

Constitutive expression of the pre-TCR enables development of mature T cells

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Abstract

Expression and signalling through the pre-TCR and the TCR $\alpha\beta$ resemble two critical checkpoints during T cell development. We investigated to which extent a pre-TCR can functionally replace mature TCR α chains during T cell development. For this purpose, transgenic mice were generated expressing the pre-TCR α (pT α) under the transcriptional control of TCR β regulatory elements. We report here on the interesting finding that constitutive pT α expression allows complete T cell maturation. The pre-TCR complex permits a subset of β -selected thymocytes to mature in the absence of TCR α into peripheral T cells (β T cells) comprising up to 10% of all lymphocytes. Lymphopenia-driven proliferation of these β T cells is similar to that of conventional $\alpha\beta$ T cells. Furthermore, β T cells proliferated and acquired effector function upon stimulation with allogeneic MHC.

Introduction

The formation of a clonally distributed antigen receptor (AgR) repertoire on B and T lymphocytes hallmarks the adaptive immune system. The antigen-binding sites of both B and T cell AgRs are shaped by heterodimerization of the amino-terminal variable (V) domains of Ig heavy and light chains, TCR α and β or TCR γ and δ chains, respectively (1). The ontogeny of B and T cells is characterized by a sequential acquisition of their clonotypic AgR chains (2–5). To compensate for the transient lack of the secondary chain, both B and T cells make use of the so-called surrogate receptor chains to form the pre-BCR and pre-TCR complexes, in which the surrogate light chain (a disulphide-linked heterodimer between a VpreB and $\lambda 5$) or the pre-TCR α (pT α) chain replaces the lacking IgL and TCR α chain, respectively (3, 6–8). Expression of the precursor forms of AgRs is associated with increased survival, rapid clonal expansion, further differentiation and rearrangement of the secondary AgR genes. The subsequent expression of clonotypic AgR complexes and their selection into the mature lymphocyte pool is accompanied by transcriptional shutdown of the surrogate AgR chains.

Regarding $\alpha\beta$ T cells, the stages of intra-thymic development can be traced by their differential expression of CD4, CD8, CD25 and CD44 (9). CD4[−]CD8[−] double-negative (DN) precursors pass successively through four stages defined as CD25[−]44⁺ (DN I), CD25⁺44⁺ (DN II), CD25⁺44[−] (DN III)

and CD25[−]44[−] (DN IV). While rearrangements of D (diversity) and J (joining) segments at the TCR β locus are found in DN I cells already, the vast majority of V (variability) to DJ rearrangements occur in resting DN III cells. If productive, the TCR β chain is disulphide linked to pT α and assembles with pre-formed CD3 components into a pre-TCR–CD3 complex, which appears at very low levels at the cell surface. The pre-TCR signals ligand independently causing the progression of late resting TCR β -negative proT cells of 'expected size' (DN III E) into 'large', cycling pre-T cells (DN III L), a process known as β -selection (10). The term β -selection has been introduced to characterize a critical checkpoint by which only those T cells are selected for further maturation that have undergone successful rearrangement of their TCR β allele (10, 11). DN III L cells initiate VJ rearrangements at the TCR α locus and continue development by down-regulation of CD25 to yield DN IV cells. After a transitional CD4[−]8⁺ immature single-positive (ISP) stage they develop into small resting, CD4⁺8⁺ double-positive (DP) thymocytes. The expansion of DN III L cells accounts for the generation of most $\alpha\beta$ T cell precursors, increasing the efficacy of TCR $\alpha\beta$ repertoire formation and counteracting the substantial cell loss associated with positive and negative selection of DP $\alpha\beta$ thymocytes (4, 12). Further differentiation and selection into mature T cell subsets require a functional VJ rearrangement at the TCR α locus and

expression of a TCR α chain capable of assembling into TCR $\alpha\beta$ -CD3 complexes. The interaction of the clonotypic TCR $\alpha\beta$ with peptide MHC (pMHC) ligands in the thymus determines the outcome for positive and negative selection.

Remarkably, the contribution of the TCR α and β chain in recognizing specific pMHC complexes varies considerably in that it can be largely determined by TCR α , TCR β or both (for review see 13, 14). According to the affinity model, negative selection or neglecton involves clonal elimination of those thymocytes that interact either too strong or too weak with pMHC, respectively (for review see 15). Positive selection is associated with the up-regulation of TCR $\alpha\beta$ /CD3 surface expression levels, transcriptional down-regulation of pT α and reduced expression of the T cell 'immaturity marker' (HSA). The final commitment into MHC class II-restricted CD4⁺8⁻ helper or MHC class I-restricted CD4⁻8⁺ cytotoxic T cell lineage is marked by the shutdown of CD8 or CD4 co-receptor expression and migration into peripheral lymphatic tissues (for review see 16).

Peripheral $\alpha\beta$ T cells derive from intermediate-affinity self-MHC-restricted, positively selected thymocytes, which represent a very small fraction of T cell precursors. Despite the fact that the pre-TCR complex uses the same signalling cascades as the TCR $\alpha\beta$, signals arising from the pre-TCR appear to be ligand independent, i.e. pre-T cell development can proceed normally in the absence of the intra- and extracellular TCR β and pT α domains (17–21) and does not require MHC expression (22).

We here addressed the developmental potential of precursor T cells, constitutively expressing the pT α chain under the transcriptional control elements of the TCR β promoter. Constitutive expression of the pre-TCR permits a subset of β -selected thymocytes to mature, giving rise to peripheral pT α /TCR β /TCR $\alpha^{-/-}$ cells. The developmental and functional potential of this novel population of peripheral T cells from pT α transgenic (tg)/TCR $\alpha^{-/-}$ mice has been addressed and the potential consequences in regard to the complexity of AgR formation are discussed.

Methods

Mice

Mice deficient in TCR α or Rag2 were purchased from Jackson Laboratories. Nude mice were obtained from Bomholtgaard as BLACK-nu and conventional C57Bl/6 mice from Charles River (Wilmington, MA, USA). Mice deficient in pT α were kindly provided by J. Fehling and H. von Boehmer (23). Mice were maintained under specific pathogen-free conditions and used for experiments at 6–8 weeks of age. All animal experiments were performed according to institutional and national guidelines.

Generation of pT α tg mice

A transgene was derived by placing a genomic fragment encompassing exon 2–4 of mouse pT α coding region under the control of TCR β transcription elements. The construct is based on a 20-kb genomic *KpnI* fragment that originates from the 36-kb insert of cosHY β 9-1.14-5 (24) and contains the rearranged HY-TCR β gene including the transcription

elements of the TCR β locus (25). The Δ V-TCR β mutant was derived by deleting most of the VDJ region, which encodes a small NH₂-terminal tag of 12 aa (comprising 6 N-terminal amino acids of V β 8.2 and 6 C-terminal amino acids of J β 2.3) and the complete constant region of TCR C β 2 (26). The Δ V-TCR β transgene was further modified by deleting a genomic 6.1-kb *Bam*HI fragment containing the TCR β constant region (C β 2) and inserting a genomic *Clal*-flanked *Eco*RV/*Hind*III fragment of 3.5 kb containing exon 2–4 of mouse pT α (27) into a unique *Clal* site located just upstream of the deleted *Bam*HI fragment. To enable release of the pT α transgene by *KpnI* digestion, the intronic *KpnI* site within pT α was destroyed. Three independent founder lines were established by microinjecting the 17-kb *KpnI* fragment into fertilized BDF1 (H2-D^b) mouse oocytes. The Δ Vtag allows differential detection of the pT α transgene and products. The genotype of pT α tg mice was determined by PCR, making use of the Δ Vtag-specific primer (CACATGGAGGCTGCAACCACTG) and a reverse pT α primer (CGGAAAGGGGTGCCAGCGATGC). Mice were back-crossed on the C57Bl/6 background for at least six generations.

Forward primer used for reverse transcription (RT)-PCR on pT α hybridizes at start of exon 2 (ATCACACTGCTGGTAGATGGA) and the reverse primer 20-bp downstream of stop codon (TCAGAGGGGTGGGTAAGATC).

Flow cytometry staining

Single cells from thymus, spleen or blood were obtained and RBCs were lysed. Cells were incubated for 15 min on ice with specific antibodies conjugated to FITC, PE, APC or biotin as indicated; biotinylated mAbs were revealed with streptavidin-PerCP. All antibodies were purchased from BD Pharmingen. Surface expression for pT α was performed according to manufacturer's protocol. Intracellular stainings for TCR β , IFN γ and Granzyme B (GrB) were performed with the Cytofix-Cytoperm Kit from Becton Dickinson (Alphen aan den Rijn, The Netherlands). Analysis was performed on a FACS Calibur using Cell Quest Software. Viable cells were gated on the basis of propidium iodide exclusions.

Foetal liver cell reconstitution and thymus transplantation

Foetal liver (14.5 dpc) cell suspensions from individual, genotyped pT α tg/TCR $\alpha^{-/-}$ embryos of pT α tg/TCR $\alpha^{-/-}$ and TCR $\alpha^{-/-}$ time mated mice were injected intravenously into sub-lethally irradiated (4 Gy) 4-week-old nude mice. Eleven weeks after the reconstitution with foetal liver cells, some of the nude mice were analysed for the presence of β T cells. A subset of the reconstituted nude mice received a thymus transplant from 15.5 dpc Rag2-deficient embryos under the kidney capsule. Six weeks after transplantation, the reconstituted and transplanted nude mice were analysed for the presence of β T cells.

Adoptive transfer

β T or $\alpha\beta$ T cells were sorted and labelled with CFSE (Invitrogen, Breda, The Netherlands). Half a million cells were injected intravenously into Rag $^{-/-}$. Seven and 16 days later, lymph nodes (LNs) and spleen were isolated and cells were stained

with anti-CD3-PE and anti-CD90-APC for flow cytometric analysis.

Stimulation of primary $\alpha\beta$ T, $\gamma\delta$ T and β T cells

Cells were sorted on the basis of CD3 ϵ , CD90 and TCR $\gamma\delta$ staining and stimulated for 3 days with either ConA (5 μ g ml⁻¹) or plate-bound anti-CD3 ϵ mAb (50 μ g ml⁻¹, clone 145.2C11). Proliferation was measured by [³H]thymidine incorporation.

Allogenic response

C57Bl/6, pT α tg/TCR $\alpha^{-/-}$ and TCR $\alpha^{-/-}$ mice were challenged intra-peritoneally with three to five million irradiated Balb/c splenocytes (50 Gy) on week 0, 2 and 6. Two weeks after the last boost, splenocytes were labelled with CFSE, and restimulated *in vitro* with irradiated (80 Gy) EL-4 (H-2^b) or P815 (H-2^d) cells at a ratio of 1 responder:10 stimulators in the presence of IL-2 (10 U ml⁻¹). Using the CFSE profile of β T cells from day 1, 3, 4 and 6, proliferation parameters such as responder frequency (the number of initial cells that are responding), burst size (the magnitude of the response) and proliferative capacity (average number of divisions of activated cells) were calculated as described (28). GrB production was determined after 10 days of *in vitro* culture and adding fresh IL-2 on day 3.

Results

Generation of tg mice expressing pT α throughout T cell development

To express the pT α chain constitutively in all T cells, a transgene was derived that places pT α under the transcriptional control elements (25) of the TCR β locus. Three founders were identified by Southern blot and PCR analysis (Fig. 1A and B).

To ensure that the tagged pT α transgene is functional, the transgene was introduced into a pT α -deficient background and T cell development in pT α tg and non-tg pT α -deficient (pT $\alpha^{-/-}$) and proficient mice was analysed. As expected, the pT α transgene compensates for the developmental block that pT $\alpha^{-/-}$ thymocytes encounter at the DN III stage of development (23, 29). The presence of tg pT α restores progression of DN III into DN IV, ISP and DP thymocytes, normalizes the ratio of CD4/CD8 subsets in pT α tg/pT $\alpha^{-/-}$ thymi and increases the cellularity from about 10% in pT $\alpha^{-/-}$ to 50% in pT α tg/pT $\alpha^{-/-}$, relative to wild-type levels (Fig. 1C). The failure of tg pT α to reconstitute the thymus cellularity completely likely relates to a general observation of reduced thymic cellularity in TCR and pT α tg mice (4). In pT $\alpha^{-/-}$ mice, the development of $\gamma\delta$ T cells is favoured. As expected, the pT α transgene restores the development of $\alpha\beta$ T cells and simultaneously reduces the frequency and number of $\gamma\delta$ T cells to wild-type levels. In conclusion, the constitutively expressed pT α transgene is capable of reconstituting β -selection in pT $\alpha^{-/-}$ mice.

Identification of a novel peripheral T cell subset in pT α tg mice

In order to reveal any obvious differences in the composition of peripheral T cell subsets in pT α tg mice compared with

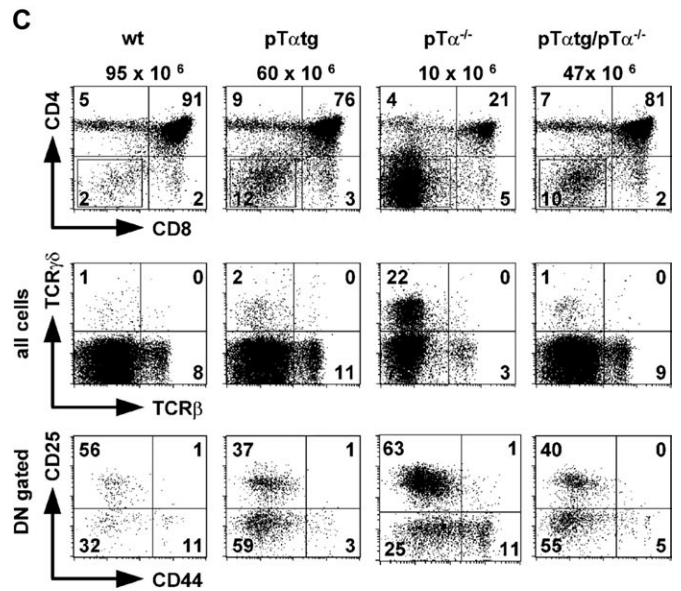
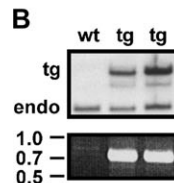
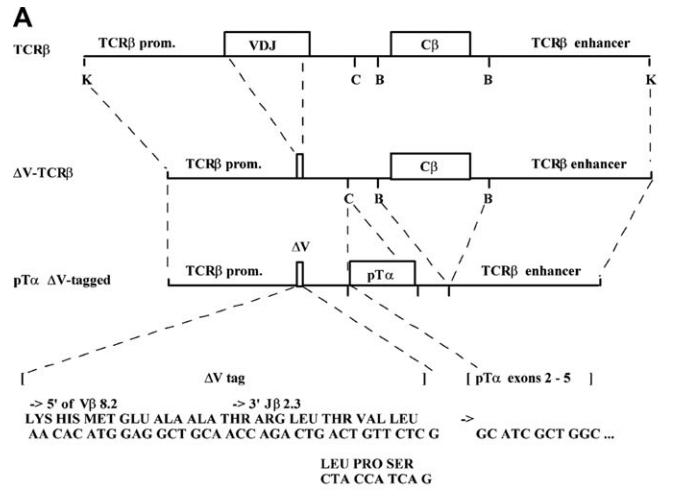


Fig. 1. (A) Derivation of pT α tg mice. Starting point for the derivation of the pT α transgene was a genomic 20-kb *Kpn*I fragment from the rearranged TCR β locus of the HY-specific clone (24). The VDJ region was almost completely deleted (26). The remaining Δ Vtag comprises 17 nucleotides of the V β 8.2 5'-region and 19 nucleotides of the J2.3 3'-region. To express pT α throughout T cell development, the C β 2 region was exchanged by the pT α gene, resulting in a tagged pT α transgene. Specifically, the Δ Vtag of 12 aa replaces the first 3 aa of the processed pT α protein. K = *Kpn*I, C = *Cl*aI and B = *Bam*HI. (B) Screening of pT α tg mice. Potential pT α tg founders were screened by Southern blotting (upper panel) of *Eco*RV digested tail DNA using a J β 2-specific probe as described and PCR analysis (lower panel). Two of three founders are shown. (C) The pT α transgene rescues thymocyte development in pT $\alpha^{-/-}$ mice. Thymocytes from wild-type, pT $\alpha^{-/-}$ and pT α tg/pT $\alpha^{-/-}$ mice were analysed by four-colour flow cytometry. Genotype and absolute cell numbers are displayed above the dot plots. Numbers within the quadrants indicate percentages.

non-tg mice, the T cell subsets of the three independent pT α tg founder lines were analysed by flow cytometry using the T cell markers CD3 ϵ -, CD4-, CD8 α - and CD90.2 (Thy1.2)- specific mAbs. Interestingly, in pT α tg mice, a unique T cell subset expressing high levels of CD90 and low level of CD3 could be identified, that is virtually absent in non-tg littermates. This subset is clearly distinguishable from conventional $\alpha\beta$ T or $\gamma\delta$ T cells expressing high levels of CD3 (Fig. 2A). Within the CD90^{high}CD3^{low} population, the majority of T cells lack CD4 and CD8 co-receptors, 20–40% express the CD8 and 1–3% express CD4. In all three founder lines, the CD90^{high}CD3^{low} population constitutes 2–5% of peripheral lymphocytes and 5–15% of T cells (Fig. 2A). Like the vast majority of mature $\alpha\beta$ T cells, these T cells resemble small lymphocytes that do not express CD25, CD44 or CD69 (data not shown). In conclusion, constitutive expression of pT α allows the development of a unique CD90^{high}CD3^{low} T cell population.

In pT α tg mice, CD90^{high}CD3^{low} T cells constitute 2–5% of peripheral lymphocytes and coexist with conventional $\alpha\beta$ T

cells. Whether their number and development are influenced in *trans* by $\alpha\beta$ T cells or in *cis* by TCR α expression was addressed by crossing the pT α tg onto a TCR α -deficient background. In pT α tg/TCR α ^{-/-} mice, conventional $\alpha\beta$ T cells do not develop. In the absence of TCR α , the frequency of CD90^{high}CD3^{low} cells increases from 2–5% to 8–10% of peripheral lymphocytes (Fig. 2B). The relative increase is accompanied by a 2- to 3-fold increase in their absolute number (from $2 \pm 0.6 \times 10^6$ to $8.7 \pm 1.0 \times 10^6$), which likely relates to a compensatory lymphopenic proliferation due to the absence of $\alpha\beta$ T cells (see below). Besides CD90^{high}CD3^{low} T cells, a CD90^{high}CD3^{high} $\gamma\delta$ T cell population is found.

The CD8 co-receptor is generally expressed as a CD8 $\alpha\alpha$ homodimer on $\gamma\delta$ T cells and as a CD8 $\alpha\beta$ heterodimer on cytotoxic $\alpha\beta$ T cells (30). The CD8⁺ subset of CD90^{high}CD3^{low} T cells expresses only the heterodimeric form of CD8 (Fig. 2B).

In summary, a constitutively expressed pT α chain gives rise to a novel subset of mature CD90^{high}CD3^{low} T cells, but fails to compensate numerically the lack of $\alpha\beta$ T cells in a

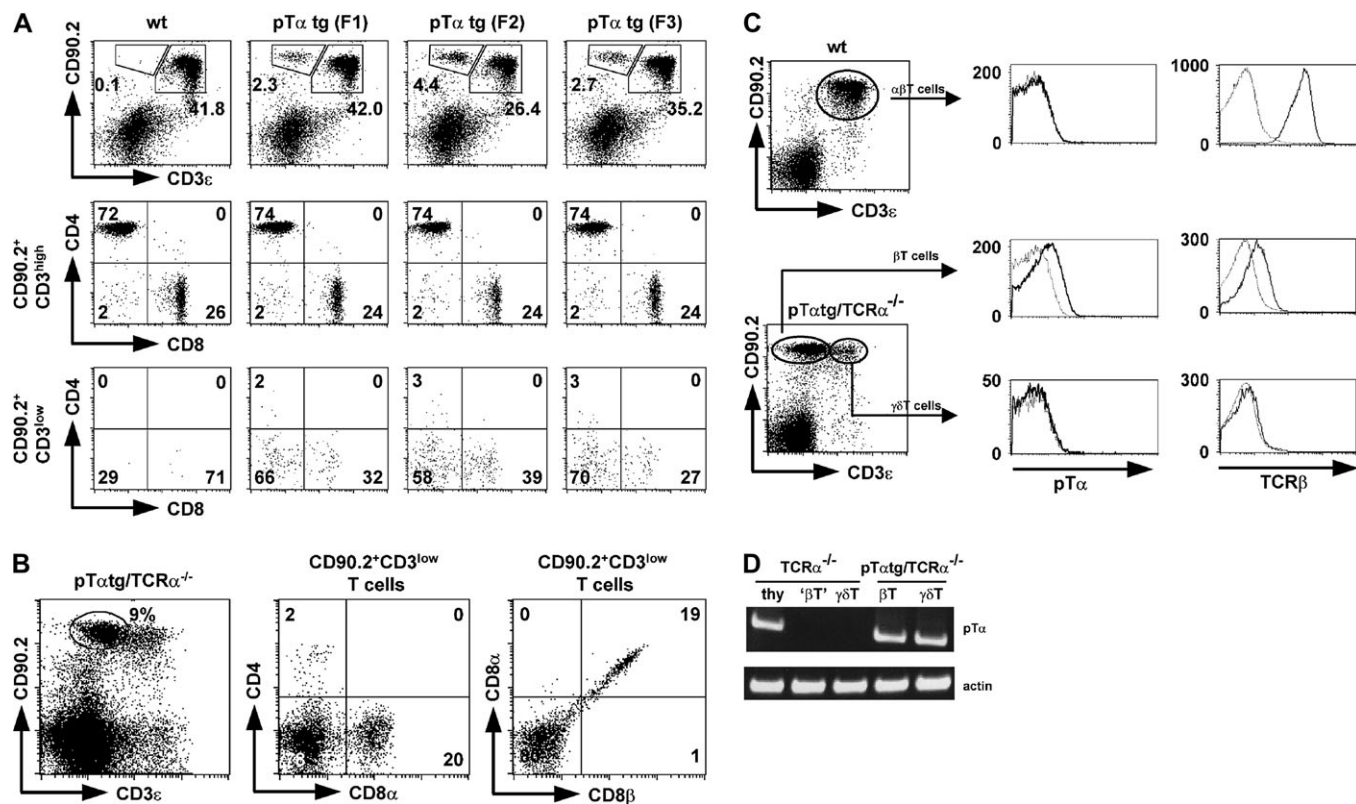


Fig. 2. (A) Identification of a novel T cell subset in pT α tg mice. Four-colour flow cytometry analysis of blood lymphocytes from wild-type mice and independent pT α tg founders. Novel T cells are identified by their low CD3 ϵ (CD3 ϵ ^{low}) and high CD90.2 expression (CD90.2^{high}) and occur at similar frequencies in all founders as indicated in each boxed area (upper panel). Co-receptor expression of conventional $\alpha\beta$ T cells (middle panel) and novel T cells (lower panel) was determined by gating on CD3 ϵ ^{high}CD90.2^{high} and CD3 ϵ ^{low}CD90.2^{high} cells, respectively. (B) CD3 ϵ ^{low}CD90.2^{high} cells develop independently of the TCR α chain and a subset expresses the CD8 $\alpha\beta$ heterodimer. The pT α transgene was introduced onto a TCR α -deficient background and pT α tg/TCR α ^{-/-} mice were analysed for the occurrence of CD3 ϵ ^{low}CD90.2^{high} cells. Co-receptor expression of splenocytes was determined by quadruple staining with CD90.2, CD3 ϵ and either CD4 and CD8 α or CD8 α and CD8 β . The co-receptor expression of gated T cells (CD3 ϵ ^{low}CD90.2^{high}) is shown in the right panels. (C) β T cells express pT α and TCR β on their surface. Splenocytes from pT α tg/TCR α ^{-/-} and wild-type mice were stained with purified pT α (black line) or mouse IgG₁ (isotype control, dashed line) in the presence of mouse Fc block, followed by biotinylated anti-mouse IgG₁ and then SA-PE. Cells were then stained with CD90.2 and CD3 ϵ . For TCR β expression, cells were stained with anti-TCR β (clone H57; black line); non-T cells served as negative control (dashed line). (D) β T cells have endogenous full-length pT α . Total RNA was isolated from thymocytes (thy) and sorted β T and $\gamma\delta$ T from TCR α ^{-/-} as well as β T and $\gamma\delta$ T from pT α tg/TCR α ^{-/-} mice. Same quantity of RNA was used for RT-PCR.

TCR α -deficient background. Based on flow cytometry, these CD90^{high}CD3^{low} T cells express low levels of TCR β and CD3 in a stoichiometric ratio at the cell surface (data not shown). As these CD90^{high}CD3^{low} T cells develop independent of the TCR α chain but express constitutively a β /pT α TCR, they are hereafter referred to as β T cells.

Surface staining showed that these CD90^{high}CD3^{low} β T cells express low levels of TCR β (Fig. 2C). Additionally, surface staining for pT α showed that β T cells express indeed pT α , in contrast to CD90^{high}CD3^{high} $\gamma\delta$ T cells and $\alpha\beta$ T cells from wild-type mice (Fig. 2C). Furthermore, β T cells express full-length pT α as determined by RT-PCR (Fig. 2D) supporting that β T cells require pT α expression. This also excludes the possibility that β T cells express a truncated splice variant of pT α lacking the extracellular domain, as has been shown in TCR β -only cells of TCR α -deficient mice (31). Interestingly, pT α transcripts do not occur in CD90^{high}CD3^{low} cells of TCR α -deficient mice explaining the absence of β T cells in those mice. The detection of tg pT α expression in $\gamma\delta$ T cells is consistent with the TCR β promoter activity in those cells.

β T cells are related to $\alpha\beta$ T cells

To examine whether β T cells are indeed thymus derived, dpc 14.5 foetal liver cells from pT α tg/TCR α ^{-/-} mice were adoptively transferred into sub-lethally irradiated nude mice, which are thymus deficient. Eleven weeks after reconstitution, the mice were analysed for the presence of β T cells (Fig. 3A). The absence of β T cells in the reconstituted nude mice implies their thymic origin. Indeed, when the pT α tg/TCR α ^{-/-} foetal liver reconstitution was followed by transplantation of dpc 15.5 foetal Rag2^{-/-} thymic lobes 6 weeks after transplantation, the peripheral T cell compartments of these mice contained, besides the expected conventional T cells from host-derived bone marrow stem cells, foetal liver stem cell-derived β T cells (Fig. 3A). Thus, the thymus transplant supports the development of β T cells clearly demonstrating the thymic origin of β T cells.

Given the finding that β T cells express low levels of TCR-CD3 complexes, we made use of CD3 and the immaturity marker CD24 to distinguish mature β T cells (CD3^{low}CD24^{low}) from mature $\alpha\beta/\gamma\delta$ thymocytes (CD3^{high}CD24^{low}) and immature thymocytes (CD3^{low}CD24^{high}) (Fig. 3B). In pT α tg mice, a small CD24^{low}CD3^{low} thymic compartment develops that is virtually absent in wild-type and TCR α ^{-/-} mice. This compartment is even more prominent in pT α tg/TCR α ^{-/-} mice. The TCR co-receptor expression pattern of these CD24^{low}CD3^{low} thymocytes is similar to the one found on peripheral β T cells. The low frequency (0.1–0.2%) of mature β T cells in pT α tg as well as pT α tg/TCR α ^{-/-} thymi suggests that the maturation of β T cells underlies rigid selection.

In summary, β T cells develop independently of conventional $\alpha\beta$ T cells and do not require TCR α chain expression. In addition, consistent with the expected requirement of a functionally rearranged TCR β locus, β T cells do not develop in pT α tg/Rag2-deficient mice (data not shown).

Proliferation and survival of β T cells in vivo and in vitro

To further elucidate whether β T cells resemble mature lymphocytes, the proliferative and survival capacity of β T cells was

compared with that of $\alpha\beta$ T cells *in vivo*. A total of 0.5 million sorted β T and $\alpha\beta$ T cells from spleens of pT α tg/TCR α ^{-/-} mice and wild-type mice, respectively, were labelled with CFSE and adoptively transferred into Rag-deficient mice. Seven and 16 days after transfer, peripheral lymphoid compartments of three recipient mice were pooled for the analysis for presence and proliferation of β T cells and $\alpha\beta$ T cells *in vivo* (Fig. 4A). The number and proliferative capacity of β T and $\alpha\beta$ T cells were very similar. The low event number is explained by the low number of transferred cells. Apparently, in this lymphopenic setting, the homeostasis of β T cells is similar to that of $\alpha\beta$ T cells. The fact that only mature T cells undergo lymphopenic proliferation in peripheral organs indicates that β T cells resemble mature T cells.

Besides homeostatic proliferation, the responsiveness of β T cells to polyclonal T cell stimuli was examined *in vitro*. Both $\alpha\beta$ and $\gamma\delta$ T cells are known to proliferate in response to the T cell-specific lectin ConA or to cross-linking of their TCR complexes with coated CD3-specific mAbs. Peripheral β T cells do not proliferate in response to ConA, but mount a normal proliferative response upon cross-linking with CD3-specific mAb (Fig. 4B). The failure of β T cells to respond to ConA suggests differential glycosylation and/or binding/cross-linking capability of ConA to the highly expressed TCR $\alpha\beta$ on T cells compared with the low levels of pre-TCR on β T cells. The fact that peripheral β T cells proliferate rather than die in response to CD3 cross-linking is an additional indication that peripheral β T cells resemble mature T cells.

β T cells respond to allogenic MHC

Apparently, a subset of thymocytes expressing the pT α chain constitutively develops in a thymus-dependent manner into mature β T cells. These β T cells derive from a large pool of DP thymocytes and share many features with conventional $\alpha\beta$ T cells. We further analysed the functional capacity of β T cells. We determined whether H-2^b-derived β T cells are capable of mounting a response to allogenic MHC. pT α tg/TCR α ^{-/-} and wild-type mice (H-2^b) were immunized three times with allogenic splenocytes (Balb/c, H-2^d) and splenocytes were re-stimulated *in vitro* with the tumour cell line P815 (H-2^d) or EL4 (H-2^b) (Fig. 5A). The percentage of cells that had been triggered to divide (responder frequency) and the mean of divisions among those cells that had divided at least once (burst size) were calculated according to Walmsley (28). Consistent with published estimates, the responder frequency of normal mice towards allogenic stimulators is in the range of 1–10% (32). Intriguingly, a subset of β T cells (4%) is capable of responding to allogenic stimulators (Fig. 5B). The CD4⁻CD8⁻, CD4⁺ or CD8⁺ β T cells participate to a similar extent in the allogenic response (data not shown). On average, antigen-reactive β T cells divide 2.4 times and $\alpha\beta$ T cells 3.6 times within 4 days (burst sizes). The proliferative capacity of $\alpha\beta$ T cells compared with β T cells was three times higher (31 versus 9, respectively).

To examine whether β T cells are capable of effector function, the production of GrB was analysed by flow cytometry following 10 days *in vitro* re-stimulation of β T and $\alpha\beta$ T cells with allogenic P815 and syngenic EL4 cells, respectively (Fig. 5C). Interestingly, 61% of CD8⁺ β T cells produce GrB, compared with 48% of CD8⁻ β T cells.

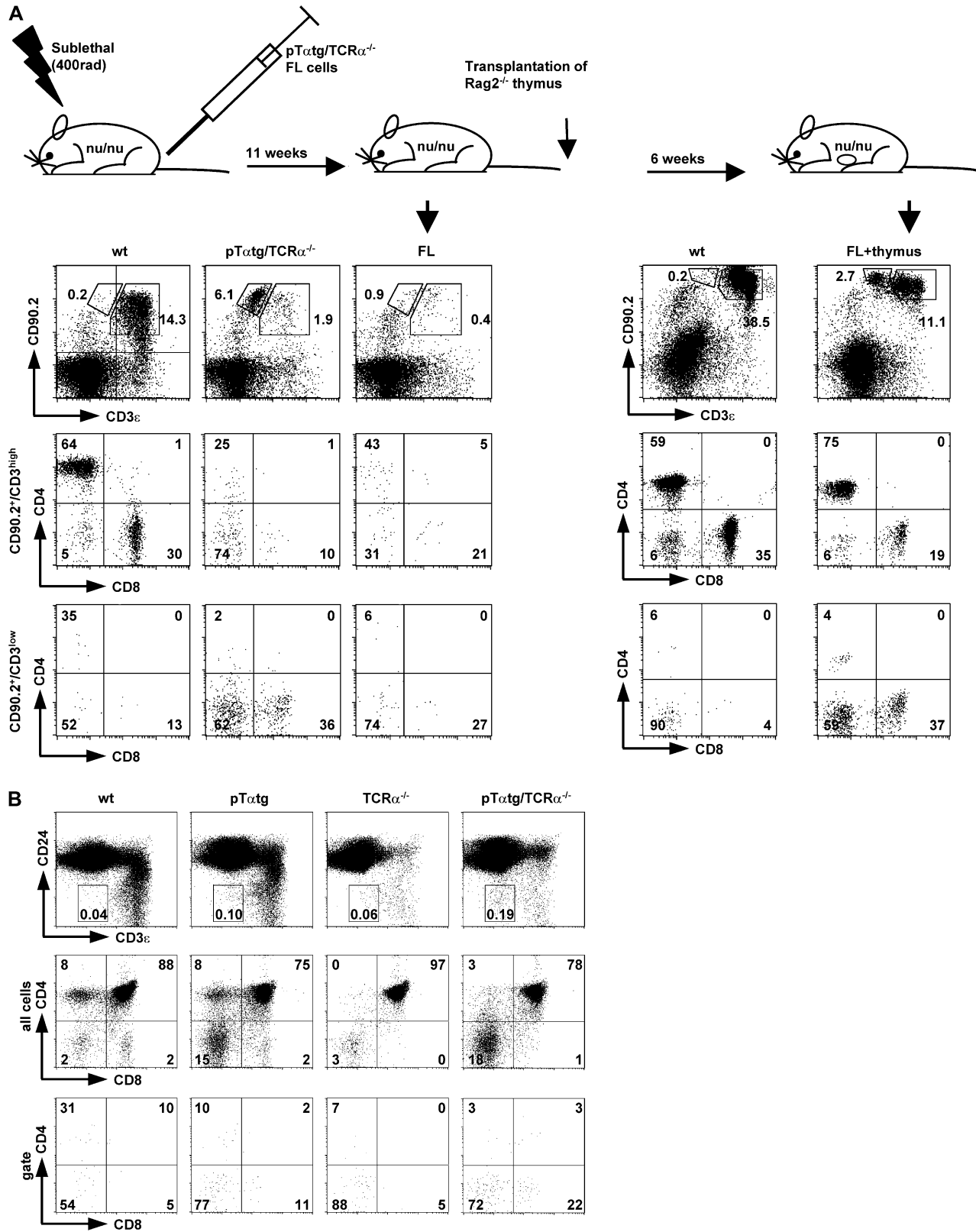


Fig. 3. (A) Development of β T cells is thymus dependent. Foetal liver (FL) cells from $pT\alpha tg/TCR\alpha^{-/-}$ embryos (day 15) were injected intravenously into sub-lethally irradiated nude mice. After 11 weeks, mice were killed to examine the occurrence of peripheral β T cells in the spleen (left part). β T cells do not develop in reconstituted nude mice. However, a thymus transplant from $Rag2^{-/-}$ in nude mice being reconstituted with $pT\alpha tg/TCR\alpha^{-/-}$ foetal liver cells enables the development of β T cells. Analysis of blood is shown after 6 weeks (right part). Co-receptor expression of conventional α β T cells (middle panel) and β T cells (lower panel) was determined by gating on $CD3\epsilon^{high}CD90.2^{high}$ and $CD3\epsilon^{low}CD90.2^{high}$ cells, respectively.

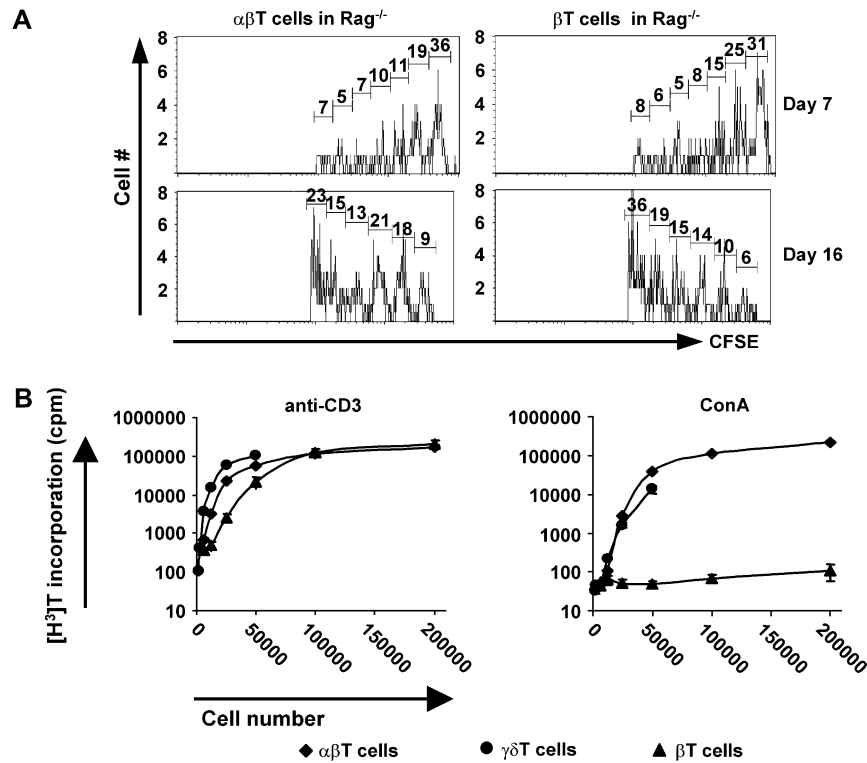


Fig. 4. (A) The homeostatic proliferation of adoptively transferred β T cells is similar to that of $\alpha\beta$ T cells. Half a million β T cells from pT α tg/TCR $\alpha^{-/-}$ and $\alpha\beta$ T cells from wild type were sorted to high purity, labelled with CFSE and injected intravenously into three $Rag^{-/-}$ mice, respectively. After 7 and 16 days, LNs from three pooled mice were analysed for the presence and proliferative capacity of β T cells and $\alpha\beta$ T cells, respectively. Depicted is one out of two independent experiments; percentages of cells in each division are shown. (B) Responsiveness of β T cells to optimal concentrations of polyclonal stimuli. $\alpha\beta$ T cells from wild-type mice and $\gamma\delta$ T and β T cells from pT α tg/TCR $\alpha^{-/-}$ were sorted to high purity and cultured at the indicated densities *in vitro* in the presence of plate-bound anti-CD3 or ConA. After 3 days, cells were pulsed with [3 H]thymidine and incorporation was determined for the cell numbers indicated ($n = 3$). Symbols indicate the T cell subsets.

Discussion

Prior to the acquisition of the second receptor chain, lymphoid precursors express surrogate IgL and pT α chains that allow IgH and TCR β chains to assemble into pre-BCR and pre-TCR complexes, respectively. In terms of structure, composition and signalling function, these precursor forms of AgRs are very similar to AgRs on mature lymphocytes and are critical checkpoints in controlling the development of precursor lymphocytes. Once, expression of the surrogate AgR is achieved, V to J rearrangement at Ig light and TCR α chain gene loci commences, which eventually leads to the replacement of the surrogate chain by Ig κ or λ light chains or the TCR α chain. Thus, the assembly of AgRs is a prerequisite for and parallels lymphocyte development. The final differentiation of bone marrow-derived IgM-expressing B cells and thymus-derived $\alpha\beta$ T cells is associated with a transcriptional termination of VpreB/ λ 5 and pT α chain expression, respectively.

While pT $\alpha^{-/-}$ mice clearly identified a critical function of the pT α chain in early T cell development (23), constitutive sur-

face expression of the pre-TCR is required to address the developmental potential beyond β -selection. Transgenic mice were derived that express pT α under transcriptional control elements of the TCR β locus, in the absence of TCR α chains. In this setup, three possibilities can be envisaged. (i) Maturation of thymocytes does not take place in the absence of the TCR α chain. (ii) Due to constitutive expression and autonomous signalling by the pre-TCR in the absence of ligand binding (17, 33), most thymocytes mature. (iii) Selective pre-TCRs are capable of interaction with MHC molecules, driving maturation of some but not all thymocytes. In the latter case, only a subset of thymocytes is expected to mature and might even be capable of responding to allogenic MHC molecules. Here, we clearly demonstrate that constitutive expression of pT α indeed allows a small fraction of β -selected DP thymocytes to mature into post-thymic, peripheral T cells. The low frequency of mature β T cells in the thymus compared with their DP precursors suggests thymic selection. In addition, β T cells develop independently of TCR α , require expression of TCR β

Left column of each panel shows the experiment group, the other columns are reference populations. One out of three sets of mice is shown. (B) Intra-thymic selection of β T cells. Positive selection of thymocytes is characterized by the up-regulation of CD3 and down-regulation of the T cell immaturity marker CD24 (HSA). To trace mature β T cells (CD3 low CD24 low) intra-thymically, anti-CD3 and anti-CD24 mAbs were used (upper panel). Thymocytes from pT α tg, TCR $\alpha^{-/-}$ and pT α tg/TCR $\alpha^{-/-}$ were stained with anti-CD3e, -CD4, -CD8 and -CD24. CD4 and CD8 expression of all cells (middle panel) and mature β T cells (lower panel) are shown.

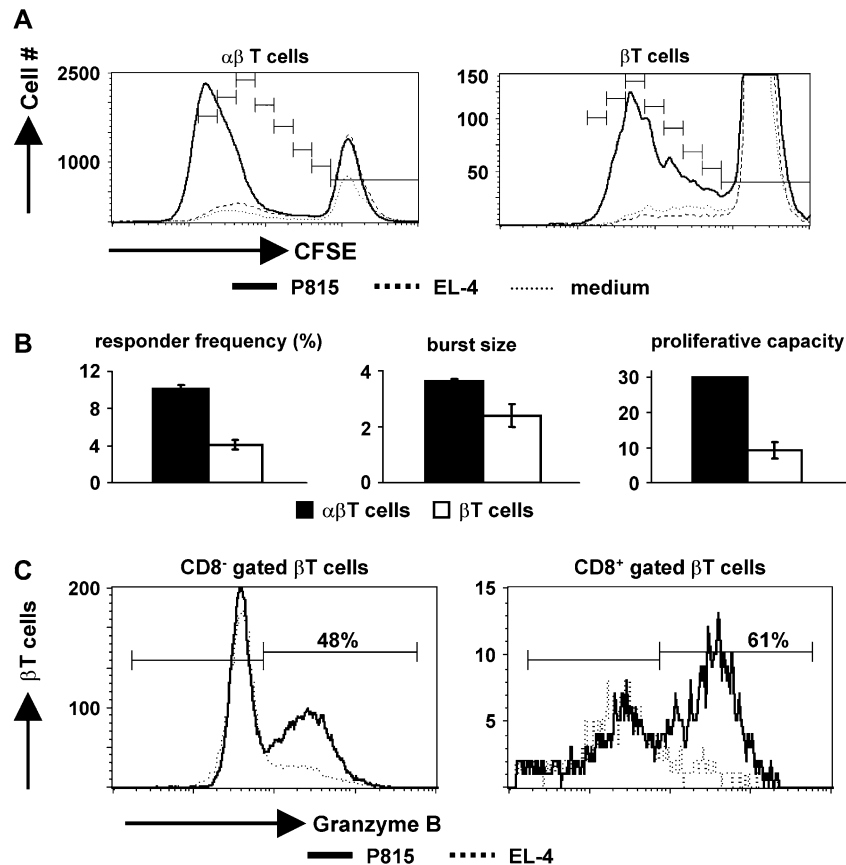


Fig. 5. (A) Allospecificity of β T cells. Respectively, three mice of wild type and pT α tg/TCR $\alpha^{-/-}$ were intra-peritoneally immunized with irradiated Balb/c splenocytes (H-2^d) on week 0, 2 and 6. Two weeks later, splenocytes were CFSE labelled and re-stimulated for 3 days *in vitro* with the syngenic EL-4 (H-2^b) tumour cell line or the allogeneic P815 (H-2^d) tumour cell line. Proliferation of gated β T cells and gated $\alpha\beta$ T cells was analysed by flow cytometry. Shown is one representative mouse out of two independent experiments. (B) Calculations of proliferative parameters. Responder frequency, burst size and proliferative capacity of antigen-specific $\alpha\beta$ T and β T cells from three mice were calculated as described previously (28). (C) Functional analysis of β T cells. Intracellular GrB staining of β T cells stimulated with allogeneic or syngenic cells are depicted. Either CD8⁻ (left) or CD8⁺ (right) are shown independently. Only upon stimulation with allogeneic cells, β T cells can produce GrB. Numbers above the margins indicate percentages of β T cells that respond to allogeneic MHC.

and differentiate intra-thymically into co-receptor-positive (CD8 $\alpha\beta$ ⁺ or CD4⁺) and -negative (CD4⁻8⁻) compartments. The increased frequency of mature β T cells in TCR α -deficient thymi most likely relates to the absence of competition of pT α and TCR α chains for dimerization with the TCR β chain. The fact that 0.2% thymic β T cells generate up to 10% of all peripheral lymphocytes and the expansion of adoptively transferred β T cells in Rag-deficient recipients strongly supports the idea that mature β T cells like conventional $\alpha\beta$ T cells can undergo homeostatic proliferation.

The pT α /TCR β -expressing thymocytes develop into CD8⁺ and some into CD4⁺ co-receptor-positive cells indicating that lineage commitment does not necessitate expression of TCR α chains. That only 1–3% of β T cells express the CD4 co-receptor suggests that pre-TCR signals do not deliver the sustained signalling thought to be required for CD4 SP differentiation (for review see 16). The V β usage of β T cells is comparable to $\alpha\beta$ T cells, suggesting that all segments can promote the development of β T cells (data not shown). No reaction was observed for β T cells in a mixed primary lymphocyte reaction of β T cells with allogeneic irradiated

Balb/c splenocytes. Yet, MHC recognition by β T cells is suggested by the ability of β T cells to respond to allogeneic MHC in a recall response, although we cannot exclude that allorecognition is due to another receptor than the pre-TCR at present. Subsequent experiments are required to address whether the pre-TCR is the relevant antigen recognition unit. These observations contrast a similar study in which CD8⁺/CD3^{ow} cells developed when a pT α transgene was placed under the proximal Ick promoter (34). Based on northern blotting from total non-sorted LN tissue, these CD8⁺ cells were interpreted to be pT α negative and capable of developing normally in an MHC-deficient environment, suggesting that recognition of MHC antigens by the pre-TCR is not required for thymic selection. Either our β T cells (Fig. 2C) differ from the Ick-driven pT α tg T cells with regard to pre-TCR expression (34), or alternatively, the pre-TCR is expressed and non-classical MHC molecules or leaky MHC class I heavy chains support the development of CD8⁺ cells. Therefore, constitutive ligand-independent tickling by the pre-TCR is quite unlikely to explain the inefficient intra-thymic maturation of β T cells, as has been previously suggested (34). Our observations that β T cells do

express the pre-TCR (Fig. 2C), resemble small resting T cells lacking expression of activation markers and seem to be capable of alloresponse (Fig. 5) are in line with the maturation of a very small subset of β -selected thymocytes into mature small resting β T cells.

Two opposing evolutionary scenarios could explain the existence of pT α : (i) the pT α chain evolved after TCR $\alpha\beta$ to fulfil its function in β -selection (35) and (ii) prior to the evolution of TCR α , the pT α /TCR β heterodimer resembled an immunocompetent AgR. Two predictions can be derived from the latter scenario. First, the pre-TCR should be able to mediate cognate antigen recognition of self-MHC molecules. Here, the pre-TCR antigen recognition would largely be determined by the TCR β chain. Second, precursor T lymphocytes expressing the pre-TCR constitutively throughout development would undergo intra-thymic selection and maturation. Our data are in favour of the second scenario.

In addition, since AgRs are generated by somatic recombination of two complex gene loci, it is very unlikely that they have evolved simultaneously. Based on these considerations, we would like to propose an alternative model, in which the sequential rearrangement of AgR chains during lymphocyte development resembles a time lapse of lymphocyte and AgR evolution. In this scenario, the development of lymphocytes from lymphoid progenitors might recapitulate the stepwise acquisition of complex AgR gene loci during evolution. Accordingly, the maintenance of the non-rearranging surrogate receptor chain genes throughout evolution reflects the necessity of lymphocytes to receive AgR signals that warrant survival at each stage of their development (2, 4, 5, 36, 37).

Crystallographic analysis of TCR $\alpha\beta$ -pMHC complexes have revealed that specific recognition of the pMHC complex by the TCR $\alpha\beta$ can be largely determined by the V α domain, the V β domain or both (13, 14, 38, 39). These data indicate that a single V β domain is principally capable of pMHC recognition. We show here that indeed the V β domain can be sufficient to allow T cell development in the absence of the TCR α chain generating immunocompetent β T cells. We speculate that with the evolution of the TCR α locus, the pT α locus got modified such that it fulfils a function in β -selection only. During this process, it might have been important to delay thymic selection and delete TCR V α -like domains within the pre-TCR. This will prevent that $\alpha\beta$ T cell precursors are selected twice, first on the basis of the pre-TCR specificity (which is not relevant) and second on the basis of the specificity of the clonotypic TCR $\alpha\beta$ (which is relevant).

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Abbreviations

AgR antigen receptor
DN double negative

DP double positive
GrB Granzyme B
ISP immature single positive
LN lymph node
pMHC peptide MHC
pT α pre-TCR α
RT reverse transcription
tg transgenic

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