V β 2 and V β 8.2 are stimulated by V β 2 and V β 8 antibodies (Fig. 3 b), suggests that both TCRs are functional on the same cell.

The superantigen staphylococcal enterotoxin B (SEB) is known to activate murine T cells carrying V β 8 chains (for review see reference 10). Proliferation of T cells from V β 8.2 single transgenic mice and from V β 2/V β 8.2 double transgenic littermates was observed (Fig. 2 b). T lymphocytes from V β 2 transgenic mice did not respond, as expected. Flow cytometry analysis showed that the population expressing high levels of V β 2 and V β 8.2 simultaneously and the one displaying high levels of V β 8.2 but lower levels of V β 2 were both stimulated (data not shown).

Peripheral T lymphocytes from nTg, TgV β 2, TgV β 8.2 and TgV β 2/V β 8.2 mice were also examined for alloreactivity. T lymphocytes from all mice responded to allogeneic stimulator cells (Fig. 2 c). No significant differences in the responses of the three above mentioned populations in double transgenic mice were observed in flow cytometry analysis (data not shown).

Our results with mice double transgenic for functionally rearranged V β 2 and V β 8.2 transgenes show that thymocytes in these mice expressed both transgenic β chains on their surface. Surprisingly, thymocytes expressing two different TCRs simultaneously were able to make their way through thymic selection to peripheral lymphoid organs. Moreover, peripheral T lymphocytes expressing both β chains on the cell surface were able to respond to stimulation by immobilized antibodies, superantigens and allogeneic stimulator cells. Thus, T lymphocytes expressing two different TCRs on their surface can be selected in vivo and are functional at least in vitro. Therefore, clonal expression of TCRs in vivo requires allelic exclusion of TCR β genes to be achieved at the level of gene rearrangement.

In contrast, allelic exclusion of TCR α genes, appears to be established primarily at a level after gene rearrangement (2, 5, 11). Posttranscriptional mechanisms, (12), such as pairing of α and β chains (8), or thymic selection may play a role. Our data indicate that these, or other postrearrangement mechanisms cannot ensure allelic exclusion of TCR β genes.

Allelic exclusion of Ig genes in B cells seem to be very similarly controlled as that of TCR genes in T cells. Functionally rearranged Ig H chain genes were found to inhibit gene rearrangement of the endogenous Ig H loci at the V_H to DJ_H level in transgenic mice (13–16). This suppression, however, was found to be less complete as for the TCR β locus. Using mutant μ chain genes as transgenes it was shown that only the membrane form of μ (μ_m) could suppress rearrangement of endogenous H chain genes, not the secretory form of μ (μ_s) (17–19). These findings suggest an active, μ_m -dependent mechanism to induce allelic exclusion. However, as for the TCR locus, little is known about the molecular basis of this signaling event.

There are also parallels between the Ig L chain locus and the TCR α genes, in that allelic exclusion of both loci can be established after gene rearrangement. Functional L chain genes alone as transgenes were not able to inhibit rearrangement of endogenous L chain genes, only complete Ig molecules were able to do so (20).

Interestingly the sizes of thymuses of TgV β 2/V β 8.2 double transgenic mice were significantly reduced as compared to thymi of normal and TgV β 2 or TgV β 8.2 single transgenic mice. The reduction in thymocyte numbers was particularly striking at 6 wk-of-age. There are many possible explanations for this finding. The expression of both transgenes in early T cell progenitors could impair differentiation of these cells. Alternatively, the expression of two different TCRs on the surface of immature thymocytes could interfere with positive selection (for instance, if one TCR is MHC class I and the other MHC class II restricted) or increase the rate of negative selection. These issues deserve further study.

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