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Dysregulated Expression of Pre-Tα Reveals the Opposite Effects of Pre-TCR at Successive Stages of T Cell Development

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The pre-TCR complex (TCRβ-pre-TCRα chain (pTα)), first expressed in a fraction of CD8−4−CD44−25+ (DN3) cells, is believed to facilitate or enable an efficient transition from the CD8−4− double-negative (DN) to the CD8+4+ double-positive (DP) developmental stage. Subsequent to pre-TCR expression, DN3 thymocytes receive survival, proliferation, and differentiation signals, although it is still unclear which of these outcomes are directly induced by the pre-TCR. To address this issue, we generated mice bearing a range of pTα transgene copy number under the transcriptional control of the p56k/ proximal promoter. All lines exhibited increased DN3 cycling, accelerated DN3/4 transition, and improved DN4 survival. However, the high copy number lines also showed a selective reduction in thymic cellularity due to increased apoptosis of DP thymocytes, which could be reversed by the ectopic expression of Bcl-2. Our results suggest that transgenic pTα likely caused apoptosis of DP thymocytes due to competitive decrease in surface TCRβ formation. These results highlight the critical importance of precise temporal and stoichiometric regulation of pre-TCR and TCR component expression.


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devlopment of the thymocyte engages with colonization of the thymic rudiment by bone marrow-derived hematopoietic precursors lacking membrane expression of the coreceptor molecules CD4 and CD8 (consequently known as double-negatives (DN)) (1, 2). DN thymocytes, while being the least numerous among the thymocyte subsets, are also developmentally the most dynamic (3–5). Those DN cells that commit to the T cell lineage will undergo numerous gene rearrangement and fate-determining events, including αβ or γδ lineage commitment and, if the αβTCR lineage is chosen, extensive proliferation and subsequent expression of CD4 and CD8.

To facilitate studies of early T cell development, DN thymocytes were further subdivided based on the cell surface expression of the Pgp-1 glycoprotein (CD44) and IL-2Rα chain (CD25) into four sequential stages of development: CD44+CD25− (DN1) → CD44+CD25+ (DN2) → CD44−CD25+ (DN3) → CD44−lowCD25− (DN4) (reviewed in Refs. 3, 4, and 6). The major changes associated with TCR gene rearrangement and cellular expansion occur at the transition from DN3 to DN4 cells. Some or all of these changes are mediated by the pre-TCR, formed by association of the TCRβ chain with the surrogate TCRα (pre-TCRα chain (pTα)) at the DN3 stage (7, 8). As a consequence, DN4 cells progress to the DP stage, where pTα expression is down-regulated as the TCRα chain becomes available to pair with TCRβ (9, 10). Subsequent expression of the αβTCR complex thus enables appropriate selection of thymocytes depending on their TCR specificity and its proper interaction with self-MHC molecules (11).

The importance of the pre-TCR was highlighted by experiments with knockout mice where the deletion of either of the pre-TCR components resulted in a severe decrease of thymic cellularity, apparently caused by an inefficient transition from DN3 to DN4 stages and/or inefficient thymocyte expansion (12, 13). Despite these clear-cut experiments, the exact mechanistic role of the pre-TCR is rather obscure. Numerous functions have been attributed to the pre-TCR, including rescue from programmed cell death, proliferation, induction of CD4 and CD8 expression, induction of TCRα locus rearrangement, and induction of TCRβ locus rearrangement (reviewed in Refs. 8 and 14). However, while the pre-TCR could directly mediate all of the above functions, it is equally plausible that it may directly induce one (albeit critical) outcome, such as rescue from apoptosis, that enables other events to occur in a pre-TCR-independent manner. In fact, evidence exists to support this view (15–17). To revisit this puzzle, we evaluated T cell development in a model where the limiting pre-TCR complex component, pTα, was constitutively overexpressed. We generated transgenic mouse lines that expressed different levels of the pTα transgene under the transcriptional control of the p56k/ proximal promoter. Results of the characterization of these mice, presented below, highlight the importance of correct developmental regulation of TCR and pre-TCR component expression and stoichiometry for optimal T cell differentiation.

Materials and Methods

Mice

Mice were bred and maintained under specific pathogen-free conditions in the Memorial Sloan-Kettering Cancer Center Research Animal Resource Center (New York, NY). Full-length pTα cDNA was inserted into the BanHI site of the expression cassette p1017 (18). The transgene was released from the vector by NotI digestion, purified, and injected into

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4 Abbreviations used in this paper: DN, double negative; pTα, pre-TCRα chain; Tg, transgene/transgenic; wt, wild type; DP, double positive; SP, single positive; FCM, flow cytometry; BrdU, 5-bromo-2′-deoxyuridine; TC, tri-color; PI, propidium iodide.
(C57BL/6 × CBA/Ca) F1 fertilized eggs. Transgenic founders were identified by Southern blot analysis of BanHI-digested genomic tail DNA using the pre-Tα cDNA as a probe. Relative copy number was determined by comparison with the construct loaded at a known copy number. The transgenic lines were backcrossed to C57BL/6J background for more than nine generations at the time of analysis, which was conducted on separate mice between 8 and 13 wk of age.

*RT-PCR*

Total RNA was isolated from mouse thymocytes using TRIzol reagent (Life Technologies, Rockville, MD). RNA was reverse transcribed using random primers following the instructions of the manufacturer (Stratagene, La Jolla, CA). PCR was performed using primers complementary to sequences in the 5′ and 3′ ends of pre-Tα (5′-CTGCAGAAGTTGCGATGATCTCCT-3′ and 5′-TCACGCGGTGTTGAGTATC-3′) and β-actin (Stratagene) as a housekeeping gene. Amplification was performed for indicated cycles at an annealing temperature of 55°C using a thermal cycling machine (PerkinElmer/Cetus, Norwalk, CT). After amplification, 10 μl of the reaction mixture was resolved on a 1.3% agarose gel, blotted to a nylon membrane (kindly provided by Dr. H. von Boehmer, Dana-Farber Cancer Institute, Boston, MA), and hybridized with a purified fragment specific for each CDNA.

*Flow cytometric analysis*

Single cell suspensions were obtained from thymus, stained with the indicated Abs, and analyzed using a FACScan instrument and CellQuest 3.1 software (BD Biosciences, Mountain View, CA). DN cells were prepared from total thymocytes by two cycles of anti-CD4 and anti-CD8 mAb plus complement-mediated depletion as previously described (19). Cell surface expression of CD markers was determined using anti-CD4-tricolor, anti-CD8 FITC, anti-CD8 PE, anti-CD25 PE, anti-CD25 biotin, anti-CD44 PE (Caltag Laboratories, San Francisco, CA), and anti-CD44 CyChrome (BD Pharmingen, San Diego, CA) mAbs. T3.70 (H-Y-specific TCRα chain) was purified from ascites and conjugated to biotin in our laboratory. PE-labeled streptavidin was purchased from Caltag Laboratories. Hybridoma (2F5) producing mAb was specific for the extracellular domain of pre-Tα and kindly provided by Dr. H. von Boehmer (Dana-Farber Cancer Institute, Boston, MA). The 2F5 mAb was purified and conjugated to biotin in our laboratory. Signal observed with this Ab was amplified using the secondary reagent streptavidin-PE-BX73 (Marteck Biosciences, Columbia, MD).

*Apoptosis assay*

Thymocytes were suspended in ice-cold PBS containing 10% FCS. Where required, DN cells were prepared as above. Cells were cultured in 10% FCS RPMI 1640 at a concentration of 1 × 10⁶ cells/ml (37°C) and aliquots were removed at 0, 12, 18, or 24 h to assess apoptosis. Surface staining was conducted with anti-CD4 tri-color (TC) plus anti-CD8 PE for total thymocytes, or with anti-CD25 PE plus anti-CD44 CyChrome for DN thymocytes. Cells were washed twice with PBS then incubated in 250 μl of binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂) containing 2.5 μl annexin V-FITC (BD Pharmingen) for 15 min at room temperature in the dark. Cells were then additionally exposed to PI (5 μl/ml of 1% paraformaldehyde containing 0.1% PMSF, 0.1 mM PMSF, 0.1 mM a-tosyl-L-lysine-chloromethyl-ketone and 5 mM iodoacetamide in TBS (10 mM Tris (pH 7.4) and 150 mM NaCl) for 30 min on ice. Lysates were centrifuged (2000 rpm for 5 min) and precleared overnight with 2 μl of normal rabbit serum, 75 μl of Zosbyrin (Zymed Laboratories, San Francisco, CA) and 25 μl of a 50% slurry of protein A-Sepharose (Sigma-Aldrich, St. Louis, MO). After centrifugation, precleared lysates were incubated with 10 μg of Ab against the C-terminal (kindly provided by Dr. D. Wiest, Fox Chase Cancer Center, Philadelphia, PA) for 2 h and then protein A-Sepharose was added to bring down the immunocomplexes. Samples were resolved by SDS-PAGE and the polyacrylamide gel was fixed, soaked in the fluorescent reagent Amplify (Amer sham Pharmacia Biotech) for 15 min, dried, and finally exposed to Kodak (Kodak, Rochester, NY) film at −80°C.

**Results**

Generation of pTα-transgenic mice

To generate mice with constitutive expression of pre-Tα, we inserted murine pTα cDNA (kindly provided by Dr. H.-J. Fehling, University of Basel, Basel, Switzerland) into the p1017 cassette (obtained from Dr. R. Perlmutter, Agen, Thousand Oaks, CA) under the transcriptional control of the p56lck proximal promoter (Fig. 1A). A NorI fragment of this construct (Fig. 1A) was micro-injected into fertilized oocytes of (C57BL/6 × CBA) F2 mice according to standard procedures, and four transgene (Tg) founder (F11, F23, F29, and F32) were obtained. Founders pTα Tg 11 and 32 expressed low Tg copy number (between two and four copies per genome), with F11 expressing less than F32 (data not shown). F11 mice are therefore referred to as pTα Tglow. Founders 23 and 29 exhibited much higher Tg copy numbers (>20 copies per genome) and are together referred to as pTα Tghigh. All the transgenic lines were backcrossed to C57BL/6J mice for over nine generations at the time of the analysis, giving rise to mouse lines 11, 23, 29, and 32. The expression of pTα mRNA was detected by RT-PCR and of the cell surface or cytosolic protein using a mAb specific for surface pTα (kindly provided by Dr. H. von Boehmer, Dana-Farber Cancer Institute), or a mAb against the C-terminal pTα peptide (generously supplied by Dr. D. Wiest, Fox Chase Cancer Center), as shown in Fig. 1, C and D. Semiquantitative RT-PCR analysis indicated that the pTα message was present at a higher level in pTα-Tg thymocytes compared with the wild type (wt); moreover, the level of mRNA expression appeared to be Tg copy dependent (Fig. 1B). As previously reported, the low cell surface expression of pTα makes its detection by flow cytometry extremely difficult. Aifantis et al. (23) have shown that a combination of biotinylated mAb 2F5 and the enhancing fluorochrome reagent streptavidin-BX73 allows pTα detection in SCB29 cells. Using the same approach, we could detect a discrete but reproducible increase of pTα surface expression on pTα-Tg DN cells (Fig. 1C). This was independently corroborated by immunoprecipitation of metabolically labeled protein lysates using a mAb directed to the pTα C terminus. As shown in Fig. 1D, pTα-Tg mice express higher levels of pTα protein compared with the wild type. Moreover, high copy number cell lines expressed more protein than the low copy number lines. We could not detect pTα expression by either method on single-positive (SP) thymocytes, nor on any of the peripheral T cell subsets (data not shown).
Thymic cellularity is reduced in mice overexpressing pTα

Tg lines 23 and 29 (pTα Tghigh) exhibited a severe decrease in thymic cellularity. Of the two low copy number cell lines, line 32 had a moderate cellularity reduction, whereas line 11 had no reduction at all (Fig. 2A). This result suggests that constitutively overexpressed pTα unexpectedly perturbs normal T cell development in a copy number-dependent manner. Reduced thymocyte numbers could be due to reduced production or to increased elimination. The CD4/CD8 profiles in pTα-Tg mice were not grossly affected, although an increase in the percentages of DN cells compared with littermate controls could be observed (Fig. 2D). More importantly, in lines 23, 29, and 32, the absolute number of pTα-Tg DN cells was not appreciably different in comparison with wild-type mice (Fig. 2B), but the absolute number of DP cells was reduced (Fig. 2C), indicating that thymic hypocellularity in these mice occurs due to a disturbance at the DP level.

pTα-Tg overexpression potentiates DN3 to DN4 transition and thymocyte proliferation

The pre-TCR complex is first expressed and is believed to exert its function in a subset of DN3 cells (8, 24). We therefore investigated in wild-type and Tg mice the biology of the DN3/4 transition, focusing on the Tg lines exhibiting low (line 11) or high Tg copy number (lines 23 and 29), which were phenotypically indistinguishable from one another. We found that the DN3/4 transition was potentiated in pTα-Tg mice compared with corresponding littermate controls (Fig. 2D, bottom, and E and F). The pTα transgene caused a copy number-dependent shift in DN4/DN3 ratios from 0.6 ± 0.4 in wild-type animals to 3.0 ± 0.8 in Tglow or Tghigh lines (Fig. 2, D and F, and data not shown).

To determine the cause of these changes in population dynamics, we assessed the proliferative potential and susceptibility to apoptosis of pTα-Tg early thymocytes. This was particularly pertinent in light of certain models of pre-TCR function, which postulate that rescue from apoptosis alone (15, 16) or rescue from apoptosis and induction of proliferation (8, 24) are induced by this receptor. Mice were injected with BrdU i.p., and the percentage of incorporation of this nucleotide analog into newly synthesized DNA in each thymocyte subset was revealed by FCM (Fig. 3). The pTα-Tg thymocytes exhibited a transgene copy number-dependent increase in BrdU incorporation among DN, DP, and SP thymocytes, as compared with non-Tg counterparts (Fig. 3A). Not surprisingly, the increase in proliferation was more evident in the DN and CD8 SP subsets, which in wild-type mice are known to be mitotically the most active (3).

Among the DN thymocyte subsets in normal mice, DN4 cells contain the bulk of proliferating cells, with DN3 cells being much less active (3, 25), so that only a fraction of DN3 cells (10–25%) are actively cycling. Consistent with these findings, we observed that 24.1 ± 7.8% of DN3 and 64.8 ± 6.3% of DN4 thymocytes labeled with BrdU in wild-type mice. By contrast, pTα overexpression induced a significant (p ≤ 0.09) increase in BrdU incorporation in DN3 cells and a somewhat decreased proliferation of DN4 cells (Fig. 3B). This decrease was significant only in pTα Tghigh DN4 cells. These data show that overexpression of pTα pronounces proliferation of the DN3 cells. It remains to be seen whether those Tg DN3 cells that proliferate are enriched in cells successfully rearranging the TCRβ locus.

The pTα transgene induces increased survival of DN4 yet apoptosis of DP cells

DP cells of pTα-Tghigh mice exhibit a marked reduction in cell numbers, decisively contributing to the overall reduction of thymic cellularity. In normal thymopoiesis, the expression of pTα is highest at DN3 and DN4 stages and is subsequently down-regulated to allow a proper interaction of successfully rearranged TCRα chains with TCRβ in DP cells. In the transgenic system using the p56lck proximal promoter, pTα expression is artistically sustained throughout intrathymic development (18). We therefore considered the possibility that this constitutive expression may interfere with DP survival and final maturation. Indeed, freshly isolated thymocytes from pTα-Tghigh mice, where the hypoplasia of the thymus is more evident, exhibited a 3- to 4-fold higher proportion of apoptotic cells compared with the wild type (Fig. 4A and results not shown). Moreover, when Tg and non-Tg thymocytes were cultured overnight in single cell suspension culture without additional stimulation, both low and high copy number DP cells showed an increase in annexin V+ cells (Fig. 4B), indicating that these thymocytes exhibit a tendency to spontaneously die by a mechanism most likely triggered before disruption of the tissue for cell isolation. Again, the effect was copy number-dependent as Tghigh DP thymocytes exhibited higher levels of annexin V+ cells than Tglow cells. By contrast, the Tg protected the immediate precursors of DP cells, the DN4 thymocytes, from apoptosis in a copy number-dependent manner (Fig. 4C). This effect was not seen in DN1, DN2, DN3, or SP thymocytes (data not shown). We conclude that the effect of pTα overexpression is stage-specific, involving increased

![FIGURE 1](http://www.jimmunol.org/) Generation of mice transgenic for the pTα molecule. A, Schematic representation of the construct used to generate pTα-Tg mice. The full-length pTα cDNA was inserted into the BamHI site of the pL107 cassette so as to be controlled by the p56lck proximal promoter. B, Detection of the Tg expression by RT-PCR using total RNA from thymocytes. For each sample, a serial 1/10 dilution of template was used. C, Cell surface expression of the pTα molecule by flow cytometry. DN cells were stained with the biotinylated Ab 2F5 (kindly provided by Dr. H. von Boehmer, Dana-Farber Cancer Institute) and streptavidin-PBXL3 as a secondary reagent and analyzed in a FACS Calibur cytometer. D, Immunoprecipitation of the pTα molecule. Total thymocytes were metabolically labeled with [35S]methionine and then immunoprecipitated with Ab against the pTα C-terminal domain (kindly provided by Dr. D. Wiest, Fox Chase Cancer Center).
proliferation of DN3 and increased survival of DN4 cells as well as increased apoptosis of DP cells. The proto-oncogene product Bcl-2 and tumor suppressor p53 exert their effects at sequential developmental points, so that apoptosis of DN3/4 cells at the pre-TCR checkpoint is controlled in a p53-dependent, Bcl-2-independent manner (26), while apoptosis of DP cells due to the lack of positive intrathymic selection proceeds largely independent of p53 but can be blocked by overexpression of Bcl-2 which neutralize the effects of the ectopic expression of pTCR in DP cells.

SP cells were produced at comparable levels in the mice from Fig. 5, except for pTCRlow × Bcl-2 mice, which had significantly more, and pTCRhigh mice, which had significantly fewer SP cells (cell numbers × 10⁶ were, for CD8 SP and CD4 SP cells, respectively: 3.1 ± 0.6 and 6.7 ± 2.1 for Bcl-2 Tg; 3.6 ± 1.0 and 8.2 ± 3.3 for pTCRlow; 7.8 ± 1.9 and 22.2 ± 8.9 for Bcl-2 × pTCRlow; 1.2 ± 0.2 and 2.5 ± 1.1 for pTCRhigh, and 2.9 ± 0.4 and 6.4 ± 0.5 for Bcl-2 × pTCRhigh mice). Two possible models can account for the observed results. Enforced expression of pTCR could provide strong signals to the DP cells, thus leading to their apoptosis by a
process akin to negative selection. Alternatively, deregulated expression of pTα could interfere with TCR assembly and thus lead to less efficient positive selection. In that regard, previous results (26, 27) suggest that the bcl-2 transgene delays DP thymocyte death but does not interfere with negative selection.

Dysregulated pTα may interfere with αβTCR formation and function

The enforced presence of overexpressed pTα in DP cells at the moment where the formation of the αβTCR is indispensable for survival and further development is likely to affect TCR chain stoichiometry. This, in turn, has the potential to decrease the efficacy of αβTCR formation and its interaction with the selecting self-MHC molecules. To test whether constitutive expression of pTα may be blocking the delivery of survival and/or selection signals, we crossed pTα-Tg mice with H-Y Tg, where the TCR specificity and selection requirements are well established. Double-Tg female mice showed a 3-fold decrease in the proportion of CD8 SP cells (Fig. 6A), with a reduction in the surface TCR-Tg chain levels within CD8 SP cells (mean fluorescence intensity for T3.70/Cd8 cells in H-Y Tg was 1344, compared with 1156 for H-Y/pTα Tg; and 190 for T3.70/Cd8* cells in H-Y Tg, compared with 88 in H-Y/pTα Tg). The effect was less pronounced in the case of Tg low mice (data not shown). This result indicates that while the pre-TCR plays an important role at earlier stages of development, at the DP stage the presence of pTα interferes with further development.

pTα could interfere with this process not only by competing with TCRα and thereby reducing the rate of assembly of αβTCR, but the presence of pTCR and TCR on the same cell could also lead to strong, deleting signals that kill DP cells. Currently we have no direct evidence to discriminate between these two alternatives. However, pTα Tg, H-Y Tg and pTα × H-Y Tg thymi all have similar numbers of DP cells (between 18 and 22 × 106 with

FIGURE 3. Cell proliferation in pTα-Tg mice measured by in vivo BrdU incorporation. Mice were injected three times 4 h apart with 1 mg of BrdU and sacrificed 12 h after the third injection. Thymi were teased and cell suspensions were analyzed for incorporation of BrdU and for the cell surface expression of CD4 and CD8 (A) or CD44 and CD25 (B), as described in Materials and Methods. Results shown are the mean ± SD of four independent experiments (*, p ≤ 0.09).

FIGURE 4. Expression of pTα induces apoptosis of DP cells but protects DN4 cells. A, Freshly isolated thymocytes were stained with annexin V-FITC and PI. Shown are the percentages of annexin V-PI (early apoptotic) cells in pTα-Tg low, pTα-Tg high, and corresponding wt littermates. B and C, Total and DN thymocytes (prepared as described in Materials and Methods) were incubated (1 × 106 cells/ml) in 10% FCS-RPMI 1640 at 37°C and aliquots were removed at 0, 12, 18, or 24 h and stained with annexin V-FITC, plus anti-CD4 PE and anti-CD8 CyChrome (for DP gating (B)) or anti-CD3/CD4/CD8 allophycocyanin, anti-CD25 PE and anti-CD44 CyChrome (for DN subset gating (C)). PI staining could not be included in these experiments due to use of the required channel for detection of other fluorochromes. Results shown are the mean of three individual experiments (SD < 10%).
overlapping SD; see Fig. 6B), suggesting that breeding of a TCR-Tg onto pTα-Tg high background does not lead to further depletion of DP cells by negative selection. By contrast, absolute numbers of CD8 SP cells are higher in pTα-Tg high × H-Y TCR-Tg double-Tg than in pTα-Tg high thymi but lower than in H-Y TCR-Tg mice (Fig. 6B), suggesting that excess of TCRα helps positive selection in double-Tg mice. This finding indirectly favors the "interference with TCR assembly" alternative, a possibility also consistent with previous observations that premature TCR expression competes with the pre-TCR in DN cells, leading to curtailed development toward DP cells (28, 29). Both observations suggest that pre-TCR and TCR stoichiometry is crucial for proper developmental progression—first for the assembly of the pre-TCR in DN thymocytes (in the absence of αβTCR) and then for the assembly of the αβTCR (in the absence of pTα-TCRβ).

Because the stoichiometry of TCR molecules apparently plays an important role in development, we evaluated whether supplying more functional TCRβ chains to the pTα Tg may further enhance the transition regulated by the pre-TCR. The presence of a rearranged TCRβ chain (Vβ5 transgenic from the OT-1 TCR), together with pTα-Tg high, did not result in appreciable correction of thymic cellularity (average cellularity of two mice in this experiment: wild type, 119 × 10^6; pTα Tg, 57 × 10^6; Vβ5, 108 × 10^6; Vβ5/pTα double-Tg, 50 × 10^6 cells), but rather in an even more efficient transition from the DN3 to DN4 stage, as judged by an overwhelming dominance of DN4 cells in the DN compartment (Fig. 7). That provision of additional TCRβ chains did not correct thymic cellularity is consistent with the conclusion that the primary defect in pTα-Tg high DP thymocytes must be in competitive disruption of αβTCR formation by the transgene. Based upon these findings, we conclude that the levels of pTα, rather than of the TCRβ chain, are limiting for the DN3 and DN4 transition in vivo. This notion is further supported by the more efficient DN3 to DN4 transition in pTα-Tg high mice.

Discussion

Due to low expression levels and difficult detection, at present the mode of operation and the precise function of the pre-TCR are still incompletely understood. Most of our knowledge was shaped by findings obtained from genetically manipulated models that were used to investigate the consequences of pre-TCR expression and signaling and to address whether the pre-TCR recognizes an extracellular ligand. In one such study, a version of pTα lacking the cytosolic domain (tailless pTα), controlled by the p56^1κ promoter (30), was shown to function nearly as efficiently as the whole molecule. Another group showed that the intracellular domain, devoid of most of the extracellular domain, also apparently functions comparably to the full molecule (31). Although both groups studied heavily overexpressed molecules (possibly subject to caveats of spontaneous aggregation and signaling), their conclusions led to the hypothesis that recognition of an extracellular ligand may not be required for the pre-TCR function. Consistent with this hypothesis, it was found that pre-TCR localizes to the liposoluble membrane microdomains ("rafts") in the absence of signals from the thymic microenvironment (32), and that ligation of the pre-TCR-associated CD3ε chain leads to calcium signaling (33). These findings strongly suggest that either there is no extracellular ligand for pre-TCR or this ligand is provided in a cell-autonomous or homotypic fashion. An even more difficult question, not decisively answered so far, is which of the changes occurring in the aftermath
of pre-TCR expression can be directly ascribed to the pre-TCR itself and which, if any, are produced by concurrent or immediately subsequent signaling via pre-TCR-independent pathways.

Experiments presented here provide a new angle to these issues owing to the presence of graded and different amounts of the transgene in different mouse lines. Our transgene was expressed under the control of the proximal \textit{lck} promoter, known to be activated in early T cell development, between the DN2 and DN4 stages (18, 34). Our results demonstrate that qualitatively and quantitatively constitutive expression of pT\textit{H9251} potentiates many of the features of early T cell development at the DN/DP interface in a strictly developmentally regulated manner. We conclude that pT\textit{H9251} must be the limiting chain of the pre-TCR complex, because providing an excess of this chain accelerated the DN3-to-DN4 transition. It is interesting that the effects of the Tg were first observed in DN3 thymocytes (in which the proximal \textit{lck} promoter first becomes activated; Ref. 34) in the form of increased proliferation, the extent of which correlated with the transgene dosage. Perhaps somewhat surprisingly, Tg DN4 cells, known to undergo major expansion in normal mice, survived better, but actually proliferated slightly less, than non-Tg cells. Again, pT\textit{α} Tg levels quantitatively correlated with increased survival and decreased proliferation. Finally, at the DP stage, the Tg caused increased apoptosis in a copy number-dependent fashion. Although this could be caused by Tg toxicity, we believe that this is not the case, because even the low (barely detectable; \( \sim 1-2 \) copies of Tg) levels of Tg caused increased DP apoptosis. Moreover, these same Tg doses did not cause apoptosis at the two preceding stages of development (DN3 and DN4), and it is difficult to envision why nonspecific toxicity would be selectively developmentally regulated. It is also possible that pT\textit{α} provides a strong signal to DP cells that alone, or synergistically with TCR\&\textit{β}, causes their apoptosis or that increased apoptosis is due to pT\textit{α} competition with TCR\&\textit{β} for TCR\&\textit{α}, leading to decreased TCR\&\textit{αβ} assembly and a consequent lack of efficient positive selection. This explanation was supported by suggestive, but not definitive, findings of reduced TCR\&\textit{α} expression and inefficient positive selection in pT\textit{α}/H-Y TCR double-Tg mice.

Overall, the results presented in this study underline the importance of correct temporal regulation of pre-TCR. Moreover, our data also point to an important role of chain stoichiometry and TCR assembly. Thus, we previously showed (see Fig. 8, middle) premature expression of TCR\&\textit{α} (as is the case in TCR-Tg mice) takes away TCR\&\textit{β} chains from the pre-TCR complex, curtailing

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**FIGURE 7.** TCR\textit{β} Tg together with the pT\textit{α} Tg enhances pre-TCR signaling. pT\textit{α} Tg\textsuperscript{high} was bred to V\textit{β5} single Tg. The CD4/CD8 (top) and CD25/CD44 profile (bottom) is shown for wt, double pT\textit{α}/V\textit{β5}-Tg, and single-Tg controls. The percentage of cells in each quadrant is indicated. Data are representative of three comparable experiments.
expansion and imprinting the mature DN developmental fate upon many of the cells that express this complex (29). By contrast, providing excess of pTα increases proliferation of DN3 cells and survival of DN4 cells, but the failure to extinguish this expression at the DP stage reduces formation of αβTCR and increases DP cell apoptosis that can be blocked by Bcl-2 overexpression. It will be of interest to further dissect the developmental effects of overexpressed pTα upon the regulation of cell cycle in the course of the DN/DP transition.

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