

INVOLVEMENT OF THE INTERLEUKIN 2 PATHWAY IN
THE REARRANGEMENT AND EXPRESSION OF BOTH
 α/β AND γ/δ T CELL RECEPTOR GENES IN HUMAN
T CELL PRECURSORS

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T cell precursors arising from hematopoietic stem cells colonize the thymus during ontogeny, where they undergo a complex maturational process involving genotypic and phenotypic changes in the expression of distinct surface molecules. Later, they migrate to the periphery as immunocompetent T cells expressing clonally distributed TCR structures (1-4). Four different TCR genes (α , β , γ , and δ) have thus far been identified and shown to be specifically rearranged and expressed throughout intrathymic T cell development (5-13). They code for two distinct types of heterodimeric TCR: the common MHC-restricted α/β heterodimer expressed on most functional T lymphocytes (14-16), and the recently described γ/δ TCR complex, expressed on a minor T cell subset (17-19). Both structures are expressed in association with the monomorphic CD3 (T3) complex, but they seem to be acquired independently by distinct intrathymic subpopulations (20, 21).

Developmental studies in mice support that the TCR- γ/δ appears first in ontogeny on early double-negative ($CD4^- CD8^-$) thymocytes. Further maturation leads to a gradual decrease of γ/δ -bearing cells. In contrast, TCR- α/β expression increases throughout T cell ontogeny concomitantly with the acquisition of CD4 and/or CD8 molecules by mature T cells, expression of TCR- γ/δ being restricted to a small population of $CD4^- CD8^-$ adult thymocytes and peripheral T cells (3, 4). These findings suggest that γ/δ -bearing $CD4^- CD8^-$ cells may define a separate T cell lineage whose intrathymic development precedes that of classical α/β mature T cells (21, 22). Nonetheless, the presence of γ gene rearrangements in mature α/β -bearing T cells (23), as well as the finding of partial β gene rearrangements in TCR- γ/δ^+ cells (22), indicate that both T cell lineages may derive from a common precursor (24). At present, however, the regulatory mechanisms underlying these developmental

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processes remain poorly understood, and precursor-product relationships involving the various intrathymic subpopulations continue to be disputed, making it difficult to establish direct correlations between the described patterns of TCR gene expression and a functional pathway of T cell development.

Here, in vitro differentiation approaches were used to analyze the precursor potential and the putative progeny of a minor population of adult human thymocytes that lack conventional T cell markers ($CD2^{-1}3^{-4}8^{-}$; i.e., $T11^{-6}3^{-4}8^{-}$) but express CD45 (i.e., T200) and CD7 molecules, suggesting that they are the most immature intrathymic progenitors (25). Moreover, only γ chain functional RNA messages are expressed in this subset, whereas α and β chain TCR genes remain in germline configuration. Interestingly enough, in vitro culture of this subpopulation in the presence of IL-2 led to an extensive cellular proliferation and the concomitant differentiation into both TCR- γ/δ^{+} and TCR- α/β^{+} thymic subsets. These data support the involvement of the IL-2 pathway in the intrathymic maturation of early T cell precursors. Furthermore, they provide a useful in vitro system to induce expression of TCR- α/β as well as TCR- γ/δ structures in developing thymocytes, making it feasible to investigate the cellular and molecular basis for T cell repertoire selection and development operating in T cell differentiation.

Materials and Methods

Antibodies. mAbs used were Na1/34, as anti-CD1a (26); B9.4, as anti-CD8 (27); HP2/6, as anti-CD4 (28); SPV-T3b, as anti-CD3 (29); 3A1, as anti-CD7 (30); GAP 8.3, as anti-CD45 (31); H108, as anti-IL-2-R (Tac) (28); W6/32, as anti-HLA class I (A, B, C) (32); anti-4F2 (30); β F1 framework mAbs to the human TCR- α/β (17) and 11F2 mAbs, which react with human TCR- γ/δ (33). Leur-5b (anti-CD2) and Leu-1 (anti-CD5) were purchased from Becton Dickinson & Co. (Mountain View, CA). WT31 mAbs, which recognize a common epitope on the human TCR- α/β and anti-HLA class II (DR) mAbs (BMA020), were obtained from Sanbio (Uden, Holland) and Beringwerke (Marburg, FRG), respectively. OKT9 (anti-T9) and OKT10 (anti-CD38) were available from Ortho Pharmaceutical (Raritan, NJ).

Rabbit anti-C γ chain heteroantiserum, raised against a synthetic peptide encompassing amino acids 137-157 of the human TCR- γ protein, were used for immunoprecipitation analyses (18, 19).

Flow Cytometry Procedures. Quantitation of the surface staining of 2×10^4 viable cells was performed using either an EPICS-C (Coulter Electronics, Hialeah, FL) or a FACStar flow cytometer (Becton Dickinson & Co.), as previously described (34). Cellular staining was performed by indirect immunofluorescence at 4°C for 30 min with saturating amounts of the corresponding mAb, followed by two washes and reincubation in the same conditions with FITC-conjugated F(ab')₂ goat anti-mouse IgG (Kallestad, Austin, TX), used as second-step reagent. Further incubations with phycoerythrin (PE)¹-coupled anti-CD2 anti-CD3, anti-CD4, or anti-CD8 (Coulter Immunology, Hialeah, FL) were performed for dual parameter analyses. Anti-mouse TCR- α/β (F23.1) mAbs (35) or PE-coupled anti-mouse CD4 (L3T4) mAbs (Becton Dickinson & Co.) were used as controls in single or double stainings, respectively.

Fractionation of Thymocyte Subpopulations. Normal human thymocytes were obtained from thymus fragments removed during corrective cardiac surgery of patients 2 mo to 5 yr old as described elsewhere (34). The isolation of the different thymocyte subpopulations was performed by incubation with specific mAbs for 30 min at 4°C, followed by 45 min at 37°C with an 1:5 dilution of noncytotoxic rabbit complement (Beringwerke) as has been previously reported (34). Mature thymocytes were immunoselected by treatment with anti-CD1a (Na1/34) mAbs plus C. Recovered viable cells ($CD2^{+}1^{-}3^{+}4^{+}/8^{+}$) were afterwards treated

¹ Abbreviations used in this paper: IPB, immunoprecipitation buffer; PE, phycoerythrin; NMS, normal mouse serum.

with anti-CD4 (HP2/6) and anti-CD8 (B9.4) mAbs plus C, allowing the enrichment in double-negative thymocytes ($CD2^+ 1^- 3^+ 4^- 8^-$). Further treatment with anti-CD3 (SPV-T3b) mAbs plus C was performed to obtain pre-T cells ($CD2^+ 1^- 3^- 4^- 8^-$), while depletion of $CD2^+$ thymocytes from this subpopulation by using anti-CD2 (Leu5b) mAbs plus C allowed the isolation of the pro-T cell subset ($CD2^- 1^- 3^- 4^- 8^-$). Viable cells from each subpopulation were recovered by centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden).

Functional Assays. Pro-T cells, immunoselected as described above, were cultured in 24-well macroplates (Costar, Cambridge, MA) (2×10^6 cells/ml) in RPMI 1640 medium (Gibco, Paisley, Scotland) containing 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% pooled and decomplexed human AB serum (complete medium), supplemented with 50 U/ml rIL-2 (Hoffman-La Roche, Basel, Switzerland). Flow cytometry of cells cultured under these conditions was carried out on different days.

Proliferative responses were analyzed by (methyl- 3H)thymidine incorporation (3H]TdR uptake). To this end, cells (pro-T cells and mature thymocytes) were cultured in flat-bottomed 96-well microtiter plates (Costar) in 0.2 ml (10^6 cells/ml) of complete medium either alone or supplemented as indicated. Mitogenic stimulation was performed with 1% PHA (PHA-M; Difco Laboratories Inc., Detroit, MI). Proliferation was measured at different periods of culture after addition of 1 μ Ci/well of 3H]TdR (Amersham Corp., Amersham, UK) for the last 18 h. Each value represents the mean of triplicate cultures.

Peripheral blood lymphocytes (PBL), isolated by Ficoll-Hypaque centrifugation, were incubated in complete medium (10^6 cells/ml) supplemented with 1% PHA, and PHA-induced blast cells were recovered after 4 d of culture.

Isolation and Analyses of Nucleic Acids. High M_r DNA was extracted from each lymphoid sample according to established procedures, was digested to completion with the indicated restriction endonucleases (Boehringer, Mannheim, FRG), and was fractionated in 0.7% agarose gels. DNA blotting, hybridization procedures, and autoradiography were performed as described (12).

Total cellular RNA was isolated using the guanidinium isothiocyanate procedure of Chirgwin et al. (36). Samples (12 μ g) were subjected to electrophoresis on 1% agarose-formaldehyde gels and transferred to nylon membranes. Hybridization and autoradiography procedures have been previously described (12).

The J γ 1 probe (37) was kindly provided by Dr. T. H. Rabbitts and TCR α (38), β (39), and γ chain (40) probes were the kind gift of Dr. T. W. Mak (Toronto, Ontario).

Immunoprecipitation Procedures. About 2×10^7 cells were surface labeled with 1–2 mCi Na- ^{125}I (Amersham Corp.), using 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Iodogen; Pierce Chemical Co., Rockford, IL) as a catalyst. Cells were lysed in 1% NP-40-containing immunoprecipitation buffer (IPB) (19). Nuclei were removed by centrifugation for 15 min at 13,000 g. Lysates were then centrifuged for 30 min at 100,000 g and precleared by three subsequent incubations with a 10% vol/vol suspension of protein A-CL4B Sepharose beads (Pharmacia Fine Chemicals) coated with normal mouse serum (NMS). Immunoprecipitations were carried out for 2–4 h by using NMS (as control), anti-TCR- α/β mAbs (β F1), or anti-C γ chain antisera as described previously (17–19). Before immunoprecipitation with anti-C γ serum, the lysate was denatured by adding SDS to 1% and heating for 5 min at 68°C, after which the lysate was diluted with 5 vol IPB, containing 1.5% NP-40. Immune complexes were recovered with protein A-Sepharose beads and samples were analyzed on 10–15% SDS-polyacrylamide gradient gels, either under nonreducing (1 mM iodoacetamide) or reducing (5% vol/vol 2-ME) conditions.

Results

Isolation and Phenotypic Characterization of Early Intrathymic T Cell Precursors. Analyses of early double-negative ($CD4^- CD8^-$) subpopulations in the adult human thymus (34, 41) led to the identification of a small subset of $CD2^- 1^- 3^- 4^- 8^-$ thymocytes (<0.5% of total thymocytes), which showed a large-sized, blast-like morphology and a phenotypic pattern similar to that of mouse early intrathymic precursors (1, 3,

4). Enrichment of this thymic subpopulation was achieved by a four-step immunoselection method using specific mAbs (anti-CD1a, -CD4 and -CD8, -CD3 and -CD2) plus complement (C). As shown in Fig. 1, highly purified (>99% pure by flow cytometry) CD2⁻ 1⁻ 3⁻ 4⁻ 8⁻ thymocytes were obtained after the isolation procedure. The hematopoietic lineage of these cells was demonstrated by their predominant expression (>98%) of the common leukocyte antigen CD45, suggesting that there are few, if any, nonlymphoid cells in the preparation. Commitment to the T cell lineage was also proved by the expression of CD7 molecules, which are thought to be the earliest marker of human T cells (30), on most (>85%) of the immunoselected thymocytes, and by the low expression (<5%) of surface structures defining other hematopoietic T cell lineages (CD13⁻, CD14⁻, CD15⁻, CD16⁻, CD19⁻, CD20⁻, PTL-1⁻) (data not shown). Indeed, this antigenic distribution resembles that of the first lymphoid cells colonizing the thymus rudiment in the human fetus at 7 wk of development (42).

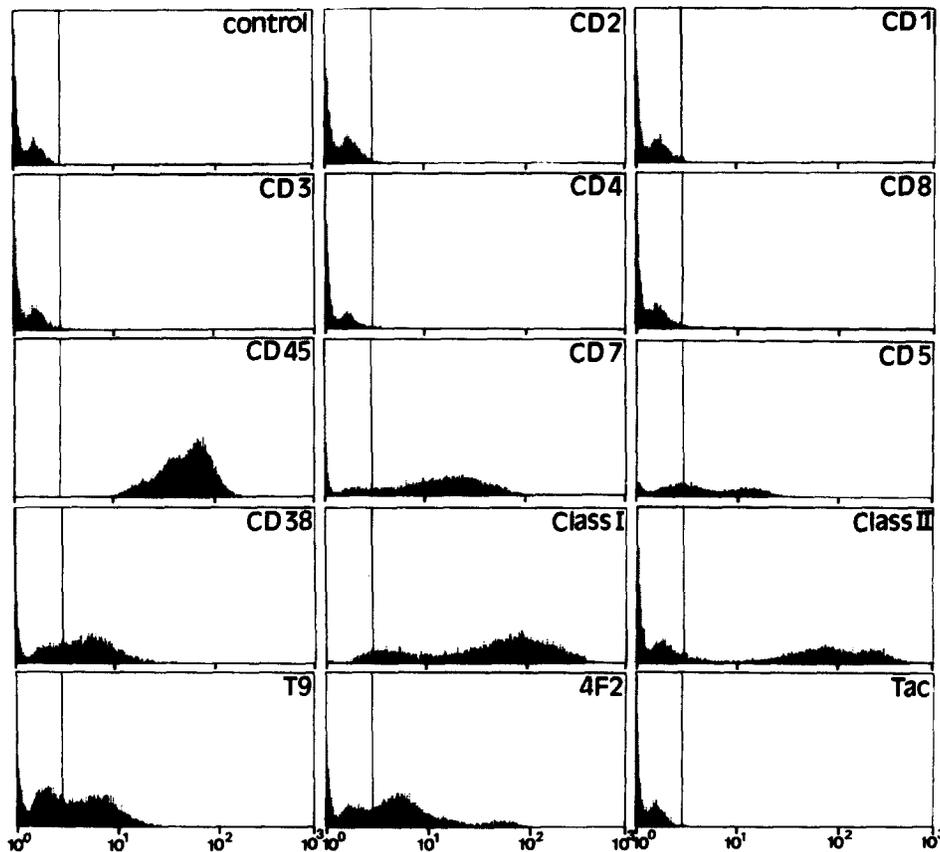


FIGURE 1. Phenotypic distribution of human intrathymic pro-T cells. Pro-T cells were isolated from total thymocytes by immunoselection with specific mAbs plus C as described in Materials and Methods. Cells were stained with the indicated mAbs followed by FITC-conjugated goat anti-mouse IgG. Immunofluorescence of 2×10^4 cells was quantitated in an EPICS-C flow cytometer. Staining with the anti-mouse TCR- α/β F23.1 mAb was used as negative control.

In agreement with the phenotype reported for mouse intrathymic progenitors (43), a variable proportion (up to 40%) of these CD45⁺ CD7⁺ thymocytes showed a weak expression of CD5 (T1) molecules (dull CD5). Moreover, expression of CD38 (T10), 4F2, and T9 (transferrin receptor, Tr) structures (up to 50%), as well as HLA class I (>95%) and class II (up to 40%) molecules, was also observed within this subset in seven independent experiments. In contrast, a low expression (<5%) of Tac molecules, defining the p55 light chain of the IL-2-R was consistently found in these cells (Fig. 1).

Taken together, the phenotypic features of these thymocytes strongly support their T lineage origin, and suggest that, in fact, they are the earliest identifiable intrathymic T cell precursors. Therefore, by applying criteria homologous to those proposed for the definition of both T and B cell lineage precursors in mice, this subset will hereafter be referred to as pro-T cells (44, 45).

Involvement of the IL-2 Pathway in the Proliferation of Intrathymic T Cell Precursors. Despite the current knowledge of the proliferative and maturational events involved in T cell development, the critical growth and activation mechanisms underlying these processes have not yet been clarified. In this regard, the physiological role of the IL-2 system in thymic proliferation and differentiation remains disputed, mainly due to controversial reports on the inability (44, 46) or capability (41, 47, 48) of precursor thymocytes to respond to IL-2 in vitro. To investigate this issue, pro-T cells were cultured in the presence of increasing amounts of rIL-2, and their proliferative potential was analyzed by [³H]TdR incorporation. As shown in Fig. 2, rIL-2 induced a great dose-dependent proliferation of pro-T cells after 7 d of culture. More importantly, as reported before for the whole double-negative thymic subset (41), IL-2-promoted proliferation of early pro-T cells was inhibited by anti-Tac mAbs in a dose-dependent way (Fig. 3), while no inhibition was observed with an irrelevant mAb used as control, showing that IL-2 is acting via its own receptor.

Interestingly, when cellular viability was scored over time in parallel cultures, a vigorous cellular proliferation exhibiting an exponential growth with an 18-24-h doubling time was consistently observed in different experiments. Maximal proliferation was achieved at days 9-12 of culture, when cellular viability was >98%, and thereafter cultures declined steadily (data not shown).

Also noteworthy is that a high spontaneous proliferation was observed in the pro-T cell subset without exogenous rIL-2, which was totally absent in CD3⁺ CD4⁺ or

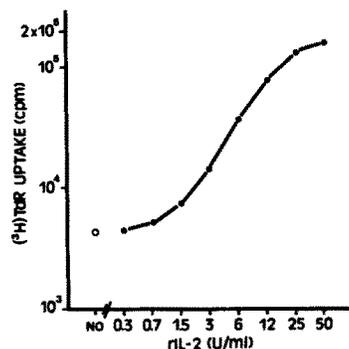


FIGURE 2. Dose-dependent IL-2-induced proliferation of pro-T cells. Pro-T cells were cultured at 10⁶ cells/ml either without (open symbols) or with (closed symbols) titrated amounts of rIL-2 (Hoffman-La Roche, Basel, Switzerland). Proliferation was quantitated in triplicate cultures by [³H]TdR incorporation at day 7. Results are representative of four independent experiments.

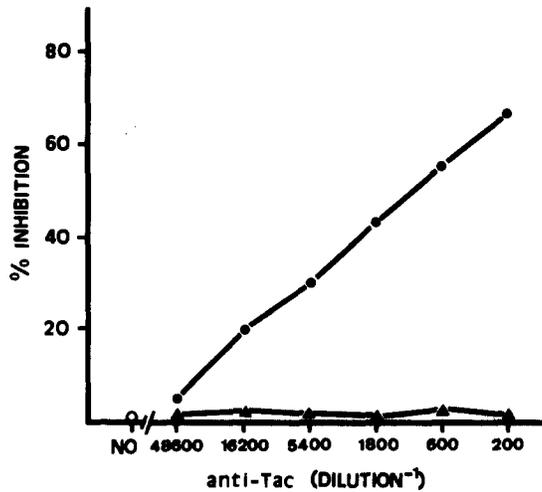


FIGURE 3. Inhibition of the IL-2-mediated proliferation of pro-T cells by anti-IL-2R (Tac) mAbs. Pro-T cells were cultured in the conditions described in Fig. 2 with 25 U/ml rIL-2 in the presence of either anti-Tac mAbs (H108) (●) or anti-mouse TCR- α/β mAbs (F23.1) used as control (▲). Both mAbs were used in ascites form. [³H]TdR uptake was measured at day 7 of culture. Results are representative of data obtained in three different experiments. The percentage of inhibition of the pro-T cell proliferation was calculated over the values obtained in the absence of mAb (157,068 \pm 1,500 cpm).

CD8⁺ (CD1-depleted) mature thymocytes (Table I). Furthermore, as shown in Table I, pro-T cells proliferated vigorously to mitogenic lectins (PHA) in the absence of exogenous growth factors, while IL-2 supplementation did not increase this proliferative response. In contrast, a lower PHA-induced proliferation was displayed by the mature thymic subset, which was significantly increased with exogenous rIL-2 (Table I). These results suggest that polyclonal activation induced by lectins might involve the production of IL-2 at the early pro-T cell developmental stage, as has already been reported for pre-T cells (49).

IL-2-promoted In Vitro Differentiation of Pro-T Cells: Acquisition of CD3-associated TCR Structures. The functional features displayed by pro-T cells support their role as early intrathymic T cell precursors. This was further assessed in vitro by analyzing the differentiation potential of this subpopulation. To this end, CD45⁺7⁺2⁻1⁻3⁻4⁻8⁻ thymocytes, cultured in the presence of rIL-2 (50 U/ml), were tested at different times for the expression of distinct differentiation molecules. Interestingly, cellular

TABLE I
Proliferative Responses of Mature Thymocytes and Pro-T Cells

Culture conditions	[³ H]TdR incorporation in:	
	Mature thymocytes	Pro-T cells
	<i>cpm</i>	
Medium	261	9,547
rIL-2 (25 U/ml)	4,042	80,438
PHA (1%)	24,997	144,828
PHA + rIL-2	234,109	139,484

CD2⁻3⁻4⁻8⁻ pro-T cells and CD2⁺1⁻3⁺4⁺8⁺ mature thymocytes, immunoselected as described in Materials and Methods, were cultured (10⁶ cells/ml) in medium alone or supplemented as indicated. Proliferation was measured at day 5. Standard deviations were \leq 15%. Results are representative of four independent experiments.

proliferation of this subset paralleled a sequential acquisition of different T cell markers. CD2 was consistently observed to be the first molecule to appear in different experiments, accounting for the intrathymic subpopulation of $CD2^+ 1^- 3^- 4^- 8^-$ pre T cells described *in vivo* (34). Acquisition of CD2 structures was followed by the expression of the CD3 complex on double-negative thymocytes ($CD2^+ 1^- 3^+ 4^- 8^-$) and the latter appearance of CD4 or CD8 molecules defining mature T cells ($CD2^+ 1^- 3^+ 4^+/8^+$). More importantly, these results were further extended at the clonal level, ruling out a possible overgrowth of contaminant mature thymocytes in the cultures (data not shown).

The distribution of these structures on the emerging populations was analyzed by two-color flow cytometry at day 8 of culture, when expression of all distinct T cell markers was already observed. The correlated expression of CD3 vs. CD2 molecules, shown in Fig. 4 A, demonstrated that CD2 was expressed on most thymocytes (80%) at this time of culture. A high proportion (63%) of the whole $CD2^+$ subset had already acquired CD3 molecules, while a smaller (37%) population of $CD2^+ CD3^-$ pre-T cells was also found. As shown before *in vivo* (34), CD3 expression did not parallel the acquisition of CD4 or CD8 molecules. Rather, in different experiments, up to 40% of $CD3^+$ thymocytes (25% in this particular experiment) showed the $CD3^+ 4^- 8^-$ double-negative phenotype (Fig. 4 B), while the remaining

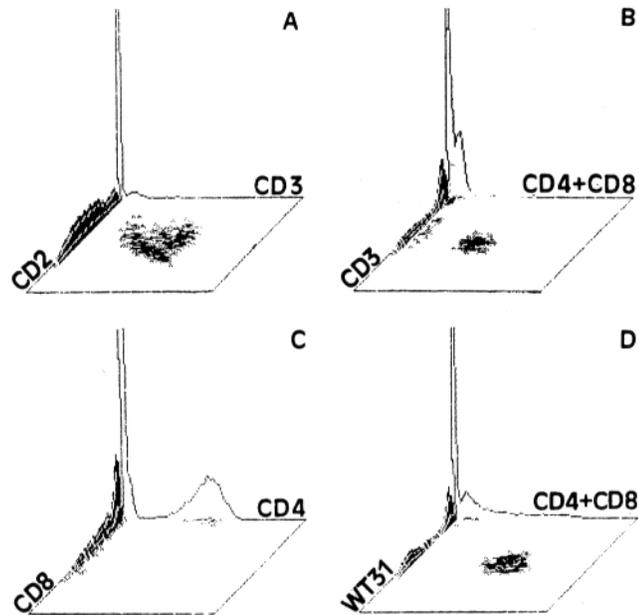


FIGURE 4. IL-2 induces the differentiation of pro-T cells and the generation of all adult intrathymic subpopulations except double-positive thymocytes. Pro-T cells, immunoselected from fresh adult human thymocytes, were cultured at 2×10^6 cells/ml in medium containing 50 U/ml rIL-2. Cellular staining was performed as described in Materials and Methods at day 8 of culture with: (A) anti-CD2 (green) plus anti-CD3 (red) mAbs; (B) anti-CD3 (green) plus a mixture of anti-CD4 and anti-CD8 (red) mAbs; (C) anti-CD8 (green) and anti-CD4 (red) mAbs; and (D) anti-TCR- α/β (WT31) (green) plus anti-CD4 and anti-CD8 (red) mAbs. Two-color immunofluorescence analyses of 2×10^4 viable cells were performed in an EPICS-C flow cytometer. The number of positive cells over background stainings observed with irrelevant mAbs anti-mouse TCR- α/β F23.1 (green) and PE-coupled anti-mouse CD4 (L3T4) (red) mAbs are represented along the vertical axis.

CD3⁺ subpopulation showed a mutually exclusive distribution of both CD4 and CD8 differentiation antigens, characteristic of mature T cells (Fig. 4 C). Special note should be also taken of the lack of expression of CD1 molecules during the whole culture period (15 d) (data not shown), suggesting that CD1⁺4⁺8⁺ double-positive thymocytes do not significantly contribute to the in vitro generation of CD4⁺ or CD8⁺ mature T cells.

On the other hand, the pattern of staining with WT31 mAbs, which recognize a common determinant on human TCR- α/β (50), was found to be similar to that observed with anti-CD3 mAbs. Most CD4⁺ or CD8⁺ thymocytes, shown to coexpress CD3 molecules (Fig. 4 B), were also WT31⁺ (Fig. 4 D). However, although acquisition of functional CD3-associated TCR- α/β is currently confined to the expression of CD4⁺ and/or CD8⁺ mature phenotypes, TCR- α/β ⁺ cells were observed within the CD4⁻CD8⁻ population (Fig. 4 D), which also contained a fraction of WT31⁻ cells.

In light of these data, another set of experiments were performed at day 8 of culture to assess the expression of CD3-associated TCR structures on the pro-T cell in vitro-derived CD3⁺ progeny. Staining with WT31 and anti-CD3 mAbs consistently demonstrated a differential expression of CD3 and TCR- α/β structures (Fig. 5 A). A significant proportion of CD3 molecules was not associated with the TCR- α/β complex. This CD3⁺WT31⁻ population seems to appear shortly before the CD3⁺WT31⁺ progeny in the differentiation assay (data not shown). In addition, both CD4⁺ (Fig. 5 B) and CD8⁺ (Fig. 5 C) mature subsets were mostly included within the TCR- α/β ⁺ population. Nevertheless, a small fraction (3%) of CD8⁺WT31⁻ cells were observed in these cultures (Fig. 5 C), whereas their CD4⁺WT31⁻ counterparts were undetectable (<1%) (Fig. 5 B).

To ascertain whether the alternative CD3-TCR complex expressed by the in vitro differentiated CD3⁺WT31⁻ subpopulation was related to the recently characterized γ/δ heterodimer, two-color flow cytometry was carried out using the mAb 11F2 that reacts with human TCR- γ/δ in native states (33). Results of a representative experiment shown in Fig. 5 D demonstrated that the percentage of CD3⁺ γ ⁺ cells (12.5%) found within the whole cultured population overlapped that of CD3⁺WT31⁻ thymocytes. According to the distribution of CD4 vs. WT31 shown in Fig. 5 B, no γ ⁺ cells were found within the CD4⁺ subset (Fig. 5 E). In contrast, a small population of CD8⁺ γ ⁺ thymocytes (2.5%) was detected in the culture, while most CD3⁺ γ ⁺ cells (10%) displayed the CD4⁻CD8⁻ double-negative phenotype. These data suggest that the IL-2 pathway is involved in the differentiation and acquisition of TCR- α/β as well as TCR- γ/δ by early T cell precursors, both TCR-bearing subpopulations accounting for the whole CD3⁺ subset.

Developmental Expression of TCR- α , - β , and γ Genes. The precursor potential of the isolated pro-T cells was further investigated at the molecular level, and the configuration of the TCR- α , - β , and - γ genes in this early subset was compared with the pattern expressed by its progeny after 8 d of culture with 50 U/ml rIL-2. In addition, the expression of the TCR genes was analyzed in different in vivo-isolated subpopulations that define subsequent maturation stages (34). To this end, total thymocytes, as well as mature (CD2⁺1⁻3⁺4⁺8⁺), CD2⁺1⁻3⁺4⁻8⁻ double-negative and pre-T cell (CD2⁺1⁻3⁻4⁻8⁻) subsets were immunoselected as described (12), being also included in the study. Rearrangements of the γ chain TCR gene were determined

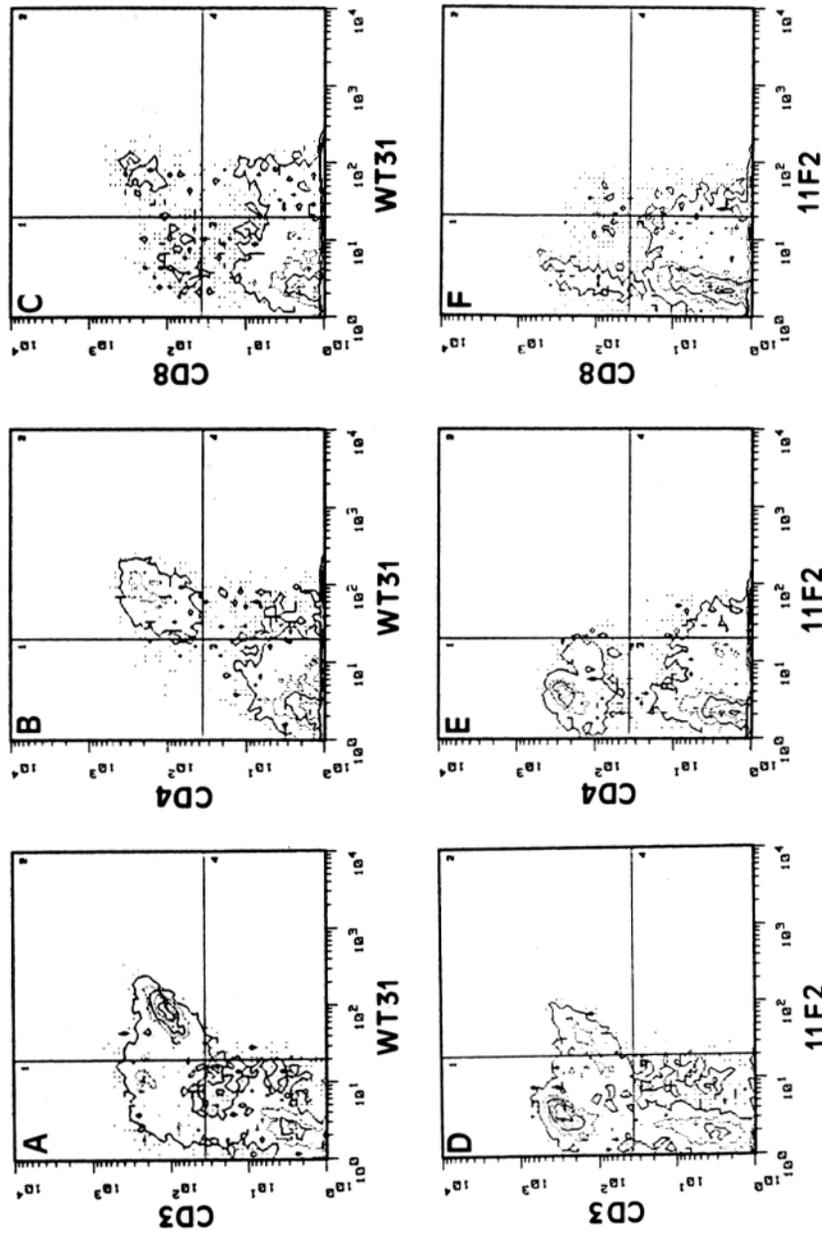


FIGURE 5. Distribution of TCR- α/β and γ/δ among the IL-2-induced progeny. Pro-T cells, cultured in the same conditions as in Fig. 4, were stained either with WT31 (anti-TCR- α/β) mAbs (green) and (A) anti-CD3 (red); (B) anti-CD4 (red) and (C) anti-CD8 (red) mAbs or with 11F2 (anti-TCR- γ/δ) mAbs (green) and (D) anti-CD3 (red); (E) anti-CD4 (red) and (F) anti-CD8 (red) mAbs. The correlated expression of TCR- α/β or γ/δ with CD3, CD4, or CD8 was analyzed on 10^4 viable cells in a FACStar flow cytometer. Based on background stainings, determined as in Fig. 4, contour plots were divided into quadrants to identify unstained cells (*lower left*), cells stained with both fluorochromes (*upper right*), and cells stained only with a single fluorochrome (*upper left and lower right*).

by Southern blot hybridization of Hind III DNA digests using a J γ 1 probe (M13H60) (37). As shown in a representative experiment in Fig. 6 A, rearrangements affecting both J γ 1 and J γ 2 gene segments must have taken place in all thymic fractions, including the early pro-T cell subset, as well as in PHA-activated peripheral T cells, as judged by the low intensity of the 2.1- and 4.5-kb germ line bands (as compared with the germline pattern displayed by the lymphoblastoid B cell line JY) and the appearance of additional new bands. Similarly, both J γ 1 and J γ 2 gene segments were rearranged in pro-T cells cultured with IL-2. In contrast, both J γ 1 alleles had undergone rearrangement in the Jurkat T-cell line, while the J γ 2 gene remained in germline configuration. Similar results were obtained using Bam HI DNA digests (data not shown).

On the other hand, analyses of the β chain gene configuration were performed using a cDNA C β 2 probe (Jur β 2) (39) (Fig. 6 B). While Eco RI DNA digests from the B cell line JY displayed the reported germline pattern (4.2- and 12.0-kb bands), rearrangement of one C β 1 allele could be detected in Jurkat T cells as a discrete fragment of 9.5 kb, the other C β 1 allele remaining in germline configuration (12.0 kb) (12). In addition, multiple distinct C β 1 rearrangements were evident in PHA-activated peripheral T cells, as well as in all thymic subpopulations analysed but pro-T cells, as judged by the marked reduction of the 12.0-kb band and the appearance of a smear of lower relative molecular mass (M_r) new bands. Since there is an Eco RI restriction site just before C β 2 (51), rearrangements affecting the C β 2 region will not be detected. Interestingly enough, although most (>95% by densitometric analyses) pro-T cells remained in germline configuration, the in vitro culture

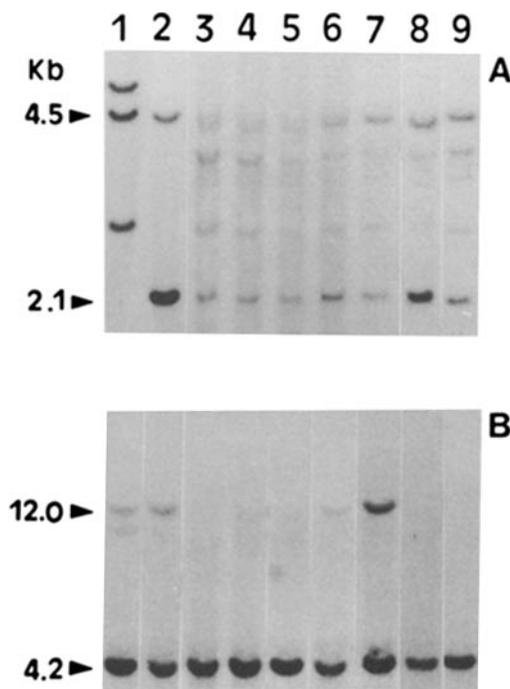


FIGURE 6. Rearrangements of γ and β chain TCR genes in different intrathymic subpopulations. IL-2-induction of β chain rearrangements in early T-cell precursors. High M_r DNA obtained from different intrathymic subsets, immunoselected from freshly thymocytes as described in materials and methods, and from pro-T cells cultured with IL-2 during 8 days, was digested with Hind III (A) or Eco RI (B) endonucleases and subjected to southern blot hybridization with γ - or β -chain TCR probes, respectively. Lane 1, Jurkat T cells; lane 2, JY B cells; lane 3, total thymocytes; lane 4, mature thymocytes; lane 5, "double negative" thymocytes; lane 6, pre-T cells; lane 7, pro-T cells; lane 8, IL-2-cultured pro-T cells and lane 9, PHA-activated peripheral T cells.

of this subset in the presence of rIL-2 resulted in the induction of multiple rearrangements of both C β 1 alleles, similar to those observed in mature thymocytes and PHA-activated peripheral T cells (Fig. 6 B). Moreover, α chain TCR gene rearrangements were not detected in both pre-T and pro-T cells, while they were observed in the pro-T cell progeny (data not shown).

The expression of genes encoding the α , β , and γ chains of the TCR complex was further assessed at the mRNA level by Northern blot hybridization of total cytoplasmic RNA. According to the pattern of gene rearrangements shown, expression of γ chain functional RNA messages (1.7 kb) was observed in all thymic subpopulations analyzed by using a C γ probe (40) (Fig. 7 A). However, a gradual decrease of γ transcripts was evident from early pro-T cells to mature thymocytes, while their expression was barely detectable in the whole thymic sample and in PHA-activated peripheral T cells, and totally absent in JY B cells. It is interesting that quite a stronger expression of γ functional messenger RNA was observed after culturing pro-T cell precursors with IL-2.

In contrast, when the same blot was hybridized with a C β 2 probe, only immature (1.0 kb) β transcripts were expressed in pro-T cells, while both nonfunctional (1.0 kb) and mature (1.3 kb) β RNA messages, representing D-J and V-D-J rearrange-

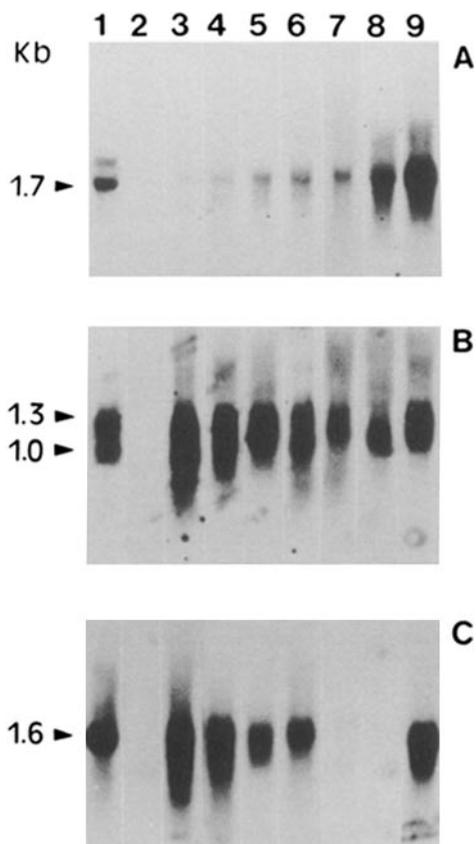


FIGURE 7. IL-2-promoted expression of functional TCR gene transcripts from early intrathymic precursors. Total RNA (12 μ g), isolated from the same samples as in Fig. 6, were electrophoresed and transferred to nylon membranes. The same blot was subsequently probed with γ - (A), β - (B) and α -chain (C) TCR probes. Lane 1, Jurkat T cells; lane 2, JY B cells; lane 3, PHA-activated peripheral T cells; lane 4, total thymocytes; lane 5, mature thymocytes; lane 6, "double negative" thymocytes; lane 7, pre-T cells; lane 8, pro-T cells and lane 9, IL-2-cultured pro-T cells.

ments, respectively (39), were already observed in pre-T cells, increasing in the double-negative subset and reaching maximum levels in mature thymocytes (Fig. 7 B). A high expression of both β RNA transcripts was also observed in unfractionated thymocytes, PHA-induced peripheral T cell blasts, and in the Jurkat T-cell line, but not in JY B cells (Fig. 7 B).

Hybridization with the α chain cDNA probe PY1.4 (38) detected mature (1.6 kb) α mRNA in total, mature and double-negative thymocytes, as well as in Jurkat cells and PHA-blasts, while α transcripts were totally absent in JY B cells and in the most early pre-T and pro-T cell subsets (Fig. 7 C). What is more important, IL-2 was able to induce in vitro a high expression of both β and α functional transcripts in the pro-T cell progeny (Fig. 7, B and C), while similar amounts of RNA were loaded in each line, as assessed by hybridization with a control actin probe (data not shown).

Similar to mouse data, these results demonstrate that TCR γ chain gene rearrangement and expression occur at very early stages of human T cell development, even before the acquisition of specific T cell markers, such as the CD2 common molecule, whereas functional TCR β chain messages are first expressed in the CD2⁺ pre-T cell subset and precede α chain transcription, which is already detected in the CD3⁺ double-negative subpopulation as reported before (12). More important, they suggest the physiological role of the IL-2 pathway in the activation and expression of TCR genes, as well as in the expansion of early T cell precursors and their progeny during intrathymic differentiation.

IL-2 Induces In Vitro Both α/β and γ/δ T Cell Lineages. To determine whether the IL-2-induced rearrangement and transcription of TCR- α , - β , and - γ genes resulted in the surface expression of α/β and/or γ/δ heterodimers among the pro-T cell progeny, immunoprecipitations were performed using the anti-TCR- α/β mAb β F1 (17), and a rabbit anti-C γ antiserum (19). SDS-PAGE demonstrated that both reagents specifically detected TCR- α/β and TCR- γ/δ , respectively, in ¹²⁵I-labeled lysates of 8-d cultured pro-T cells (Fig. 8). Under nonreducing conditions in lane C the TCR- α/β heterodimer can be seen, with an M_r of $\sim 80 \times 10^3$. Under reducing conditions the interchain disulphide bond is disrupted, resulting in a mobility of both α and

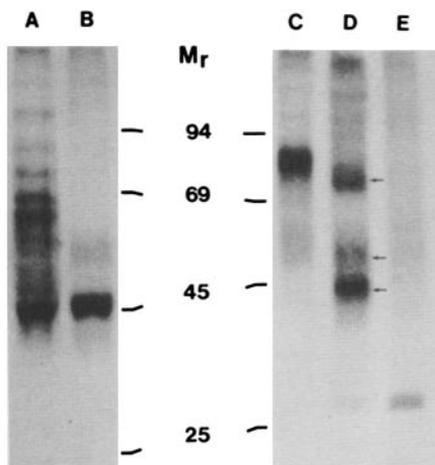


FIGURE 8. Cell surface expression of TCR- α/β and - γ/δ molecules in human pro-T cells differentiated in vitro with IL-2. Pro-T cells, cultured with IL-2 (50 U/ml) during 8 d, were ¹²⁵I surface labeled and subjected to immunoprecipitation with anti-TCR- α/β (β F1) mAbs or anti-C γ serum and analyzed by SDS-PAGE under reducing (lanes A, B), or non-reducing (lanes C-E) conditions. (lane A) β F1; (lane B) anti-C γ ; (lane C) β F1; (lane D) anti-C γ ; (lane E) normal mouse serum control, immunoprecipitate made from denatured lysate. Relative molecular mass (M_r) is indicated ($\times 10^{-3}$).

β chains at $\sim 45 \times 10^3 M_r$. The chains have not been labeled with equal intensity. Immunoprecipitation with anti-C γ serum resulted in the detection of three bands under nonreducing conditions at 75, 55, and $45 \times 10^3 M_r$ (lane D, arrows). Under reducing conditions, bands were detected at 55 and $45 \times 10^3 M_r$ only. Therefore, it is most likely that the $75 \times 10^3 M_r$ band represents the disulphide-linked TCR- γ/δ , which consists of γ and δ chains with M_r of $40\text{--}45 \times 10^3$. The $55 \times 10^3 M_r$ band would represent the described (18) $55 \times 10^3 M_r$ γ chain, which is part of a non-disulphide-linked receptor, while the material migrating at an M_r of $\sim 45 \times 10^3$ under nonreducing as well as reducing conditions, represents a non-disulphide-linked TCR- γ/δ , which contains a γ chain of $\sim 45 \times 10^3 M_r$, as has also been described (20). It is known, that the δ chain cannot easily be detected upon labeling with ^{125}I .

Discussion

Despite the wide diversity of cellular subsets accounting for the whole thymic compartment, increasing agreement has been reached among different laboratories on the correlation of these subpopulations with discrete stages of development (1, 3, 4). Phenotypic, functional, and genetic approaches led to the identification of the earliest mouse intrathymic progenitors, and their differentiation potential was demonstrated mainly by *in vivo* transfer experiments and *in vitro* organ cultures (1, 3, 4). Yet, the physiological mechanisms involved in the maturation of these precursors are not well known.

In the present study we have approached this issue in humans on the basis of the functional features of a small subset of CD2⁻1⁻3⁻4⁻8⁻ adult thymocytes. The precursor potential of this thymic subpopulation was suggested by: (a) its phenotypic distribution, similar to that of mouse fetal and adult intrathymic progenitors; (b) the finding of both α and β chain genes of the TCR in germline configuration; and (c) their ability to generate phenotypic and functionally mature T cells.

Also relevant in functional terms is the observation of large-sized cycling cells in this compartment, as well as the expression of different activation molecules (4F2, T9, HLA-DR), suggesting that they are *in vivo*-activated cells. These data underline a critical point in T cell development, namely the growth "signals" involved in the intrathymic differentiation process. In this regard, the expression of receptors for the IL-2 growth molecule in early double-negative thymocytes, shown to contain the precursors of mature T cells (1), has been reported in mouse (47, 52) and humans (41), and the potential role of the IL-2 pathway in T cell development has been suggested in both systems (34, 53-56).

Nevertheless, a low expression of Tac molecules was consistently observed in the pro-T cell subset, arguing against a putative involvement of the IL-2 molecule in promoting the proliferation of this subpopulation. Functional analyses, however, clearly demonstrated the IL-2-dependent growth of pro-T cells in the absence of any *in vitro* activation stimuli, and moreover, in the absence of exogenous IL-2 supplementation. This apparent discrepancy may be explained on the basis of the recent molecular characterization of the IL-2-R as a heterodimeric structure composed of two polypeptide chains, p55 (L, identified by anti-Tac mAbs) and p75 (H), which bind IL-2 with low and intermediate affinity, respectively, whereas high affinity binding sites for IL-2 arise from complex formation of L and H chains (57-59). Interestingly,

binding studies performed in our laboratory have demonstrated that intermediate affinity binding sites are expressed constitutively in pro-T cells, while high affinity IL-2-R can be detected in the more differentiated Tac⁺ pre-T cell subset (54 and Toribio, M. L., J. C. Gutierrez, L. Pezzi, M. A. R. Marcos, and C. Martinez-A, manuscript in preparation). It is thus tempting to speculate that p75 chains are expressed on early pro-T cells before p55 chains, this molecule being responsible for the marked IL-2-dependent proliferation of this subset. In fact, recent studies in human peripheral T cells showed that the IL-2-p75 interaction alone is sufficient to trigger the complete T cell growth response (60).

Therefore, expression of high affinity IL-2-R seems to be a developmentally controlled process that may be involved in the regulation of the T cell growth/differentiation rate. In this regard, the role of IL-2 in regulating the expression of its own receptors may be envisaged in the light of several reports, showing that IL-2 can both augment the expression of Tac molecules, and decrease the number of high affinity binding sites (60). Experimental support for this notion stems from functional studies showing that Tac⁻ pro-T cells acquire high levels of Tac molecules upon culture in the presence of rIL-2, without any in vitro activation stimuli, and Tac expression gradually decreases during the culture period (unpublished results). This is further extended in vivo by the demonstration that up to 50% of pre-T cells, regarded as the immediate progeny of pro-T cells, express Tac molecules, whereas no IL-2-R (Tac) expression can be observed in mature thymocytes (34). The proposed transient expression of IL-2-R during T cell development is in agreement with recent reports in mice (56).

An important point emerging from this hypothesis is the availability of IL-2 within the thymic microenvironment at an early developmental stage. In this regard, we have recently shown the ability of pre-T cells to produce IL-2 constitutively as well as upon activation with mitogenic lectins (49). These findings can be extended now to pro-T cells in view of their spontaneous and PHA-induced proliferation. Therefore, these results are consistent with an autonomous activation of the IL-2 pathway at the early pro-T cell developmental stage, when membrane structures other than the CD3-TCR complex might be involved. In this sense, the functional role that alternative molecules like CD45, CD7, CD5, and 4F2 play early in T cell development needs to be assessed. Nonetheless, whatever molecule is involved, the requirement of accessory cells in the activation process can not be ruled out given the presence of a minor proportion (<5%) of macrophage contaminants in the isolated pro-T cell subset.

The relevance of these findings is further strengthened by providing direct evidence that IL-2 is involved in the generation of mature T lymphocytes from pro-T cells. It is noteworthy that the in vitro-induced differentiation process parallels a sequential acquisition of CD2, CD3, and CD4 or CD8 molecules, accounting for the in vivo-characterized pre-T cell, double-negative, and mature intrathymic subsets, respectively (12). All these subpopulations, which most likely reflect sequential developmental stages, can be observed after 8 d in our culture conditions. Moreover, generation of mature T cells during the culture correlates with the decrease in pro-T cells, pre-T cells, and double-negative thymocytes (data not shown). However, CD1⁺CD4⁺CD8⁺ double-positive thymocytes were not observed among the pro-T cell progeny, while in vivo studies and in vitro organ cultures in mice suggest that

they are derived from early thymic precursors (1, 3). This discrepancy may be explained by a hampering of the expansion of this subset by our *in vitro* culture conditions as evidence has been provided of the inability of $CD1^+4^+8^+$ thymocytes to proliferate in response to IL-2 (61). Therefore, alternative growth and differentiation factors such as IL-3 and IL-4 may also play a role in the differentiation of human T cell precursors, as has already been suggested in mice (45, 62).

Taken together, our results suggest the involvement of the IL-2 pathway in promoting the differentiation of precursor pro-T cells into $CD4^+$ or $CD8^+$ mature lymphocytes throughout a committed developmental pathway in which pre-T cells and double-negative thymocytes may be envisaged as transitional maturation stages. Nevertheless, an important criticism to these results arises from the possibility that a small contaminating subset of mature thymocytes is actually outgrowing the isolated pro-T cells. This can be ruled out considering previously reported data showing the low proliferative potential of the mature thymic compartment in response to IL-2 (Table I and reference 34). Furthermore, recent studies on the frequency of pro-T cells growing under these culture conditions, as well as phenotypic analyses at the clonal level, completely exclude this possibility (unpublished results).

Finally, the TCR- α , - β , and - γ gene configuration displayed by the *in vivo*-isolated intrathymic subpopulations further supports the results observed *in vitro*, once more stressing the sequential activation of TCR- γ , - β , and - α genes during T cell development. In fact, comparison of the TCR gene patterns displayed by early intrathymic pro-T cells and their progeny strongly suggest that the IL-2 pathway plays a relevant role in promoting not only the proliferation of T cell progenitors, but also the functional rearrangement and expression of TCR genes, leading to the acquisition of both TCR- α/β and TCR- γ/δ structures. Moreover, the distribution of both TCR types among the pro-T cell progeny resembles the pattern observed *in vivo*, namely in that TCR- α/β are expressed in mature $CD4^+$ or $CD8^+$ thymocytes, as well as in $CD4^- CD8^-$ double-negative thymocytes (12), while TCR- γ/δ are mostly restricted to the double-negative subset and to a small fraction of $CD8^+ CD4^-$ thymocytes (17-20).

It is also remarkable that TCR- γ/δ expressed by the pro-T cell progeny are encoded in part by either $C\gamma 1$ or $C\gamma 2$ gene segments, as judged by the presence of both disulphide-linked and non-disulphide-linked structures, respectively (63, 64). In addition, the differences in size of TCR γ chains (55 and $40-45 \times 10^3 M_r$) detected among the non-disulphide-linked receptors reflect the polymorphism described for the $C\gamma 2$ gene, where triplications or duplications of the second exon of the C gene segment have been reported (64). Therefore, IL-2-promoted *in vitro* differentiation of human T cell precursors results in the generation of all distinct TCR types previously identified in T cell acute lymphoblastic leukemias (65), providing the appropriate framework to establish the regulatory mechanisms underlying the acquisition and selection of mature T cell repertoires.

Summary

In this report, we have undertaken the phenotypic, functional and molecular characterization of a minor (<5%) subpopulation of adult thymocytes regarded as the earliest intrathymic T-cell precursors. Pro-T cells were immunoselected and shown to express different hematopoietic cell markers ($CD45$, $CD38$, $CD7$, $CD5$) and some

activation-related molecules (4F2, Tr, HLA class II), but lack conventional T cell antigens ($CD2^{-}1^{-}3^{-}4^{-}8^{-}$). TCR- γ RNA messages are already expressed at this early ontogenic stage, while α and β chain TCR genes remain in germline configuration. In vitro analyses of the growth requirements of pro-T cells demonstrated the involvement of the IL-2 pathway in promoting their proliferation and differentiation into $CD3^{+}CD4^{+}$ or $CD8^{+}$ mature thymocytes. Moreover, during the IL-2-mediated maturation process rearrangements and expression of both α and β chain TCR genes occurred, and resulted in the acquisition of α/β as well as γ/δ (either disulphide-linked or non-disulphide-linked) heterodimeric TCR among the pro-T cell progeny.

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