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Immunology

Involvement of the interleukin 4 pathway in the generation of functional γδ T cells from human pro-T cells

(CD3 complex/T-cell development)

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ABSTRACT **We have used the technique of *in situ* hybridization to investigate the transcription of genes encoding the CD3 complex and the lymphokine interleukin 4 (IL-4) by human pro-T cells—i.e., cells that phenotypically resemble those T-cell precursors that colonize the thymus during early intrathymic development. $CD1^-2^-3^-4^-7^+8^-45^+$ pro-T cells isolated from postnatal thymi via immunoselection with a panel of specific monoclonal antibodies are already committed to the T-cell lineage because most of them transcribe the genes encoding the δ and ε chains of the CD3 complex. About half of such pro-T cells synthesize IL-4 mRNA in the absence of any exogenous stimulation. Upon culture with IL-4, pro-T cells extensively proliferate and differentiate into functionally competent, mature γδ T cells expressing a T-cell receptor repertoire similar to that of γδ T cells that can be found in postnatal thymus. The IL-4 response of pro-T cells is not mediated by induction of the interleukin 2 (IL-2)–IL-2 receptor pathway and, unlike IL-2-driven T-cell differentiation, does not require the presence of stromal cells. Taken altogether, these findings suggest that an autocrine IL-4-mediated pathway might be implicated in early thymocyte differentiation—namely, in the generation of T cells bearing the γδ T-cell receptor.**

Fetal liver T-cell precursors colonize the thymic rudiment, giving rise to cells that express either γδ or αβ T-cell receptor (TCR) heterodimers. In mice, γδ TCR^+ cells predominate early in fetal development, whereas αβ TCRs account for the majority of mature peripheral T cells in the adult. In humans, the characterization of hematopoietic precursors that colonize the fetal thymus at 7–9 weeks of gestation has allowed definition of the phenotype of the earliest intrathymic precursors. These progenitor cells display the pan-leukocyte common antigen CD45 and the earliest T-cell marker CD7 but lack surface expression of CD2, components of the CD3–TCR complex, CD4, and CD8 (1, 2). An analogous population referred to as pro-T cells (3) has been isolated from postnatal thymus. Pro-T cells that constitutively express the β chain of the interleukin 2 receptor (IL-2R) (4), respond to interleukin 2 (IL-2) by proliferation and differentiation, provided thymic stroma cells are in the culture (5, 6).

Recently, mouse fetal thymocytes have been shown to constitutively express interleukin 4 (IL-4) at the mRNA level (6). Mouse $Thy\text{-}1^+$ $CD4^-CD8^-$ thymocytes grow and differentiate into $CD8^+$ cytolytic mature T cells after prolonged culture with IL-4 (7), and adult double-negative thymocytes proliferate in response to IL-4 (8). In humans, it has been shown that IL-4 displays growth factor activity for bone marrow cells (9), total thymocytes (10), and $CD2^+3^-4^-8^-$ immature thymocytes (pre-T cells) (11, 12). These data, together with our previous results showing that IL-4 is a growth and differentiation factor for immature $CD45^+7^+2^+1^-3^-4^-8^-$ thymocytes (pre-T cells) (11), prompted us to investigate the role of IL-4 in human pro-T-cell differentiation.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs) and Fluorometric Analysis. The following mAbs were used: Na1/34, anti-CD1a (13); Leu 5b, anti-CD2 (Becton Dickinson); SPV-T3b or Leu 4b, anti-CD3 (ref. 14 and Becton Dickinson, respectively); HP2/6, anti-CD4 (15), B9.4, or Leu 2a, anti-CD8 (ref. 16 and Becton Dickinson, respectively); 3A1, anti-CD7 (17); Mo1, anti-CD11b (Coulter); Mo2, anti-CD14 (Coulter); Leu11, anti-CD16 (Becton Dickinson); anti-HNK-1 (18); GAP 8.3, anti-CD45 (15); W6/32, anti-HLA class I (19); Edu-1, anti-HLA class II DR (20); MAR108, anti-α chain of IL-2R (21); YTA-1, anti-p75 protein (22); TCR-δ-1, anti-δ TCR (23); WT31, anti-αβ TCR (Sanbio, Uden, The Netherlands); δ-TCS-1, anti-variable region δ1 (Vδ1)-related determinant TCR (T-Cell Sciences, Cambridge, MA); BB3, anti-Vδ2 TCR (24); TiγA TCR (25). Cells were stained for fluorocytometric analysis as described (26).

Isolation of Thymocyte Populations. Thymocyte suspensions were obtained from thymus fragments removed during corrective cardiac surgery from 1 mo to 3-year-old patients. Pro-T cells were immunoselected by subsequent treatment with mAbs recognizing CD1a, CD2, CD3, CD4, and CD8 for 30 min at 4°C, followed by 45-min incubation at 37°C with a 1:4 dilution of noncytotoxic baby rabbit complement (Sera-Lab, Sussex, U.K.). Viable cells were recovered by centrifugation on Ficoll/Hypaque (Pharmacia), treated again with the corresponding mAbs, and incubated 30 min at 4°C with magnetic beads coated with affinity-purified anti-mouse IgG (Dynabeads M-450, Dynal, Oslo). This procedure allowed the routine isolation of highly purified cell preparations (>99% pure).

Cell Cultures. Pro-T cells were cultured in 24-well macroplates (Costar) at 1.5×10^6 cells per ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM Hepes, 10% decomplemented pooled human AB serum and antibiotics, in the presence of the indicated concentrations of IL-4 (Sandoz Pharmaceutical) or IL-2 (Hoffman–La Roche). For measuring proliferative responses, cells were cultured in flat-bottomed 96-well microtiter plates (Costar) in 0.2 ml (10^6 cells per ml). The proliferation was measured after addition of 1 μCi per well (1 Ci = 37 GBq) of [^{3}H]thymidine for the last 18 hr of culture. Each value represents the mean of triplicates. SDs were <10%. Cytotoxicity of pro-T cells or their

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Abbreviations: IL-2, interleukin 2; IL-4, interleukin 4; IL-2R, IL-2 receptor; mAb, monoclonal antibody; TCR, T-cell receptor; V, variable region.

progeny was evaluated in a 4-hr ^{51}Cr-release assay as described (6).

Limiting Dilution Analysis. Purified human pro-T cells were cloned as described (11). Briefly, pro-T cells were diluted in IL-4 (160 units/ml) and phorbol 12-myristate 13-acetate (0.02 ng/ml)-supplemented medium containing irradiated [6000 rad (1 rad = 0.01 Gy)] peripheral blood leukocytes (1×10^6 per ml) and Epstein–Barr virus-transformed B cells (1×10^6 per ml). Cells were cultured at a density of 10, 3, or 1 cell per well in Terasaki microwell plates (25 μl per well) during 7–10 days. The growing cells were expanded in the same conditions in round-bottom 96-well microtiter plates (200 μl per well) and restimulated once per week.

***In Situ* Hybridization.** All probes used for *in situ* hybridization were cDNA fragments subjected to random-primed DNA labeling with digoxigenin-11-dUTP (Boehringer Mannheim). The cDNA probes encoding CD3δ (27) and CD3ε (28) were donated by C. Terhorst (Dana–Farber Cancer Institute, Boston). The cDNA probes encoding CD3γ (29) and CD3ζ (30) and IL-4 (31) were from M. J. Crumpton (Imperial Cancer Research Fund, London), R. Klausner (National Institutes of Health, Bethesda, MD), and K. Arai (DNAX Research Institute, Palo Alto, CA), respectively. Freshly isolated pro-T cells ($5–10 \times 10^4$ cells per slide) were subjected to *in situ* hybridization with antidigitoxine antibody conjugated to alkaline phosphatase as described (32).

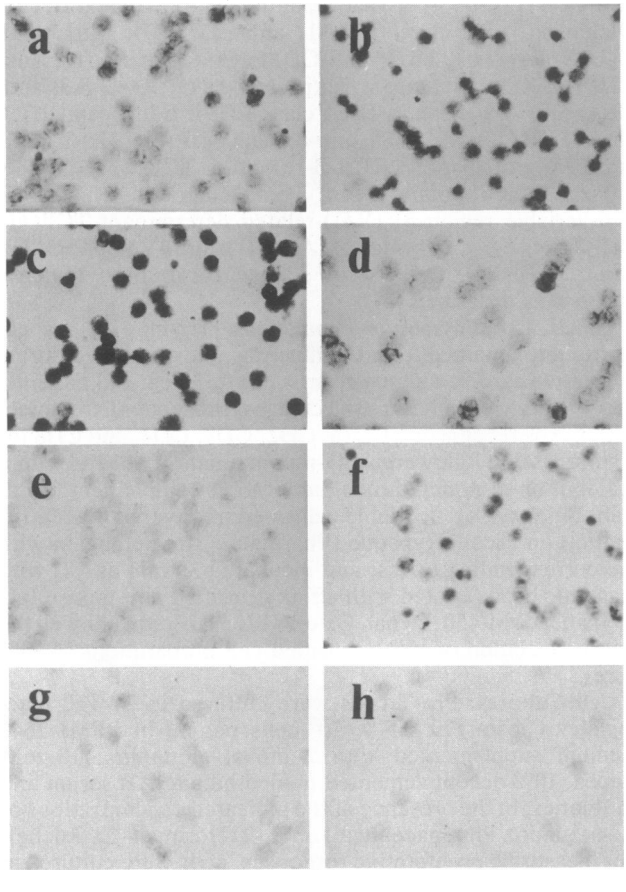

FIG. 1. Analysis of CD3 and IL-4 transcription in intrathymic pro-T cells. Freshly purified pro-T cells (10^5 cells per slide) were hybridized *in situ* with cDNA digoxigenin-11-dUTP-labeled probes corresponding to the genes encoding the monomorphic chains of CD3 complex γ (*a*), δ (*b*), ε (*c*), ζ (*d*), IL-2Rα (Tac) (*e*), and IL-4 (*f*). Human pro-T cells cultivated *in vitro* for 18 days with exogenous IL-4 hybridized *in situ* with an IL-4 probe (*g*) or fresh pro-T cells hybridized with a mouse immunoglobulin probe (J11) (*h*) as controls. Representative fields were photographed. (×470.)

RESULTS AND DISCUSSION

Human Intrathymic Pro-T Cells Are Committed to T-Cell Lineage and Transcribe the IL-4 Gene. Pro-T cells ($CD45^+7^+1^-2^-3^-4^-8^-$) (6), the most immature T-cell subpopulation contained in postnatal human thymus, were enriched by a four-step immunoselection method with mAbs (specific for CD1a, CD2, CD3, CD4, and CD8) plus noncytotoxic complement and magnetic beads. Highly purified pro-T cells, representing 0.15–0.3% of total thymocytes, express CD45 (99%) and variable amounts of CD7 (50–75% in 12 experiments). This population lacks the expression of differentiation antigens specific for T cells, such as CD1, CD2, CD3, CD4, CD8, $\alpha\beta$-TCR, $\gamma\delta$-TCR, or IL-2Rα markers specific for myelomonocytic cells (CD11b, CD14), and the natural killer surface molecules CD16 and HNK-1. Expression of CD3-encoding genes is restricted to cells of the T-lymphocyte lineage (14)—with the exception of the ζ chain, which is also present in $CD3^-$ natural killer cells (32)—and is one of the first events that commits to the T-cell lineage, preceding the expression of TCR genes (28, 33, 34). As shown in Fig. 1, most freshly isolated pro-T cells synthesize mRNA for the δ and ε CD3 genes (>90%, Fig. 1). In contrast, the transcription of the γ and ζ genes is limited to 30 ± 5% and 15 ± 5% of total pro-T cells, respectively. These data, together with our previous findings that pro-T cells have rearranged and transcribe the TCR-γ gene but have the TCR-α and -β genes still in germ-line configuration (3–6), infer the irreversible, albeit early T-cell lineage commitment of this thymic subpopulation. As shown in Fig. 1, 50% of pro-T cells express IL-4 mRNA, whereas IL-4 production is restricted to 1–2% of polyclonally activated peripheral T cells (35), total thymocytes, or mature T cells generated from pro-T cells by culture with IL-4.

IL-4 Promotes the Growth and Differentiation of Human Pro-T Cells into $\gamma\delta$ T Cells via a Pathway Independent of IL-2. Fig. 2 shows that optimal concentrations of IL-4 (160 ng/ml) (11) stimulate the proliferation of pro-T cells in the absence of additional costimulatory signals that would be necessary to render mature peripheral T cells IL-4 responsive (36). We

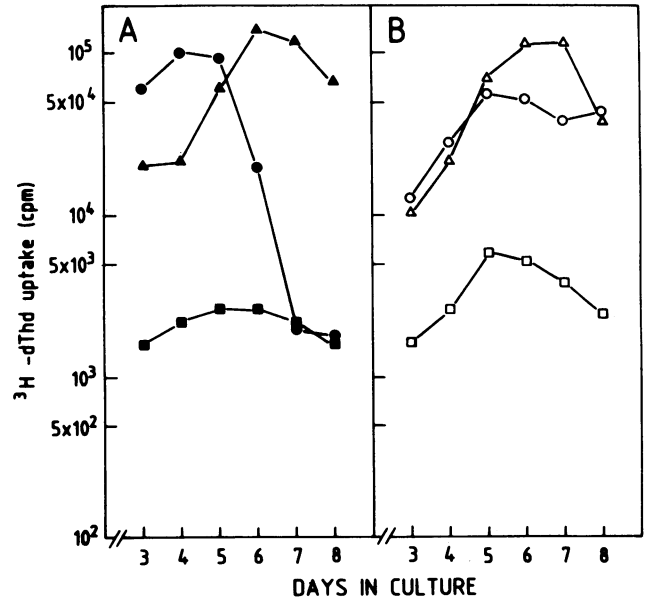

FIG. 2. IL-4 promotes growth of human pro-T cells. Pro-T cells (10^6 cells per ml) were cultured without lymphokines (□, ■) or with IL-4 at 160 ng/ml (△, ▲) or IL-2 at 50 units/ml (○, ●). Triplicate cultures were grown without (*A*) or with (*B*) 1:200 dilution of anti-Tac mAb (H108; ref. 15). Proliferation was measured by [^{3}H]thymidine (^{3}H-dThd) uptake at the indicated days.

Table 1. Acquisition of T-cell markers on pro-T cells cultured in the presence of recombinant IL-4

Day of culture	Phenotype expression, %								
	CD7	CD2	CD3	αβ-TCR	γδ-TCR	CD4	CD8	YTA-1	Tac
A									
3	69	10	3	1	3	0.1	0.5	35	5
5	75	41	13	5	10	3	6	28	24
9	81	82	57	20	36	12	32	55	22
14	89	85	78	25	56	7	44	98	11
B									
15	96	92	85	18	68	7	45	95	10

Pro-T cells (A) and $CD7^+$ pro-T cells (B) were cultured at 1.5×10^6 cells per ml in medium containing human recombinant IL-4 at 160 ng/ml. Quantitative flow cytometry of 2×10^4 viable cells was done as described at the indicated days.

have shown previously that an autocrine IL-2 pathway participates in the expansion of thymic pro-T cells (3, 4); however, IL-2 is not implicated in the proliferative response to IL-4 because the growth-promoting effect of IL-4 on pro-T cells was not affected by addition of saturating amounts of mAb directed against the IL-2Rα chain (Fig. 2). In contrast, anti-IL-2Rα inhibited the initial (3 days) IL-2-driven proliferation of pro-T cells, although at later stages (7–8 days), high doses of exogenous and/or endogenous IL-2 may stimulate cell growth via the intermediate affinity IL-2R (IL-2Rβ) (4). Upon culture with IL-4, $CD45^+7^+1^-2^-3^-4^-8^-$ pro-T cells first acquired a pre-T cell phenotype ($CD2^+1^-3^-4^-8^-$, day 3), followed by the expression of the CD3–TCR complex ($CD2^+1^-3^+4^-8^-$) and, ultimately, acquired the accessory molecules $CD4^+$ and $CD8^+$ (Table 1). A preferential differentiation into cells bearing γδ-TCR and CD8 at the expense of αβ TCR and CD4 was consistently observed in the presence of IL-4. After 18 days of culture in the presence of IL-4, cells display a stable phenotype, and only a small subset (3%) remains undifferentiated (Fig. 3). Ninety-six percent of the IL-4-cultured pro-T cells coexpress CD45 and CD7, and most of the cells express CD2 (90%) and the CD3–TCR complex (80%); CD8 expression (40%) dominates over CD4 (6%). The observation that IL-4 promotes the expression of CD8 in human mature $CD4^+$ clones (37), as well as in mouse fetal pro-T cells (38), is in line with this finding. A high proportion of cells exhibit a double-negative $CD4^-CD8^-$ phenotype (38%), and a small subpopulation (2–7% in six experiments) displays a double-positive ($CD4^+CD8^+$) phenotype.

Three findings exclude the notion that the acquisition of T-cell markers seen with IL-4 is from an overgrowth of mature contaminant T cells: (*i*) in the absence of costimulatory signals, mature γδ T cells do not grow in IL-4-containing medium (11); (*ii*) as much as 10–20% of pro-T cells proliferate in response to IL-4 as estimated by extrapolation of the growth curve or limiting dilution analysis (11); (*iii*) with IL-4, pro-T cells acquire cytotoxic activity after only 5 days of *in vitro* culture (data not shown); and (*iv*) the differentiation can be obtained at clonal level (see below).

Most $CD3^+$ T cells generated with IL-4 (55–90% in six experiments) express the γδ-TCR, and only a minor population (5–20%) bears the αβ-TCR. These γδ-TCR^+ T cells exhibit a low (0–10%) expression of CD5 (data not shown). This contrasts with the CD5 expression normally found in many peripheral blood T cells (39) and on the $\alpha\beta^+$ subset generated in our cultures (6). The reciprocal distribution of αβ and γδ-TCR-bearing T cells was found in the $CD3^+$ progeny of IL-2-supported pro-T-cell cultures, whereas the αβ-TCR predominates over the γδ TCR (6) and CD4 over CD8 expression (11). Fig. 3 shows that the γδ T-cell population arising from pro-T cells after culture with IL-4 for 18 days is composed of $CD8^+$ (25%) and $CD4^-CD8^-$ cells (30%). No γδ $CD4^+$ cells were detected in five independent experiments. TCR $\alpha\beta^+$ cells obtained in the same conditions preferentially express CD8 (15%), although double-negative

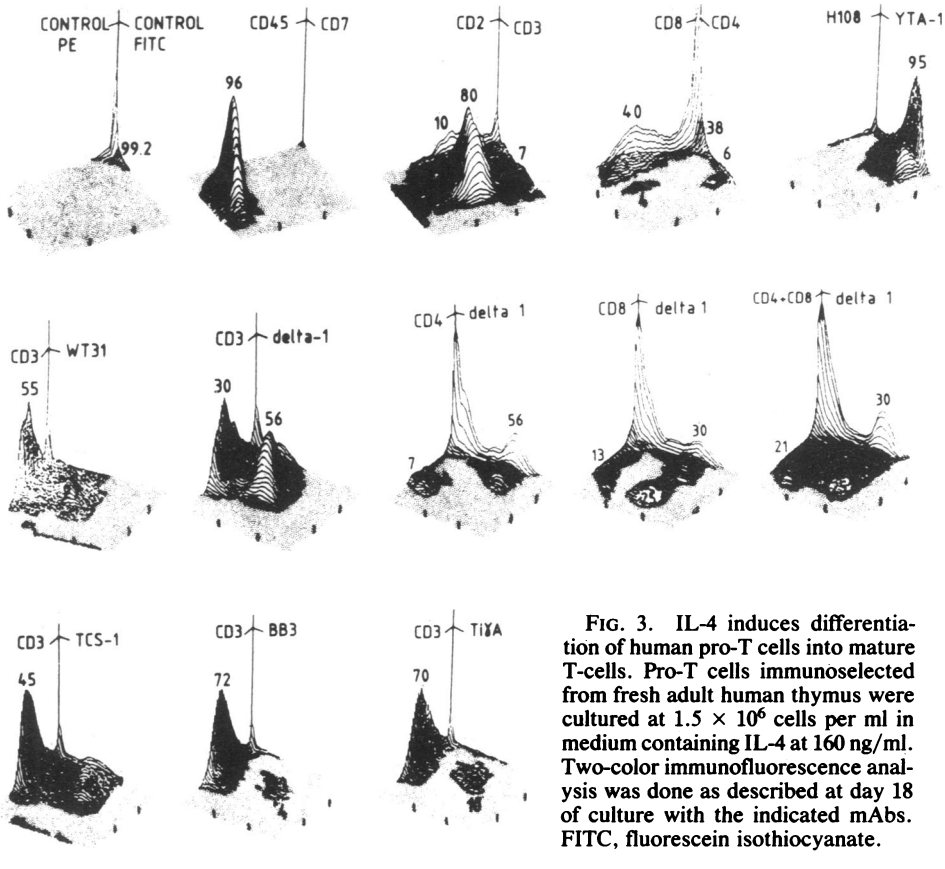

FIG. 3. IL-4 induces differentiation of human pro-T cells into mature T-cells. Pro-T cells immunoselected from fresh adult human thymus were cultured at 1.5×10^6 cells per ml in medium containing IL-4 at 160 ng/ml. Two-color immunofluorescence analysis was done as described at day 18 of culture with the indicated mAbs. FITC, fluorescein isothiocyanate.

(9%) and $\alpha\beta$ CD4$^+$ T cells (5%) were encountered (data not shown).

In response to IL-2, pro-T cells only proliferate or differentiate when they are cocultivated with stroma cells (5). To address the question as to whether IL-4 responsiveness depends upon similar requirements, CD7$^+$ and CD7$^-$ subsets were purified by incubation of the total pro-T cells with anti-CD7 mAb and subsequent separation with anti-mouse-immunoglobulin-coated magnetic beads. Freshly isolated CD7$^+$ pro-T cells express CD45 (99%), whereas among the CD7$^-$ subset (mostly stromal cells), only 3.1% expresses CD45. Upon culture in the presence of IL-4 at 160 ng/ml, CD7$^-$ cells rapidly die, and after 6 days of culture, no viable cells were recovered. In contrast, the CD7$^+$ population, which is practically devoid of stromal elements, proliferates and differentiates into mature T cells. The phenotypic characterization of the CD7$^+$ pro-T-cell progeny after 18 days in culture (Table 1B) revealed an antigenic profile similar to cultured total pro-T cells (Table 1A and Fig. 3).

Analysis of the $\gamma\delta$ T-Cell Repertoire Generated with IL-4. As shown in Fig. 3, the variable region (V)γ repertoire expressed by *in vitro*-generated $\gamma\delta$ T cells is characterized by a dominant expression of Vδ1 in comparison with Vδ2, thus differing from adult peripheral blood lymphocytes where anti-Vδ2 and anti-Vδ1 recognize two-thirds and one-third of TCR-$\gamma\delta^+$ lymphocytes, respectively (39). However, this pattern correlates with the predominance of Vδ1$^+$ cells among $\gamma\delta$ thymocytes (40) and neonatal peripheral blood lymphocytes (41). Interestingly, the number of Vδ1$^+$ and Vδ2$^+$ cells (36 ± 4%) is superated by the cells reacting with the anti-constant region (C)δ mAb (55%), suggesting the existence of extra Vδ products among T cells generated *in vitro* with IL-4.

The phenotypes of clones generated from human pro-T cells with IL-4 are listed in Table 2. After a 6-week culture, 49 clones developed from 520 seeded wells. Most (93%) express $\gamma\delta$ TCR on the surface, whereas only 4% bear $\alpha\beta$ TCR. Two-thirds of the clones express Vδ1, only one clone expresses Vδ2, and nearly one-third are negative for both Vδ1 and Vδ2. Expression of CD8 is unevenly distributed among clones differing in Vδ1 and Vγ9 expression. Whereas the majority of Vγ1$^+$Vδ9$^-$ cells are CD4$^-$CD8$^-$ (18 clones among 43), half of the Vγ1$^+$Vδ9$^+$ cells are CD4$^-$CD8$^+$. A few $\gamma\delta^+$ clones coexpressing CD4 and CD8 were detected in all experiments; these cells die in culture, whereas all other phenotypes are stable. Cells expressing the phenotype of progenitor pro-T cells (one clone) or pre-T cells (two clones) were rarely encountered.

Table 2. Phenotype of clones derived from human pro-T cells cultured with IL-4

Phenotype of clone								
CD2	CD3	$\gamma\delta$	V$_\delta$1/J$_\delta$1/2	V$_\delta$2	V$_\gamma$9	CD4	CD8	Frequency
+	+	+	+	−	−	−	−	18
+	+	+	+	−	−	−	+	3
+	+	+	+	−	+	−	−	4
+	+	+	+	−	+	−	+	4
+	+	+	+	−	+	+	+	1
+	+	+	−	−	+	−	+	1
+	+	+	−	+	−	−	−	1
+	+	+	−	−	−	−	−	13
−	−	−	−	−	−	−	−	1
+	−	−	−	−	−	−	−	1
−	+	+	−	−	−	+	−	1
+	+	+	−	−	−	−	+	1

Pro-T cells were seeded as described, giving rise to 0.3 clone per culture. After a 6-week propagation in IL-4-containing medium, cells were subjected to fluorocytometric analysis. Clones bearing a comparable $\gamma\delta$ repertoire or lacking $\gamma\delta$ TCR were grouped. Within $\gamma\delta^+$ groups, cells differ only in the expression of accessory molecules (CD4, CD8). In the $\gamma\delta^-$ group, one clone exhibits a pro-T cell phenotype (negative for CD2, CD3, CD4, and CD8), one resembles pre-T cells (positive only for CD2), and two express $\alpha\beta$ TCR. J, joining region.

Acquisition of Immunocompetence by Pro-T-Cell Progeny. Fresh pro-T cells do not lyse K-562 and P815 cells either with or without anti-CD3 antibodies (ref. 7 and data not shown). Upon culture with IL-4, these cells acquire strong cytolytic activity detectable with anti-CD3 mAbs (Fig. 4). Acquisition of lytic capacity correlates with differentiation into mature T cells and peaks after 4–5 days in culture. In contrast to the pro-T-cell progeny generated in IL-2-driven cultures, cells harvested from IL-4-driven cultures do not display any (or minimal) natural killer activity. These results reveal the distinct functional behavior of the pro-T-cell progeny recovered from IL-4- and IL-2-supported cultures.

CONCLUDING REMARKS

We provide evidence for involvement of IL-4 in the development of human T-cell precursors. In response to IL-4, human pro-T cells undergo several rounds of cell division and differentiate into functionally competent mature $\gamma\delta$ TCR$^+$ lymphocytes *in vitro*, a process not involving the IL-2 pathway previously implicated in the generation of $\alpha\beta$ T cells. The constitutive transcription of the IL-4 gene by up to 45% of pro-T cells and their IL-4 responsiveness suggest involvement of an auto- or paracrine IL-4–IL-4R pathway in the *in vivo* generation of $\gamma\delta^+$ T lymphocytes. Recently, interleukin 7 has been postulated to just accelerate realization of a pre-established cell maturation program in thymocytes (i.e., transition from $\gamma\delta^{dull}$ stage to $\gamma\delta^{bright}$ stage) (42). In contrast, our data reflect true cell differentiation—i.e., *de novo* expression of surface components (CD3/TCR $\gamma\delta$; CD2, CD4 and/or CD8, IL-2Rα) as a result of irreversible commitment to successive developmental stages.

The different quantitative outcome of pro-T cell cultures set up with IL-2 and IL-4, as well as the distinct lytic activity displayed by the pro-T-cell progeny obtained in the presence of IL-2 and IL-4, suggest that either different subpopulations of pro-T cells use distinct growth factors or that the same cell

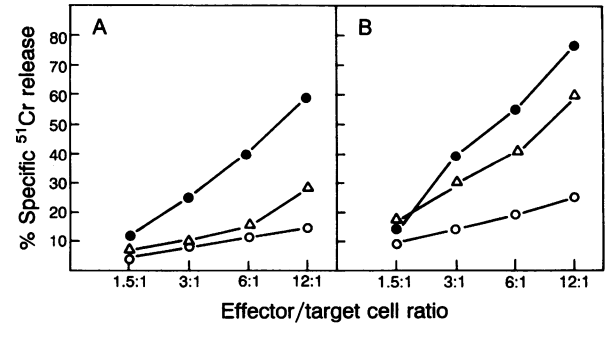

FIG. 4. Induction of cytotoxic activity in human pro-T cells cultured with IL-4. Cytotoxic activity of human pro-T cells after 14 days in culture (1.5 × 10^6 cells per ml) with IL-4 at 160 ng/ml (*A*) or IL-2 at 50 units/ml (*B*) was tested in a 4-hr ^{51}Cr-release assay, either with (●) or without (○, △) anti-CD3 at 1 μg/ml with P815 (○, ●) and K-562 (△) tumor cells as targets. The phenotypes of the effector cells upon *in vitro* differentiation with IL-4 were 95.6% CD2$^+$, 95% CD3$^+$, 90% TCR-δ-1$^+$, 2% WT31$^+$, 40% CD8$^+$, 3% CD4, 72% TCS-1$^+$, and with IL-2 were 97% CD2$^+$, 87% CD3$^+$, 23% TCR-δ-2$^+$, 70% WT31$^+$, 28% CD8$^+$, 42% CD4$^+$, and 13.5% TCS-1$^+$.

responds to different lymphokines at different stages of intrathymic development. It is tempting to correlate early transcription of the IL-4 gene with the fact that γδ TCR expression precedes the αβ TCR. Moreover, it is possible that interleukins could act through activation of factors that interact with the α or γ gene-associated silencer (43, 44). Although growth factors are crucial for *in vivo* proliferation and differentiation of pro-T cells, additional signals might be required—e.g., for induction of TCR-gene rearrangement. Whereas the *in vitro* rearrangement of α and β TCR genes and the generation of αβ TCR-bearing lymphocytes may require direct interaction with thymic epithelial components (5), the development of γδ T cells is independent of nonlymphoid (i.e., $CD7^-$) cells, possibly because the γ and δ TCR genes are already rearranged in this cell population (ref. 6 and unpublished results). These findings may be integrated into a model according to which, upon migration from the bone marrow to the thymus, pro-T cells face a series of developmental choices. Upon contact with stromal cells, a sequential TCR rearrangement, as well as lymphokine production and growth factor-receptor expression, occurs. Because interleukin production and lymphokine-receptor expression are tightly controlled during development, the timing of such stimuli may be critical in determining whether cells expressing the γδ or αβ TCR will be preferentially expanded.

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