Identification of a Late Stage of Small Noncycling pTα Pre-T Cells as Immediate Precursors of T Cell Receptor α/β+ Thymocytes

By César Trigueros,* Almudena R. Ramiro,* Yolanda R. Carrasco,* Virginia G. de Yebenes,* Juan P. Albar,‡ and María L. Toribio*

From the *Centro de Biología Molecular "Severo Ochoa," and the ‡Departamento de Immunología y Oncología, Centro Nacional de Biotecnología, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Summary

During thymocyte development, progression from T cell receptor (TCR)β to TCRα rearrangement is mediated by a CD3-associated pre-TCR composed of the TCRβ chain paired with pre-TCRα (pTα). A major issue is how surface expression of the pre-TCR is regulated during normal thymocyte development to control transition through this checkpoint. Here, we show that developmental expression of pTα is time- and stage-specific, and is confined in vivo to a limited subset of large cycling human pre-T cells that coexpress low density CD3. This restricted expression pattern allowed the identification of a novel subset of small CD32 thymocytes lacking surface pTα, but expressing cytoplasmic TCRβ, that represent late non-cycling pre-T cells in which recombination activating gene reexpression and downregulation of T early α transcription are coincident events associated with cell cycle arrest, and immediately preceding TCRα gene expression. Importantly, thymocytes at this late pre-T cell stage are shown to be functional intermediates between large pTα+ pre-T cells and TCRα/β+ thymocytes. The results support a developmental model in which pre-TCR–expressing pre-T cells are brought into cycle, rapidly downregulate surface pre-TCR, and finally become small resting pre-T cells, before the onset of TCRα gene expression.

Key words: pre-T cells • pTα • noncycling • recombination activating gene • T early α

The pre-TCR from the endoplasmic reticulum/cis-Golgi compartment (8). The question remains as to whether pre-TCR signaling is triggered by binding to an extracellular ligand or, alternatively, as proposed recently (9), whether pre-TCR complexes become constitutively active as soon as they reach the plasma membrane, where signaling molecules are available. In this latter situation, pre-TCR activity might be regulated by control of membrane expression. However, extremely low levels of the pre-TCR complex (~100-fold lower than those of the TCRα/β on mature T cells) appear to reach the plasma membrane of immature thymocytes (10), a fact that has hindered the development of monospecific anti-pre-TCR reagents and, hence, the study of pre-TCR expression patterns on normal thymocytes.

Current data support the notion that one of the first consequences of pre-TCR expression is the induction of a cell cycle progression that results in the greatest expansion in cell numbers that occurs in the developing thymus (1, 11). In mice, this process is associated with differentiation of CD44+CD8+ into CD44−CD8− double negative (DN)
towards a more precise definition of the stages involved in proliferating DP thymocytes (19, 20). However, progress in understanding the stages is limited by the lack of a precise definition of the stages involved in the development of T cells. TCR expression is an obligatory early event in the opening of the TCR-CD3 complex, which is likely to be an important component of the checkpoint that controls cell-cycle progression. However, the TCR or TCR-CD3 complex is not detectable until the CD44 

Immunohistochemical analysis (25) was performed with an anti-pT B1 Ab that recognizes an exposed epitope of the native human CD3/pT B1 Ab. CD3/pT B1 Ab was detected by sequential staining with a rabbit polyclonal Ab (BMA031 [reference 22]; provided by Dr. R. Kurrle, Behringwerke AG, M arburg, Germany) and anti-pT B1 Ab (25; provided by Dr. M. Brenner, Brigham and Women's Hospital, Boston, MA) and anti-CD3 (25; provided by Dr. M. Brenner, Brigham and Women's Hospital, Boston, MA). The pre-TCR induced cell cycle transition is, in turn, associated with the downregulation of RAG-1 and RAG-2 gene transcription (16). RAG-2 protein expression is hampered because previous attempts to demonstrate surface expression of the pre-TCR complex throughout normal thymocyte development have been unsuccessful. In this study, analysis performed with a polyclonal rabbit Ab that recognizes an exposed epitope of the native human CD3/pT B1 Ab protein revealed a restricted pattern of surface CD3/pT B1 Ab expression during normal human pre-T cell development. On the basis of surface CD3/pT B1 Ab expression and cell size, we have identified a novel subset of small pre-T cells that lack surface CD3/pT B1 Ab expression and are mostly in a non-cycling state. Transition to this developmental stage is shown to be associated with the induction of specific developmental events that precede expression of the TCR-CD3 complex. Interestingly, small pT B1 Ab noncycling pre-T cells are shown to be functional intermediates between large pT B1 Ab-bearing pre-T cells and the first thymocytes expressing the mature TCR-CD3 complex.

Materials and Methods

Isolation of Thymocyte Subsets. Postnatal thymocytes isolated from thymus samples removed during corrective cardiac surgery of patients aged 1 mo to 3 yr were fractionated by centrifugation on stepwise Percoll density gradients (LKB, Uppsala, Sweden), as described elsewhere (21). Thymocytes from the 1.068 and 1.08 density layers were designated as large and small thymocytes, respectively. Large thymocytes were depleted (>99% purity) of mature T cells (CD4/CD8), B cells, N K cells, and myeloid cells by two rounds of treatment with magnetic beads (Dynabeads Dynal A.S., O slo, Norway) coupled to the following mAbs: anti-CD4 (BM A031 [reference 22]; provided by Dr. R. Kurrle, Behringwerke AG, M arburg, Germany) and anti-CD8 (BM A031 [reference 22]; provided by Dr. R. Kurrle, Behringwerke AG, M arburg, Germany). Thymocytes from the 1.068 fraction were purified by depletion of CD3 low DP thymocytes. Small thymocytes were then isolated from the 1.068 fraction after two rounds of fractionation on Percoll gradients, followed by depletion of T, B, N K, and myeloid cells, and anti-CD8 sorting, as described above. Such large TCR-CD3/CD8 B DP thymocytes consisted almost entirely (95–99%) of CD3 low cells.

Small thymocytes recovered from the 1.08 density layer were depleted of TCR-CD3/CD8 B DP thymocytes (either CD3 low or CD3 high) by anti-CD14 (Caltag Laboratories, Inc.) were first incubated with the anti-pT B1 Ab, followed by treatment with anti-CD3, large CD3 low DP thymocytes were isolated from the 1.08 fraction recovered after two rounds of fractionation on Percoll gradients, followed by depletion of T, B, N K, and myeloid cells, and anti-CD8 sorting, as described above. Such large TCR-CD3/CD8 B DP thymocytes consisted almost entirely (95–99%) of CD3 low cells.

Small thymocytes recovered from the 1.08 density layer were depleted of TCR-CD3/CD8 B DP thymocytes (either CD3 low or CD3 high) by anti-TCR-CD3/CD8 B magnetic bead depletion as described above for large cells. CD3 low cells (termed small CD3 low DP thymocytes) were then isolated from the recovered population (>99% CD3 low CD8 B) by anti-CD3 bead depletion (Dynal A.S.). Mature TCR-CD3/CD8 B single positive (SP) thymocytes were isolated as described previously (15).

Flow Cytometry Analysis. Directly labeled mAbs against CD3 (Leu4-PE) and CD8 (Leu2a-FITC) were obtained from Becton Dickinson; anti-CD4 (CD4-PE-Cy5) mAbs were purchased from Caltag Laboratories, Inc. (San Francisco, CA). A PE-labeled mAb against the human TCR V B1 family (24) was obtained from Serotec Ltd. (Kidlington, O xford, U.K.). Unlabeled mAbs against monomorphic determinants of either TCR-CD3/CD8 (BM A031) or TCR-CD3/CD8 (BM A031), or against the human TCR V 12.1 (25; provided by Dr. M. Brenner), were used in combination with goat anti-mouse FITC or PE-coupled F(ab)2 Ig (Caltag Laboratories, Inc.). Isotype-matched irrelevant mAbs (Caltag Laboratories, Inc.) were used as negative controls.

For detection of cytotoxic TCR B, cells were stained with the anti-TCR-CD3/CD8 B mAb J5 (26; provided by Dr. M. Brenner), as described elsewhere (15). Surface expression of pT B1 Ab was determined by sequential staining with a rabbit polyclonal Ab (ED-1) derived in this study (see below), plus FITC-conjugated goat anti-rabbit F(ab)2 Ig (Southern Biotechnology Associates, Inc., Birmingham, AL). Preimmune rabbit serum was used as negative control. For peptide competition, peptide (100 µg/ml) was first incubated with the anti-pT B1 Ab for 1 h at room temperature. Stained cells were analyzed in a flow cytometer (EPICS XL; Coulter Corp., Hialeah, FL) as described previously (15). Cell cycle analyses were performed by flow cytometry using a doublet discrimination function in cells treated with 0.05% digitonin (Sigma Chemical Co., St. Louis, MO), washed, and stained.
with 50 μg/ml of propidium iodide (PI; Sigma Chemical Co.), as described elsewhere (15).

Generation of Polyclonal Anti-human pTα Abs. A synthetic peptide corresponding to the human pTα sequence 61–82 (15), with an additional Cys in the COOH-terminal region, was coupled to Mâleimide-activated KLH following the manufacturer’s instructions (Pierce Chemical Co., Rockford, IL). R Abs were immunized with 1 mg of the peptide-KLH conjugate in CFA (Difco Laboratories Inc., Detroit, MI) and boosted 30 and 50 d later with 0.5 mg of immunogen in IFA (Difco Laboratories Inc.). The animals were bled 10 d after the last booster injection, and the sera were purified by affinity chromatography (Sulfolinic Coupling gel; Pierce Chemical Co.) and tested for antipeptide reactivity with a horseradish peroxidase-labeled polyclonal goat anti-rabbit IgG (Nycodemed Amersham plc, Little Chalfont, Bucks, UK) plus-pheno-lylene diamine dihydrochloride (OPD; Sigma Chemical Co.).

cT Transfections and Immunofluorescence Assays. c-myc tagging was performed by PCR amplification of a complete pTα cDNA contained in the Bluescript-KS II plasmid (Stratagene Inc., La Jolla, CA) with the sense 5'-GGG CCC GGA TCC ATA TGG CCG GTA CAT GGC TG-3' and antisense 5'-GGG CCC GGA TCC GTA CAT GGC TG-3'. The fragment obtained by HindIII-EcoRI restriction enzyme digestion and ligation into the BamHI site of the Bluescript-KS II plasmid (Clontech, Palo Alto, CA) was cloned into the BamHI site of the pcDNA3 plasmid vector. An EcoRI-Inot restriction fragment from the pTα-EGFP vector was subsequently ligated into the pCDNA3 plasmid vector (Invitrogen Corp., Carlsbad, CA). The cT CRα (V, 12, 1) full-length cDNA (AV1251) was the gift of Dr. J.A. López de Castro (Centro de Biología Molecular “Severo Ochoa”), The pTα-green fluorescent protein (GFP) fusion was carried out by PCR amplification of a complete pTα cDNA with the sense 5'-GGG CCC GGA TCC ATA TGG CCG GTA CAT GGC TG-3' and antisense 5'-GGG CCC GGA TCC GTA CAT GGC TG-3'. The fragment obtained by HindIII-EcoRI restriction enzyme digestion and ligation into the BamHI site of the pcDNA3 plasmid vector. COS cells were transfected by electroporation with 5 μg of pSR-pTα-myc plasmid plus 20 μg of carrier pUC-19 plasmid DNA (Promega Corp., Madison, WI), at 250V, 960 μF, at 25°C (Southern Biotechnology Associates, Inc.). The coverslips were sequentially incubated with 0.5 mg of immunogen in IFA (Difco Laboratories Inc., Detroit, MI) and boosted 30 and 50 d later with 0.5 mg of immunogen in IFA (Difco Laboratories Inc.).

Reverse Transcription PCR Analysis. Total RNA (1 μg) was reverse-transcribed into cDNA according to the manufacturer’s protocol (Boehringer Mannheim, Mannheim, Germany). Equivalent amounts of cDNA among different samples were estimated by reverse transcription (RT)-PCR carried out for 18, 21, and 25 cycles with β-actin primers as described previously (15). Titration of cycle number allowed us to perform densitometric analyses (Bio-imaging BAS 1500; Fujifilm, Kanagawa, Japan) under nonsaturating conditions. Vα degenerate primers used in combination with Cα primers enabled the amplification of all known human Vα segments, as described (32). Specific amplifications were detected by Southern blotting with a Cα probe (29).

Hybrid Human/Mouse T lymphoma cultures. In the vitro generation of mature TCRα/β human T cells was analyzed using a modification of the previously described hybrid human/mouse fetal thymic organ culture (hut/mo FT OC [33]). In brief, thymi removed from 15-d-old embryos of Swiss mice were precultured for 5–6 d in the presence of 1.35 mM dGuo (Sigma Chemical Co.). The thymic lobes were then washed and cocultured in hanging drops in Terasaki plates (Nunc, Inc., Roskilde, Denmark) with either large CD3+ (106 cells/lobe) or small CD3- (106 cells/lobe) human pre-T cells. After 2 d, lobes were transferred to filters (Millipore Corp., Bedford, MA), which were layered over gelfoam rafts and cultured in IMDM supplemented with 2% human AB serum and 5% FCS (GIBCO BRL, Paisley, UK). Surface staining of human cells was performed at the indicated culture periods, and flow cytometric analyses were then performed on electronically gated CD45+ human cells.

Results

Surface CD3 Expression and Cell Size Define Distinct Subsets of Human TCRα/β- DP Thymocytes. We have previously identified a subset of large cycling CD4+CD8+ human thymocytes in which the TCRβ chain is expressed as part of a complex distinct from the mature αβ TCR, likely the pre-TCR (15). According to their size, such TCRα/β- DP thymocytes could be selectively isolated from the fraction of large cells recovered from Percoll density gradients (approximately one third of total unfractonated thymocytes), whereas conventional TCRα/β+ DP thymocytes were more common with the small-sized cell fraction (around two thirds of total thymocytes [15]). Since human thymocytes typically coexpress CD3 and the αβ TCR in stoichiometric amounts, CD3 expression studies similarly defined a differential distribution of cell subsets among Percoll-fractionated thymocytes (Table 1). However, analysis of the correlated expression of CD3 versus Cα (PY 1.4 [29]) or Cβ (Jurβ2 [30]) regions (provided by Dr. T.W. Mak, The Ontario Cancer Institute, Toronto, Ontario, Canada). The RAG-1 and RAG-2 cDNA probes (31) were the gift of Dr. L.A. Turk (The Howard Hughes Medical Institute, Ann Arbor, MI), and the human TCRα cDNA probe was derived in our laboratory (15). The TEA probe was generated by PCR amplification (sense primer 5'-TGG ATG GAT AGA GAC AGA TGG TG-3' and antisense primer 5'- CCT GCC CTT GGG AAT AAT AGG-3') of the K562 erythroleukemia genomic DNA and cloning in a pMOS Blue-T vector (Nycodemed Amersham plc). The fragment obtained by HindIII-EcoRI restriction enzyme digestion was used for Northern blotting. The same blot was subsequently stripped and hybridized with a β-actin probe (15).
CD3 low thymocytes, essentially all CD3 low small cells (15–20% of total small thymocytes) coexpressed the CD3 low TCR. However, small CD3− thymocytes were phenotypically similar to CD3+ large cells in that they expressed neither the α/β nor the γ/δ TCR (Fig. 1, and data not shown). As both large and small CD3low thymocytes displayed a homogeneous CD4+CD8− DP phenotype (see below), they were phenotypically indistinguishable except for the expression of α/β TCR on small DP thymocytes, but not on large DP thymocytes.

Surface expression of pT α chain C can be detected with anti-pT α Ab. The above results prompted us to investigate whether large thymocytes with the CD3low TCR α/β− phenotype do represent pre-T cells expressing the pre-TCR. However, this issue was difficult to approach because no appropriate reagents such as anti-pT α Abs or Abs able to recognize the human TCR β chain on the cell surface were available. Consequently, Abs were raised in rabbits against a synthetic peptide contained in the extracellular Ig-like domain of the human pT α molecule (15). The specificity of the affinity-purified antisera was then assayed by immunofluorescence microscopy of COS cells transfected with a pT α cDNA, tagged with a c-myc epitope that is recognized by the specific 9E10 mAb. Results in Fig. 2A show that one of these anti-pT α antisera (ED-1) was reactive against all c-myc+ transfectedants (top panels), and that both anti-c-myc and anti-pT α reagents displayed an identical intracellular recognition pattern (bottom panels), thus confirming the anti-pT α specificity of the ED-1 antisera.

To determine whether the anti-pT α antisera was also able to recognize the pT α chain when expressed on the cell surface, we next derived pT α stable transfectants from the human T cell line SU-P-T1, which expresses TCR β (Vp11) in the absence of a functional TCR α chain and, hence, lacks surface TCR α/β heterodimers (34). As a summary of these results, we have developed a sensitive method to detect pT α expression on the surface of human thymocytes. Postnatal thymocytes were fractionated into large and small cells on Percoll density gradients. CD3−, CD3low, CD3int, and CD3bright subsets from each fraction were analyzed for cell size by flow cytometry (mean FSC ± SD of 10 independent experiments). Unfractionated total thymocytes are included for comparison.

**Table 1.** FSC analysis and relative cell numbers of human thymocyte subsets as defined by their CD3 expression levels

| Subset          | T total thymocytes | Large thymocytes | Small thymocytes |
|-----------------|--------------------|------------------|------------------|
|                 | relative no. (%)   | mean FSC ± SD    | relative no. (%) | mean FSC ± SD | relative no. (%) | mean FSC ± SD |
| CD3−            | 22 ± 4.8           | 357 ± 12.1       | 28 ± 4.9         | 358 ± 12.0  | 19 ± 2.2         | 333 ± 17.2   |
| CD3low          | 21 ± 4.5           | 377 ± 14.3       | 25 ± 5.6         | 395 ± 19.5  | 17 ± 2.4         | 342 ± 22.3   |
| CD3int          | 31 ± 3.4           | 335 ± 22.3       | 13 ± 2.7         | 346 ± 20.0  | 45 ± 4.6         | 313 ± 14.3   |
| CD3bright       | 24 ± 4.6           | 340 ± 11.6       | 29 ± 5.1         | 330 ± 12.2  | 17 ± 6.1         | 329 ± 11.1   |

Postnatal thymocytes were fractionated into large and small cells on Percoll density gradients. CD3−, CD3low, CD3int, and CD3bright subsets from each fraction were analyzed for cell size by flow cytometry (mean FSC ± SD of 10 independent experiments). Unfractionated total thymocytes are included for comparison.
pressed low but detectable levels of CD3, but were unreactive with the BMA031 mAb which recognizes a common epitope of the TCR α/β dimer (22). In contrast, both TCR α/β and CD3 were detected on SUP-T1 clones stably transfected with a TCR α chain (Vα12.1), whose expression could be followed with the anti-Vα12.1 mAb 6D6 (25). Interestingly, a reciprocal expression pattern was observed when surface staining was performed with the anti-pTα Ab plus anti-CD3. Thus, pTα (GFP+) transfectants, but not TCR α (Vα12.1+) transfectants, were reactive with the anti-pTα antiserum and coexpressed CD3 in stoichiometric amounts (Fig. 2 B). It is worth noting that expression of the endogenous TCR β could be specifically detected with an anti-Vβ1 mAb (24) on both cell types. Strikingly, levels of TCR β expressed on pTαtransfectants were consistently lower than those on TCR α-expressing clones, although in both cases, either pTα or TCR α was coexpressed with TCR β in stoichiometric amounts (Fig. 2 B). As a whole, these data suggest that the ED-1 antiserum was able to specifically detect pTα-containing surface complexes which likely comprise CD3-associated TCR β-pTα heterodimers, the hallmark of the pre-TCR complex.

Surface pTα Expression Is Restricted In Vivo to Large-sized CD3low TCR α/β–DP Thymocytes. Having established that the anti-pTα antiserum recognized specifically pTα-containing surface complexes, we wished to examine whether pTα was actually expressed on the surface of TCR α/β–primary thymocytes. To this end, Percoll-fractionated large and small DP thymocytes depleted of CD3int and CD3bright cells (including both TCR α/β– and TCR γδ– cells) were analyzed by flow cytometry for their reactivity with the anti-pTα Ab. As expected, both isolated DP cell subsets were exclusively composed of CD3– and CD3low cells (Fig. 3 A). CD3low thymocytes made up ~50 and 30% of the large- and small-sized DP thymocytes, respectively. Of these, only small CD3low thymocytes coexpressed the α/β TCR (see above), albeit at low levels, suggesting that they were representative of the developmental onset of TCR α/β expression. As shown in Fig. 3 A, such cells were unreactive with the anti-pTα Ab. Expression of pTα was negative as well on CD3– DP thymocytes, regardless of their cellular size. In contrast, essentially all large CD3low cells displayed a low but detectable reactivity with the anti-pTα Ab, thus providing direct evidence that pTα-containing complexes are expressed in vivo on the surface of normal pre-T cells. That this low level staining is specific was demonstrated by showing that it could be completely inhibited by the specific pTα peptide (Fig. 3 B). It is worth noting that pTα and CD3 were coexpressed on large CD3low thymocytes in a stoichiometric-like fashion similar to that observed on SUP-T1 pTα transfectants (Fig. 2 B), suggesting that the pTα-containing complex expressed on the former cells did correspond to the CD3-associated pre-TCR.

Developmental Status of Subsets of TCR α/β–DP Thymocytes. The above results allowed a novel subdivision of the TCR α/β–DP compartment into three individual subsets of thymocytes defined as large DP CD3–, large DP CD3low, and small DP CD3–. Because pTα expression was

Figure 2. Specificity of anti-pTα ED-1 antiserum by immunofluorescence microscopy and flow cytometric analysis of pTα transfectants. (A) COS cells were transfected with a human pTα cDNA tagged with a c-myc epitope. After fixation and permeabilization, cells were sequentially stained with the affinity-purified anti-pTα ED-1 rabbit antiserum and the 9E10 anti-c-myc mAb. FITC-coupled goat anti-rabbit IgG and Texas red-coupled goat anti-mouse IgG were used as second Abs, respectively. Original magnification: (A) ×400 (top panel) and ×630 (bottom panel). (B) SUP-T1 human T-lineage cells were transfected either with a productively rearranged human TCR α (Vα12.1) cDNA or with a pTα-GFP cDNA. Stable transfectants, characterized, respectively, as Vα12.1– or GFP+ by flow cytometry (shaded monoparametric histograms) were assayed for their reactivity with anti-TCR α/β and anti-CD3 mAbs (top biperametric histograms), or with ED-1 anti-pTα antiserum and anti-CD3 (middle histogram). Coexpression of endogenous TCR β chain (Vβ1) with either pTα or TCR α (Vα12.1) was assayed on pTα-GFP and TCR α transfectants, respectively (bottom histograms). Background values were determined with preimmune rabbit serum and isotype-matched irrelevant mAbs.

pTα-GFP chimeric protein was used in these studies, reactivity of the anti-pTα antiserum could be analyzed by flow cytometry on stable transfectants traced by their GFP expression. As shown in Fig. 2 B, such GFP+ transfectants ex...
restricted to large DP CD3low thymocytes, we wanted to investigate further the developmental status of the distinct pTα+ and pTα− populations in order to improve definition of their precursor-product relationships. To this end, the three cell subsets were independently isolated and examined for their respective patterns of TCRβ, TCRα, and pTα gene expression. Northern blot analysis shown in Fig. 4 A revealed that both the 1.3-kb mature and the 1.0-kb immature TCRβ transcripts were expressed in the three subsets of TCRαβ− DP thymocytes, regardless of their cellular size and CD3 phenotype. In contrast, TCRα transcription was undetectable in all of them, but occurred at high levels in mature SP thymocytes included as control. As expected, CD4+CD8+ DP thymocytes were stained with ED-1 anti-pTα rabbit anti-serum after PBS (left) or specific pTα peptide (right) incubation (shaded areas). Unshaded areas, Background fluorescence.

**Figure 3.** Analysis of surface pTα chain expression on normal human thymocytes. CD4+CD8+ DP cells were isolated from Percoll-separated large and small thymocytes after depletion of CD3+ and CD3− cells (A). Each cell subset was independently analyzed by two-color flow cytometry for CD4 versus CD8 expression (top panels), and for their reactivity with anti-TCRαβ and anti-CD3 mAbs (middle panels), or with anti-pTα and anti-CD3 (bottom panels). Background values were obtained with preimmune rabbit serum and isotype-matched irrelevant mAbs. (B) Peptide competition assay. Large DP TCRαβ− thymocytes were stained with ED-1 anti-pTα rabbit antisera (left) or specific pTα peptide (right) incubation (shaded areas). Unshaded areas, Background fluorescence.

**Figure 4.** Analysis of transcriptional regulation of relevant genes involved in pre-T cell development. (A) Northern blots of total RNA isolated from the indicated populations were sequentially hybridized with the cDNA probes indicated (left). β-Actin mRNA expression served as internal control. (B) RT-PCR analysis of TCRα gene transcription. C DNA samples were amplified by using a pan-Vα primer in concert with a C-specific primer. Equivalence of cDNA among different samples was assessed by RT-PCR using β-actin primers under nonoptimizing conditions. Sizes of the bands are indicated at the right (kb [A] or bp [B]).
TCR β chain expression (1, 11). Therefore, the prediction would be that all cells downstream of the pre-TCR-expressing pre-T cell stage should show evidence of β-selection. To address this issue, pre-T cell subsets were independently analyzed by flow cytometry for their DNA content as well as expression of cytoplasmic TCR β protein. Results shown in Fig. 5A revealed that essentially all (>90%) large pTα+ as well as small CD3+ pre-T cells expressed cytoplasmic TCR β; therefore, both cell subsets comprise β-selected pre-T cells. Unexpectedly, however, only 50–80% of large CD3+DP thymocytes (50% in this particular experiment) expressed cytoplasmic TCR β, whereas the remaining 30–50% were TCR β−. Such a differential expression of cytoplasmic TCR β defined two distinct cell subsets of large CD3+ pre-T cells which could thus be placed on either side of the β-selection process.

Formal support for this notion came from additional flow cytometric studies that addressed directly the cell cycle status of either the TCR β− or the TCR β+ subsets of large CD3+ pre-T cells. As shown in Fig. 5B, double staining with anti-TCRβ and PI demonstrated that essentially all (>90%) large CD3+pre-T cells lacking TCR β were arrested in the G2/M phase of the cell cycle, whereas, as expected of β-selected thymocytes, TCR β+CD3+ pre-T cells featured a high proportion (up to 55%) of cells in S/G2/M. This is consistent with 30% of bulk CD3+ pre-T cells being in S/G2/M (Fig. 5A). Therefore, TCR β− large pre-T cells are strong candidates for cells immediately before β-selection, and most likely immediately downstream of the CD4+CD8−CD3− precursor stage, which was essentially composed of TCR β− thymocytes (>95%) displaying only a background level (<10%) of cells in S/G2/M (Fig. 5A). As expected of β-selected thymocytes, large pTα+pre-T cells were highly enriched in cycling cells (~55% in S/G2/M). However, TCR β expression could not be associated with an active cycling state in small CD3− pre-T cells. Rather, these cells typically displayed only background levels of cells in S/G2/M (~15%), with a substantial fraction of them (~50%) in the G2/M phase (Fig. 5A).

As an additional indicator of their resting state, small CD3− pre-T cells were shown to display exclusively the fast hypophosphorylated form of retinoblastoma (not shown). We thus concluded that most, if not all, small CD3−DP thymocytes are noncycling pre-T cells that have already passed through β-selection. This, in turn, suggests that β-selected large pre-T cells may normally lose surface pre-TCR expression and return to slow cycle conditions before the onset of TCR α gene expression. As a whole, these data provide strong evidence that small resting pre-T cells represent the latest pre-T cell stage in human thymocyte development, immediately upstream of conventional DP TCR α/β+ resting thymocytes.

Small CD3−Pre-T Cells Are Functional Intermediates between Large CD3low Pre-T Cells and TCR α/β+DP TThymocytes. To seek direct evidence that small CD3−DP thymocytes represent the normal progeny of large pre-TCR-expressing pre-T cells in the pathway of T cell differentiation, highly purified large CD3low pre-T cells (~98% pure) were analyzed for their developmental fate in a hybrid hu/mo FTOC system. The pattern of differentia-
tion from several experiments was identical (Fig. 6 A): the rapid appearance of CD3- DP cells (up to 60% by day 5 in this experiment) with minimal differentiation into TCR α/β+ cells (>5%), followed by the generation of a major population of conventional DP thymocytes that coexpressed CD3 and the α/β TCR at low to intermediate levels (85% by day 17), and the later appearance of small numbers of mature SP thymocytes (not shown). FSC analysis of the cells harvested on day 5 in the experiment shown in Fig. 6 A revealed that, by this stage, the cells that remained CD3low had kept their original size, whereas the CD3- cells generated in the lobes were significantly smaller (mean FSC: 450 vs. 410, respectively). However, by day 17, essentially all large cells had reverted to small cells, and thus, all TCR α/β+ progeny generated by this time (85%) were similar in size to the remaining (15%) CD3- DP cells (mean FSC: 330, 335, and 329, for CD3-, TCR α/βlow, and TCR α/βint cells, respectively). Interestingly, total yields of viable human cells increased progressively during the initial phase of culture, resulting in a 15–20-fold increase of absolute cell numbers by days 5–7, but cellular recoveries then stabilized or increased modestly (up to 2–3 times) through the next 10–12 d, and declined steadily thereafter.

The above data indicate that cell division in thymus lobes reconstituted with large CD3low pre-T cells is extensive and skewed to the early stages of culture. Therefore, the high yields of TCR α/β+ DP progeny in FTOC are mostly a reflection of cellular expansion of blast precursors, presumably before transition to small CD3- pre-T cells. This in turn suggests that differentiation into TCR α/β+ DP cells can occur in the absence of cell division from small noncycling CD3- pre-T cells. To provide direct evidence of precursor activity, we tested the capacity of highly purified (>98%) populations of small CD3- DP thymocytes to produce TCR α/β+ progeny in the FTOC system. As shown in Fig. 6 B, a high proportion of both TCR α/βlow (20%) and TCR α/βint (50%) progeny was already seen in the thymic lobes at day 1, the earliest sampling time. However, the number of TCR α/β+ progeny did not increase in absolute terms thereafter, an expected finding considering that all cells recovered by day 1 were small-sized cells (mean FSC: 300, 293, and 290, for CD3-, TCR α/βlow, and TCR α/βint cells, respectively). Thus, although kinetics of TCR α/β+ cell generation were more pronounced with small CD3- than with large CD3low pre-T cells, total cell yields were substantially lower with the small CD3- pre-T cell fraction. Based on the above results, we concluded that small CD3- DP thymocytes represent functional intermediates between large pre-T cells and TCR α/β+ DP thymocytes.

Discussion

Considerable progress has recently been made in defining the role that preantigen receptors, namely the pre-B cell receptor and the pre-TCR, play in lymphocyte development. It is now established that both receptors direct in an analogous way the survival, expansion, and clonality of pre-B and pre-T lymphocytes by triggering cell cycle activation and the simultaneous downregulation of RAG genes (10, 11). However, less is known about the mechanisms that control terminal differentiation of lymphocyte precursors thus selected, especially in the T cell lineage. This can be partly attributed in both mice and humans to the lack of experimental data concerning regulation of pre-TCR expression on the surface of primary thymocytes, a fact that

Figure 6. Phenotypic analysis of the cellular progeny generated after differentiation of large CD3low and small CD3- pre-T cells in a hybrid hu/mo FTOC. Large CD3low (A) and small CD3- (B) DP thymocytes, isolated as described in Materials and Methods, were analyzed by three-color flow cytometry after the indicated days of culture. Phenotypic analyses were performed on electronically gated human cells characterized as CD45+ (not shown). Background fluorescence was determined by staining with isotype-matched irrelevant mAbs.
has hampered the definition of the developmental stages involved in the transition from TCR\(\beta\) to TCR\(\alpha\) rearrangement. In this study, analysis performed with a polyclonal rabbit Ab that recognizes an exposed epitope of the native human pT\(\alpha\) protein has provided evidence for a restricted pattern of surface pT\(\alpha\) expression during normal T cell development in humans. Surface pT\(\alpha\) versus CD3 expression, together with cell cycle analyses, enabled a novel subdivision of the whole compartment of TCR\(\beta\)-expressing pre-T cells into three distinct subsets of increasing maturity, and allowed the identification of a late stage of small noncycling pre-T cells representing the immediate precursors of TCR\(\alpha/\beta\)-bearing thymocytes. The definition of the precursor-product relationships between such pre-T cell subsets, together with the characterization of the stage-specific events associated with the developmental onset of TCR\(\alpha\) gene expression, namely exit from cell cycle, reexpression of RAG genes, and downregulation of TCR\(\alpha\) germline transcription, collectively support the developmental scheme depicted in Fig. 7.

The distinct pre-T cell stages defined in our model are all included within a subset of CD4\(^+\)CD8\(^+\)DP thymocytes that lack the mature \(\alpha/\beta\) TCR and represent, as a whole, the downstream progeny of CD4\(^+\)CD8\(^-\)CD3\(^-\) thymocyte precursors (15). About one third of such DP TCR\(\alpha/\beta\)-thymocytes are larger in size than the remaining two thirds and, thus, the two cell types have been defined, respectively, as large and small pre-T cells. Although pT\(\alpha\) transcription is common to all pre-T cell stages, surface expression of the pT\(\alpha\) protein is shown to be restricted to a limited fraction (50%) of large-sized pre-T cells that coexpress small but stoichiometric amounts of CD3. As neither pT\(\alpha\) nor CD3 is detectable on the rest of the large and small pre-T cells, the coexpression of both molecules seems to define the particular subset of primary pre-T cells in which the pT\(\alpha\) chain is paired with TCR\(\beta\) and associates with CD3 to form the pre-TCR. However, attempts to demonstrate coexpression of surface TCR\(\beta\) in vivo were unsuccessful, essentially because neither anti-TCR\(\beta\) mAbs useful for flow cytometry nor anti-pT\(\alpha\) reagents suitable for biochemical studies are yet available. Despite this, the possibility that TCR\(\beta\)-pT\(\alpha\) heterodimers associated with CD3 are indeed expressed on pT\(\alpha^+\) pre-T cells is strongly supported by several independent findings: (a) low but stoichiometric amounts of pT\(\alpha\) and CD3 were specifically coexpressed with endogenous TCR\(\beta\) on pT\(\alpha\) transfectants derived from a TCR\(\alpha\)-deficient cell line; (b) we have previously shown that heterodimeric complexes containing TCR\(\beta\) without TCR\(\alpha\) could be immunoprecipitated from unfractionated large DP TCR\(\alpha/\beta\)-thymocytes (15); (c) others have noticed that large thymocytes from TCR\(\alpha\)-deficient and TCR\(\beta\) transgenic RAG-1 mutant mice express low but stoichiometric amounts of surface TCR\(\beta\) and CD3 (13), similar to what has been reported for mouse thymocytes from which a CD3-associated pT\(\alpha\)-TCR\(\beta\) heterodimeric complex has recently been characterized (14); and (d) to date, no surface pT\(\alpha\) expression has been described without association with TCR\(\beta\) and CD3.

![Figure 7](image_url)  
*Figure 7.* Proposed model of pre-T cell development in the human thymus. ic, Intracytoplasmic.
Therefore, expression of surface pre-TCR complexes is proposed in our model to be confined to the minor subset of large pTα+ CD3+ pre-T cells, whereas large pre-T cells lacking detectable amounts of surface pTα and CD3 (~50% of all large DP TCRα/β− thymocytes) are proposed to be homogeneously negative for pre-TCR expression (Fig. 7). However, the latter cells represent a heterogeneous population in which a major fraction (50–80%) have already passed β-selection, as indicated by their high expression levels of intracellular TCRβ (11), whereas the remaining cells (20–50%) still lack cytoplasmic TCRβ and may thus represent intermediates between CD4+CD8+CD3− thymocytes and the first β-selected pre-T cells. It is highly likely that such intermediates include the pool of precursor thymocytes undergoing rearrangements at the TCRβ locus, although they may also include cells carrying nonproductive Vβ-Dβ-Jβ joints on both TCRβ loci, which may thus be destined to die. Both possibilities, illustrated in Fig. 7, are compatible with the hypothesis that the human pre-TCR does not participate, as does its murine counterpart, in the transition to the DP stage (2, 3, 18). Rather, expression of CD8 appears to precede pre-TCR expression during human T cell development.

An important aspect of our study was the observation that virtually all large pre-T cells with cytoplasmic TCRβ, whether or not they display surface pTα chain expression, were actively engaged in cell cycle, a characteristic previously associated with the process of β-selection (11). Conversely, DP thymocytes lacking TCRβ protein were non-dividing cells arrested at G0/G1. The finding that up to 40% of cycling, β-selected pre-T cells did not express the putative pre-TCR is apparently difficult to reconcile with the current idea that cell cycle activation involves signaling mediated through the pre-TCR (2, 3, 11). However, the possibility that undetectable, but functional, amounts of the pre-TCR are expressed on the surface of such cycling pTα− pre-T cells cannot be formally excluded. Alternatively, it is likely that, as proposed for pre-B cells at the equivalent developmental point (35), β-selected pre-T cells rapidly downregulate expression of the pre-TCR from the cell surface while they are still in cycle. In this latter situation, it could be expected that the maintained expression of surface pre-TCR in a short developmental window is both necessary and sufficient to provide a sustained proliferation signal that would allow pre-T cells to undergo a great cellular expansion before turning back to slow cycle conditions. Supporting this hypothesis, results from a recent study have provided evidence that such a proliferation phase corresponds in mice to nine rapid cell divisions per 4 d and end at the small resting DP thymocyte stage (36). This concurs with our finding that a major fraction (about two thirds) of β-selected pre-TCR− pre-T cells in humans are small-sized, nondividing cells. Interestingly, although such small pre-T cells do not yet express the mature α/β TCR, they already transcribe low levels of the TCRα gene. Thus, they are proposed to define the developmental point at which onset of TCRα gene rearrangement and transcription occurs, and are placed in our model at the latest pre-T cell stage, immediately upstream of the first TCRα/β− expressing DP thymocytes (Fig. 7).

Consistent with the above proposal, we found that indicators of Vα-Jα recombination activity, such as RAG gene reexpression and downregulation of TEA transcription, are coincident and stage-specific events induced after entry of late pre-T cells into the pool of small, resting cells. Thus, it was observed that expression of RAG genes, which is turned down after pre-TCR signaling (11, 18), is regained in small pre-T cells, allowing rearrangements at the TCRα locus to be initiated at this stage. Further, the demonstration that germline transcription of TCRα spans all cycling pre-T cell stages but drops significantly in resting pre-T cells also supports the concept that these cells are actively rearranging their Vα genes. Similarly, IgL chain gene rearrangement is restricted to small, resting pre-B cells that represent the equivalent precursor stage along the B cell pathway (35). In contrast to the proposal that small resting TCRα- DP thymocytes are functional intermediates in the T cell differentiation pathway, it is currently assumed that these cells represent the large pool of end-stage products of failed rearrangement attempts. However, recently published data have shown that small noncycling TCRα− DP thymocytes in the mouse are actually the physiological targets of the multiple rearrangements that occur at the TCRα locus (20), and are subject to positive selection (21, 37). Direct evidence of the physiological relevance of small resting CD3+ pre-T cells in humans was further provided by the demonstration that these cells are functional intermediates between large pTα+ pre-T cells and TCRα/β+ DP thymocytes. Accordingly, as shown previously in mice (19, 20), surface expression of the mature CD3−TCRα/β complex can be first detectable on small nonproliferating DP thymocytes.

As a whole, our results suggest that, after pre-TCR−mediated cellular expansion, β-selected large pre-T cells may normally downregulate surface pre-TCR expression and return to slow cycle conditions before the onset of TCRα gene expression. The proposed pattern of pre-TCR expression differs from previous hypothetical models in mice postulating that mature TCRα/β and pre-TCR complexes are coexpressed on the cell surface of late pre-T cells (10). However, it is still possible that some of these cells are cotranscribing pTα and TCRα genes. It is tempting to speculate that, in that situation, both molecules compete with each other for dimerization with TCRβ, the affinity of TCRα being higher than that of pTα. Alternatively, as proposed in mice, another still unknown component of the pre-TCR (i.e., the hypothetical VpreT) might be already shut off at the earliest TCRα− stages, hence preventing surface expression of the whole pre-TCR complex (3). Finally, it must be stressed that the restricted pattern of surface pTα expression shown in this study closely resembles that of the surrogate light chain of the pre-B cell receptor (35, 38). This emphasizes the similarities of early developmental events associated with the transient expression of both preantigen receptors during T and B cell development.
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Address correspondence to María L. Toribio, Centro de Biología Molecular “Severo Ochoa,” CSIC-UAM, Facultad de Biología, Universidad Autónoma de Madrid, Cantoblanco 28049, Madrid, Spain. Phone: 34-1-3978076; Fax: 34-1-3978087; E-mail: mtoribio@trasto.cbm.uam.es

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