A Role for Interleukin 4 in the Differentiation of Mature T Cell Receptor γ/δ^+ Cells from Human Intrathymic T Cell Precursors

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Summary

We have analyzed the effect of human recombinant interleukin 4 (rIL-4) on the growth and differentiation of human intrathymic pre-T cells ($CD7^+2^+1^-3^-4^-8^-$). We describe that this population of T cell precursors proliferates in response to rIL-4 (in the absence of mitogens or other stimulatory signals) in a dose-dependent way. The IL-4-induced proliferation is independent of the IL-2 pathway, as it cannot be inhibited with an anti-IL-2 receptor α chain antibody. In our culture conditions, rIL-4 also promotes the differentiation of pre-T cells into phenotypically mature T cells. Although both CD3/T cell receptor (TCR)-α/β^+ and CD3-γ/δ^+ T cells were obtained, the preferential differentiation into TCR-γ/δ^+ cells was a consistent finding. These results suggest that, in addition to IL-2, IL-4 plays a critical role in promoting growth and differentiation of intrathymic T cell precursors at early stages of T cell development.

Interleukin 4 (IL-4), also known as B cell–stimulating factor 1 (BSF-1)[1] (1, 2), was originally defined by its ability to induce the proliferation of anti-IgM-activated B cells (3). However, this T cell–derived lymphokine is now known to display diverse biological effects on a broad array of cell types, including T lymphocytes (4, 5), mast cells (5), macrophages (6), and hematopoietic progenitor cells (7).

In T cells, the functional role of IL-4 as a growth factor alternative to IL-2 has been proposed (8). Thus, IL-4 is able to support proliferation of several T cell lines (5, 9, 10), and promotes T cell growth upon activation by phorbol esters or lectins, both in mice (11–13) and humans (14). In addition, IL-4 appears to be involved in the generation of specific cytolytic activity (15, 16), as well as in the induction of CD8 expression in human $CD4^+$ T cell clones (17), suggesting a putative role for this lymphokine as a cytotoxic T cell differentiation factor specific for $CD8^+$ lymphocytes (18).

Recently, accumulating evidence indicates that IL-4 may also be involved in T cell development. As previously described for IL-2, several groups have shown the growth-promoting activity of IL-4 in different subsets of adult thymocytes (14, 19, 20). More importantly, IL-4 appears to induce both growth and differentiation of fetal intrathymic $Thy\text{-}1^-CD4^-CD8^-$ T cell precursors into $CD8^+$ cytolytic T cells (21). These data, together with the observation that production of IL-4 (19, 22, 23), as well as expression of specific IL-4 receptors (IL-4R) (20), occurs at early intrathymic developmental stages in the mouse, suggest that IL-4 may play an important role in T cell ontogeny.

In this report, the potential effects of IL-4 on the generation of mature T cells from human intrathymic T cell precursors have been investigated, comparing them with those promoted by IL-2. Our results show that adult $CD3^-4^-8^-$ thymocytes (pre-T cells) proliferate in vitro in response to IL-4 through an IL-2-independent pathway. Moreover, IL-4 stimulation promotes the differentiation of pre-T cells into $CD3^+$ mature T lymphocytes preferentially expressing TCR-γ/δ, whereas mature T cells generated in response to IL-2 mostly express the TCR-α/β. These results suggest that the selective use of either IL-4 or IL-2 pathways by T cell precursors may be crucial in the generation of γ/δ or α/β T cell lineages, respectively, during T cell development.

Materials and Methods

mAbs. Na1/34, anti-CD1a (24); B9.4, anti-CD8 (25); HP2/6, anti-CD4 (26); SPV-T3b, anti-CD3 (27); 3A1, anti-CD7 (28); GAP 8.3, anti-CD45 (29); and W6/32, anti-HLA class I (A, B, C) (30) mAbs were used as culture supernatants. MAR108, a mAb raised against the p55-kD component of the IL-2R (26), was purified by protein A–Sepharose and conjugated with biotin. YTA-1, a mAb that appears to recognize the p75 IL-2R component (31), was used after purification by protein A–Sepharose. Edu-1, a monomorphic mAb anti-HLA class II DR (32) and TCR-δ-1 mAbs, raised against the δ chain of the TCR-γ/δ (33), were obtained from ascites. WT31 mAbs, which recognize the TCR-α/β, and δTCS-1 mAbs, which

[1] *Abbreviation used in this paper:* BSF-1, B cell–stimulating factor 1.

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recognize a Vδ1 related determinant, were obtained from Sanbio (Uden, Holland) and T Cell Sciences (Cambridge, MA), respectively. PE conjugated anti-CD1 and anti-CD2 mAbs were obtained from Coulter Immunology (Hialeah, FL) and PE-coupled anti-CD3, anti-CD4, and anti-CD8 mAbs, from Becton and Dickinson & Co. (Mountain View, CA).

Quantitative Flow Cytometry. Quantitation of the surface staining of 5–10 × 10^4 viable cells was performed with an Epics-Profile II (Coulter Electronics, Hialeah, FL) flow cytometer. Cellular staining was performed by indirect immunofluorescence at 4°C for 30 min with saturating amounts of the corresponding mAbs, followed by incubation with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (Kallestad Laboratories, Inc., Austin, TX) used as second-step reagent in single stainings, as previously described (34). For dual parameter analysis, cells were further incubated with PE-conjugated mAbs. Irrelevant isotype-matched mAbs from Becton and Dickinson & Co. were used as negative controls. The data were analyzed for representation using the Flowsys program developed by Dr. L. Pezzi.

Isolation of Thymocyte Populations. Normal human thymocyte suspensions were obtained from thymus fragments removed during corrective cardiac surgery in patients 1 mo to 5 yr old. Viable thymocytes were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifugation. Intrathymic T cell precursors were immunoselected by treatment with mAbs plus noncytotoxic rabbit complement from Beringwerke (Marburg, FRG), as described before (34). Briefly, fresh thymocytes were incubated with anti-CD1a for 30 min at 4°C, followed by a 45-min incubation at 37°C with a 1:5 dilution of complement. Recovered $CD1^-$ mature thymocytes were afterwards treated with anti-CD4, anti-CD8, and anti-CD3 mAbs plus complement, in the same conditions, to obtain pre-T cells ($CD2^+1^-3^-4^-8^-$). Viable cells were recovered by centrifugation on Ficoll-Hypaque. Either mature thymocytes or pre-T cells were further treated with the corresponding mAbs and incubated for 30 min at 4°C with magnetic beads coated with affinity-purified sheep anti-mouse IgG (Dynabeads M-450; Dynal, Oslo, Norway), following the manufacturer's recommendations. As described before (35), separation using this procedure allowed the isolation of highly purified cell preparations (>99% pure) of mature thymocytes and pre-T cells.

Cell Cultures. Intrathymic subpopulations were cultured in 24-well macroplates (Costar, Cambridge, MA) at 8 × 10^5 cells/ml in RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, 10 mM Hepes, 10% decomplemented pooled human AB serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of the indicated concentrations of rIL-2 (Hoffman-La Roche, Basel, Switzerland) or rIL-4 (Sandoz, Basel, Switzerland). Cell growth was followed by daily counting of viable cells under the microscope.

Proliferative responses were analyzed by methyl-[^{3}H] thymidine incorporation. Cells 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 with lymphokines, as indicated in each assay. Proliferation was measured at different periods of culture after addition of 1 μCi/well of [^{3}H]thymidine (Amersham Corp., Amersham, UK) for the last 16–24 h. Each value represents the mean of triplicate cultures. The SDs were ≤15%. The frequency of IL-4-reactive human pre-T cells was evaluated by limiting dilution analysis. Basically, pre-T cells were diluted in medium containing 10^6/ml EBV-transformed B cells, limiting the number of responding pre-T cells to around one, and grown in Terasaki micro wells in 25 μl in the presence of rIL-4. Frequencies of IL-4-reactive pre-T cells were calculated from the number of cultured cells that were yielded in 63% of all growing cultures, and thus, contained one reactive cell.

Results

IL-4 Promotes the Proliferation of Human Intrathymic T Cell Precursors. To investigate the capacity of IL-4 to induce growth of human T cell precursors, we isolated a highly purified population (hereafter referred to as pre-T cells; reference 35) that represents the 1–3% of total thymocytes. As shown in Fig. 1, this subpopulation displayed a marked expression of the CD45 common antigen, whereas CD7 (known to be the earliest T cell marker; reference 36) and CD2 molecules were expressed on most (82 and 72%, respectively), but not all, pre-T cells. Expression of other T cell antigens, including CD1, CD3, CD4, and CD8, as well as TCR-α/β or -γ/δ structures, was not detected. In addition, very few cells (1%) were stained with YTA-1 mAb, which appears to recognize the p75 component of the IL-2R (IL-2R-β) (31), and a low IL-2R-α expression was detected (5%).

The proliferation of pre-T cells was assessed by [^{3}H]thymidine uptake after 4 d of culture with increasing amounts of rIL-4. As shown in Fig. 2, rIL-4 induces a dose-dependent growth response in the absence of mitogenic lectins or other activation agents. Indeed, the addition of PMA to the cultures set up in rIL-4 did not significantly increase the IL-4 promoted growth, although earlier response kinetics were observed (data not shown).

Under these culture conditions, IL-4 responsiveness was also observed in mature thymocytes ($CD1^-3^+4^+/8^+$) (Fig. 3 *A*), as well as in double-negative ($CD1^-3^+4^-8^-$) and in double-positive ($CD1^+3^+4^+8^+$) thymic subsets (A. Bárcena et al., manuscript in preparation). However, proliferative responses displayed by immature pre-T cells were higher than those observed for mature thymocytes (Fig. 3, *A* and *C*). These data agree with previous results on the differential growth requirements of early and mature intrathymic subpopulations (37).

Studies on the kinetics of IL-2- and IL-4-induced responses revealed different behaviors in pre-T cells. As shown in a representative experiment depicted in Fig. 3 *C*, IL-4-promoted responses were always delayed in comparison with those induced by IL-2. Thus, maximal responses to IL-2 were observed at day 3 of culture, whereas IL-4-promoted growth reached maximal levels at day 7 in this particular experiment (Fig. 3 *C*). In contrast, the kinetics observed for mature T cells in response to IL-2 and IL-4 were more similar (Fig. 3 *A*). Cellular viability of pre-T cells was also scored over time in parallel cultures set up either in IL-2 or IL-4. This study revealed that an exponential growth with a calculated doubling time of 48 h was sustained for 7–9 d in both culture conditions. However, the IL-4-promoted growth declined after this period, whereas proliferation induced by IL-2 increased exponentially until day 9–12, and thereafter, cultures declined steadily (35; and data not shown).

IL-4-mediated Proliferation of Pre-T Cells Is Independent of the IL-2 Pathway. Human T cell precursors have been shown to constitutively produce IL-2, express variable levels of IL-

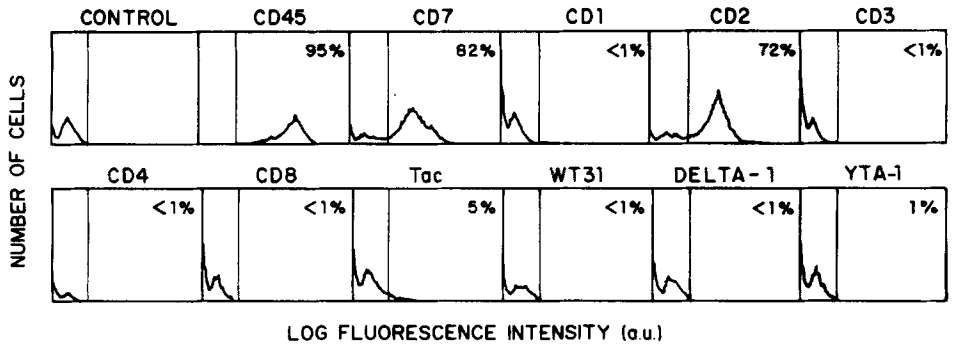

Figure 1. Phenotypic characterization of human intrathymic pre-T cells. Pre-T cells were isolated from postnatal thymic samples, as described in Materials and Methods. After staining with the indicated mAbs, immunofluorescence of 5 × 10^4 cells was quantitated in an Epics-Profile II flow cytometer. Staining with an irrelevant mouse mAb was used as negative control.

2R, and, therefore, display an autocrine IL-2-supported growth (37–39). To ascertain whether the growth of pre-T cells induced by rIL-4 was mediated by IL-2 produced in the cultures, the proliferation of pre-T cells in response to either rIL-4 or rIL-2 was assayed in cultures set up in the presence or in the absence of mAbs raised against the α chain of the IL-2R (anti-Tac). As shown in Fig. 3, *B* and *D*, the anti-Tac mAb did not affect the proliferation induced by IL-4 in pre-T cells and mature thymocytes (included as control in the assay). The effectiveness of the antibody is demonstrated by the marked inhibition of the IL-2-promoted proliferation in both populations. These data provide evidence for the involvement of IL-4 in the growth and expansion of early human intrathymic T cell precursors by an IL-2-independent pathway.

IL-4 Promotes the Differentiation In Vitro of Pre-T Cells into Mature T Cells. Taking advantage of the observed role of IL-4 in promoting the proliferation of intrathymic pre-T cells, experiments were performed to analyze whether IL-4 could be also effective in inducing differentiation of T cell precursors in vitro. Acquisition of different T cell markers, including CD4, CD8, and TCR, was assessed by flow cytometry, after culture of pre-T cells with optimal doses of rIL-4 (160 ng/ml). Parallel studies were performed in cultures set up in the presence of rIL-2 (50 u/ml), known to induce the maturation in vitro of this intrathymic subset and the generation of both $CD4^+$ and $CD8^+$ TCR-bearing mature T cell subsets (34).

Pre-T cells growing either in rIL-4 or rIL-2 gave rise to mature T cells mostly expressing CD3 molecules (Fig. 4 and Table 1). However, significant differences with regard to the development of the two major α/β and γ/δ T cell lineages were detected in both culture conditions. As shown in Fig. 4, while 85% of $CD3^+$ T cells generated in the presence of rIL-4 expressed the TCR-γ/δ (recognized by TCR-δ-1 mAb), only 5% of cells were stained with anti-TCR-α/β (WT31) mAb. Among TCR-γ/δ^+ cells, 46% of them used the Vδ1 gene segment, recognized by δTCS-1 mAb (data not shown). Analysis of the correlated expression of TCR-γ/δ vs. CD4 or CD8 accessory molecules indicated that most TCR-γ/δ^+ cells (46%) displayed a double-negative ($CD4^-CD8^-$) phenotype. In addition, $CD8^+$ cells were also observed among the TCR-γ/δ^+ subset (36%), while no TCR-γ/δ^+ $CD4^+$ cells were detected in different experiments (Fig. 4). In contrast, both $CD4^+$ and $CD8^+$ mature phenotypes were observed within the TCR-α/β^+ population (data not shown). The generation of a small number of TCR-α/β- vs. TCR-γ/δ-bearing mature T cells was a consistent finding in all cultures set up in the presence of rIL-4. Variable levels of TCR-α/β expression (up to 25%) were detected in other experiments (Table 1). In all of them, expression of the TCR-α/β paralleled the acquisition of the CD4 and CD8 molecules by pre-T cells, although a preferential differentiation into $CD8^+$ cells was consistently observed (Table 1). In addition to $CD4^+$ and $CD8^+$ TCR-α/β^+ mature T cells, TCR-α/β^+ thymocytes were also observed within the $CD4^-CD8^-$ population (data not shown).

Regarding the expression of CD2 molecules in the IL-4-induced pre-T cell progeny, also worthy of note is the observation of a variable proportion of cells (20% in this experiment; Fig. 4), characterized by the expression of the TCR complex, in the absence of CD2 antigens ($CD2^-CD3^+$). Assuming that the frequency of TCR-α/β^+ cells is 5% of total cells, the $CD2^-CD3^+$ subpopulation will be mainly constituted by TCR-γ/δ^+-bearing T cells.

Analyses of the distribution of the two types of TCRs in cultures set up in the presence of rIL-2 revealed results opposite to those observed with rIL-4. TCR-α/β^+ cells, expressing either CD4 or CD8 molecules, accounted for the majority of $CD3^+$ thymocytes (85%) generated in rIL-2, although TCR-γ/δ^+ cells were also detected (12%) (Table 1). Both $CD4^+$ and $CD8^+$ TCR-α/β^+ populations were observed in these cultures. However, $CD4^+$ cells (84%) (bearing the TCR-α/β complex, data not shown) were significantly expanded in contrast to the preferential differen-

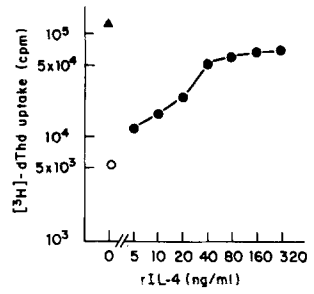

Figure 2. rIL-4 promotes the proliferation of $CD7^+CD2^+$ $CD1^-3^-4^-8^-$ pre-T cells in a dose-dependent way. Pre-T cells (10^6 cells/ml) were cultured in RPMI medium, either with (●) or without (○) titrated amounts of rIL-4. Proliferation induced by IL-2 (50 U/ml) was included as control (△). [^{3}H]Thymidine incorporation was quantitated at day 4 in triplicate cultures. Results are representative of four independent experiments. SDs were ≤15%.

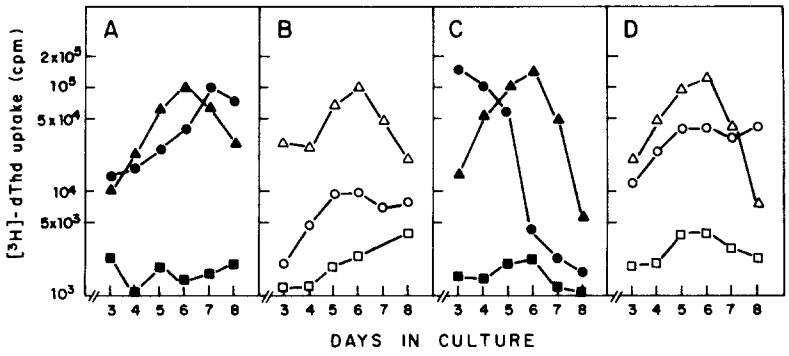

Figure 3. Proliferation of mature thymocytes and pre-T cells in response to rIL-4 vs rIL-2. Mature thymocytes (*A* and *B*) and pre-T cells (*C* and *D*) (10^6 cells/ml) were cultured in medium (□, ■) rIL-4 (160 ng/ml) (△, ▲), or rIL-2 (50 U/ml) (○, ●) either in the presence (*open symbols*) or in the absence (*filled symbols*) of 1:200 dilution of anti-Tac mAb (H108). Proliferation was quantitated by [^{3}H]thymidine uptake at the indicated days of culture. Results are representative of three independent experiments.

tiation into CD8$^+$ cells detected in cultures set up with rIL-4 (Fig. 4 and Table 1). As observed with rIL-4, a significant proportion of TCR-γ/δ^+ cells generated in rIL-2 expressed CD8 molecules, whereas most of them were included within the CD4$^-$CD8$^-$ subset, and no CD4$^+$ TCR-γ/δ^+ cells were detected (data not shown).

In light of previous studies (39) showing that activation of IL-2R-α genes is induced in vitro in T cell precursors growing in rIL-2, experiments were performed to ascertain whether upregulation of IL-2R was also involved in the IL-4-induced differentiation process. Phenotypic analyses of the expression of both α and β IL-2R components revealed that, in contrast to the high expression of IL-2R-α observed after culture in the presence of rIL-2, very low levels of IL-2R-α molecules were displayed by pre-T cells cultured with rIL-4 (Table 1). However, reactivity with YTA-1 mAb was observed in most of pre-T cells (95%) cultured with rIL-4, even when IL-2R-α expression was not detectable (Fig. 4), as well as in IL-2-cultured pre-T cells (Table 1). In both differentiation systems, expression of IL-2R-α correlated with the levels of HLA-DR molecules induced in pre-T cells (Table 1).

Finally, the fact that IL-4 promotes the differentiation of pre-T cells preferentially into TCR-γ/δ-bearing T cells led us to investigate the ability of this lymphokine to induce the selective proliferation of TCR-γ/δ^+ mature T cells from PBL. To this end, we chose a normal healthy donor possessing a high frequency of γ/δ^+ T cells (20% of total lymphocytes). PBL were cultured in presence of 160 ng/ml of rIL-4 and 70 U/ml of rIL-2, the latter included as a control of experiment. The proliferation of the cells, assessed by [^{3}H]thymidine uptake, and the phenotypic distribution of the TCR-α/β^+- and -γ/δ^+-bearing T cells (assessed by flow cytometry) took place after 4 and 7 d of culture.

The results of a representative experiment are shown in Table 2. The peripheral blood mature T cells do not grow in response to IL-4, and the frequency of TCR-γ/δ^+ T cells was not modified after culture with this lymphokine. In contrasts, the IL-2-promoted proliferation of PBL is parallel to a selective expansion of TCR-γ/δ^+ T cells, as previously described (40). These results demonstrate that the appearance of γ/δ^+ T cells in the progeny of IL-4- cultured pre-T cells cannot be explained by the overgrowth of small mature contaminant T cells. Therefore, an IL-4-promoted differentiation of pre-T cells into TCR-γ/δ^+-bearing T cells is required.

To formally rule out that outgrowth of rare contaminants with mature phenotype can account for the differentiation of pre-T cells upon in vitro culture with IL-4, we have undertaken the following set of experiments: (*a*) daily character-

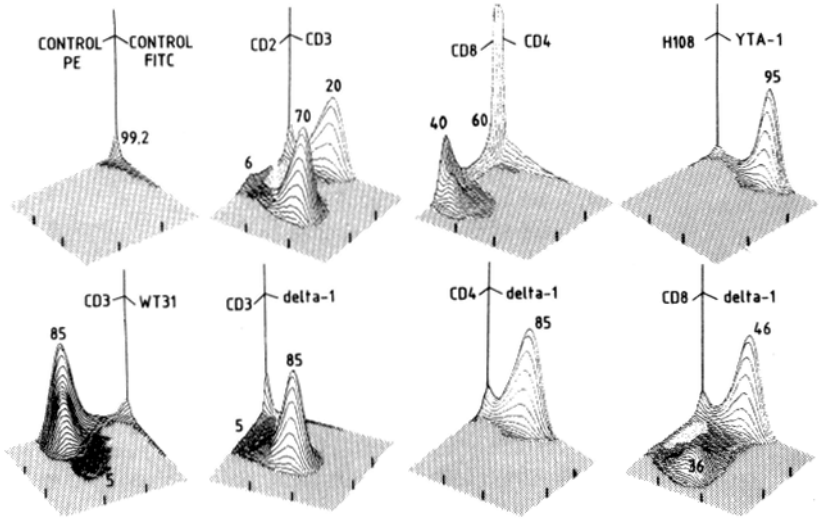

Figure 4. In vitro differentiation of human pre-T cells cultured with IL-4. Pre-T cells immunoselected from fresh adult human thymocytes were cultured at 2 × 10^6 cells/ml in medium containing 160 ng/ml of rIL-4. Cellular staining was performed, as described in Materials and Methods, at day 9 of culture with the indicated mAbs. Two-color immunofluorescence analyses of 2 × 10^4 viable cells were performed in an Epics-Profile II flow cytometer. The number of positive cells over background stainings observed with irrelevant mAbs are represented. The data were analyzed for representation using the FlowSys program.

Table 1. *Differentiation In Vitro of Intrathymic Pre-T Cells in rIL-4 and rIL-2*

Culture conditions	Day of culture	Cell recovery*	Expression of surface T cell markers											
			CD45	CD7	CD1	CD2	CD3	CD4	CD8	WT31	δ-1	TAC	YTA-1	HLA-DR
		%							%					
–	0		86	55	<1	52	<1	<1	<1	<1	<1	5	14	35
rIL-4	14	175	99	98	ND	100	80	26	49	25	60	12	98	30
rIL-2	14	233	99	99	ND	100	97	84	20	85	12	67	95	59

Pre-T cells (2 × 10^6 cells/ml) were cultured in complete medium supplemented with 160 ng/ml of rIL-4 or 50 U/ml of rIL-2. The phenotype of the cells was assessed by direct or indirect immunofluorescence and quantitative flow cytometry (Epics-Profile II). Results show the percentage of cells positive for each marker.

* Cell recovery is the cell number after 14 d culture/initial cell number.

ization of the growing T cells; and (*b*) repeating of the experiments under limiting dilution conditions. The results depicted in Fig. 5 allow us to draw the following conclusions. The extrapolation of time zero of the exponential growth curves of the cultures reveals that the number of growth-initiating T cell precursors per culture ranges from 10 to 20% of the starting cells (Fig. 5 *B*), which agrees with the frequency of responding cells in cultures performed under limiting dilution conditions (Fig. 5 *A*) (5–15%). Interestingly enough, the frequency of growth-initiating pre-T cells per culture in the presence of IL-2 or IL-4 is the same (not shown). However, the doubling time and the clone size of platted cells in both types of cultures is quite different. Thus, while in the presence of IL-2 pre-T cells divide every 36–40 h, in the presence of IL-4, the doubling time is 48–50 h. This division time agrees with the measures obtained in mass cell cultures. Early phenotypic characterization of clones derived by limiting dilution analysis showed a more frequent differentiation into the TCR-γ/δ-bearing lymphocytes. In fact, the phenotypic characterization of the proportion of limiting dilution cultures yield 70–80% and 20–30% of TCR-γ/δ- and α/β-bearing lymphocytes, respectively.

Discussion

Recent studies of embryonic and adult human thymus have identified $CD45^+7^+2^+1^-3^-4^-8^-$ pre-T cells as an early ontogenic subset comprising the precursors of mature T cells (34, 36). At present, the regulation of the complex process leading to TCR rearrangement and differentiation of T cell precursors into $CD3^+$ mature T cells remains poorly understood. In humans, the development of in vitro model systems of differentiation has been a useful tool to study cellular and molecular events involved in such a process. From these studies, evidence has been obtained for the role that the IL-2 pathway plays in the generation of both TCR-α/β- and TCR-γ/δ-bearing T cell lineages (41). In addition, recent reports in mice showing that intrathymic T cell precursors are able to produce (19, 22) and respond to (19–21) IL-4 after cellular stimulation indicate that IL-4 could be an alternative growth factor to IL-2, involved in T cell differentiation.

In this study, in vitro differentiation approaches, similar to those previously described for IL-2 (34, 39), have been used to analyze the potential effects of IL-4 on human pre-T cells. Our results show that pre-T cells are able to proliferate in response to rIL-4 in a dose-dependent way. Although the ability

Table 2. *Phenotypic Analysis and Proliferation of PBL Cultured in rIL-2 and rIL-4*

Day of culture	Culture conditions	[^{3}H]Thymidine uptake	Expression of surface T cell markers						
			CD45	CD2	CD3	CD4	CD8	WT31	δ-1
		cpm				%			
0	–	–	93	76	74	40	15	63	18
4	rIL-4	300 ± 29	97	80	77	50	20	57	19
4	rIL-2	44,428 ± 600	96	86	84	44	28	67	20
7	rIL-4	971 ± 147	98	94	90	47	25	77	19
7	rIL-2	135,501 ± 2,136	98	96	82	17	35	45	35

PBL (5 × 10^5 cells/ml) were cultured in complete medium supplemented with 160 ng/ml of rIL-4 or 70 U/ml of rIL-2. The proliferative responses were analyzed at indicated days of triplicate cultures after addition of 1 μCi/well of [^{3}H]thymidine for the last 18 h, and the phenotype of the cells was assessed by direct or indirect immunofluorescence and quantitative flow cytometry. The results show the percentage of cells positive for each marker.

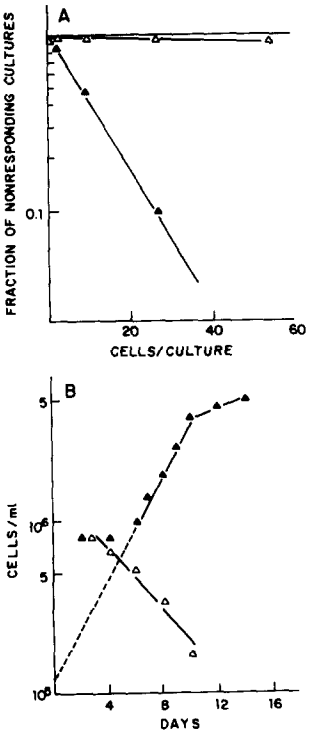

Figure 5. (*A*) Titration of IL-4-reactive human pre-T cells. The indicated number of cells were grown in the presence (*filled symbols*) or in the absence (*open symbols*) of 160 ng/ml of IL-4. Each point in the figure is based on assays of 60 cultures. (*B*) Growth of IL-4-reactive human pre-T cells. Human pre-T cells (8×10^5/ml) were incubated in the presence (*filled symbols*) or in the absence (*open symbols*) of 160 ng/ml of rIL-4, and the increase in cell number was measured with time of stimulation.

of IL-4 to promote proliferation of postnatal immature thymocytes in mice is well documented (19, 20, 42), in all cases so far reported, IL-4-induced proliferation required cellular activation with mitogenic agents. In contrast, human pre-T cells did not appear to require costimulatory signals to respond to IL-4, and, indeed, addition of PMA to the cultures did not significantly increase IL-4-promoted growth (data not shown). These findings must be discussed in the light of recent studies showing that mouse $CD4^-CD8^-$ T cell precursors constitutively expressed low levels of high affinity receptors for IL-4, which increased approximately eightfold after cellular activation with PMA (20). These data suggest that functional levels of IL-4R, capable of mediating growth signal transduction, may be expressed constitutively on human pre-T cells. In agreement, IL-4-promoted proliferation of mouse fetal thymocytes has been reported at the clonal level (43, 44). Alternatively, IL-4/IL-4R interaction on pre-T cells may result in an upregulation of high affinity IL-4R, leading to cellular proliferation. The recent observation that IL-4 alone is able to induce IL-4R expression on human PBMC at levels similar to those obtained by anti-CD3 activation (45) further supports the latter hypothesis. Therefore, availability of IL-4 within the thymic microenvironment appears to be critical in the regulation of the physiological growth responses mediated by IL-4. Results showing that by 15 d of gestation in the mouse up to 45% of thymocytes constitutively produced IL-4 mRNA (23) provide evidence for the involvement of IL-4 in the proliferation of T cell precursors, as proposed earlier for IL-2 (35, 41).

Analyses of the growth-promoting effect of IL-4 on pre-T cells revealed that this process must be independent of the IL-2 pathway. In fact, antibodies specific for the α component of the IL-2R were not able to block the proliferation induced by IL-4. However, recent studies showing that IL-4 downregulates the number of high affinity IL-2Rs in B and T cell lines suggested an interaction between the two pathways (46). This possibility could explain the synergistic effect of both lymphokines on the proliferative response induced in T cells (14, 46, 47). Whether or not IL-4 is also responsible for the low IL-2R-α expression detected in the pro-T cell progeny remains to be established. The answer to this issue must wait until much more information on the populations of T cell precursors responding to IL-4 and IL-2 is available. Therefore, different T cell precursor subsets may respond to distinct lymphokines, or, alternatively, the same intrathymic population may utilize both growth pathways. To date, evidence obtained at the clonal level with 14-d fetal thymocytes indicates that the same fetal precursor can use both IL-2 and IL-4 as growth factor (43, 44).

Results reported here suggest that human early intrathymic T cell precursors are able to respond to both lymphokine pathways. Our data showed that IL-4 responsiveness resulted not only in proliferation but also in differentiation of pre-T cells. Moreover, a predominant differentiation of pre-T cells into TCR-γ/δ^+-bearing mature T cells was induced by IL-4, whereas IL-2-responding pre-T cells preferentially differentiate into TCR-α/β^+ T cells.

A common question that arises upon analysis of pre-T cell cultures concerns the possibility that mature cells escape both antibody plus complement and magnetic beads coated with anti-mouse Ig treatments in the starting population, and expand to account for the phenotypic pattern observed. The following findings, presented here and elsewhere (39), cannot be easily reconciled with such an interpretation. First, precursor and mature populations were distinct with regard to their expression of CD3, CD4, CD7, CD8, and CD45 antigens. Second, the expression of CD3 antigen clearly precedes that of CD4 and CD8. Third, as shown here, mature γ/δ T cells do not grow in IL-4. Fourth, effector function, mainly killing activity, was acquired after 4 d in culture, at which point the activity per cell rose 250-fold in 2 d (data not shown). Fifth, the frequency of responding cells, calculated by limiting dilution analysis and by the extrapolation to day 0 of the exponential growth curves, is at least 20–40 times higher than the number of possible contaminants detected by flow cytometry. Sixth, the production of IL-4 and the expression of IL-4R by thymic pre-T cells and not by mature thymocytes (J. L. de la Pompa et al., manuscript in preparation).

Our data can be interpreted on the basis of the differences in the kinetics of fetal thymocytes expressing IL-4R and producing IL-4 or IL-2 in ontogeny (23), as well as of the developmental regulation of TCR-γ/δ and -α/β gene expression. We proposed that two different pathways may be involved in the generation of mature T cells from intrathymic precursors. At early stages of development, availability of IL-4 within the thymic microenvironment would induce IL-4R-bearing progenitors to grow and to differentiate preferentially

into TCR-γ/δ^+ cells. Later on, activation of the IL-2 pathway (IL-2 production and IL-2R expression) would result in generation of TCR-α/β^+ cells. The reported involvement of an IL-2-dependent autocrine pathway of growth in T cell development (39) suggests a similar autocrine behavior for IL-4. Therefore, differentiation into another T cell lineage at a given ontogenic stage would actually depend on the available local concentrations of IL-2 and IL-4, as well as on the proportion of T cell precursors expressing IL-2R vs. IL-4R throughout intrathymic T cell development.

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