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CD8$^+$ T Lymphocytes in Double $\alpha\beta$ TCR Transgenic Mice. I. TCR Expression and Thymus Selection in the Absence or in the Presence of Self-Antigen[1]

Nicolas Legrand and Antonio A. Freitas[2]

We derived Rag2-deficient mice bearing two rearranged $\alpha\beta$ TCR transgenes, one specific for the HY male Ag and the second specific for the gp33-41 peptide of lymphocytic choriomeningitis virus, both restricted to the MHC H-2D^b class I molecule. We found that, in female double transgenic (DTg) mice, most CD8 T cells express only the TCRβ chain from the aHY transgene. By comparing the mRNA species for both β-chains, we observed that in T cells from DTg mice the aHY TCRβ chain transcripts are abundant, whereas the anti-lymphocytic choriomeningitis virus TCRβ chain transcripts are rare. In contrast to TCRβ chain expression, most of the T cells from DTg mice express two TCRα chains. We examined the thymus selection of the dual-receptor CD8 T cells in the presence of self-Ag. We found that the presence of a second TCRα chain allows a significant number of CD8 T cells expressing a self-reactive receptor to escape central deletion and migrate to the peripheral pools of male mice. Differences in TCR and coreceptor expression between female and male MoaHY and DTg mice suggest that peripheral T cell survival requires an optimal level of signaling, which implies a process of "adaptation" of lymphocyte populations to the host environment. ***The Journal of Immunology*****, 2001, 167: 6150–6157.**

Antigen receptors of B and T lymphocytes are subjected to allelic exclusion (1). In general, for T cells the TCRβ chain gene segments start to rearrange on one chromosome and continue on the second chromosome only when the first attempt resulted in a nonproductive gene (2). Productive rearrangement results in the expression of a TCRβ chain, which alone (3) or in association with the pre-TCRα (pTα)[3] chain (4, 5) during the early phases of T cell development is essential for TCRβ chain allelic exclusion. Allelic exclusion of the TCR, however, is never fail proof. Different studies indicate that ~1% of the mature $\alpha\beta$ T cells contain two productive TCRβ alleles (6, 7). Differential kinetics of TCRβ chain expression, however, may still contribute to inhibition of the expression of one of the two productive rearrangements. Thus, TCRβ chains may compete for limited amounts of pTα (5) and/or the CD3 complex, and in this case it is possible that the first β-chain expressed preempts surface expression of a second β-chain. At the protein level, earlier expression of one TCRβ chain may also change the efficiency of the association and expression of distinct TCR heterodimers (8). In contrast to the TCRβ locus, TCRα chain rearrangements proceed simultaneously in both chromosomes (9) and ~30% of the human $\alpha\beta$ T cells express two TCRα chains (10–12). In normal mice, 30% of $\alpha\beta$ T cells show two rearranged α-chains (10) and a variable fraction express two α-chains, suggesting that the likelihood of generation of T cells with dual specificity is elevated.

Potentially the allelically included T cells could play an important role in autoimmunity. In dual-receptor cells, a second self-reactive TCR could bypass negative selection in the thymus, by virtue of its lower expression. If a non-self Ag stimulates the naive dual-receptor T cells, these T cells once activated would acquire a lower threshold of activation, respond to self-peptide/MHC, and cause disease. Several in vivo experimental models have examined this possibility. Studies on the susceptibility to spontaneous autoimmune diseases of nonobese diabetic mice hemizygous for the TCRα locus did not support the role of dual α T cells in autoimmune diabetes (13). However, in different mouse models, co-expression of two TCR transgenes rescues self-reactive T cells from tolerance induction and allows their exit into the periphery (14, 15). These cells could be stimulated in vitro to anti-self effector functions via the second receptor (14), and their in vivo presence was correlated to the induction of autoimmune diabetes when the relevant Ag was expressed by the pancreatic tissue (15).

The role of dual-receptor T cells in autoimmune diseases, however, is strictly dependent in their ability to maintain surface expression of two functional receptors and to be positively selected in the thymus. Because thymus positive selection requires receptor engagement by MHC molecules (16), it is likely that, in the presence of two receptors bearing a different specificity, each may impose some constraints to the selection of dual-receptor T cells. Indeed, it was recently shown that reduced specific receptor density results in reduced thymus positive selection of dual-receptor T cells (17). To examine these questions, we studied thymus T cell selection and TCR expression in Rag2-deficient animals bearing two complete rearranged $\alpha\beta$ TCR transgenes. One receptor is specific for the HY male Ag (18, 19) and the second is specific for the gp33-41 peptide of lymphocytic choriomeningitis virus (LCMV) (20), both restricted to the same MHC H-2D^b class I molecule.

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[3] Abbreviations used in this paper: pTα, pre-TCRα; LCMV, lymphocytic choriomeningitis virus; Tg, transgenic; DTg, double transgenic; MFI, mean fluorescence intensity; LN, lymph node; SP, single positive; BM, bone marrow; DN, double negative; DP, double positive.

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Materials and Methods

Mice

C57BL/6 mice transgenic (Tg) for the anti-HY TCR (VαT3.70;Vβ8.2) (18, 19), or the P14 TCR (Vα2;Vβ8.1) (20), were crossed into a Rag2-deficient background (21). The mice obtained (MoaHY and MoP14) were intercrossed to give rise to MoaHY.MoP14 double-transgenic (DTg) mice. All these strains were maintained in specific pathogen-free isolators at the Centre de Développement des Techniques Avancées pour l'Expérimentation Animale-Centre National de la Recherche Scientifique (Orléans, France). B6.CD3$\epsilon^{-/-}$ mice (22) were from the Centre de Développement des Techniques Avancées pour l'Expérimentation Animale-Centre National de la Recherche Scientifique. Double CD3$\epsilon^{-/-}$H-2D$^{b-/-}$ mice (23) were maintained specific pathogen-free in our animal facilities at the Pasteur Institute and at the Centre de Développement des Techniques Avancées pour l'Expérimentation Animale.

Flow cytometry

The following mAbs were used: anti-CD8α (53-6.7), anti-Vα2 (B20.1), pan-TCRβ chain (H57-597), anti-Vβ8.1/2 (MR5-2), anti-CD3ϵ (145-2C11), anti-CD4 (L3T4/RM4-5), anti-CD69 (H1.2F3), anti-CD25 (PC61), and anti-CD24/HSA (M1/69) from BD PharMingen (San Diego, CA) and anti-CD44 (IM781) and anti-CD62L (MEL14) from Caltag Laboratories (San Francisco, CA). The anti-VαT3.70 and the F23.2 anti-Vβ8.2 were from B. Rocha (Institut National de la Santé et de la Recherche Médicale U345, Institut Necker, Paris, France). Cell-surface staining was performed with the appropriate combinations of FITC, PE, TRI-Color (Caltag Laboratories), PerCP (BD Biosciences, San Jose, CA), Biotin, and APC-coupled Abs. Biotin-coupled Abs were revealed by APC-, TRI-Color-, or PerCP-coupled streptavidin. Dead cells were excluded by light-scattering gating. All analyses were performed with a FACScalibur (BD Biosciences) interfaced to Macintosh CellQuest software (Apple Computer, Cupertino, CA). For TCR co-internalization analysis, spleen cells from Tg mice were incubated (37°C, 5% CO_2) in NaN_3-free balanced salt solution for different periods of time in the presence of Abs specific for either one of the Tg TCRα or TCRβ chains. At the end of the incubation periods, cells were washed in 0.2% NaN_3 and kept in the dark at 4°C. Next, the cells were stained with fluorescent Abs directed against TCR chains and were analyzed. Results are expressed as a percentage of the geometric mean of fluorescence intensity (MFI), compared with control cells incubated in absence of the first capping Ab.

Immunoscope

We followed the Immunoscope analysis previously described (24). In brief, total RNA extracted from lymph node (LN) CD8 T cells of the different Tg mice by TRIzol (Life Technologies, Mannheim, Germany) was treated with DNase I (Boehringer Mannheim, Mannheim, Germany). From each RNA sample, cDNA was prepared using $(dT)_{12-18}$ and Superscript II reverse transcriptase (Life Technologies). The cDNAs for the different TCRβ chains were amplified by PCR (40 cycles) using primers specific for the TCRVβ8 family (5′-TCC CTG ATG GRT ACA AGG CC-3′) and TCR-Cβ2 (5′-GCC AGA AGG TAG CAG AGA CCC-3′). The resulting PCR products were used for a run-off reaction (two cycles) using a nested fluorescent primer specific for the TCR-Cβ2 segment (5′-CCT GGG TGG AGT CAC ATT TCT C-3′). Finally, the run-off products were resolved on an automatic 373A DNA sequencer (Applied Biosystems, Foster City, CA), and the results were analyzed using the Immunoscope software (24).

Bone marrow (BM) chimeras

Host mice were lethally irradiated (900 rad) in a ^{137}Ce source and injected i.v. with 2–4 $\times$ 10^6 BM cells. Thymus, spleen, inguinal, and mesenteric LN cell suspensions were prepared, and the number and phenotype of the cells were evaluated.

In vitro proliferation assays

Spleen cells from Tg mice were incubated in 96-well plates (10^5 cells/well) at 37°C, 5% CO_2, in a final volume of 200 μl in complete RPMI 1640 medium supplemented with 10% FCS (Boehringer Mannheim). Cells were stimulated with Con A (Sigma-Aldrich, St. Louis, MO), anti-CD3 (BD PharMingen), or the TCR Tg specific peptides gp33-41 (25) (KAVYNFATM) and *Smcy*-3 peptides (26) (KCSRNRQYL) purchased from Neosystem (Strasbourg, France) and used with a >95% purity. After 1–4 days of culture, cells were pulsed overnight with 0.5 μCi of [^{3}H]thymidine (ICN Pharmaceuticals, Costa Mesa, CA).

Results

CD8$^+$ T cells in female $\alpha\beta$ TCR DTg Rag2-deficient mice

Mice expressing Tg TCRs specific for the male HY Ag (aHY) and for the gp33-41 peptide of the LCMV (P14) were crossed with B6.Rag2$^{-/-}$ mice to obtain two lines of mice harboring homogeneous populations of CD8$^+$ T cells all expressing the same TCR specificity. Monoclonal MoaHY (Vβ8.2$^+$VαT3.70$^+$) and MoP14 (Vβ8.1$^+$Vα2$^+$) mice were intercrossed to obtain MoaHY.MoP14 (DTg) mice bearing two MHC H-2D^b class I-restricted TCR transgenes.

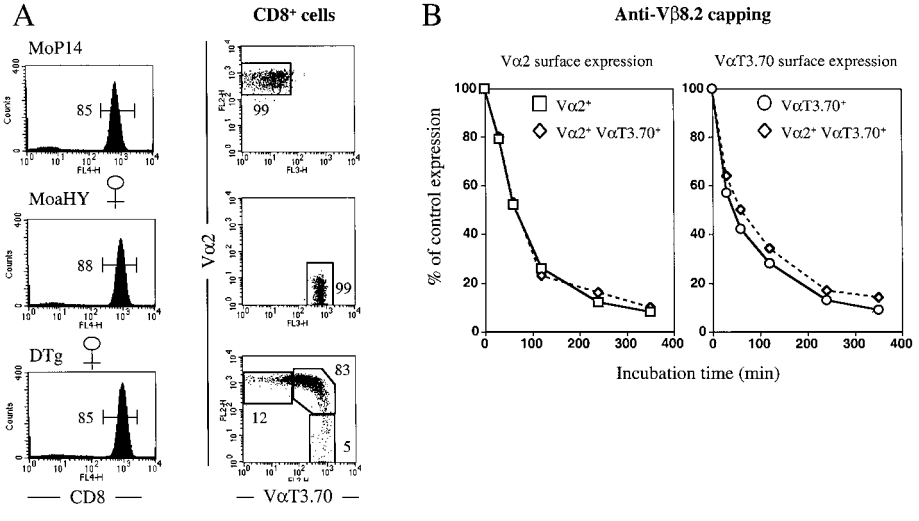

FIGURE 1. Peripheral T cells in MoP14, MoaHY and MoaHY.MoP14DTg female mice. *A*, Expression of CD8 (histograms) and of Vα2 and VαT3.70 TCR transgenes by gated peripheral LN CD8 T cells (dot plots). *B*, Spleen cells from DTg females were incubated for 6 h in the presence of a first anti-Vβ8.2 capping Ab. The expression of the TCRα at different times after incubation was compared with the TCRα expression in absence of the first Ab. The left graph shows the kinetics of Vα2 internalization (expressed as a percentage of MFI of control cells) among the Vα2$^+$VαT3.70$^-$ (□) and Vα2$^+$VαT3.70$^+$ (◇) T cells. The *right graph* shows VαT3.70 down-regulation in Vα2$^-$VαT3.70$^+$ (○) and Vα2$^+$VαT3.70$^+$ (◇) T cells. We must mention that, in CD8 T cells from monoclonal mice, down-regulation of a TCRβ chain correlates with the TCRα chain disappearance and vice versa. Thus, pre-incubation with T3.70 does not internalize the TCR of T cells from MoP14 origin, and pre-incubation with the P14-α chain-specific B20 Ab does not internalize the aHY TCR in T cells from MoaHY mice. Similarly, the Vβ8.2 Ab specific for the aHY TCR did not internalize the P14 TCR (data not shown).

In the DTg female mice, dual TCRα chain-expressing (Vα2$^+$T3.70$^+$) cells represent the majority (~80%) of the peripheral CD8 T cells (Fig. 1*A*). About 20% of the CD8 T cells express a single Vα chain; i.e., they are either Vα2$^+$T3.70$^-$ (~5–15%) or Vα2$^-$T3.70$^+$ (~5–15%) (Fig. 1*A*). Expression of VαT3.70 was lower in DTg than in MoaHY mice, but varied as in aHY.Rag$^+$ TCR Tg mice, where endogenous TCRα chains can be co-expressed (18). The vast majority (99%) of the peripheral CD8 T cells express the aHY TCR Vβ8.2 chain. Only <1% of the total CD8 T cells are Vβ8$^+$8.2$^-$Vα2$^+$T3.70$^-$, corresponding to the P14 TCR (data not shown). Although detection of T cells expressing each of the β-chains from the Vβ8 family can be done using the F23.1, KJ16, and F23.2 Abs (27), we found that direct analysis of dual TCRβ expression was not conclusive due to the lack of a Vβ8.1 monospecific Ab. The assembly of the TCR transgenes at the cell surface of the DTg T cells was studied by following the kinetics of co-internalization of the different TCR Tg chains (Fig. 1*B*). Pre-incubation of DTg cells with anti-Vβ8.2 promotes the complete internalization of both VαT3.70$^+$ and Vα2$^+$ chains, suggesting that the Vβ8.2 aHY TCRβ Tg chain associates with both the aHY and the P14 TCRα Tg chains. These results also indicate that, in the DTg cells, equivalent levels of surface expression of two TCRβ chains must be a rare event. Studies on the in vitro response of DTg CD8 T cells to specific peptides provided further evidence of the poor expression of the Vβ8.1 P14 TCR transgene. Thus, whereas the dual-receptor T cells proliferated in response to the *Scmy-3* HY peptide (Fig. 2, *A* and *B*), the P14-specific gp33-41 LCMV peptide failed to induce proliferation of DTg cells (Fig. 2*C*). However, it induced the early CD69 expression in 50% of Vα2$^+$T3.70$^+$ cells (Fig. 2*D*), indicating that those cells were still capable of peptide recognition and of a partial agonistic response (28).

In summary, in female DTg mice, most dual-TCR CD8 cells are Vβ8.2$^+$Vα2$^+$T3.70$^+$. A minor fraction of the cells may also express low levels of P14 Vβ8.1 chain, which are insufficient to induce cell proliferation by the gp33-41 peptide and only induce up-regulation of CD69 expression in a fraction of the DTg cells.

Thymus T cell development in $\alpha\beta$ TCR (DTg) Rag2$^{-/-}$ female mice

To understand the cellular mechanisms that could lead to the exclusion of one TCRβ chain transgene, we compared T cell development in the thymus of the DTg females, MoaHY females, and MoP14 mice (Fig. 3). The total number of thymocytes (~75 $\times$ 10^6 cells) and the double negative (DN) (10%), double positive (DP) (80%), and single positive (SP) CD8$^+$ (10%) distributions were similar in all mice (Fig. 3*A*). The composition of the DN populations as well as their levels of TCR expression (Fig. 3*B*) varied. In both MoaHY and DTg mice, CD44$^-$CD25^{high} populations were absent, and all DN T cells, CD44$^-$CD25^{int} and CD44$^-$CD25$^-$, expressed high levels of the aHY TCRβ Tg Vβ8.2. In the MoP14 mice, CD44$^-$CD25^{high} cells were present and they did not express TCR. Expression of the P14 TCRβ transgene was first detected among CD44$^-$CD25^{int} cells, but the frequency of these cells with TCR^{high} expression was much lower than that found at similar stages of differentiation in the thymus of both MoaHY and DTg mice. Therefore, the aHY TCRβ transgene was expressed earlier than the P14 TCRβ chain. This earlier expression, probably before the CD44$^-$CD25^{high} stage, induces the rapid transition of immature thymocytes into CD44$^-$CD25^{int} DN cells and the disappearance of the CD44$^-$CD25^{high} DN population. The kinetics of expression of the TCRα chain transgenes also differed. In MoaHY females, all CD44$^-$CD25^{int} DN cells expressed high levels of VαT3.70, whereas in the MoP14 thymus, Vα2 expression was low in CD44$^-$CD25^{int} DN cells and high levels of expression could only be detected among CD44$^-$CD25$^-$ DN cells.

In conclusion, the presence of a TCR transgene leads to the rapid transition of the thymus cells through the earlier steps of DN differentiation. The differential kinetics for the expression of the two TCRβ chain transgenes show that T cell maturation occurs faster in MoaHY and DTg than in MoP14 mice. The earlier expression of the aHY TCRβ transgene may lead to the functional exclusion of the second P14 TCRβ Tg.

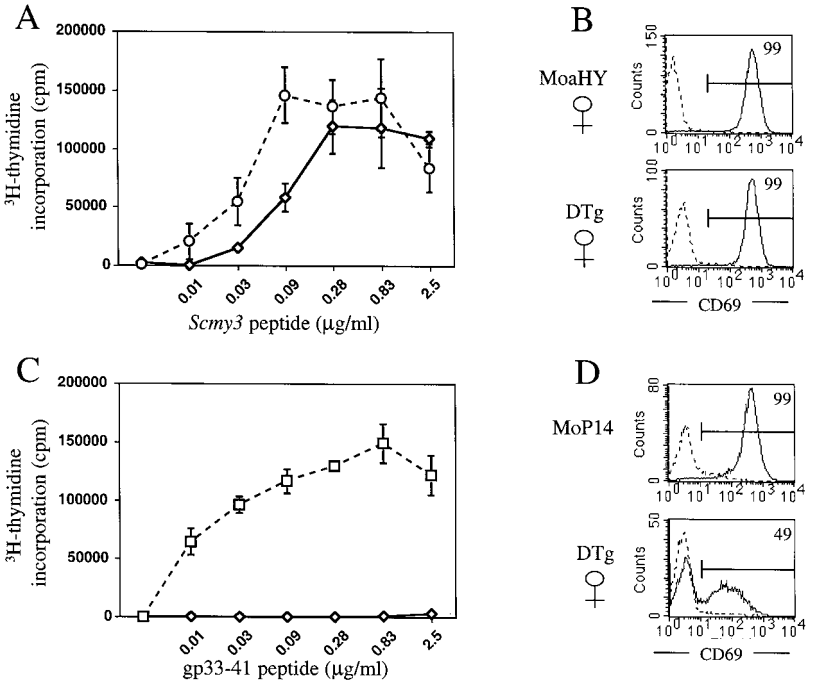

FIGURE 2. In vitro stimulation of T cells from MoP14, MoaHY, and MoaHY.MoP14 DTg mice. *A*, Spleen T cells from MoaHY female (○) and DTg female (◇) mice were stimulated in cultures with the *Scmy3* aHY TCR-specific peptide. Results show the [^{3}H]thymidine incorporation at day 2 of culture obtained in one typical experiment (mean of triplicate ± SD). *B*, Peptide activated (solid line) and nonactivated (dotted line) cells were stained at 24 h for the surface expression of the early activation marker CD69. The percentage of CD69$^+$ cells is indicated inside each histogram. *C* and *D*, The same analysis was performed for T cells from MoP14 mice (□) and DTg female (◇) using the gp33-41 P14 TCR-specific peptide.

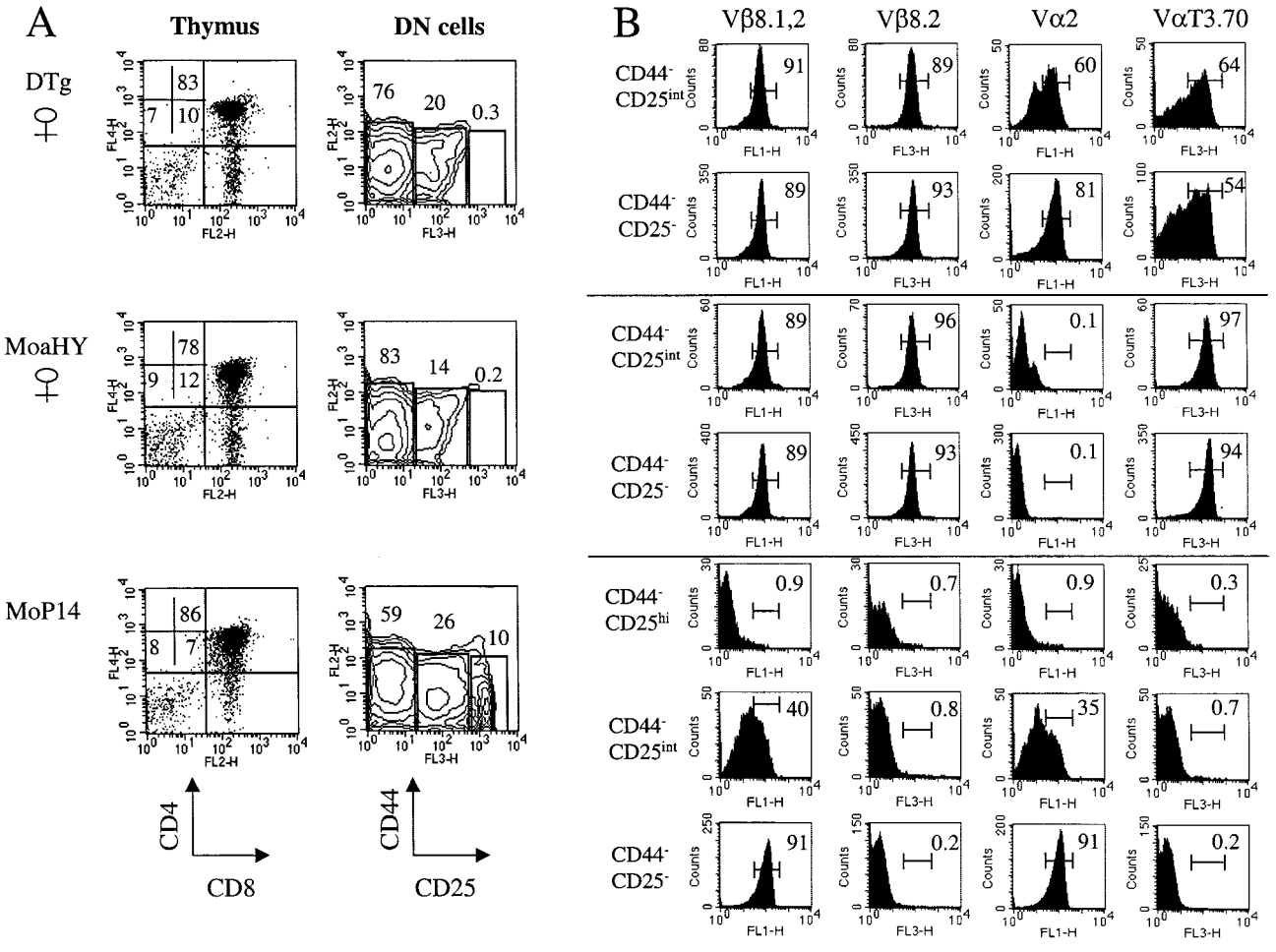

FIGURE 3. T cell development in the thymus of single- and double-TCR Tg Rag2-deficient female mice. *A*, Thymus cells from MoaHY.MoP14DTg, MoaHY, and MoP14 mice were stained for the expression of CD4 and CD8 (*left panels*). The relative proportions of the different DN, DP, and SP populations are shown in the quadrants inside each dot plot. The *right panels* show the pattern of expression of CD25 and CD44 among the gated DN thymocytes. *B*, The histograms show the expression of the Vβ and Vα TCR Tg chains among the different subpopulations of DN thymocytes defined according to intensity of expression of CD25 and CD44.

Decreased P14 TCRβ chain mRNA levels in dual $\alpha\beta$TCR Tg CD8 T cells

The exclusion of the P14 TCRβ transgene could occur at the transcription and/or at a posttranscription (protein) level. The high sequence homology between the two Tg TCRβ chains prevented RT-PCR analysis of the specific mRNA transcripts. However, the size of the hypervariable CDR3-like region of the two chains differs. Therefore, it was possible to evaluate the frequency of the TCRβ chain transcripts using a method of PCR amplification and primer extension with fluorescent oligonucleotides (24). We must mention that we used the same amplification and extension primers for both TCRβ chains. By measuring the area corresponding to the transcripts of each β-chain from a 1:1 mixture of both transgene T cells, we found that the level of the P14 TCRβ Tg mRNA was 3- to 4-fold lower than that of the aHY TCRβ transgene (Fig. 4). Correspondingly, the surface expression of TCRβ by mature CD8 T cells is 20–30% lower in MoP14 mice than in MoaHY mice. The lower level of transcription of the P14 transgene may be due to the types of promoters and transgene vectors used. Indeed, the aHY vector includes both the TCRβ chain promoter and the TCRβ chain enhancer, whereas the P14 vector uses an MHC class I promoter and the Ig H chain enhancer, without regulatory elements specific for TCR expression (19, 20).

In dual $\alpha\beta$TCR Tg CD8 T cells, the transcripts for the aHY TCRβ chain were 9–10 times more frequent than those of the P14 TCRβ chain (Fig. 4). It is possible that the presence of TCR regulatory elements favors a stronger and earlier expression of the aHY TCRβ chain that subsequently reduces transcription or stability of the mRNA for the late Vβ8.1 chain of the P14 transgene. In summary, our results suggest that exclusion of the P14 TCRβ chain in dual $\alpha\beta$TCR Tg CD8 T cells occurs at the message level.

DTg T cells are H-2D^b restricted

Do new combinations of TCR chains due to the random association between the different TCR Tg chains confer a new MHC restriction specificity to the MoaHY.MoP14 DTg cells? Lymphocyte development in the thymus of the DTg females only gives rise to CD4$^-$CD8$^+$ SP cells (Fig. 3*A*). The lack of CD4 SP cells indicates that any potential association between α- and β-chains from the two TCR transgenes does not permit positive selection by MHC class II. Selection of DTg cells in the absence of the aHY and P14 TCR MHC class I H-2D^b restricting element was also investigated. In female CD3$^{-/-}$H-2D$^{b-/-}$ chimeras reconstituted with BM cells from female DTg donors (Fig. 5), the number of thymocytes is reduced. The few CD8$^+$ SP cells present in the thymus are immature because

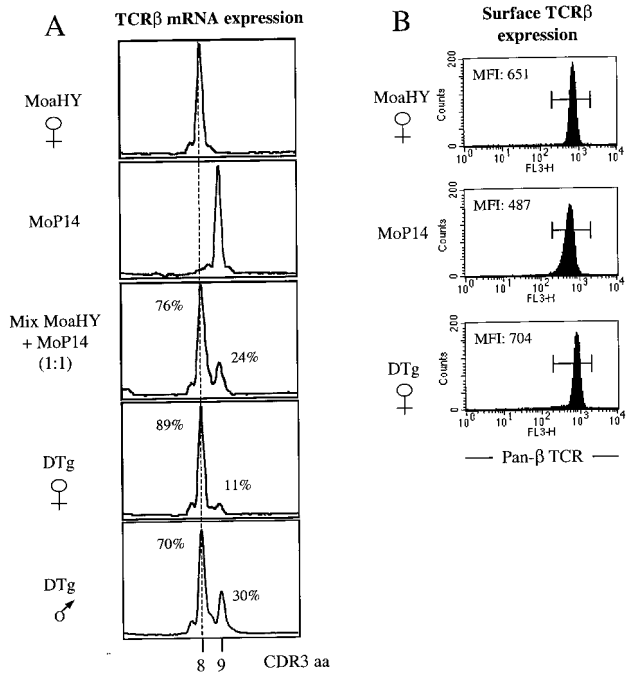

FIGURE 4. TCRβ chain expression in single- and double-TCR Tg Rag2-deficient female mice. *A*, The aHY and P14 TCRβ chain mRNA expression was studied by Immunoscope, using lymph node cells from MoaHY, MoP14, and MoaHY.MoP14DTg female and male mice. A 1:1 mixture of LN CD8 T cells from MoaHY and MoP14 mice was used to compare the relative transcription of both TCRβ chain transgenes. *B*, CD8$^+$ T cells from MoaHY, MoP14, and MoaHY.MoP14DTg mice were stained for total TCRβ chain surface expression using a panβ Ab (H57). The MFI obtained is indicated in each histogram.

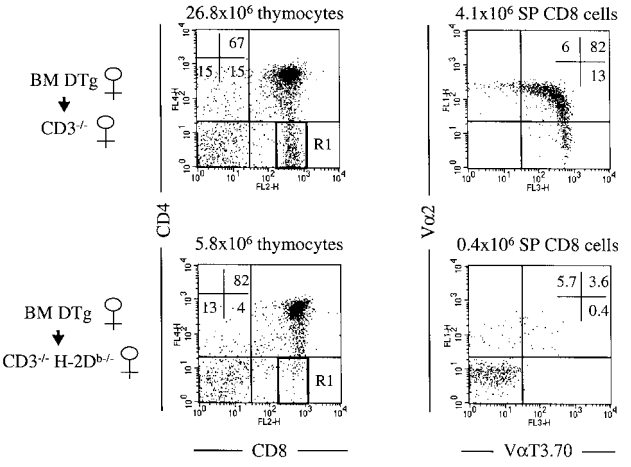

FIGURE 5. Development of MoaHY.MoP14DTg T cells in BM chimeras. Irradiated CD3$\epsilon^{-/-}$ (*top*) or CD3$\epsilon^{-/-}$H-2D$^{b-/-}$ mice (*bottom*) were reconstituted with BM cells from DTg donors. Two months later, T cell development was examined. The panels show the expression of CD4 and CD8 in the thymus of both BM chimeras and the expression of Vα2 and VαT3.70 among the SP CD8$^+$ cells (R1 gate). Note the rarity of SP TCR$^+$ cells in the CD3$\epsilon^{-/-}$H-2D$^{b-/-}$ hosts. The relative proportions of the DN, DP, and SP populations are shown in the quadrants inside each dot plot. The total numbers of thymocytes and SP CD8 thymus cells are indicated above each dot plot.

TCR^{high}CD8$^+$ SP cells are almost absent (Fig. 5) and the number of peripheral CD8 T cells is 20-fold lower (i.e., $<0.5 \times 10^6$ cells) than in CD3$^{-/-}$H-2D$^{b+/+}$ chimeras. The few peripheral CD8 SP cells present express the aHY Vβ8.2 and the P14 Vα2 chains (data not shown). From these results, we conclude that, in DTg mice, dual-receptor expression does not hinder thymus T cell positive selection and that SP CD8 cells are restricted to MHC H-2D^b class I molecules.

Thymus selection of dual-receptor CD8 T cells in the presence of self-Ag

We next investigated whether the presence of two TCR transgenes could rescue cells from deletion in the presence of male HY self-Ag. We compared T cell development in the thymus of MoaHY and DTg male mice. In MoaHY male mice, the presence of the HY Ag blocks thymus T cell differentiation at the DN to DP transition. The number of thymocytes is reduced to 5×10^6, of which 92% are DN (18). In the thymus of DTg males, T cell development is also impaired, but in contrast to MoaHY male mice, the presence of the second Vα2 TCR transgene rescues a significant fraction of cells into the DP compartment (Fig. 6, *A* and *B*). This allows the selection of cells co-expressing both P14-specific Vα2 and low levels of HY-specific VαT3.70 into the SP CD8 T cell compartment (Fig. 6*B*). At the periphery of the MoaHY males, 70% of the peripheral TCR$^+$ cells are CD8$^-$CD4$^-$ DN (Fig. 7*A*), and the few TCR$^+$ CD8$^+$ T cells present (4×10^6) express low levels of TCR (18). In the DTg males, TCR$^+$ DN cells are practically absent and we recovered 3-fold more peripheral CD8$^+$ T cells (12×10^6), of which most (~80%) express two TCRα chains (Fig. 7*A*) and diminished levels of CD8β (data not shown). In contrast with DTg females (Fig. 1*A*), single Vα2$^+$T3.70$^-$ cells are more abundant (~20%) and Vα2$^-$T3.70$^+$ cells are rare ($\leq$2%). We examined the density of expression and the assembly of the different TCR chains in DTg cells from male mice. Compared with dual TCRα cells from DTg females, the expression of the VαT3.70 TCR chain, though variable, was consistently reduced (compare Figs. 1*A* and

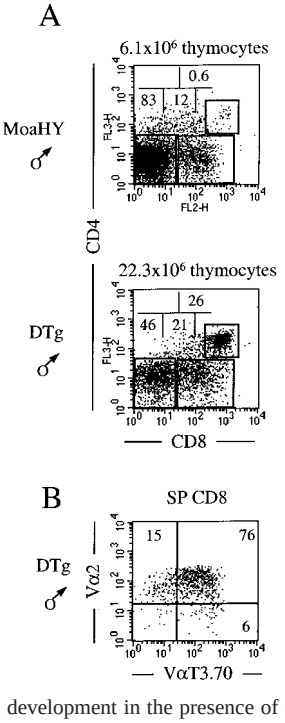

FIGURE 6. T cell development in the presence of the HY male Ag. *A*, Thymocytes from MoaHY and DTg male mice were stained for CD4 and CD8 surface expression. The relative proportions of the different DN, DP, and SP populations are shown in the quadrants inside each dot plot. *B*, The dot plots show the expression of Vα2 and VαT3.70 chains by SP CD8 thymus cells in DTg male mice.

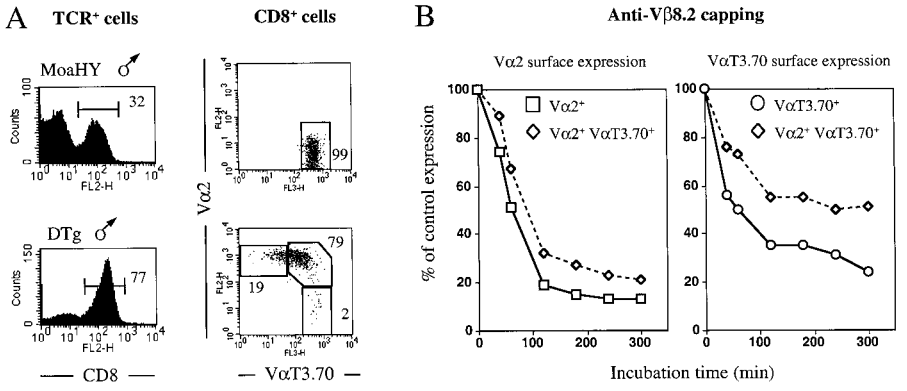

FIGURE 7. Peripheral T cells in MoaHY and MoaHY.MoP14DTg male mice. *A*, Expression of CD8 (histograms) by gated TCR$^+$ LN cells and of Vα2 and VαT3.70 TCR transgenes by gated peripheral LN CD8 T cells (dot plots). *B*, Spleen cells from DTg male mice were incubated for 5 h in the presence of a first anti-Vβ8.2 capping Ab. The expression of the TCRα at different times after incubation was compared with the TCRα expression in absence of the first Ab. The *left graph* shows the kinetics of Vα2 internalization (expressed as a percentage of MFI of control cells) among the Vα2$^+$VαT3.70$^-$ (□) and Vα2$^+$VαT3.70$^+$ (◇) T cells. The *right graph* shows VαT3.70 down-regulation in Vα2$^-$VαT3.70$^+$ (○) and Vα2$^+$VαT3.70$^+$ (◇) T cells.

7*A*). Pre-incubation of dual receptor cells from DTg males with anti-Vβ8.2 Abs fails to internalize 50% of the VαT3.70 chains (Fig. 7*B*). This observation contrasts with the results obtained with dual-receptor cells from DTg females and suggests an increased expression of the P14 Vβ8.1, which associates with the VαT3.70 chains at the surface (Figs. 1*B* and 7*B*). Indeed, the level of the P14 TCRβ Tg mRNA in cells from DTg male mice is higher than in cells from DTg female mice (Fig. 4*A*). Thus, deletion of dual-receptor T cells bearing the aHY TCR is abrogated, allowing the selection of the cells expressing low levels of the self-reactive TCR and higher levels of the second non-self-reactive P14 TCR (15). These findings show that most peripheral CD8$^+$ T cells in DTg males are Vβ8.1$^{+/low}$8.2$^+$Vα2$^+$T3.70^{low}. We conclude that in male DTg mice, the presence of a second TCR transgene prevents deletion of cells expressing a self-reactive receptor. The types and the levels of receptor expression differ from those observed in female mice, indicating a process of adaptation of the population of CD8 T cells determined by their antigenic microenvironment.

Discussion

The role of dual-receptor T cells in autoimmune diseases is dependent on the ability of the dual TCR cells to escape negative selection, to be positively selected in the thymus, and to survive and remain functionally active in the peripheral pools. In the present study, we investigated the thymus selection of dual-receptor CD8 T cells from B6.Rag2$^{-/-}$ mice Tg for two complete TCRs, namely the α- and the β-chains of the anti-LCMV P14 TCR and of the anti-male Ag aHY TCR (18, 20).

In the MoaHY.MoP14 DTg mice, thymus T cell development only gives rise to CD8 T cells, indicating that the association between the different $\alpha\beta$-chains of the two TCR transgenes was not able to generate MHC class II-restricted CD4 T cells. The lack of significant T cell development in MHC class I H-2D$^{b-/-}$ chimeras further demonstrates that the all TCR chain combinations that can be generated from the two transgenes are restricted only to the H-2D^b molecule. These results are not due to the inability of the different TCR chains to associate (29). Indeed, the study of the co-internalization of the TCR chains in CD8 cells from female and male DTg mice shows that the two β-chains and the two α-chains of both transgenes can form $\alpha\beta$ heterodimers in all possible combinations. These observations contrast with previous reports, in which DTg mice using TCR transgenes restricted to MHC class I and MHC class II or to two different MHC class I molecules were shown to express a new MHC specificity (14, 17, 30). The fidelity of the dual MoaHY.MoP14 TCR transgene cells to the H-2D^b restricting element allows comparing the selection of dual- and single-receptor T cells, without the biases that could be introduced by the generation of a new MHC restriction specificity.

In female DTg mice, whereas ~80% of the peripheral CD8 T cells express two TCRα chains, the vast majority of the CD8 T cells only express one TCRβ chain; i.e., they functionally "excluded" one of the TCRβ chain transgenes. Indeed, several findings suggest that, although 99% of the CD8 T cells from DTg mice are Vβ8.2$^+$, most cells either do not express or poorly express the Vβ8.1 chain of the P14 TCR. First, internalization of the Vβ8.2 chain of the aHY TCR transgene leads to complete co-internalization of both Vα chains. Secondly, in vitro stimulation of CD8 T cells from DTg mice with the LCMV gp33-41 peptide failed to induce cell proliferation. Because the peripheral pools of MoP14 mice contain a high number of CD8 T cells, the absence of the P14 β-chain in DTg mice is not due to its poor expression or the inability of cells expressing it to be positively selected. Moreover, in DTg mice a minority (1%) of CD8 T cells are Vβ8$^+$8.2$^-$Vα2$^+$T3.70$^-$; i.e., they express only the P14 Vβ8.1 chain. By comparing the kinetics of expression of the Tg TCR chains during T cell development, we found that, in MoaHY mice, 96% of the CD44$^-$CD25^{int} DN cells already express the TCRβ chain, whereas, in MoP14 mice, expression of the TCRβ chain occurs at a later CD44$^-$CD25$^-$ stage. The different kinetics of TCR expression may relate to the type of vectors used. The aHY transgene contains both the TCRβ chain promoter and enhancer, whereas the P14 transgene uses an MHC class I promoter and an IgH enhancer (19, 20). The presence of the correct TCRβ chain enhancer justifies both the earlier and the higher transcription of the aHY TCRβ chain. By comparing the mRNA species of both β-chains, we observed that the TCRβ chain transcripts in the CD8 T cells from the MoP14 mice are less abundant than in the CD8 T cells from the MoaHY mice. Accordingly, TCRβ chain surface expression was found to be lower in MoP14 CD8 T cells than in MoaHY CD8 T cells. More importantly, in the dual-receptor T cells, the P14 β-chain transcripts are very poorly represented (10% of total). These results suggest that, in dual-receptor T cells, silencing of the P14 β-chain occurs at the mRNA level. The exact mechanisms that regulate the levels of the P14 β-chain transcripts remain unknown. Double-TCR transgenic mice have been previously shown to produce cells with mutual exclusion of the TCR

transgenes posttranscriptionally (31) or which express both receptors equally (32, 33). The effect on P14 TCRβ chain allelic exclusion could be due to the earlier and stronger expression of the aHY β-chain that may affect expression of the second P14 β-chain transgene at the posttranscriptional level. It may reduce the stability and the half-life of the P14 β-chain transcripts. It may compete for limited amounts of pTα or for the CD3 complex required for surface receptor expression. It may also inhibit transcription of the second P14 β-chain transgene. In the latter case, any possible regulatory mechanisms involved in suppressing transcription of the P14 β-chain might act through the IgH enhancer used in the P14 vector. In the rare Vβ8$^+$8.2$^-$Vα2$^+$T3.70$^-$ CD8 T cells, the reverse must occur because these cells express only the P14 Vβ8.1 chain and exclude the aHY β-chain. These effects may simply reflect vagaries of the TCR constructs. However, all these mechanisms are selective for the β-chain transgene, in that they do not affect expression of the P14 TCRα transgene under the control of the same promoter and enhancer (20), suggesting that they may have some physiological relevance. Examples of silencing of one transgene have also been reported in mice that bear two complete rearranged Ig transgenes (34, 35). Overall, these findings indicate that the presence of two productive β-chain rearrangements (6) does not necessarily correlate with double β-chain expression, as shown for the TCRα locus (29). This represents yet another mechanism for functional TCRβ chain exclusion. As a result of this process, most of the CD8 T cells from the DTg mice express one TCRβ associated with two TCRα chains, like ~30% of the peripheral T cells from a normal mouse.

What happens to the development of dual-receptor-expressing T cells in the presence of the male HY self-Ag? Thymus T cell development has been shown to be particularly sensitive to changes in TCR expression levels (17, 36). In male DTg mice, the presence of a second TCR transgene reduces the expression of the aHY TCR, allowing the appearance of significant numbers of DP thymocytes and TCR^{high}CD8$^+$ SP T cells. During the DP to SP transition, only the cells expressing lower levels of CD8 and of the aHY TCR are positively selected. SP cells in the thymus and CD8$^+$ cells at the periphery of DTg males mostly express two Vα chains, as in DTg females, but in contrast to the female mice, single T3.70$^+$ cells are rare and single Vα2$^+$ cells are more abundant. The lack of complete internalization of the TCRα chains after anti-Vβ8.2 treatment indicates that the dual-receptor cells express increased levels of the P14 Vβ8.1 chain. In agreement, P14 β-chain transcripts were more represented in the cells from DTg male mice than in those from DTg females. Overall, the presence of the HY Ag leads to the counterselection of cells bearing high levels of the aHY-specific receptors both in DTg and in MoaHY mice. Alternatively, cells may modulate expression of specific receptors as a function of the levels of Ag encountered in the developing environment.

In contrast with MoaHY males, in which TCR$^+$CD8$^-$ (DN) cells represent 60% of the peripheral TCR$^+$ cell pool (18), in DTg male mice most peripheral TCR$^+$ cells are CD8$^+$. It has been claimed that, in aHY Tg mice, the peripheral DN cells belong to $\gamma\delta$ T cell lineage (37, 38) and that expression of the $\gamma\delta$ TCR receptor could ensure maturation and survival of the cells. Our current observations in Rag2$^{-/-}$ MoaHY mice demonstrate that expression of $\gamma\delta$ TCRs is not required for the survival of the TCR$^+$ DN cells. Overall, these findings suggest that successful lymphocyte survival at the periphery requires optimal levels of signaling. Thus, in male MoaHY the presence of an excess of self-Ag counter selects CD8$^+$ cells and allows the peripheral accumulation and survival of aHY TCR$^+$ T cells, which do not express the CD8 or CD4 coreceptors. In DTg males, in the presence of the same levels of self-Ag, T cells expressing CD8 can accumulate due to the dilution of the aHY TCR. In fact, the few peripheral TCR$^+$ DN cells that survive in DTg male mice express higher levels of the aHY-specific TCR (data not shown).

We show here, by using double-TCR Tg mice, that TCRβ chain usage is tightly controlled and that mechanisms other than classical allelic exclusion can affect expression of a second TCRβ chain transgene. In contrast, control of TCRα chain is loose, allowing the co-expression of two TCRα chain transgenes. As a consequence, the majority of CD8 T cells from the MoaHY.MoP14 DTg female and male mice, like ~30% of the peripheral T cells from a normal mouse (11, 12), express one TCRβ chain associated with two different TCRα chains. Therefore, these DTg mice provide a unique model for studying the in vivo functional behavior of the dual-TCRα CD8 T cells. We examined the thymus selection of the dual-receptor CD8 T cells in the presence of self-Ag. We found that the presence of a second TCRα chain allows a significant number of CD8 T cells expressing a self-reactive receptor to escape central deletion and migrate to the peripheral pools of male mice. Differences in TCR and coreceptor expression between female and male MoaHY and DTg mice suggest that peripheral T cell survival requires an optimal level of signaling, which implies a process of "adaptation" of lymphocyte populations to the host environment.

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