Pre-TCRα and TCRα Are Not Interchangeable Partners of TCRβ during T Lymphocyte Development

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Abstract

In contrast with the αβ T cell receptor (TCR), the pre-TCR spontaneously segregates to membrane rafts from where it signals in a cell-autonomous fashion. The disparate behaviors of these two receptors may stem either from differences inherent to the distinct developmental stages during which they are expressed, or from features intrinsic and unique to the receptor components themselves. Here, we express TCRα precisely at the pre-TCR checkpoint, at levels resembling those of endogenous pre-TCRα (pTα), and in the absence of endogenous pTα. Both in isolation and more dramatically when in competition with pTα, TCRα induced defective proliferation, survival, and differentiation of αβ T lymphocyte precursors, as well as impaired commitment to the αβ T lymphocyte lineage. Substitution of TCRα transmembrane and cytoplasmic domains with those of pTα generated a hybrid molecule possessing enhanced competitive abilities. We conclude that features intrinsic to the pre-TCR, which are absent in TCRα, are essential for its unique function.

Key words: T lymphocyte subsets • cell lineage • receptor-mediated signal transduction • receptor antigen • T-cell γδ

Introduction

Thymocytes harboring a productive rearrangement at the TCRβ locus generate a TCRβ molecule that pairs covalently with the invariant pre-TCRα (pTα) and noncovalently with CD3 signal–transducing molecules, resulting in formation of a pre-TCR complex at the cell surface. The failure to identify an extracellular ligand for the pre-TCR, as well as the functional capabilities of a mutant pre-TCR complex lacking all extracellular domains (1), could indicate that pre-TCR signaling may be initiated in a manner independent of extracellular ligation. Indeed, pre-TCR complexes on the surface of a SCID murine thymoma cell line localize to membrane rafts, from where they signal in a cell-autonomous fashion (2). The ensuing phosphorylation and activation of $p56^{Lck}$ and ZAP-70 (2), mobilization of intracellular Ca^{2+} stores (3), and nuclear translocation of nuclear factor κB and NFAT transcription factors (3, 4) presumably mediate survival, proliferation, differentiation, and TCRβ allelic exclusion during αβ T lymphocyte development (5). The constitutive internalization and ubiquitin-mediated proteasome-dependent degradation of the pre-TCR complex terminates this signal transduction process (6, 7).

In sharp contrast, TCRαβ complexes localize to membrane rafts, from where they trigger signal transduction cascades only in response to stimulation with costimulatory molecules and agonist peptides presented by MHC molecules (8, 9). Like the pre-TCR, TCRαβ complexes are constitutively internalized. However, in contrast to the pre-TCR, unstimulated TCRαβ complexes are recycled back to the cell surface (10, 11). Only in response to stimulation are internalized TCRαβ complexes degraded (12).

The lack of pre-TCR–induced survival, proliferation, and differentiation is evident in $pT\alpha^{-/-}$ mice, even though some cells still pass the pre-TCR–controlled checkpoint by virtue of their ability to form TCRγδ and TCRαβ complexes (13, 14). Whether or not a TCRα molecule can functionally replace pTα in the pre-TCR complex is a matter of considerable controversy.

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Abbreviations used in this paper: DN, $CD4^{-}8^{-}$ double negative; EGFP, enhanced green fluorescent protein; FTOC, fetal thymic organ culture; HSA, heat-stable antigen; pTα, pre-TCRα; WTpTα, wild-type pTα.

One view suggests that the cell-autonomous nature of pre-TCR signaling is dependent on the identity of the TCRβ partner component. Several papers document that, in contrast with pre-TCR expression, premature expression of a TCRαβ complex promotes a "γδ-like" T cell lineage fate, and results in impaired proliferation and differentiation to the $CD4^+8^+$ stage of thymocyte development (15–18). More specifically, some evidence has implicated the cytoplasmic domain unique to pTα as essential for pre-TCR function. Provision of a wild-type pTα transgene to $pT\alpha^{-/-}$ mice restored pre-TCR-induced proliferation, survival, and differentiation better than a transgene lacking the pTα cytoplasmic domain or the proline-rich regions thereof (19). Pulse chase experiments documented cell-autonomous internalization and degradation of TCR and CD3ε surface receptor components in cell lines expressing pre-TCR components, but not in those expressing TCRγδ or TCRαβ components (7). Mutagenesis studies identified the cytoplasmic domain of human pTα as essential for cell-autonomous receptor internalization and degradation (6). These latter studies further implied that cell-autonomous constitutive internalization might represent one mechanism by which pre-TCR surface expression and signaling is self-regulated. An apparent requirement for strict regulation of surface expression level may also exist in the analogous cell-autonomous pre–B cell receptor signaling cascade (20).

The alternative view implies that the cell-autonomous nature of pre-TCR signaling depends on the developmental stage at which the receptor is expressed rather than on qualities inherent to the TCRβ partner component itself. This view suggests that pTα represents merely a "surrogate TCRα molecule," whose function is restricted to stabilizing surface expression of a productively rearranged TCRβ molecule. Indeed, a recent paper implicated elevated raft content, stronger capacitative Ca^{2+} entry, and increased extracellular signal-related kinase activation as factors generating a unique developmental environment in $CD4^-8^-$ double negative (DN) 3 thymocytes (21).

Thus far, observations concerning the potential interchangeability of pTα and TCRα have been inconclusive. Utilization of TCRα and TCRβ transgenes (14, 16–18), or a TCRα transgene expressed at developmental stages different from those of endogenous pTα (15, 22), has precluded a direct assessment of the performance of a TCRα molecule expressed at precisely the same developmental stage as that of endogenous pTα. Although controlled by the $p56^{Lck}$ proximal promoter, the wide variation among founders in the expression of transgenes encoding either pTα or pTα substituted with the connecting peptide, transmembrane, and cytoplasmic domains of TCRα made difficult a comparison between these two transgenes (23). The proliferative potential of $pT\alpha^{-/-}$ fetal thymocytes retrovirally transduced with either pTα or TCRα may have been obscured by analysis of their proliferation in fetal thymic lobes (21), which is much reduced when compared with that of thymocytes developing in situ. Finally, analyses of the reconstitution of empty adult or fetal thymi in which there is no competition for resources or niche space may grossly overestimate the ability of TCRα molecules to mimic the functions of pTα.

Here, we analyze the interchangeability of WTpTα (WTpTα) with TCRα, and with a TCRα/pTα hybrid molecule consisting of the extracellular domain of TCRα joined to the transmembrane and cytoplasmic domains of pTα. By placing each transgene under the control of the $p56^{Lck}$ proximal promoter, we ensured that each potential TCRβ partner component is expressed at an equal and relevant stage of thymocyte development. By crossing each transgene onto the $pT\alpha^{-/-}$ genetic background, we allowed each receptor to perform in the absence of endogenous pTα. Furthermore, by introducing equal numbers of precursors expressing different receptors into a single thymus, we forced them to compete for available space and resources, revealing differences in receptor function that may be obscured by analyses of the developmental potential of cells that express only a single type of receptor in a noncompetitive environment.

We found that the TCRα molecule was unable to fully restore the proliferation, survival, differentiation and αβ T cell lineage commitment induced by the WTpTα molecule. When placed in direct competition, the superiority of WTpTα became more dramatically apparent with regard to proliferation and progression to the $CD4^+8^+$ stage of thymocyte development. Substitution of the transmembrane and cytoplasmic domains of TCRα with those of pTα generated a TCRα/pTα hybrid molecule exhibiting enhanced performance in competition with WTpTα when compared with TCRα, supporting the view that some of the cell-autonomous nature of pre-TCR signaling is bestowed on the complex by properties unique and intrinsic to the pTα molecule.

Materials and Methods

Mice and Cell Lines. C57Bl/6 and $Rag^{-/-}\gamma c^{-/-}$ mice were purchased from Taconic Farms. $pT\alpha^{-/-}$ mice were generated as described previously (13). All mice were kept in specific pathogen-free animal facilities at the Dana-Farber Cancer Institute. All animal procedures were performed in compliance with the guidelines of the Dana-Farber Cancer Institute Animal Resources Facility, which operates under regulatory requirements of the U.S. Department of Agriculture and Association for Assessment and Accreditation of Laboratory Animal Care. The $58\alpha^-\beta^+$ T cell hybridoma (provided by F. Grassi, Institute for Research in Biomedicine, Bellinzona, Switzerland; reference 28) was maintained in IMDM containing 10% FBS, penicillin-streptomycin, and 0.1% β-mercaptoethanol.

Generation of Transgenic Mice. WTpTα and pTα-PROΔ transgenic mice were generated as described previously (19, 24). The TCRα/pTα hybrid construct was generated by PCR of an N15 TCRα cDNA template with the primers TCRaBamup, 5′-ACGGATCCTTTCCACCATGAACATGCGTCC-3′, and TCRalo, 5′-AGGAATTCTGAAAGTTTAGGTTCATATCTGT-3′; and a pTα cDNA template with the primers pTaEcoup, 5′-CAGAATTCCTGGCTGAGCCTACTGCGCCTGCT-3′, and pTaBamlo, 5′-TGGGATCCAGGGGTGGGTAAGATCTAA-3′.

The two amplification products were cut with EcoRI, and the cohesive EcoRI sites were fused. cDNA encoding the TCRα chain of the N15 TCR (provided by L. Clayton, Dana-Farber Cancer Institute, Boston, MA) was inserted pUC1017 vector, 3′ to the $p56^{Lck}$ proximal promoter. $p56^{Lck}$ proximal promoter TCRα or TCRα/pTα hybrid-hGH minigene fragments released by NotI digestion were microinjected into fertilized eggs. Founders were screened for transgene insertion by amplification of tail DNA with the primers lckp, 5′-AACCCAGTCAGGAGCTTGAA-3′; mus3, 5′-CATCGAGCAGAAGCAGTTTGA-3′; and TCRalo. pTα deficiency was assessed by amplification of tail DNA with the primers pTaF, 5′-TCACAGTGCTGGTAGATGGAAGG-3′; pTaKOR, 5′-GTTTGCTCGACATTGGGTGG-3′; and pTaWTR, 5′-GGCTCAAGAGATAACCTGAACCATG-3′.

Antibodies and Reagents. Anti-CD8, anti-CD4, anti-TCRγδ, anti-TCRβ, anti-CD25, anti-CD44, anti-CD24 (heat-stable antigen [HSA]), anti–Gr-1, anti–Ter-119, anti-Dx5, anti-CD19, anti-CD3ε, anti-CD45.1, anti-CD45.2, and Annexin V were purchased from BD Biosciences. Each mAb was either biotinylated or directly conjugated to FITC, PE, cychrome, or allophycocyanin fluorophores. Cychrome- or allophycocyanin-conjugated streptavidin (BD Biosciences) was used to reveal staining with biotinylated mAbs. Surface staining of thymocytes and BM was performed as described previously (14, 19). Cells were analyzed using a FACSCalibur™ flow cytometer (Becton Dickinson) and sorted using a MoFlo cell sorter (DakoCytomation). Propidium iodide was obtained from Sigma-Aldrich.

RT-PCR. High pure RNA isolation kit (Roche) was used to isolate thymocyte RNA. SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) was used to generate cDNA. Fivefold serial dilutions of cDNA were used for semi-quantitative PCR analysis of transgene and actin levels with the primers lckp; mus3; A2, 5′GGCACCCCCTTTCCGTCTCT-3′; TCRalo; actinup, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; and actinlo, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′.

Competition Assay. BM cells lacking the lineage markers Gr-1, Ter-119, Dx5, CD19, TCRβ, and CD3ε were sorted from WTpTα, TCRα, TCRα/pTα hybrid, pTα-PROΔ, and $pT\alpha^{-/-}$ mice. 1–2 × 10^5 cells of each population were mixed and injected intravenously into the tail vein of each irradiated (500 rad) $Rag^{-/-}\gamma c^{-/-}$ mouse. Thymic reconstitution was analyzed 5 wk after injection.

Retroviral Infection. WTpTα, TCRα, or TCRα/pTα hybrid constructs, upstream of an internal ribosomal entry site and enhanced green fluorescent protein (EGFP; CLONTECH Laboratories, Inc.), were cloned into a modified Moloney murine leukemia virus-based retroviral vector (provided by R. Mulligan, Children's Hospital, Boston, MA). Retroviral supernatants were generated as described previously (19). 10^5 $58\alpha^-\beta^+$ T cell hybridoma cells were infected on ice for 3 h in the presence of 8 μg/ml polybrene.

Online Supplemental Material. Surface expression of TCRβ protein on $CD4^-CD8^-CD25^+CD44^-$ (DN3) thymocytes from WTpTα and transgenic mice was analyzed using anti-TCRβ from BD Biosciences using a FACSCalibur™ flow cytometer (Becton Dickinson). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20031973/DC1.

Results

Generation of Transgenic Mice. To directly compare the performance of WTpTα with TCRα, constructs encoding the TCRα component of the N15 TCR (Vα8), or a TCRα/pTα hybrid molecule consisting of the extracellular domain of the N15 TCRα molecule fused to the trans-

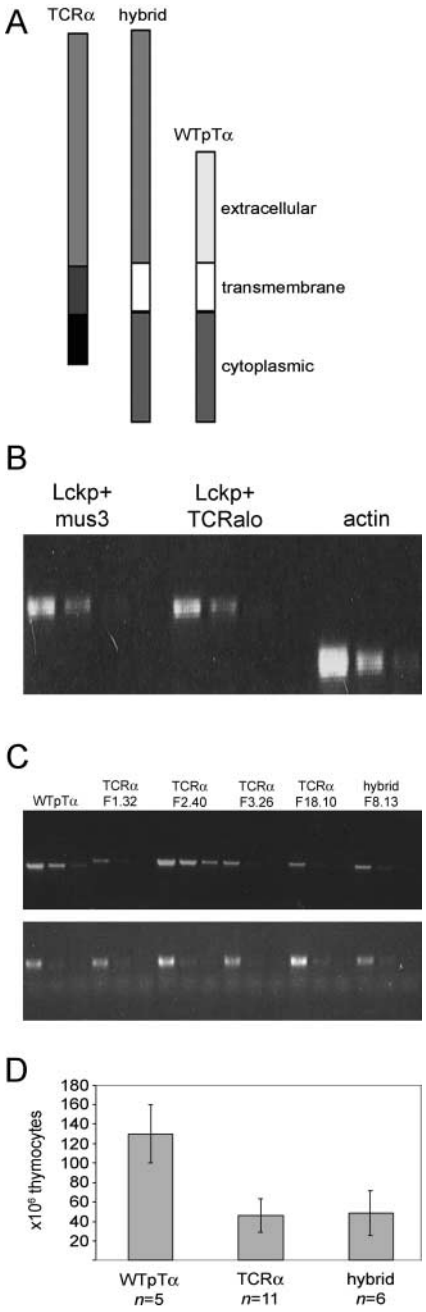

Figure 1. Generation of transgenic mice. (A) Structures of the WTpTα, TCRα, and TCRα/pTα hybrid molecules. (B) Semi-quantitative RT-PCR analysis of primer efficiency with β-actin control. Fivefold serial dilutions of the same sample of TCRα/pTα hybrid whole thymocyte cDNA were used in all samples. Lckp recognizes transcript $p56^{Lck}$ proximal promoter sequences, mus3 recognizes WTpTα sequences, and TCRalo recognizes TCRα sequences. (C) Semi-quantitative RT-PCR analysis of transgene expression (top) in transgenic founders with β-actin controls (bottom). Fivefold serial dilutions of whole thymocyte cDNA were used. (D) Total thymocyte numbers in transgenic mice. Multiple founders of each transgenic construct were analyzed. Mice were analyzed at 3–5 wk of age.

membrane, and cytoplasmic domains of pTα were placed under the control of the $p56^{Lck}$ proximal promoter (Fig. 1 A). A previously described founder containing two to three copies of a WTpTα transgene (F63), which was also controlled by the $p56^{Lck}$ proximal promoter, was selected for comparison (19, 24). This WTpTα founder was provided by the lab of J. Nikolich-Zugich (Oregon Health and Science University, Beaverton, OR). All founders expressing each transgene were crossed to the $pT\alpha^{-/-}$ background.

To ensure that TCRα and TCRα/pTα hybrid transgenes were expressed at or near levels of the WTpTα transgene as well as physiological levels of endogenous pTα transcripts, RNA prepared from total thymocyte suspensions was analyzed by semi-quantitative RT-PCR (Fig. 1 C). A single pair of primers was used to demonstrate similar levels of transcript expression of the WTpTα transgene in DN3 thymocytes of this founder with those of endogenous pTα in DN3 thymocytes of C57Bl/6 mice (19). A common 5′ primer (Lckp), which recognizes a portion of the $p56^{Lck}$ proximal promoter, was used to amplify all transgene transcripts. Distinct 3′ primers that recognize the cytoplasmic domain of pTα (mus3) and the extracellular region of TCRα (TCRalo) were used to quantify WTpTα and TCRα transgene transcripts. The inclusion of both of these domains in the TCRα/pTα hybrid molecule permitted use of either 3′ primer in the amplification of TCRα/pTα hybrid transcripts. To ensure the validity of a comparison of different transgenes amplified with distinct 3′ primers, we compared the amplification efficiency achieved by each primer pair using the same sample of TCRα/pTα hybrid whole thymocyte cDNA as a template (Fig. 1 B). Using these primer pairs of comparable efficiency, four founders of TCRα transgenic mice and two founders of TCRα/pTα hybrid transgenic mice expressing transgene transcripts at levels similar to those of the WTpTα transgene (F63) were selected for analysis. With regard to surface expression of TCRβ, $CD4^-CD8^-CD25^+CD44^-$ (DN3) thymocytes from WTpTα and TCRα transgenic mice were indistinguishable (Fig. S1 available at http://www.jem.org/cgi/content/full/jem.20031973/DC1).

Neither TCRα nor TCRα/pTα Hybrid Molecules Functionally Replace pTα in the pre-TCR Complex. When compared in noncompetitive conditions with WTpTα, neither TCRα nor the TCRα/pTα hybrid molecule successfully recapitulated pre-TCR function. In contrast with that of the endogenous pTα promoter, the activity of the $p56^{Lck}$ proximal promoter persists in the $CD4^+8^+$ stage of thymocyte development. Expression of a WTpTα transgene controlled by the $p56^{Lck}$ proximal promoter resulted in a copy number–dependent increase in apoptosis of $CD4^+8^+$ thymocytes, and a concomitant decrease in thymic cellularity (24). Although this effect was severe in transgenic founders containing >20 copies of the WTpTα transgene, little or no reduction in thymic cellularity was apparent in low copy number transgenic founders. Accordingly, the WTpTα transgenic founder used in the present work contained two to three transgene copies (24). Cellularity of thymi in TCRα and

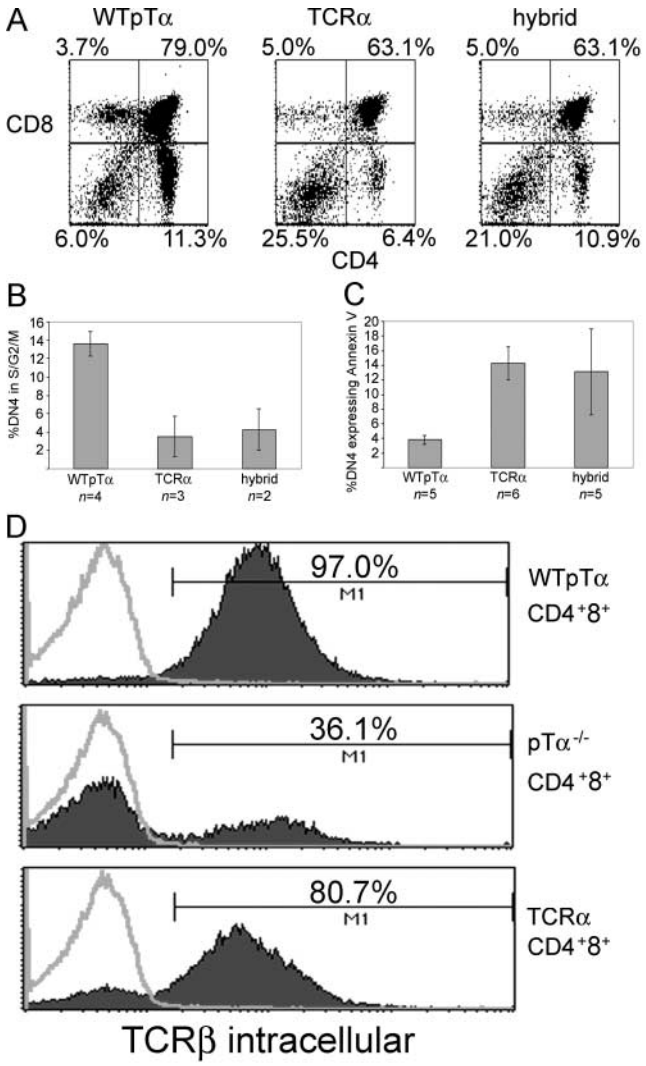

Figure 2. Defective differentiation, cell cycle entry, and survival of thymocytes expressing TCRα and hybrid transgenes. (A) Dot plots depict the CD4 versus CD8 profile of a minimum of five 3–5-wk-old mice of each transgenic construct. Multiple founders were analyzed. (B) Percentage of sorted $Gr\text{-}1^-Ter\text{-}119^-Dx5^-CD19^-TCR\gamma\delta^-CD4^-CD8^-CD25^-CD44^-$ (DN4) thymocytes in the S/G2/M phases of the cell cycle. DNA content was analyzed by propidium iodide staining. (C) Percentage of DN4 thymocytes expressing externalized phosphatidyl serine apoptosis marker as revealed by staining with Annexin V. (D) Percentage of $CD4^+8^+$ thymocytes expressing intracellular TCRβ (shaded histogram). Overlay represents intracellular staining with isotype-matched control antibody.

TCRα/pTα hybrid transgenic mice was only one third of that in WTpTα mice (Fig. 1 D). The fraction of thymocytes remaining at the $CD4^-8^-$ stage of development was increased three- to fourfold in TCRα and TCRα/pTα hybrid thymi, whereas the proportion of thymocytes in the $CD4^+8^+$ compartment was decreased (Fig. 2 A).

To exclude the possibility that most $CD4^+8^+$ thymocytes in TCRα transgenic mice were generated by transgenic TCRα–endogenous TCRγ4 complexes (22), rather than by transgenic TCRα–endogenous TCRβ complexes, $CD4^+8^+$ thymocytes from WTpTα, $pT\alpha^{-/-}$,

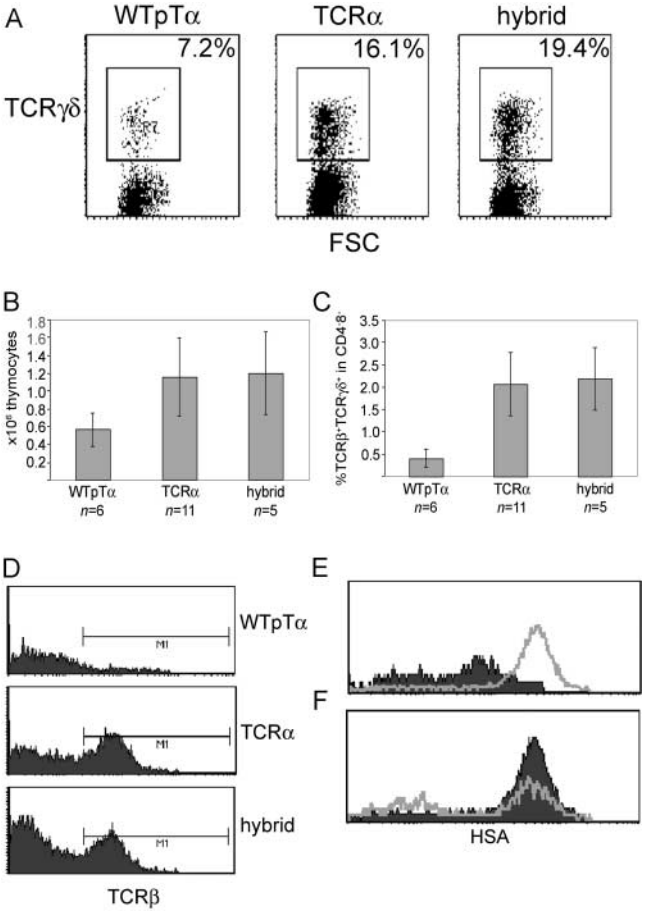

Figure 3. Defective αβ T lineage commitment induced by TCR complexes containing TCRα and hybrid molecules. Proportion (A) and absolute number (B) of $CD4^-8^-$ thymocytes expressing surface TCRγδ. (C) Percentage of $CD4^-8^-$ thymocytes coexpressing TCRγδ and TCRαβ on the cell surface. (D) Histograms depict the $CD4^-8^-TCR\beta^+$ population representative of a minimum of five 3–5-wk-old mice of each transgenic construct. Multiple founders were analyzed. (E) Surface HSA expression on $CD4^-8^-TCR\beta^+$ thymocytes in TCRα (overlay) and WTpTα (fill) mice. (F) Surface HSA expression of $CD4^-8^-TCR\beta^+$ (shaded histogram) and $CD4^-8^-TCR\gamma\delta^+$ (overlay) thymocytes in TCRα mice.

and TCRα mice were stained for intracellular TCRβ chains. If the majority of $CD4^+8^+$ in TCRα mice were selected for progression to the $CD4^+8^+$ stage by TCRαγ4 complexes, a large proportion of these $CD4^+8^+$ would fail to express intracellular TCRβ chains (22). However, >80% of the $CD4^+8^+$ thymocytes in TCRα mice expressed intracellular TCRβ (Fig. 2 D), indicating that they were selected for progression to the $CD4^+8^+$ stage by TCRαβ complexes, rather than by TCRαγ complexes.

Pre-TCR signaling in DN3 thymocytes induces downregulation of CD25 surface expression, survival, entry into the cell cycle, and progression through the DN4 stage, as well as acquisition of CD4 and CD8 surface expression (5). Propidium iodide staining of DN4 thymocytes sorted from TCRα and TCRα/pTα hybrid mice revealed a threefold reduction in the proportion of this population in the S/G2/M phases of the cell cycle (Fig. 2 B), whereas Annexin V staining identified a three- to fourfold increase in the proportion of cells expressing the apoptotic surface phenotype characterized by externalized phosphatidyl serine (Fig. 2 C). Collectively, these data implicate deficits in cell cycle entry and survival initiated by TCR complexes containing TCRα or TCRα/pTα hybrid molecules as two reasons for the observed thymic hypocellularity.

Defective αβ T Cell Lineage Commitment Induced by TCRα and TCRα/pTα Hybrid Molecules. In accordance with the behavior of endogenous pTα (13, 25), provision of the WTpTα transgene to $pT\alpha^{-/-}$ mice reduced the proportion and absolute number of thymocytes expressing surface TCRγδ complexes (19). When compared with the $CD4^-8^-$ compartment in WTpTα thymi, both the proportion (Fig. 3 A) and absolute number (Fig. 3 B) of $TCR\gamma\delta^+$ thymocytes were elevated in mice expressing TCRα or TCRα/pTα hybrid transgenes. In contrast to the pre-TCR, receptors substituted with TCRα and TCRα/pTα hybrid molecules lack the ability to rescue $CD4^-8^-$ thymocytes from commitment to the γδ TCR lineage.

Previous papers investigating the effects of premature TCRα expression using TCRαβ (17, 18) or TCRα (15) transgenic mice identified a population of $CD4^-8^-TCR\beta^+TCR\gamma\delta^+$ cells in some transgenic lines. However, differences in the developmental stage at which expression of the various TCR transgenes began made a conclusion about the αβ lineage commitment capacity of TCRαβ complexes difficult. To more definitively resolve this issue, we examined the $CD4^-8^-TCR\beta^+TCR\gamma\delta^+$ populations in mice expressing WTpTα and TCRα molecules at precisely the same developmental stage. When expressed at the pre-TCR checkpoint, the TCRα molecule generated a four- to fivefold larger population of $CD4^-8^-TCR\beta^+TCR\gamma\delta^+$ thymocytes than does the WTpTα molecule (Fig. 3 C), further supporting the notion that TCRαβ complexes lack the robust αβ lineage commitment capacity possessed by pre-TCR complexes.

In addition to the abnormally increased population of $CD4^-8^-TCR\beta^+TCR\gamma\delta^+$ thymocytes, an unusually large population of $CD4^-8^-TCR\beta^+TCR\gamma\delta^-$ thymocytes was observed in mice expressing TCRα and TCRα/pTα hybrid transgenes (Fig. 3 D). In contrast to the $HSA^{lo}CD4^-8^-TCR\beta^+TCR\gamma\delta^-$ population observed in C57Bl/6 (26) and WTpTα mice, the $CD4^-8^-TCR\beta^+TCR\gamma\delta^-$ thymocytes in TCRα and TCRα/pTα hybrid mice bore an immature HSA^{hi} surface phenotype (Fig. 3 E). In fact, their HSA^{hi} surface phenotype most closely resembled that of thymic HSA^{hi} $TCR\gamma\delta^+$ thymocytes (Fig. 3 F). Collectively, these observations raise the possibility that TCRαβ complexes may substitute more effectively for the TCRγδ complex than for the pre-TCR.

Differences in Pre-TCR and TCRαβ Functionality Become Much More Apparent under Competitive Conditions. Analysis of the ability of precursors expressing each transgene merely to fill empty thymi, in an environment of unlimited resources and niche space, might obscure functional differences between each TCR complex. To directly assess the

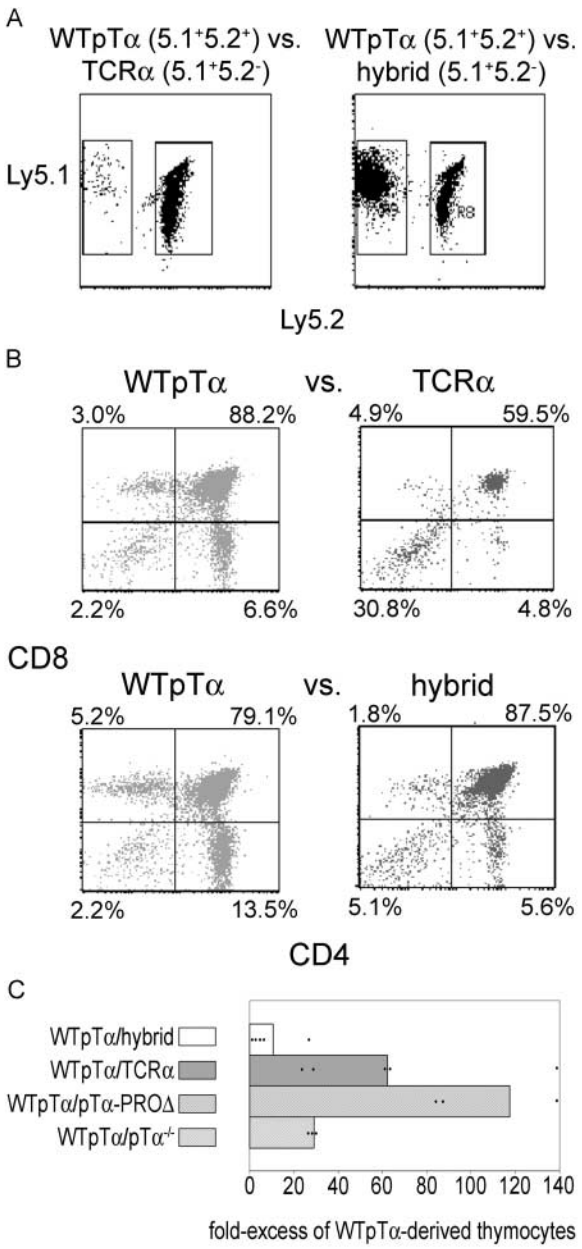

Figure 4. Competitive ability of TCRα, hybrid, and WTpTα-derived thymocytes. (A) Dot plots depict apparent domination of WTpTα-derived thymocytes when in direct competition with TCRα and TCRα/pTα hybrid–derived thymocytes. (B) Dot plots depict the CD4 versus CD8 surface profile of WTpTα, TCRα, and TCRα/pTα hybrid–derived thymocytes during competitive thymic reconstitution. (C) Average fold excess of WTpTα-derived thymocytes during competitive thymic reconstitution, normalized to any fold excess $CD4^-8^-25^-44^+$ (DN1) cells in either population. WTpTα versus $pT\alpha^{-/-}$ competition is included as a negative control to illustrate background. Dots depict individual mice for each type of competition.

ability of TCRα and TCRα/pTα hybrid molecules to function as the partner of TCRβ in the pre-TCR complex, we forced precursors expressing each of these molecules to compete with those expressing WTpTα. To this end, $1–2 \times 10^5$ lineage-negative BM cells from mice expressing TCRα TCRα/pTα hybrid transgenes or $pT\alpha^{-/-}$ mice were sorted and mixed with equal numbers of lineage-negative BM cells sorted from WTpTα mice. Crossing TCRα, TCRα/pTα hybrid, and WTpTα transgenes onto the $pT\alpha^{-/-}$ $Ly5.1^+Ly5.2^-$ or $Ly5.1^+Ly5.2^+$ background allowed us to identify thymocytes derived from each donor. Each BM cell mixture was injected into the tail vein of irradiated $Rag^{-/-}\gamma c^{-/-}$ recipients. Reconstituted thymi were analyzed 5 wk after injection.

The differences visible between thymi of mice expressing WTpTα and TCRα transgenes became much more apparent in the competition assay. Although in noncompetitive assays, WTpTα transgenic thymi contained on average only threefold more thymocytes than did TCRα transgenic thymi, WTpTα-derived thymocytes dominated TCRα-derived thymocytes in the competitive reconstitution assay by an average of >60-fold (Fig. 4, A and C). The proportion of WTpTα-derived donor thymocytes that progressed to the $CD4^+8^+$ developmental stage was also far greater than that of the competing TCRα-derived population (Fig. 4 B). $pT\alpha^{-/-}$-derived thymocytes were dominated by WTpTα-derived thymocytes by an average factor of thirty (Fig. 4, A and C). This slight (twofold) difference in performance between thymocytes derived from TCRα and $pT\alpha^{-/-}$ BM could be the consequence of transgenesis and was not statistically significant ($P > 0.09$). Therefore, provision of a TCRα transgene failed to give any competitive advantage to a $pT\alpha^{-/-}$ population.

Properties Inherent to pTα Bestow Some Competitive Ability on TCRα. In contrast with the noncompetitive analyses, the competitive reconstitution assays revealed clear differences in the abilities of cells expressing TCRα and TCRα/pTα hybrid molecules to compete with cells expressing WTpTα molecules. In comparison with the 60-fold domination of WTpTα-derived thymocytes over their TCRα competitors, TCRα/pTα hybrid–derived thymocytes were on average dominated by WTpTα-derived competitors by only a factor of ten (Fig. 4, A and C). Similarly, when compared with TCRα-derived thymocytes, a larger proportion of TCRα/pTα hybrid–derived thymocytes progressed to the $CD4^+8^+$ developmental stage (Fig. 4 B). To confirm the significance of the pTα cytoplasmic domain specifically at the pre-TCR checkpoint, the performance of a pTα molecule lacking cytoplasmic proline-rich motifs was placed in direct competition with WTpTα. When controlled by the $p56^{Lck}$ proximal promoter, expression of this pTα-PROΔ on a $pT\alpha^{-/-}$ genetic background resulted in defective β selection and αβ T lineage commitment (19). pTα-PROΔ–derived thymocytes were dominated by WTpTα-derived competitors by a factor of >100 (Fig. 4 C). The TCRα/pTα hybrid molecule mediated passage through the $CD4^-8^-CD25^+44^-$ (DN3) pre-TCR checkpoint as efficiently as did the WTpTα molecule (Fig. 5). In contrast, thymocytes expressing a pTα molecule lacking the proline-rich region of its cytoplasmic tail (pTα-PROΔ) accumulated at the DN3 developmental stage during forced competition with thymocytes expressing WTpTα (Fig. 5).

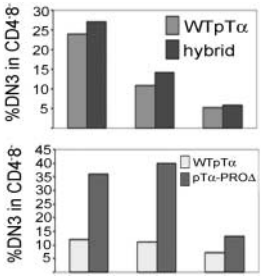

Figure 5. Enhanced performance of hybrid at pre-TCR checkpoint requires intact pTα cytoplasmic domain. Percent of $CD4^-8^-$ thymocytes expressing $CD25^+CD44^-$ (DN3) surface phenotype in each competing population. Three individual mice are shown for each competition.

Together, these observations solidify the importance of pTα, specifically its intact cytoplasmic domain, in securing successful passage through the pre-TCR checkpoint.

"pTα-like" Performance of TCRα/pTα Hybrid Molecule Is Altered by the Presence of the Extracellular Domain of TCRα. Although the TCRα/pTα hybrid performance was superior to that of the TCRα and pTα-PROΔ molecules, it remained inferior to that of WTpTα. As the only difference between the TCRα/pTα hybrid and WTpTα molecules is the presence of the extracellular domain of TCRα in the former, perhaps properties inherent to this domain are detrimental to the function of the pTα-derived portion of the TCRα/pTα hybrid molecule.

Specifically, the more efficient pairing of TCRβ with TCRα than with pTα (27) is attributed to the presence of a second extracellular Vα domain in TCRα, which is absent in pTα. In addition, pairing with TCRβ is influenced by differences in the position of the interchain cystine residue within the connecting peptide regions of TCRα and pTα. The addition of a Vα domain to the extracellular portion of pTα generated a molecule capable of outcompeting wild-type pTα for pairing with TCRβ molecules in the ER (27). The distance between the transmembrane domain and the cystine residue involved in covalent linkage with TCRβ is longer in TCRα than in pTα. Relocation of this TCRα cystine to the position of the pTα cystine was accompanied by loss of efficiency in pairing with TCRβ, to the degree that this mutant TCRα molecule, when expressed in the same cell as a wild-type TCRα molecule, could not compete for pairing with limited TCRβ molecules (27). By virtue of its TCRα extracellular domain and connecting peptide sequence, the hybrid may outcompete pTα for pairing to TCRβ in the ER. This higher efficiency of pairing may better protect TCRβ monomers from degradation in the ER, resulting in higher levels of hybrid TCR complexes at the thymocyte surface. The apparent strict regulation of pre-TCR surface expression, mediated by its constitutive internalization and ubiquitin-mediated proteasome-dependent degradation (6, 7), highlights the potential link between receptor surface level and function. Thus, deviation from the exquisitely low level of surface receptor expression characteristic of the pre-TCR checkpoint could alter the intensity of signals received by DN3 thymocytes.

Indeed, symmetrical TCR complexes containing TCRα and TCRα/pTα hybrid molecules, both of which contain a second extracellular Vα domain as well as TCRα connecting peptide sequences, were expressed at levels higher than those of normal pre-TCR complexes on the surface of the $58\alpha^-\beta^+$ T cell hybridoma (28), which is a variant of a DO-11.10.7 mouse T cell hybridoma that does not express functional TCRα or TCRβ chains (Fig. 6). Thus, the possibility exists that higher surface levels might impair the pTα-like function of TCRα/pTα hybrid TCR complexes.

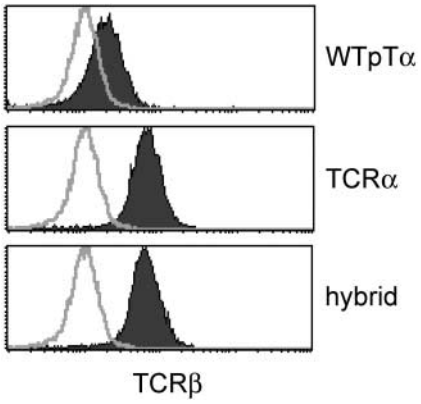

Figure 6. Influence of the extracellular region of TCRα on the behavior of the hybrid molecule. TCRβ levels on the surface of the $58\alpha^-\beta^+$ T cell hybridoma are influenced by the second extracellular domain TCRα. $58\alpha^-\beta^+$ hybridoma cells were infected with retroviruses encoding WTpTα, TCRα, or TCRα/pTα hybrid molecules, followed by an internal ribosomal entry site and enhanced green fluorescent protein (EGFP). Infected cells were identified by EGFP expression. Shaded histograms represent TCRβ surface staining on cells infected with retroviruses expressing WTpTα, TCRα, or TCRα/pTα hybrid molecules. The overlay represents TCRβ staining on cells infected with a control retrovirus expressing EGFP alone. The $58\alpha^-\beta^+$ T cell hybridoma is a variant of the DO-11.10.7 mouse T cell hybridoma that does not express functional TCRβ or TCRα chains (28).

Discussion

Here, we show that replacement of pTα with TCRα precisely at the pre-TCR developmental checkpoint generated a TCRαβ complex incapable of inducing normal β selection processes. TCRα substitution resulted in an increase in apoptosis and decrease in proliferation of DN4 thymocytes, a defect in progression to the $CD4^+8^+$ developmental stage, and a failure to rescue thymocytes from the γδ T cell lineage. The similarities between these defects and those observed in $pT\alpha^{-/-}$ mice emphasize the inability of TCRα to functionally replace pTα as the partner of TCRβ in the pre-TCR complex. Although visible in noncompetitive conditions, the differences in functional capacity between pre-TCR and TCRαβ complexes became much more dramatically apparent when placed in direct competition with each other. Addition of the pTα transmembrane and cytoplasmic domains to the extracellular domain of TCRα generated a TCRα/pTα hybrid molecule possessing enhanced abilities that became evident only when placed in direct competition with WTpTα. The competitive performance of a pTα molecule lacking the

proline-rich regions of the cytoplasmic domain (pTα-PROΔ) was inferior to that of both the TCRα/pTα hybrid and TCRα molecules, suggesting that possession of an intact pTα cytoplasmic domain was required for the improved competitive ability displayed by the TCRα/pTα hybrid molecule.

Although the TCRα/pTα hybrid performance was superior to that of the TCRα and pTα-PROΔ molecules, it remained inferior to that of WTpTα. One possible explanation for this discrepancy is that the higher surface expression levels of TCRα/pTα hybrid TCR complexes may impair their pTα-like function (Fig. 6). The strict regulation of pre-TCR surface expression, mediated by its constitutive internalization and ubiquitin-mediated proteasome-dependent degradation (6, 7), highlights the significance of the potential link between receptor surface level and function. Alternatively, the pTα-like function of TCRα/pTα hybrid TCR complexes may be affected by the strength of association between TCRα/pTα hybrid molecules and TCRζ chains. Sequences unique to the connecting peptide of TCRα, which are present in the TCRα/pTα hybrid but absent in WTpTα, are required for strong association with TCRζ chains (29). Their potentially stronger association with TCRζ chains might allow TCRα/pTα hybrid TCR complexes to activate or augment signaling pathways that are silent or attenuated during normal pre-TCR signaling. A final possibility is that the second extracellular Vα domain in the TCRα/pTα hybrid molecule may allow, in addition to higher levels of surface expression, recognition of MHC molecules in the thymic microenvironment. In contrast, the asymmetrical nature of the pre-TCR ectodomain precludes recognition of MHC molecules. The observed spontaneous segregation of pre-TCR complexes to glycolipid-enriched microdomains, which contain a high concentration of signaling molecules (2), may be involved in the cell-autonomous nature of pre-TCR signal initiation. Recognition of thymic MHC molecules might interfere with spontaneous membrane segregation processes, resulting in altered receptor signaling capacity.

Whether or not TCRα can substitute for pTα in the pre-TCR complex has been a matter of some controversy and discussion. Conflicting views placing significance either on the receptor components themselves or on the developmental stage at which they are expressed have been the subject of numerous investigations. However, three factors have precluded a complete assessment of the interchangeability of pTα and TCRα at this point of development. These factors are as follows: (a) expression of TCRβ and/or TCRα transgenes at developmental stages different than that of endogenous pTα, either in the presence (15–18, 22) or absence (14) of endogenous pTα; (b) analyses of the ability of precursors to fill empty thymi with no competition for niche space or resources; and (c) use of fetal thymic organ culture (FTOC) systems that may not reveal the full proliferative potential of precursors (21). Here, we observe the performance of a TCRα molecule expressed in the absence of pTα, at the same relevant developmental stage and RNA levels as endogenous pTα, in adult thymi, both by itself and in direct competition for thymic space and resources.

Utilization of $p56^{Lck}$ proximal promoter elements ensured a uniform temporal regulation of WTpTα, TCRα, TCRα/pTα hybrid, and pTα-PROΔ transgene expression. Thus, our results fail to support the notion that the developmental stage at which the pre-TCR is expressed, rather than the identity of the receptor components themselves, is the factor on which pre-TCR function is most dependent. A recent work (21), reaching the opposite conclusion, compared the development of $pT\alpha^{-/-}$ fetal thymocytes retrovirally transduced with either WTpTα or TCRα in FTOC. The short time of incubation in FTOC (2 d), which may have obscured differences in the full proliferative potential of precursors retrovirally transduced with each receptor component, raises a question regarding the validity of the conclusion that these two molecules are functionally equivalent at the pre-TCR checkpoint.

Its failure to rescue $CD4^-8^-$ thymocytes from commitment to the γδ T cell lineage may be perhaps the most significant deficiency in the repertoire of "pre-TCR-like" skills possessed by a TCRαβ complex. Specifically, this defect highlights a potential reason for the precise temporal segregation of pTα and TCRα expression during thymocyte development. By virtue of its failure to direct immature $CD4^-8^-$ thymocytes through the normal β selection processes vital to the development of mature $CD4^+$ and $CD8^+$ T lymphocytes, a TCRα molecule expressed at the pre-TCR checkpoint could instead direct young $CD4^-8^-$ thymocytes into a pathway leading to an immature γδ-like fate.

Our data in TCRα transgenic mice and previous data in $pT\alpha^{-/-}TCR\delta^{-/-}$ mice (14) indicate that a prematurely expressed TCRα chain can generate some limited numbers of TCRβ-selected $CD4^+8^+$ thymocytes and argues that the αβ TCR, rather than a putative TCRγ4/TCRα heterodimer (22), is involved in the selection of $CD4^+8^+$ thymocytes.

The requirement for the cytoplasmic domain of pTα at the pre-TCR checkpoint might reside in its ability to recruit the pre-TCR complex to glycolipid-enriched membrane microdomains rich in signaling molecules. An interaction of the proline-rich region of the pTα cytoplasmic domain with Src homology 3 domains of raft-localized Src kinases could result in both raft localization and, via disruption of the inhibitory Src kinase intramolecular loop, in cell-autonomous activation of Src kinase activity. Alternatively, the pTα cytoplasmic domain might be required for constitutive internalization and degradation of pre-TCR complexes (6, 7), thereby regulating and maintaining the exquisitely low levels of pre-TCR complexes normally observed on the thymocyte surface.

Future studies analyzing differences in genetic and biochemical profiles induced by expression of WTpTα and TCRα at the pre-TCR checkpoint may provide insight as to why TCRα is unable to substitute for pTα at this crucial stage of thymocyte development.

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