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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 TCRαβ resemble two critical checkpoints during T cell development. We investigated to which extent a pre-TCR can functionally replace mature TCRα chains during T cell development. For this purpose, transgenic mice were generated expressing the pre-TCRα (pTα) under the transcriptional control of TCRβ regulatory elements. We report here on the interesting finding that constitutive pTα expression allows complete T cell maturation. The pre-TCR complex permits a subset of β-selected thymocytes to mature in the absence of TCRα into peripheral T cells (βT cells) comprising up to 10% of all lymphocytes. Lymphopenia-driven proliferation of these βT cells is similar to that of conventional αβT cells. Furthermore, βT 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, TCRα and β or TCRγ 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 λ5) or the pre-TCRα (pTα) chain replaces the lacking IgL and TCRα 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 αβT 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 TCRβ 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 TCRβ chain is disulphide linked to pTα 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 TCRβ-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 TCRβ allele (10, 11). DN III L cells initiate VJ rearrangements at the TCRα 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 αβT cell precursors, increasing the efficacy of TCRαβ repertoire formation and counteracting the substantial cell loss associated with positive and negative selection of DP αβ thymocytes (4, 12). Further differentiation and selection into mature T cell subsets require a functional VJ rearrangement at the TCRα locus and

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expression of a TCRα chain capable of assembling into TCRαβ–CD3 complexes. The interaction of the clonotypic TCRαβ with peptide MHC (pMHC) ligands in the thymus determines the outcome for positive and negative selection.

Remarkably, the contribution of the TCRα and β chain in recognizing specific pMHC complexes varies considerably in that it can be largely determined by TCRα, TCRβ or both (for review see 13, 14). According to the affinity model, negative selection or neglection 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 TCRαβ/CD3 surface expression levels, transcriptional down-regulation of pTα 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 αβT 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 TCRαβ, 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 TCRβ and pTα domains (17–21) and does not require MHC expression (22).

We here addressed the developmental potential of precursor T cells, constitutively expressing the pTα chain under the transcriptional control elements of the TCRβ promotor. Constitutive expression of the pre-TCR permits a subset of β-selected thymocytes to mature, giving rise to peripheral pTα/TCRβ/$TCR\alpha^{-/-}$ cells. The developmental and functional potential of this novel population of peripheral T cells from pTα 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 TCRα or Rag2 were purchased from Jackson Laboratories. Nude mice were obtained from Bomholtgaard as BLACK-nu and conventional C57Bl/6 mice from Charles River (Wilmingtion, MA, USA). Mice deficient in pTα 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 pTα tg mice

A transgene was derived by placing a genomic fragment encompassing exon 2–4 of mouse pTα coding region under the control of TCRβ transcription elements. The construct is based on a 20-kb genomic *Kpn*I fragment that originates from the 36-kb insert of cosHYβ9-1.14-5 (24) and contains the rearranged HY-TCRβ gene including the transcription elements of the TCRβ locus (25). The ΔV-TCRβ mutant was derived by deleting most of the VDJ region, which encodes a small NH_2-terminal tag of 12 aa (comprising 6 N-terminal amino acids of Vβ8.2 and 6 C-terminal amino acids of Jβ2.3) and the complete constant region of TCR Cβ2 (26). The ΔV-TCRβ transgene was further modified by deleting a genomic 6.1-kb *Bam*HI fragment containing the TCRβ constant region (Cβ2) and inserting a genomic *Cla*I-flanked *Eco*RV/*Hin*dIII fragment of 3.5 kb containing exon 2–4 of mouse pTα (27) into a unique *Cla*I site located just upstream of the deleted *Bam*HI fragment. To enable release of the pTα transgene by *Kpn*I digestion, the intronic *Kpn*I site within pTα was destroyed. Three independent founder lines were established by microinjecting the 17-kb *Kpn*I fragment into fertilized BDF1 ($H2\text{-}D^b$) mouse oocytes. The ΔVtag allows differential detection of the pTα transgene and products. The genotype of pTα tg mice was determined by PCR, making use of the ΔVtag-specific primer (CACATGGAGGCTGCAACCAGACTG) and a reverse pTα primer (CGGAAAGGGGGTGCCAGCGATGC). Mice were back-crossed on the C57Bl/6 background for at least six generations.

Forward primer used for reverse transcription (RT)–PCR on pTα 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 pTα was performed according to manufacturer's protocol. Intracellular stainings for TCRβ, IFNγ 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 pTα tg/$TCR\alpha^{-/-}$ embryos of pTα 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 βT 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 βT cells.

Adoptive transfer

βT or αβT 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 αβT, γδT and βT cells

Cells were sorted on the basis of CD3ε, CD90 and TCRγδ staining and stimulated for 3 days with either ConA (5 μg ml^{-1}) or plate-bound anti-CD3ε mAb (50 μg ml^{-1}, clone 145.2C11). Proliferation was measured by [^{3}H]thymidine incorporation.

Allogenic response

C57Bl/6, pTα tg/TCRα$^{-/-}$ and TCRα$^{-/-}$ 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 re-stimulated *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 βT 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 pTα throughout T cell development

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

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

Identification of a novel peripheral T cell subset in pTα tg mice

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

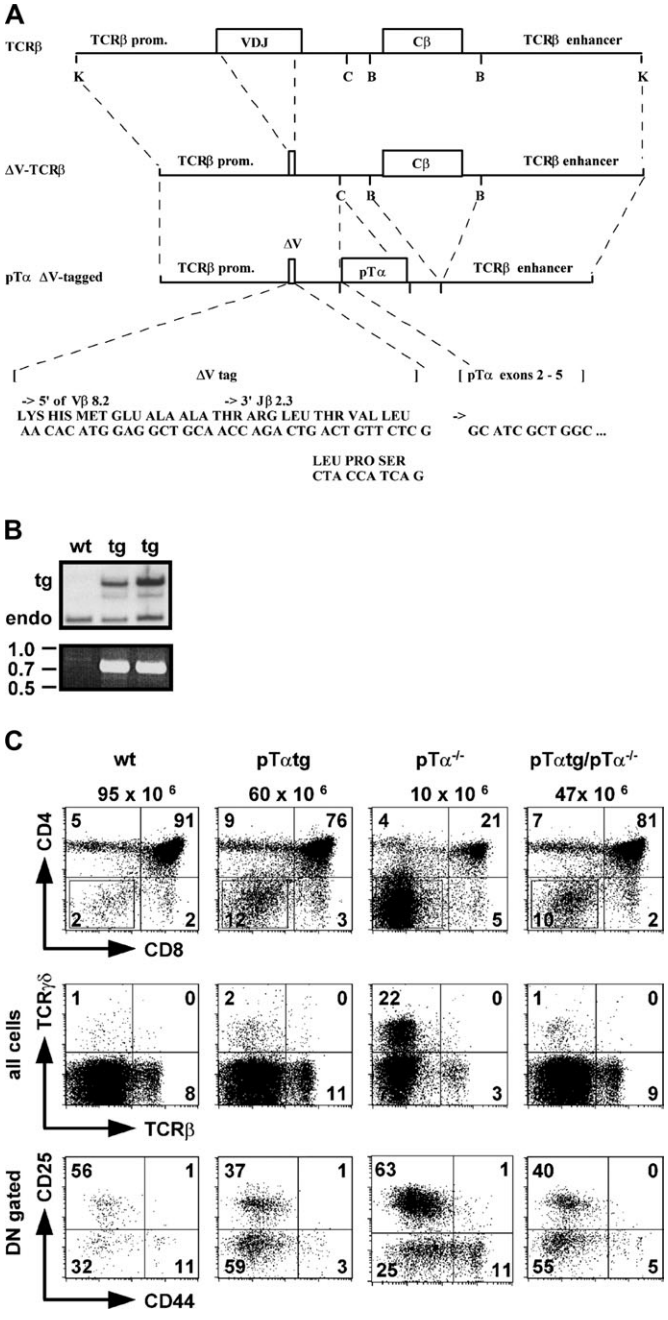

Fig.1. (A) Derivation of pTα tg mice. Starting point for the derivation of the pTα transgene was a genomic 20-kb *Kpn*I fragment from the rearranged TCRβ locus of the HY-specific clone (24). The VDJ region was almost completely deleted (26). The remaining ΔVtag comprises 17 nucleotides of the Vβ8.2 5′-region and 19 nucleotides of the J2.3 3′-region. To express pTα throughout T cell development, the Cβ2 region was exchanged by the pTα gene, resulting in a tagged pTα transgene. Specifically, the ΔVtag of 12 aa replaces the first 3 aa of the processed pTα protein. K = *Kpn*I, C = *Cla*I and B = *Bam*HI. (B) Screening of pTα tg mice. Potential pTα tg founders were screened by Southern blotting (upper panel) of *Eco*RV digested tail DNA using a Jβ2-specific probe as described and PCR analysis (lower panel). Two of three founders are shown. (C) The pTα transgene rescues thymocyte development in pTα$^{-/-}$ mice. Thymocytes from wild-type, pTα$^{-/-}$ and pTα tg/pTα$^{-/-}$ 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 pTα tg founder lines were analysed by flow cytometry using the T cell markers CD3ε-, CD4-, CD8α- and CD90.2 (Thy1.2)- specific mAbs. Interestingly, in pTα 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 αβT or γδT 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 αβT cells, these T cells resemble small lymphocytes that do not express CD25, CD44 or CD69 (data not shown). In conclusion, constitutive expression of pTα allows the development of a unique $CD90^{high}CD3^{low}$ T cell population.

In pTα tg mice, $CD90^{high}CD3^{low}$ T cells constitute 2–5% of peripheral lymphocytes and coexist with conventional αβT cells. Whether their number and development are influenced in *trans* by αβT cells or in *cis* by TCRα expression was addressed by crossing the pTα tg onto a TCRα-deficient background. In pTα tg/$TCR\alpha^{-/-}$ mice, conventional αβT cells do not develop. In the absence of TCRα, 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 αβT cells (see below). Besides $CD90^{high}CD3^{low}$ T cells, a $CD90^{high}CD3^{high}$ γδT cell population is found.

The CD8 co-receptor is generally expressed as a CD8αα homodimer on γδT cells and as a CD8αβ heterodimer on cytotoxic αβT 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 pTα chain gives rise to a novel subset of mature $CD90^{high}CD3^{low}$ T cells, but fails to compensate numerically the lack of αβT cells in a

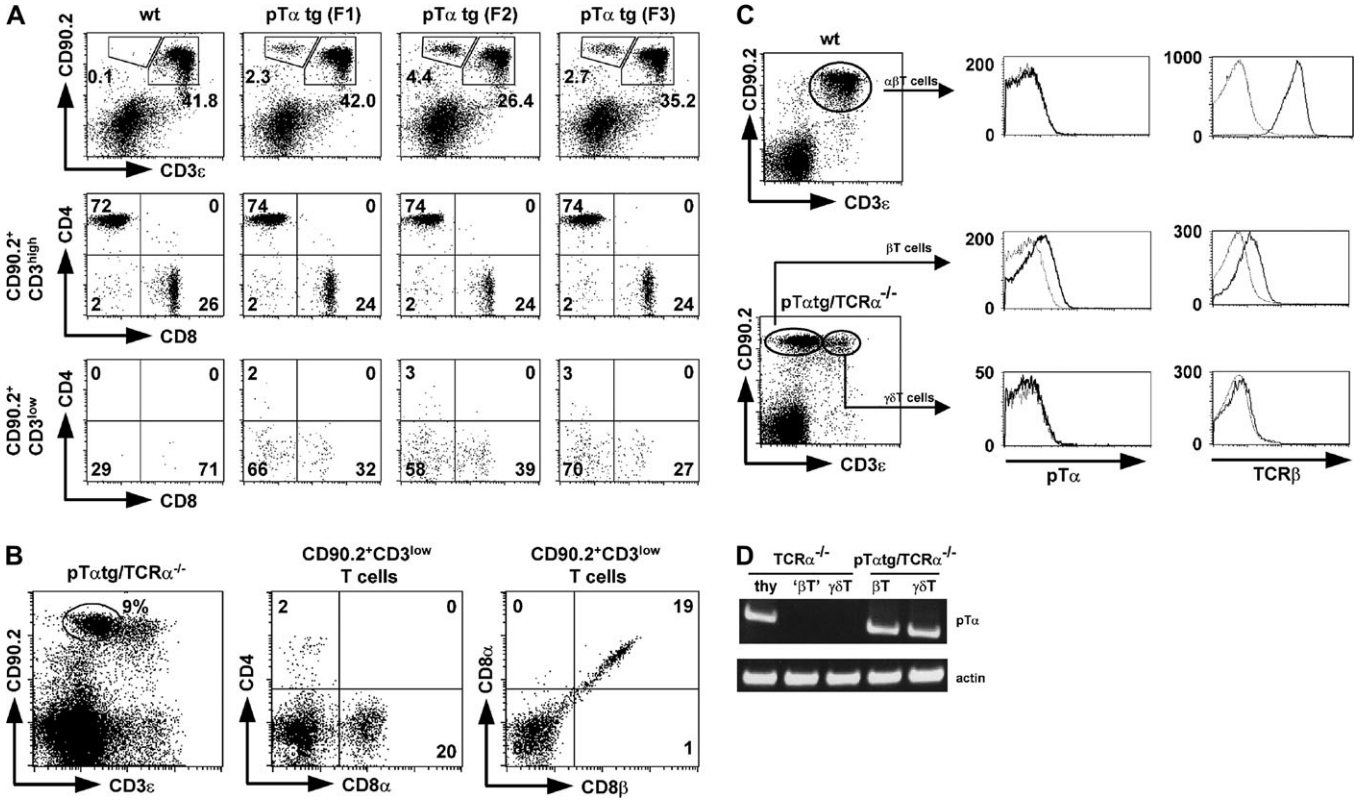

Fig. 2. (A) Identification of a novel T cell subset in pTα tg mice. Four-colour flow cytometry analysis of blood lymphocytes from wild-type mice and independent pTα tg founders. Novel T cells are identified by their low CD3ε ($CD3\varepsilon^{low}$) and high CD90.2 expression ($CD90.2^{high}$) and occur at similar frequencies in all founders as indicated in each boxed area (upper panel). Co-receptor expression of conventional αβT cells (middle panel) and novel T cells (lower panel) was determined by gating on $CD3\varepsilon^{high}CD90.2^{high}$ and $CD3\varepsilon^{low}CD90.2^{high}$ cells, respectively. (B) $CD3\varepsilon^{low}CD90.2^{high}$ cells develop independently of the TCRα chain and a subset expresses the CD8αβ heterodimer. The pTα transgene was introduced onto a TCRα-deficient background and pTα tg/$TCR\alpha^{-/-}$ mice were analysed for the occurrence of $CD3\varepsilon^{low}CD90.2^{high}$ cells. Co-receptor expression of splenocytes was determined by quadruple staining with CD90.2, CD3ε and either CD4 and CD8α or CD8α and CD8β. The co-receptor expression of gated T cells ($CD3\varepsilon^{low}CD90.2^{high}$) is shown in the right panels.(C) βT cells express pTα and TCRβ on their surface. Splenocytes from pTα tg/$TCR\alpha^{-/-}$ and wild-type mice were stained with purified pTα (black line) or mouse IgG_1 (isotype control, dashed line) in the presence of mouse Fc block, followed by biotinylated anti-mouse IgG_1 and then SA–PE. Cells were then stained with CD90.2 and CD3ε. For TCRβ expression, cells were stained with anti-TCRβ (clone H57; black line); non-T cells served as negative control (dashed line). (D) βT cells have endogenous full-length pTα. Total RNA was isolated from thymocytes (thy) and sorted 'βT' and γδT cells from $TCR\alpha^{-/-}$ as well as βT and γδT from pTα tg/$TCR\alpha^{-/-}$ mice. Same quantity of RNA was used for RT–PCR.

TCRα-deficient background. Based on flow cytometry, these CD90^{high}CD3^{low} T cells express low levels of TCRβ 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 TCRα chain but express constitutively a β/pTα TCR, they are hereafter referred to as βT cells.

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

βT cells are related to αβT cells

To examine whether βT cells are indeed thymus derived, dpc 14.5 foetal liver cells from pTα tg/TCRα$^{-/-}$ 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 βT cells (Fig. 3A). The absence of βT cells in the reconstituted nude mice implies their thymic origin. Indeed, when the pTα tg/TCRα$^{-/-}$ 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 βT cells (Fig. 3A). Thus, the thymus transplant supports the development of βT cells clearly demonstrating the thymic origin of βT cells.

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

In summary, βT cells develop independently of conventional αβT cells and do not require TCRα chain expression. In addition, consistent with the expected requirement of a functionally rearranged TCRβ locus, βT cells do not develop in pTα tg/Rag2-deficient mice (data not shown).

Proliferation and survival of βT cells in vivo and in vitro

To further elucidate whether βT cells resemble mature lymphocytes, the proliferative and survival capacity of βT cells was compared with that of αβT cells *in vivo*. A total of 0.5 million sorted βT and αβT cells from spleens of pTα tg/TCRα$^{-/-}$ 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 βT cells and αβT cells *in vivo* (Fig. 4A). The number and proliferative capacity of βT and αβT 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 βT cells is similar to that of αβT cells. The fact that only mature T cells undergo lymphopenic proliferation in peripheral organs indicates that βT cells resemble mature T cells.

Besides homeostatic proliferation, the responsiveness of βT cells to polyclonal T cell stimuli was examined *in vitro*. Both αβ and γδ 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 βT 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 βT cells to respond to ConA suggests differential glycosylation and/or binding/cross-linking capability of ConA to the highly expressed TCRαβ on T cells compared with the low levels of pre-TCR on βT cells. The fact that peripheral βT cells proliferate rather than die in response to CD3 cross-linking is an additional indication that peripheral βT cells resemble mature T cells.

βT cells respond to allogenic MHC

Apparently, a subset of thymocytes expressing the pTα chain constitutively develops in a thymus-dependent manner into mature βT cells. These βT cells derive from a large pool of DP thymocytes and share many features with conventional αβT cells. We further analysed the functional capacity of βT cells. We determined whether H-2^{b}-derived βT cells are capable of mounting a response to allogenic MHC. pTα tg/TCRα$^{-/-}$ 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 βT cells (4%) is capable of responding to allogenic stimulators (Fig. 5B). The CD4^{-}CD8^{-}, CD4^{+} or CD8^{+} βT cells participate to a similar extent in the allogenic response (data not shown). On average, antigen-reactive βT cells divide 2.4 times and αβT cells 3.6 times within 4 days (burst sizes). The proliferative capacity of αβT cells compared with βT cells was three times higher (31 versus 9, respectively).

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

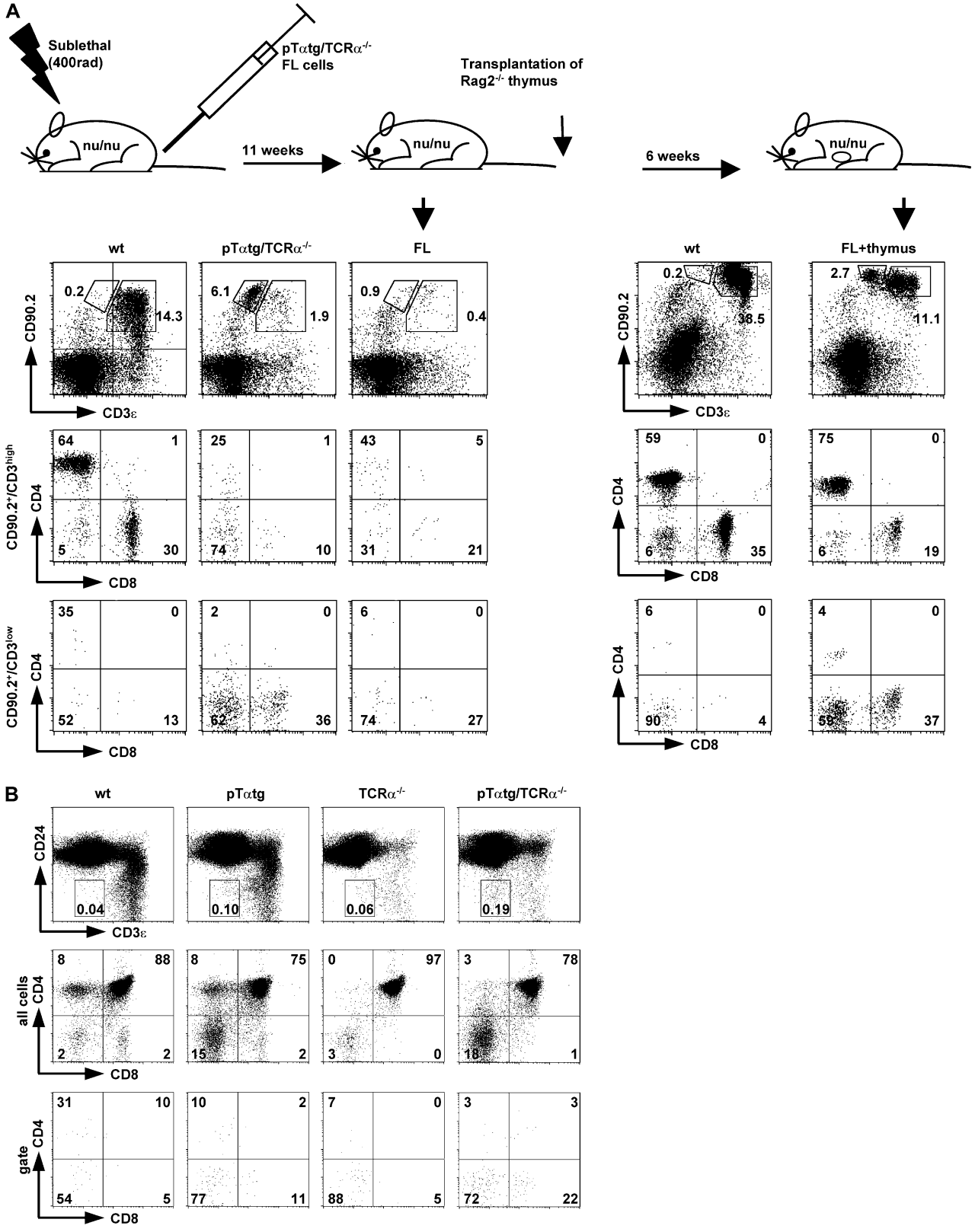

Fig. 3. (A) Development of βT cells is thymus dependent. Foetal liver (FL) cells from pTα tg/TCRα$^{-/-}$ embryos (day 15) were injected intravenously into sub-lethally irradiated nude mice. After 11 weeks, mice were killed to examine the occurrence of peripheral βT cells in the spleen (left part). βT cells do not develop in reconstituted nude mice. However, a thymus transplant from Rag2$^{-/-}$ in nude mice being reconstituted with pTα tg/TCRα$^{-/-}$ foetal liver cells enables the development of βT cells. Analysis of blood is shown after 6 weeks (right part). Co-receptor expression of conventional αβT cells (middle panel) and βT cells (lower panel) was determined by gating on CD3ε^{high}CD90.2^{high} and CD3ε^{low}CD90.2^{high} cells, respectively.

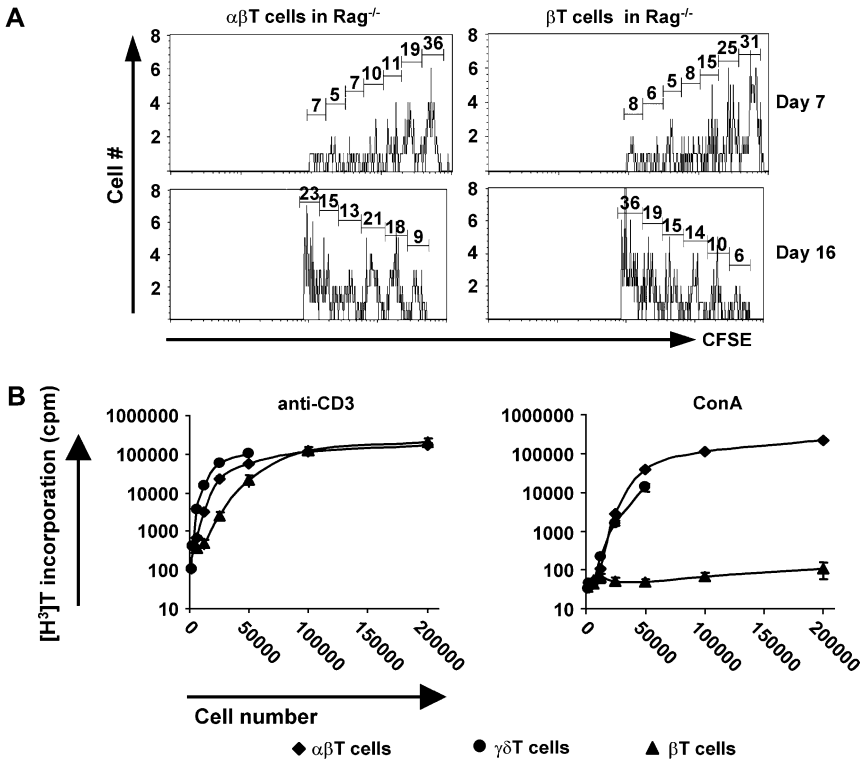

Fig. 4. (A) The homeostatic proliferation of adoptively transferred βT cells is similar to that of αβT cells. Half a million βT cells from pTα tg/TCRα$^{-/-}$ and αβT cells from wild type were sorted to high purity, labelled with CFSE and injected intravenously into three Rag$^{-/-}$ mice, respectively. After 7 and 16 days, LNs from three pooled mice were analysed for the presence and proliferative capacity of βT cells and αβT cells, respectively. Depicted is one out of two independent experiments; percentages of cells in each division are shown. (B) Responsiveness of βT cells to optimal concentrations of polyclonal stimuli. αβT cells from wild-type mice and γδT and βT cells from pTα tg/TCRα$^{-/-}$ 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 pTα chains that allow IgH and TCRβ 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 TCRα chain gene loci commences, which eventually leads to the replacement of the surrogate chain by Igκ or λ light chains or the TCRα 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 αβT cells is associated with a transcriptional termination of VpreB/λ5 and pTα chain expression, respectively.

While pTα$^{-/-}$ mice clearly identified a critical function of the pTα chain in early T cell development (23), constitutive surface expression of the pre-TCR is required to address the developmental potential beyond β-selection. Transgenic mice were derived that express pTα under transcriptional control elements of the TCRβ locus, in the absence of TCRα chains. In this setup, three possibilities can be envisaged. (i) Maturation of thymocytes does not take place in the absence of the TCRα 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 pTα indeed allows a small fraction of β-selected DP thymocytes to mature into post-thymic, peripheral T cells. The low frequency of mature βT cells in the thymus compared with their DP precursors suggests thymic selection. In addition, βT cells develop independently of TCRα, require expression of TCRβ

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 βT 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 βT cells (CD3ε^{low}CD24^{low}) intra-thymically, anti-CD3 and anti-CD24 mAbs were used (upper panel). Thymocytes from pTα tg, TCRα$^{-/-}$ and pTα tg/TCRα$^{-/-}$ were stained with anti-CD3ε, -CD4, -CD8 and -CD24. CD4 and CD8 expression of all cells (middle panel) and mature βT cells (lower panel) are shown.

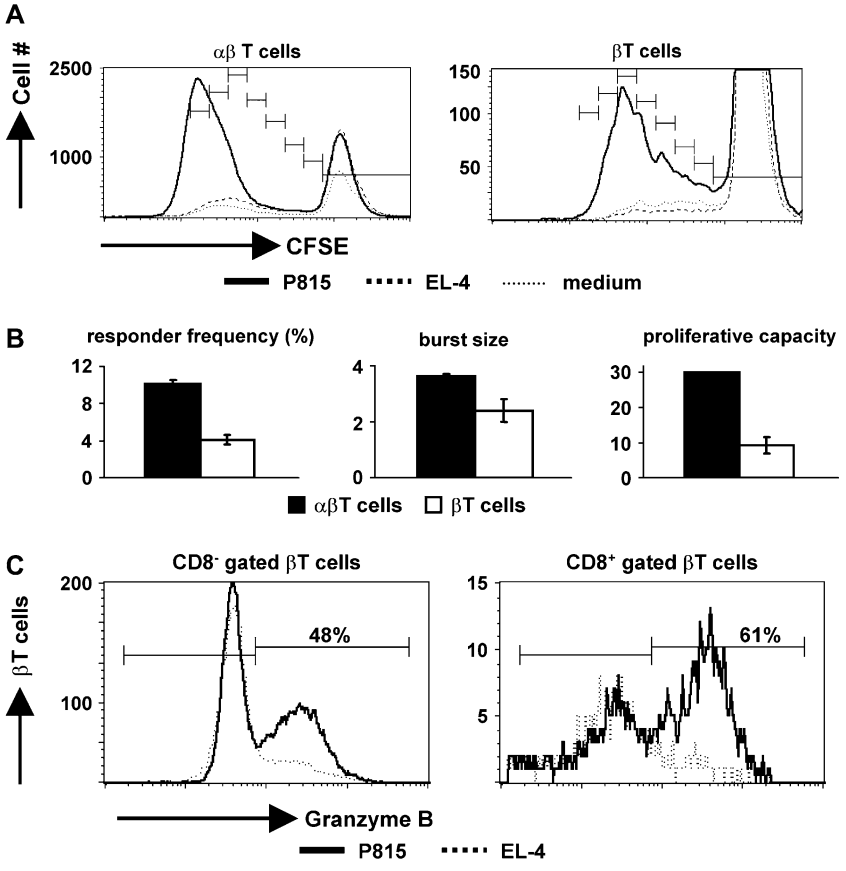

Fig. 5. (A) Allospecificity of βT cells. Respectively, three mice of wild type and pTα tg/TCRα$^{-/-}$ were intra-peritoneally immunized with irradiated Balb/c splenocytes (H-2^{d}) on week 0, 2 and 6. Two weeks later, splenocytes were CFSE labelled and re-stimulated for 3 days *in vitro* with the syngenic EL-4 (H-2^{b}) tumour cell line or the allogeneic P815 (H-2^{d}) tumour cell line. Proliferation of gated βT cells and gated αβT 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 αβT and βT cells from three mice were calculated as described previously (28). (C) Functional analysis of βT cells. Intracellular GrB staining of βT cells stimulated with allogeneic or syngenic cells are depicted. Either CD8^{-} (left) or CD8^{+} (right) are shown independently. Only upon stimulation with allogenic cells, βT cells can produce GrB. Numbers above the margins indicate percentages of βT cells that respond to allogenic MHC.

and differentiate intra-thymically into co-receptor-positive (CD8αβ^{+} or CD4^{+}) and -negative (CD4^{-}8^{-}) compartments. The increased frequency of mature βT cells in TCRα-deficient thymi most likely relates to the absence of competition of pTα and TCRα chains for dimerization with the TCRβ chain. The fact that 0.2% thymic βT cells generate up to 10% of all peripheral lymphocytes and the expansion of adoptively transferred βT cells in Rag-deficient recipients strongly supports the idea that mature βT cells like conventional αβT cells can undergo homeostatic proliferation.

The pTα/TCRβ-expressing thymocytes develop into CD8^{+} and some into CD4^{+} co-receptor-positive cells indicating that lineage commitment does not necessitate expression of TCRα chains. That only 1–3% of βT 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 Vβ usage of βT cells is comparable to αβT cells, suggesting that all segments can promote the development of βT cells (data not shown). No reaction was observed for βT cells in a mixed primary lymphocyte reaction of βT cells with allogenic irradiated Balb/c splenocytes. Yet, MHC recognition by βT cells is suggested by the ability of βT cells to respond to allogenic 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^{low} cells developed when a pTα transgene was placed under the proximal lck promotor (34). Based on northern blotting from total non-sorted LN tissue, these CD8^{+} cells were interpreted to be pTα 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 βT cells (Fig. 2C) differ from the lck-driven pTα 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 βT cells, as has been previously suggested (34). Our observations that βT 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 βT cells.

Two opposing evolutionary scenarios could explain the existence of pTα: (i) the pTα chain evolved after TCRαβ to fulfil its function in β-selection (35) and (ii) prior to the evolution of TCRα, the pTα/TCRβ 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 TCRβ 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 TCRαβ–pMHC complexes have revealed that specific recognition of the pMHC complex by the TCRαβ can be largely determined by the Vα domain, the Vβ domain or both (13, 14, 38, 39). These data indicate that a single Vβ domain is principally capable of pMHC recognition. We show here that indeed the Vβ domain can be sufficient to allow T cell development in the absence of the TCRα chain generating immunocompetent βT cells. We speculate that with the evolution of the TCRα locus, the pTα 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 Vα-like domains within the pre-TCR. This will prevent that αβT 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 TCRαβ (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
pTα	pre-TCRα
RT	reverse transcription
tg	transgenic

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