In Vitro Expansion of CD3/TCR$^-$ Human Thymocyte Populations That Selectively Lack CD3δ Gene Expression: A Phenotypic and Functional Analysis

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Summary

Highly purified CD1$^-$3$^-$4$^-$8$^-$ human thymocytes were obtained by panning techniques combined with cell depletion with antibody-coated magnetic beads. Most of these cells expressed cytoplasmic CD3 antigen, as assessed by mAbs known to react with the CD3ε chain. After culture with low doses of PMA (0.5 ng/ml) and subsequent addition (at 24 h) of recombinant interleukin 2 (rIL-2; 100 U/ml) cells underwent extensive proliferation (40–60-fold of the initial cell input after 2 wk). The majority of the proliferating cells were CD3$^-$TCR$^-$. The remaining cells (5–40%) were represented by CD3$^+$TCR γ/δ^+ (BB3$^-$A13$^+$) cells. Further removal of CD3$^+$TCR-γ/δ^+ cells resulted in highly purified CD3$^-$ populations that further proliferated in culture with no substantial phenotypic changes. When CD3$^+$ thymocytes were cultured under the same experimental conditions, only CD3$^+$ TCR-α/β^+ cells could be detected, thus indicating that PMA did not affect the surface expression of the CD3/TCR complex, but rather induced preferential growth of CD3$^-$ thymocytes. Surface marker analysis of cultured CD3$^-$ thymocytes showed that they were homogeneously CD7$^+$, whereas low proportions of cells expressed CD2 and CD8 antigens. Among the natural killer (NK) cell markers, CD56 was highly expressed by all cells, whereas CD16, CD57, CD11b, NKH2, and GL183 were absent. Importantly, these cells were different from peripheral NK cells, as 80–95% of them expressed cytoplasmic CD3 antigen. Functional analysis revealed a strong cytolytic activity against both NK-sensitive (K562) and NK-resistant (M14, Daudi) human target cells. In a redirected killing assay against the FcγR$^+$ P815 cells, mAbs specific for triggering molecules including CD3, CD2, and CD16 failed to augment target cell lysis, while a strong cytolytic effect was induced by PHA. In addition, PHA alone or in combination with PMA induced tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) (but not IL-2) production by CD3$^-$ thymocytes. Cloning of fresh CD1$^-$3$^-$4$^-$8$^-$ thymocytes in the presence of PMA and rIL-2 resulted in CD3$^-$CD56$^+$ clones that displayed a pattern of cytolytic activity and lymphokine production similar to that of the polyclonal populations. Northern blot analysis of transcripts coding for CD3/TCR molecules revealed the presence of CD3ζ, ε, and γ transcripts, while CD3δ was undetectable. Mature transcripts for both γ and δ TCR chains could be detected, whereas no TCR-α mRNA and only a truncated (1.0 kb) form of TCR-β mRNA were revealed. These data suggest that CD3$^-$ cultured thymocytes may be, at least in part, representative of a T cell maturation stage that precedes the surface expression of the CD3/TCR molecular complex. Southern blot analysis of the TCR-δ gene rearrangements revealed that the cultured CD3$^-$ thymocytes retained a germline configuration.

T cell development within the thymus is characterized by the progressive acquisition of functionally relevant and/or lineage-specific molecules (1–14). Three major, phenotypically defined subsets have been identified on the basis of the expression of the CD1, CD4, CD8, and CD3/TCR differentiation antigens. A small (1%) CD1$^-$3$^-$4$^-$8$^-$ subset that expresses CD7 and CD2 antigens is generally thought to include the most immature thymocytes (1, 4, 6). A second subset,

consisting of the CD1$^+$ thymocytes (60–85%), includes cells that coexpress the CD4 and CD8 antigens (1, 4, 6, 15). The third subset is represented by "mature" surface CD3/TCR$^+$ thymocytes that express either CD4 or CD8 antigens (1, 4, 6).

In humans, the small subset characterized by the CD1$^-$3$^-$4$^-$8$^-$ surface phenotype (and by the presence of cytoplasmic CD3ϵ chain) has been extensively studied in vitro in order to analyze its ability to undergo phenotypic and functional maturation (16–22). These studies have suggested that CD1$^-$3$^-$4$^-$8$^-$ thymocytes, under appropriate culture conditions (20, 21), undergo differentiation and give rise to the two major subsets of peripheral T lymphocytes expressing either TCR-α/β or -γ/δ. However, a precise analysis of the sequential stages occurring during T cell maturation (from CD1$^-$3$^-$4$^-$8$^-$ towards CD3$^+$TCR$^+$ cells) has been hampered primarily by the limited number of CD1$^-$3$^-$4$^-$8$^-$ cells available after thymocyte fractionation. In addition, it has been reported that after culture these cells rapidly acquire surface CD3/TCR (17, 18).

In the present study we have defined culture conditions that allow extensive proliferation of CD3$^-$ thymocyte populations and clones without maturation into surface CD3$^+$ TCR$^+$ cells. These cells expressed cytoplasmic CD3ϵ chain and acquired surface CD56 antigen, although they lacked other NK cell markers. They expressed effector functions including cytolytic activity and lymphokine production. Analysis of mRNA for CD3 or TCR chains revealed the presence of full-length transcripts for both TCR-γ and -δ chains and the expression of transcripts for CD3ζ, ϵ and γ but not for CD3δ chain. Analysis of the TCR-δ gene rearrangements revealed a germline configuration.

Materials and Methods

Antibodies and Reagents. mAbs Leu4 (anti-CD3), Leu2a (anti-CD8), Leu3a (anti-CD4), Leu9 (anti-CD7), Leu5b (anti-CD2), Leu1 (anti-CD5), TCR-1 (anti-WT31), Leu19 (anti-CD56), LeuM5 (anti-CD11b), Leu7 (anti-CD57), Leu11c (anti-CD16), anti-HPCA-1 (anti-CD34), anti-IL-2 receptor (anti-CD25), and anti-HLA class II were purchased from Becton Dickinson (Mountain View, CA). OKT6 (anti-CD1) and OKT3a (anti-CD3) mAbs were from Ortho Immune Diagnostic (Raritan, NJ). Anti-TCRδ1 was purchased from T Cell Sciences Inc. (Cambridge, MA). Anti-HLA class I mAb (W6/32) was from Dakopatts (Glostrup, Denmark). CK248 (anti-CD28) (23), CD2-1 (anti-T11-2) (24), CD2-9 (anti-T11-1) (24), Mar206 (anti-T11-1) (25), VD4 (anti-CD16) (26), KD1 (anti-CD16) (26), A13 (anti-Vdelta-1) (27, 28), BB3 (anti-Vdelta-2) (28, 29), JT3A (anti-CD3) (30), J90 (anti-CD11a) (30), and GL183 (31) mAbs were obtained in our laboratory. FITC-conjugated goat anti-mouse IgG was purchased from Cappel Laboratories (Cochranville, PA). NKH2 and UCHT-1 mAbs were generous gift of Drs. T. Hercend (Institut Gustave Roussy, Villejuif, France) and P. C. L. Beverley (Imperial Cancer Research Fund Human Tumour Immunology Group, London, UK), respectively. PMA was from Sigma Chemical Co. (St. Louis, MO). PBS, PHA, RPMI 1640 medium, FCS, L-glutamine, penicillin-streptomycin were purchased from Gibco Laboratories (Grand Island, NY). rIL-2 was kindly provided by Cetus Corp. (Emeryville, CA).

Isolation of CD1$^-$3$^-$4$^-$8$^-$ Thymocytes. Normal human thymocytes were obtained from thymus fragments removed during cardiac surgery of patients 2 mo to 4 yr old as described (21). The isolation of CD1$^-$3$^-$4$^-$8$^-$ thymocytes (<1% of total thymus cell suspensions) were obtained after negative depletion by panning using saturating amounts of anti-CD1 (OKT6), anti-CD3 (Leu4), anti-CD4 (Leu3a), and anti-CD8 (Leu2a) mAbs (21). After panning, the contamination with "positive" cells was 10–25% (as assessed by immunofluorescence). Cell suspensions were further depleted of "positive" cells by using magnetic beads coated with anti-mouse IgG (Oxoid Italiana S.p.A., Milan) as described (32). Both cell purification steps were performed at 4°C. After this second step, cells recovered were >99.9% CD1$^-$3$^-$4$^-$8$^-$. These highly purified thymocyte suspensions were used in further experiments.

Generation of CD3$^-$ Cell Lines from CD1$^-$3$^-$4$^-$8$^-$ Thymocytes. Highly purified CD1$^-$3$^-$4$^-$8$^-$ thymocytes were cultured in 200 μl of RPMI 1640 medium supplemented with 10% of FCS (Gibco Laboratories) in 96 U-bottomed microplates (Greiner, Nurtingen, FRG) either in the presence or in the absence of 0.5 ng/ml of PMA (Sigma Chemical Co.) or with 0.5 μg/ml of PHA (Gibco) for 24 h. After this period, 100 μl of culture medium was discarded and 100 μl of rIL-2–containing (100 U/ml) (Cetus Corp.) medium (with no PHA or PMA) was added. Within 2–5 d evident cell growth could be observed. Cultures were repeatedly splitted by adding 100 μl of the same IL-2-containing medium. Surface marker analysis of stimulated CD1$^-$3$^-$4$^-$8$^-$ thymocytes was performed at different time intervals. After 10–15 d of culture, high proportions (60–95%) of CD3$^-$ proliferating cells could be detected in cultures pulsed with PMA. These cultures were further depleted of CD3$^+$ cells by using antibody-coated magnetic beads. The resulting populations contained <0.1% CD3$^+$ cells. These highly purified CD3$^-$ cells proliferated in culture for up to 8 wk and maintained their CD3$^-$ phenotype.

Flow Cytometry Analysis. The techniques used have been described in detail elsewhere (23). Briefly, aliquots of 10^5 cells were stained with the corresponding mAb followed by FITC-conjugated goat anti-mouse Ig (Cappel Laboratories). Control aliquots were stained with an unrelated mAb (Becton Dickinson). The samples were analyzed on a flow cytometer (FACStar, Becton Dickinson) gated to exclude no viable cells. The presence of cytoplasmic CD3 antigen into CD3$^-$ proliferating thymocytes was performed as follows: 10^5 cells were fixed in formaldehyde (Sigma Chemical Co.) (3.7% in PBS) for 5 min at room temperature (RT), washed twice, and then permeabilized with 0.01% (final concentration) of N-P40 (Sigma Chemical Co.) for 5 min at RT. Treated cells were extensively washed in PBS (Gibco Laboratories) and then stained with different anti-CD3 mAbs (Leu4, UCHT-1, OKT3a) followed by the FITC-conjugated goat anti-mouse Ig. Under these conditions, no detectable change of the cell size (as assessed by forward scatter and side scatter analysis) could be observed. In addition, no modification of the pattern of reactivity with several mAbs could be detected after cell treatment.

Cloning of CD1$^-$3$^-$4$^-$8$^-$ Thymocytes. Cloning of highly purified CD1$^-$3$^-$4$^-$8$^-$ thymocytes was performed under limiting dilution conditions (33) in 96-well U-bottomed plates (Greiner) in the presence of 10^5 irradiated PBMC as feeder cells and 0.5 ng/ml of PMA (Sigma Chemical Co.), as previously described (33). After 24 h 100 U/ml rIL-2 was added to each well. IL-2 supplementation was repeated at 4–5-d intervals. Evident cell growth could be detected after 12–18 d of culture. Clones were maintained in culture for up to 1 mo.

Functional Assays. Cytolytic activity of polyclonal or clonal CD3$^-$ thymocytes was tested in a 4-h ^{51}Cr-release assay as previously described in detail (34). Briefly, NK-sensitive (K562) or NK-

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resistant (Daudi, M14) tumor cell lines were labeled with 100 μCi/10^6 cells of sodium chromate (34) and seeded into 96-well V-bottomed plates (Greiner) at 5 × 10^4 cells/well. $CD3^-$ thymocytes were used as effector cells at E/T ratios ranging from 25:1 to 6:1 in a final volume of 200 μl of RPMI 1640. After 4 h, 100 μl of supernatant was removed from each well and counted in a gamma counter (Gamma 5500; Beckman Instruments, Fullerton, CA) for the assessment of ^{51}Cr release. The percent specific release was determined as previously described (34). In a redirected killing assay, the P815 ($Fc\gamma R^+$) murine mastocytoma, used as target cells, were seeded at an E/T ratio of 1:1. The cytolytic test was performed in medium alone, or in the presence of 0.5 μg/ml of PHA or 20 μl of culture supernatant of various mAbs including MAR206, CD2-9, VD4, and JT3A. To evaluate the production of TNF-α, IFN-γ, and IL-2, $CD3^-$ thymocytes were stimulated for 36 h in culture medium in either the presence or absence of PMA (0.5 ng/ml) and/or PHA (0.5 μg/ml). Culture supernatants were tested for TNF-α, IFN-γ, and IL-2 activity by enzyme immunoassays (T Cell Sciences).

Southern and Northern Blot Analysis. DNA was extracted from the B cell leukemia line Raji and a $CD3^-$ proliferating thymocyte population, then digested with the BamHI restriction enzyme. DNA (5 μg) was electrophoresed through 0.6% agarose and transferred to Gene Screen Plus (New England Nuclear, Boston, MA) filters described by Southern (35). The transferred DNA was covalently attached to an air-dried membrane by 1-min exposure to 1,200 μW/cm of UV light. The filter was prehybridized, hybridized, and washed as described (36). The probe used was the 1.5-kb EcoRI fragment of the TCR-δ cDNA (37). RNA was prepared, size fractionated by electrophoresis in agarose gels containing formaldehyde, and subsequently blotted and hybridized as previously described (36). The probes used were the following: a 1.1-kb EcoRI fragment of the TCR-α cDNA clone pY1.4 (38), a 0.77-kb PstI fragment of the TCR-β cDNA clone IUR-β 2 (39), a 1.6-kb EcoRI fragment of the TCR-γ cDNA clone HGPO2 (40), a 1.5-kb EcoRI fragment of the TCR-δ cDNA (37), a 0.7-kb EcoRI fragment of the CD3γ cDNA clone pJ6T3γ-2 (41), a 0.9-kb OxaNI/BamHI fragment CD3ε cDNA clone pCD3H (42), a 0.8-kb PstI fragment of CD3δ cDNA, derived from clone 25 (kindly provided by Dr. Tak W. Mak, Ontario Cancer Institute, Toronto, Ontario), and two 30-mer oligonucleotides overlapping by 10 complementary bases corresponding to the CD3ζ cDNA (43) (GGCCTGCTGGATCCCAAACTCTGCTACCTG and ATGAAGAGGATTCCATCCAGCAGGTAGCAG). Probes were labeled as described (36). Blots were exposed to x-ray films using intensifying screens at −70°C.

Results

Isolation of $CD1^-3^-4^-8^-$ Thymocytes. $CD1^-3^-4^-8^-$ thymocyte populations were obtained by the combined use of panning techniques and cell depletion with antibody-coated magnetic beads. This double-step cell isolation yielded highly purified $CD1^-3^-4^-8^-$ populations containing <0.1% contaminating cells. In different experiments, the cell yield was 0.2–0.6% of the starting unfractionated thymocyte cell suspension. Purified $CD1^-3^-4^-8^-$ thymocyte populations contained a high proportion of surface $CD7^+$ cells and variable proportions of $CD2^+$ cells (50–60%). Virtually no $CD25^+$, $CD16^+$, $CD56^+$, and $CD11b^+$ cells could be detected (Fig. 1). In agreement with previous reports, most of the $CD1^-3^-4^-8^-$ thymic cells expressed intracytoplasmic

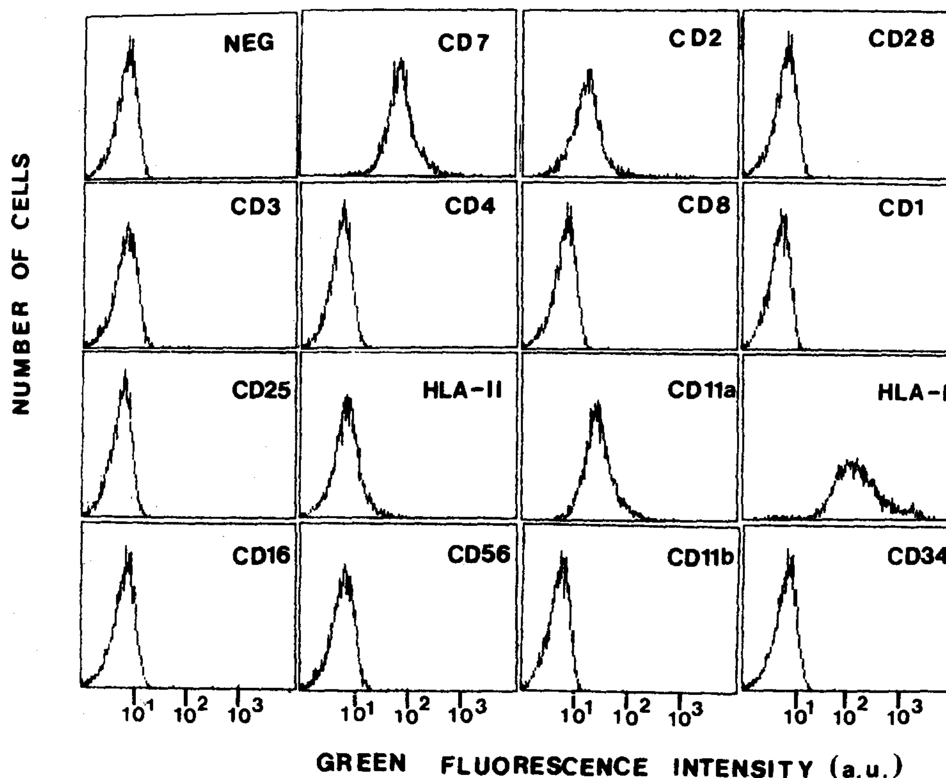

Figure 1. Surface phenotype of $CD1^-3^-4^-8^-$ resting thymocytes. $CD1^-3^-4^-8^-$ thymocytes were isolated from total thymocytes by panning techniques combined with cell depletion with antibody-coated magnetic beads, as described in Materials and Methods. Cells were stained with the mAbs indicated, followed by FITC-conjugated goat anti-mouse IgG. In the upper left quadrant (negative control) cells were stained with an irrelevant mAb. Samples were analyzed on a FACStar and results are expressed as log green fluorescence intensity vs. number of cells. Data are representative of six independent experiments.

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CD3 antigen, as assessed by both fluorescence microscopy and cytofluorometric analysis (following cell membrane permeabilization) (not shown). It should be noted that the anti-CD3 mAbs used are known to specifically react with the CD3ε chain (44).

In Vitro Expansion of CD1⁻3⁻4⁻8⁻ Thymic Cells. Given the high degree of purity of the $CD1^-3^-4^-8^-$ thymic populations, we further attempted to define culture conditions suitable for their vitro expansion. To this aim, cells were stimulated in the presence of rIL-2 (100 U/ml), or PMA (0.5 ng/ml) or PHA (1 μg/ml) added at time 0. All the stimulated thymocyte populations were then expanded using culture medium containing IL-2 but not PHA or PMA. At different culture intervals, cells were analyzed for their proliferating ability as well as for their surface phenotype. A strong proliferative response could be detected after 48–72 h in the presence of either PHA or PMA plus rIL-2. Three- fivefold less proliferation was detected in the presence of rIL-2 alone (not shown). Consistently, at later culture intervals, maximal cell expansion was detectable in cultures stimulated with PMA and rIL-2. For example, as shown in Fig. 2, the cell number recovered after 2 wk was ~50-fold higher than the initial cell input. On the other hand, cell recovery in cultures containing rIL-2 alone or rIL-2 and PHA was consistently lower. In some experiments, cells were continuously grown in culture for up to 8 wk with no substantial decrease in cell proliferation. It should be noted that due to the rate of cell growth, the starting PHA or PMA concentrations were 100-1,000-fold diluted in culture medium.

The cell surface phenotype expressed by purified $CD3^-$ thymocyte populations cultured under the different conditions described was analyzed. In agreement with previous reports, cells cultured in the presence of rIL-2 alone or IL-2 plus PHA largely resulted in $CD3^+WT31^+$ cells. No cells expressing TCR-γ/δ (as assessed by mAbs BB3, A13, and TCR-δ-1) could be detected under these culture conditions. On the other hand, culture of $CD3^-$ thymocytes in the presence of PMA and IL-2 gave rise (after 2 wk of culture) to cell populations containing 60–95% $CD3^-$ cells (not shown). Interestingly, the minor $CD3^+$ cell fractions were homogeneously composed of $A13^+BB3^-$ cells, thus representing the TCR-γ/δ^+ subset utilizing the Vδ1 gene segment (45). These data indicate that the type of triggering signal provided to $CD1^-3^-4^-8^-$ thymocyte populations appears to influence the cell surface phenotype resulting after culture. More importantly, in the presence of PMA and rIL-2 it was possible to obtain an extensive proliferation of cells that expressed the original $CD3^-TCR^-$ surface phenotype. The above data suggest that the combination of low doses of PMA and IL-2 induced extensive $CD3^-$ thymocyte proliferation, without substantial phenotypic changes. Since PMA has been reported to downregulate the surface expression of the CD3/TCR complex in mature T lymphocytes (46), we investigated whether low doses of PMA used in order to induce thymocyte proliferation (0.5 ng/ml) could also have a similar effect on $CD3^+$ thymocytes. To this aim, unfractionated, $CD3^-$ and $CD3^+$ thymocyte populations were stimulated with PMA (0.5 ng/ml) and rIL-2. An extensive proliferation could be detected with all cell populations tested. However, surface marker analysis performed at day 14 showed that in cultures derived from the $CD3^+$ thymocyte fraction, virtually no $CD3^-$ cells could be detected. In addition, proliferating cells were mostly characterized by the $CD3^+$ $WT31^+$ surface phenotype. Interestingly, large proportions of $CD3^-$ cells could be derived not only from purified $CD3^-$ thymocytes, but also from unfractionated populations. In the latter case, $CD3^+$ cells represented 20–40% of recovered cells and were composed of both TCR-α/β^+ (5–20%) and TCR-γ/δ^+ cells (10–25%) (not shown). Taken together, these data indicate that the combined use of PMA and rIL-2 induces preferential growth of $CD3^-$ thymocytes without affecting the surface expression of CD3/TCR complex in $CD3^+$ thymocytes.

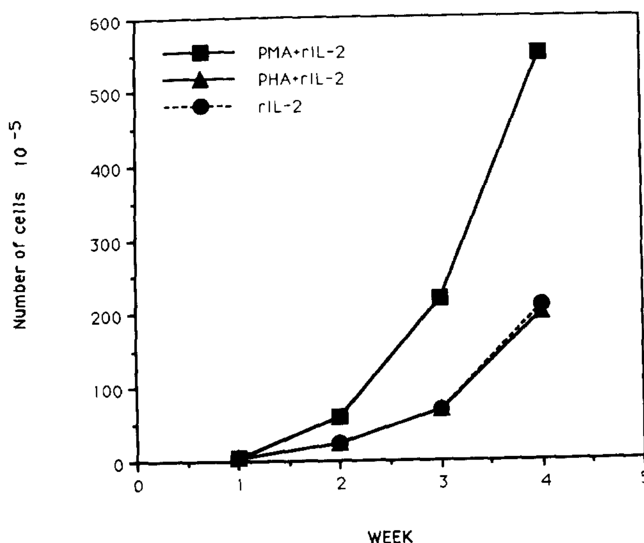

Figure 2. Cell recovery upon culture of $CD1^-3^-4^-8^-$ thymocytes in the presence of different stimuli. 10^5 Purified $CD1^-3^-4^-8^-$ thymocytes were cultured in culture medium alone (●), or in the presence of PHA (▲) (0.5 μg/ml) or PMA (■) (0.5 ng/ml). In all instances rIL-2 was added after 24 h. Cell recovery was evaluated after different culture intervals. Similar data were obtained in three other independent experiments.

Phenotypic Analysis of Cultured CD3⁻ Thymocytes. $CD3^-$ thymocytes obtained by in vitro culture with PMA and rIL-2 were further analyzed for their phenotypic and functional properties, after further depletion of $CD3^+$ cells. The resulting populations contained <0.1% $CD3^+$ cells. Analysis of surface antigens (Fig. 3) revealed that cells were homogeneously $CD7^+$, whereas variable (usually low) proportions of cells expressed CD2 antigen. The expression of CD2 antigen was detected by the use of several mAbs recognizing different epitopes of the CD2 molecule (T11-1, T11-2). Cells also lacked surface expression of CD1, CD4, CD28 (Fig. 3), CD5, TCR-γ/δ, and TCR-α/β molecules (not shown), while a minor proportion (<10%) expressed CD8 antigen (Fig. 3). Because the thymocyte populations examined, similar to peripheral $CD3^-$ NK cells, lacked surface CD3/TCR complex, we analyzed a number of surface markers

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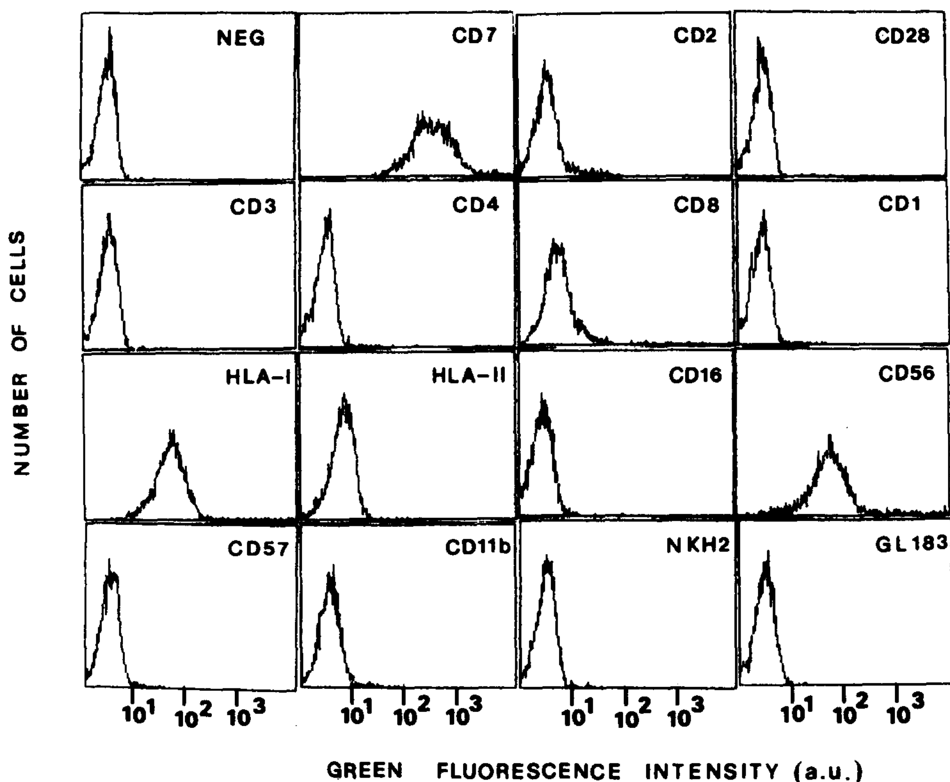

Figure 3. Surface phenotype of cultured CD3⁻ thymocytes. Cells were stained with the indicated mAbs followed by FITC-conjugated goat anti-mouse IgG. The upper left quadrant (negative control) represents cells stained with an irrelevant mAb. Samples were analyzed on a FACStar and results are expressed as log green fluorescence intensity vs. the number of cells. Data are representative of four independent experiments.

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expressed by NK cells. Cells were homogeneously stained by anti-CD56 mAb, but they did not express CD16, CD57 (Leu7), CD11b, NKH2, and GL183 surface antigens (Fig. 3). In addition, different from peripheral blood NK cells, the majority (80–95%) of cultured $CD3^-$ thymocytes were found to express cytoplasmic CD3 antigen (Fig. 4).

Functional Analysis of Cultured $CD3^-$ Thymocytes. We next analyzed whether cultured $CD3^-$ thymocytes expressed effector cell functions such as cytolytic activity and lymphokine production. In conventional ^{51}Cr-release assays, these cells displayed a strong cytolytic activity against NK-sensitive (K562) and NK-resistant (M14, Daudi) human target cells (shown in Table 1). We also analyzed the ability of these cells to lyse murine mastocytoma P815 cells in a redirected killing assay using PHA or mAbs specific for "triggering" cell surface molecules, including CD3, CD2, and CD16. As shown in Table 2, while addition of PHA significantly augmented target cell lysis, none of the stimulatory mAbs had any effect.

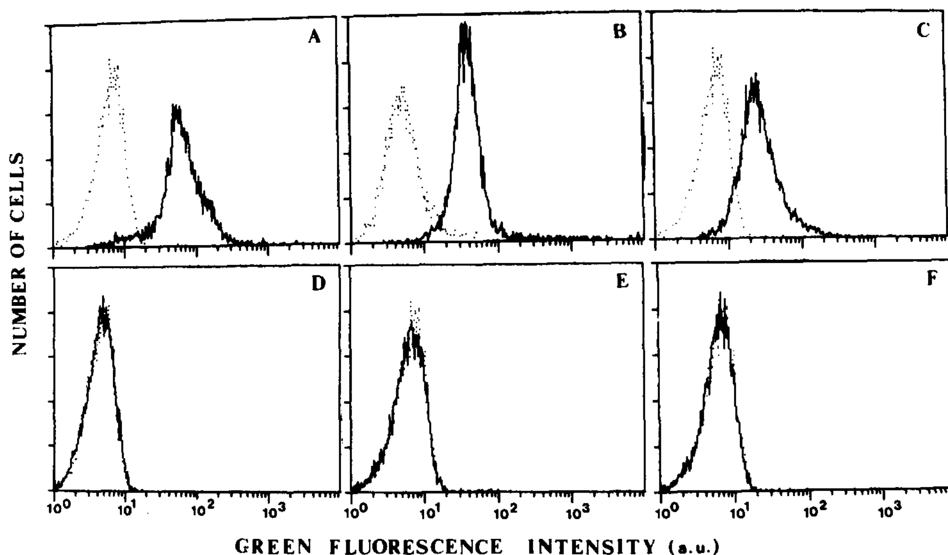

Figure 4. Cultured $CD3^-$ thymocytes express cytoplasmic CD3 antigen. Cultured $CD3^-$ thymocytes (*A–C*) were analyzed for cytoplasmic CD3 in comparison to cultured $CD3^-CD16^+$ peripheral blood–derived populations (*D–F*). Cells were stained with Leu4 (*A*, *D*), UCHT-1 (*B*, *E*), or OKT3a (*C*,*F*) mAbs and analyzed at the FACS after cell fixation and membrane permeabilization as described in Materials and Methods. Dotted lines represent cells stained with an unrelated mAb followed by the FITC-conjugated reagent. Data are representative of six independent experiments.

Table 1. *Cytolytic Activity of Polyclonal or Clonal CD3⁻ Thymocytes against NK-sensitive or NK-resistant Tumor Target Cells*

CD3$^-$TCR$^-$ thymocytes	Target cell: K562 at E/T ratio of: 25:1	12:1	6:1	M14 at E/T ratio of: 25:1	12:1	6:1	Daudi E/T ratio of: 25:1	12:1	6:1
Polyclonal population 1*	100‡	85	70	85	70	55	90	70	60
Polyclonal population 2	95	80	65	75	60	45	81	65	50
Clone Th1	70	55	40	75	60	55	75	55	50
Clone Th2	100	75	50	90	70	53	95	70	42

* Polyclonal populations and clones were homogeneously CD3$^-$TCR$^-$, expressed CD7 and CD56, but lacked CD16 and CD57 surface antigens.
‡ Results are expressed as percent of specific ^{51}Cr release.

These results are in line with the data of cytofluorometric analysis showing lack of surface expression of CD3 and CD16 molecules and low levels of CD2. These results also indicate that PHA could induce CD3$^-$ thymocyte triggering upon interaction with surface molecules different from CD3, CD2, and CD16.

Analysis of lymphokine production in response to either PHA or PHA and PMA revealed that cultured CD3$^-$ thymocytes produced both IFN-γ and TNF-α (Table 3), but not IL-2 (not shown). As opposed to peripheral CD3$^-$ NK cells, the addition of exogenous rIL-2 did not significantly increase the release of IFN-γ and TNF-α (not shown).

Clonal Analysis of CD1$^-$3$^-$4$^-$8$^-$ Thymocytes. Since culture of CD1$^-$3$^-$4$^-$8$^-$ thymocytes in the presence of PMA and rIL-2 induced maximal cell proliferation in bulk culture, we investigated whether the same requirements could be appropriate also for inducing clonal growth under limiting dilution (33). The clonal efficiencies obtained were 1–3% in four different experiments. Phenotypic analysis of clones showed that the majority were CD3$^-$TCR$^-$CD56$^+$. Clones were analyzed for their cytolytic activity and lymphokine production. They were found to express a cytolytic pattern similar to that of bulk CD3$^-$ thymocyte populations (see representative CD3$^-$ clones in Tables 1 and 2). All CD3$^-$ thymocyte clones analyzed produced both IFN-γ and TNF-α, but not IL-2. The IFN-γ and TNF-α production by two representative clones are shown in Table 3.

Analysis of Transcripts Coding for TCR and CD3 Chains in CD3$^-$ Cultured Thymocytes. The availability of relatively large numbers of proliferating CD3$^-$ thymocytes prompted us to analyze the expression in these cells of transcripts coding for either CD3γ, δ, ε, and ζ chains or TCR-α, -β, -γ, and

Table 2. *Effect of PHA or mAbs Specific for Triggering Molecules on the Cytolytic Activity of Polyclonal or Clonal CD3⁻ Thymocytes Against P815 Target Cells*

Effector cell	Stimuli added to the cytolytic test: None	PHA	MAR206	CD2-9	MAR206$^+$ CD2-9	JT3A	VD4
Th1*	5‡	55	4	3	3	1	2
Th2	4	65	2	1	2	2	1
Clone Th1	3	70	1	0	2	2	0
Clone Th2	4	35	3	4	3	2	3
Clone AP25.1	6	90	4	5	62	85	3
Clone RS12.5	4	85	47	50	75	2	100

* Th1 and Th2 are representative CD3$^-$ thymocyte populations cultured in vitro for 4 wk. Th clones 1 and 2 are two representative CD3$^-$TCR$^-$CD56$^+$ clones derived from highly purified CD1$^-$3$^-$4$^-$8$^-$ thymocytes. Clones AP25.1 and RS12.5 are two representative PB-derived clones expressing the CD3$^+$TCR-α/β$^+$CD8$^+$ or the CD3$^-$CD16$^+$ surface phenotype, respectively.
‡ Results are expressed as percent specific ^{51}Cr release at an E/T ratio of 1:1. MAR206 (anti-T11-1), CD2-9 (anti-T11-1), JT3A (anti-CD3), and VD4 (anti-CD16) mAbs were used at the final concentration of 20 ng/ml.

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Table 3. *TNF-α and IFN-γ Production by Polyclonal or Clonal CD3⁻ Thymocytes*

CD3⁻TCR⁻ thymocytes*	Stimuli added to the cytolytic test							
	None		PMA		PHA		PHA + PMA	
	TNF-α‡	IFN-γ§	TNF-α	IFN-γ	TNF-α	IFN-γ	TNF-α	IFN-γ
Th1	<10	<1	<10	<1	300	50	1,000	100
Th2	<10	<1	<10	<1	450	80	2,000	300
Clone Th1	<10	<1	<10	<1	250	40	1,500	200
Clone Th2	<10	<1	<10	<1	300	70	1,000	100

* 10^5 thymocyte cultures or clones (CD3⁻CD56⁺) were cultured in triplicate microwells (final volume 200 μl/well) for 36 h at 37°C in the presence of one or another of the stimuli indicated. PHA was used at 0.5 μg/ml and PMA at 0.5 ng/ml as a final dilution. Supernatant fluids were collected and tested for TNF-α and IFN-γ content using an enzyme immunoassay as described in Materials and Methods.
‡ TNF-α is expressed as pg/ml.
§ IFN-γ is expressed as U/ml.

-δ chains. As assessed by Northern blot analysis, in CD3⁻ cultured thymocytes the levels of the transcripts for CD3ε and ζ chains were comparable to those of Jurkat or Peer T cell lines used as positive controls. More importantly, the CD3γ transcript, known to be absent in CD3⁻ peripheral blood NK cells, was also expressed. On the other hand, no expression of CD3δ transcript could be detected (Fig. 5). Regarding the transcripts for TCR genes, a 1.8-kb and a weaker 2.3-kb transcript for TCR-δ gene were detected. Southern blot analysis revealed that the TCR-δ gene retained the germline configuration (Fig. 6). Moreover, a longer exposure of the Northern blot for TCR-δ revealed faint bands of 1.3- and 0.8-kb transcripts. Furthermore, these cells expressed a transcript of 1.6 kb for TCR-γ, and a 1.0-kb (truncated) TCR-β transcript. In addition, no TCR-α transcript was detectable.

It is noteworthy that the 2.3-kb and 1.3-kb transcripts for the TCR-δ gene (47) and the 1.6-kb mRNA of the TCR-γ gene had a size similar to the functional mRNAs found in the TCR-γ/δ⁺ Peer cell line.

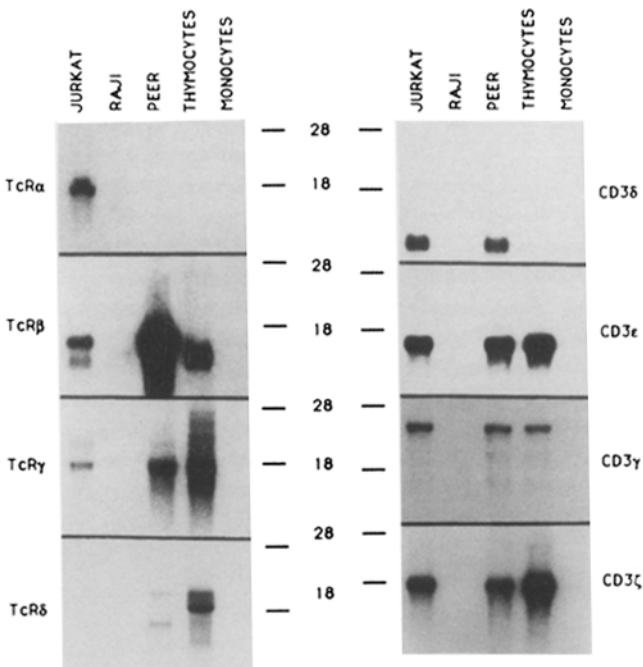

Figure 5. Northern blot analysis of transcripts coding for TCR and CD3 chains in CD3⁻ cultured thymocyte populations. Total RNA (5 μg) isolated from a cultured CD3⁻ thymocyte population, a CD3⁺TCR-α/β Jurkat T cell line, a CD3⁺TCR-γ/δ⁺ Peer T cell line, a Raji B cell line, and peripheral monocytes was treated as described in Materials and Methods and hybridized with the corresponding probes, as indicated. The 28S and 18S rRNA subunits used as markers are indicated.

Discussion

In this study, by applying appropriate culture conditions, we could obtain an extensive cell proliferation starting from CD1⁻3⁻4⁻8⁻ thymocytes. More importantly, the prolifer-

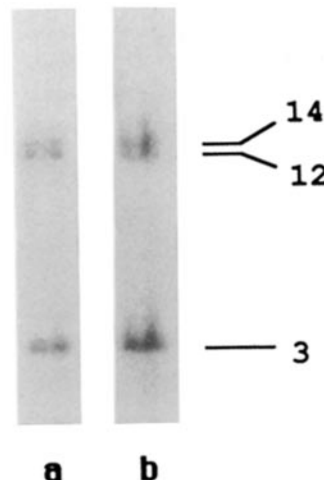

Figure 6. Southern blot analysis for TCR-δ gene rearrangements. 5 μg of DNA was isolated from the B cell line Raji (lane *a*) or a CD3⁻ proliferating thymocyte population (lane *b*). Restriction patterns after DNA digestion with BamHI indicate that the TCR δ chain gene of CD3⁻ thymocytes is in a germline configuration. The sizes of the fragments are indicated.

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ating thymocytes maintained their original $CD3^-TCR^-$ surface phenotype and yielded cell numbers suitable for further phenotypic and functional studies as well as for analysis of transcripts of CD3/TCR genes. Thus, by the analysis of both polyclonal or clonal populations, we could demonstrate that $CD1^-3^-4^-8^-$ thymocytes acquire effector lymphocyte functions, including cytolytic activity and lymphokine production. In addition, they appear to belong to a T cell differentiation stage characterized by the expression of all the transcripts for CD3 chains, with the exception of CD3δ chain.

The minor thymocyte fraction expressing the $CD1^-3^-4^-8^-$ surface phenotype has been extensively studied in vitro to analyze its maturation potential and to achieve a better definition of the stages of human T cell development. Along this line, purified $CD1^-3^-4^-8^-$ thymocytes have been shown to differentiate in culture into $CD3^+$ TCR-α/β^+ as well as $CD3^+$ TCR-γ/δ^+ cells, thus giving rise to the two major subsets of peripheral T lymphocytes (6–22). In the present study we have defined culture conditions that allow proliferation of $CD3^-$ thymocytes without substantial maturation into surface $CD3^+TCR^+$ cells. Thus, whereas addition of rIL-2 alone or in combination with PHA resulted in the preferential expansion of $CD3^+$ cells (mostly TCR-α/β^+), the use of low doses of PMA (0.5 ng/ml) plus rIL-2 (after 24 h) resulted in an extensive proliferation of $CD3^-$ thymocytes. Proliferating cells contained 5–40% $CD3^+$ TCR-γ/δ^+ cells, but no $CD3^+$ TCR-α/β^+ cells. Further removal of $CD3^+$ cells yielded highly purified $CD3^-$ cell populations that proliferated in response to rIL-2 and maintained their $CD3^-$ surface phenotype in culture for up to 8 wk. That the lack of surface expression of CD3/TCR complex did not simply reflect a downregulation induced by PMA is indicated by the following considerations. First, the doses of PMA reported to induce downregulation of CD3/TCR molecules were considerably higher (10 ng/ml) (46). More importantly, $CD3^+$ thymocytes cultured in the presence of PMA and rIL-2 failed to give rise to $CD3^-$ cells, but rather they were represented by surface $CD3^+$ TCR-α/β^+ cells. We can therefore conclude that the doses of PMA used in our study induced (in conjunction with rIL-2) the preferential proliferation of $CD3^-$ thymocytes. In view of the major differences detected in the surface phenotype of thymocyte populations proliferating in response to PMA or PHA, we analyzed the effect of PHA and IL-2 in $CD3^-$ polyclonal or clonal thymocytes that had been pulsed with PMA. Although not shown, no expression of CD3/TCR could be detected, thus indicating that PHA and IL-2 do not represent stimuli capable of inducing maturation of these cells.

Surface phenotype analysis of the cultured $CD3^-$ thymocyte populations showed the expression of CD56 antigen, known to be expressed by peripheral NK cells. Similar to cultured peripheral NK cells, $CD3^-$ thymocytes displayed a strong cytolytic activity against both NK-sensitive and NK-resistant target cells. However, major differences were represented by the lack of expression of NK cell markers such as CD16, CD57, and GL183. In addition, cultured thymocytes expressed cytoplasmic CD3 antigen, as detected by several mAbs known to react with the CD3ϵ chain (44). In no instance under the same experimental conditions did fresh or cultured CD3- peripheral NK cells express cytoplasmic CD3 antigen (Fig. 4). The lack of surface expression of NK-specific triggering molecules was further confirmed by experiments of re-directed killing using anti-CD16 mAbs. Interestingly, PHA augmented cytolysis of P815 target cells, whereas none of the stimulatory mAbs directed to known triggering surface molecules had any effect. These data suggest that PHA may activate the lytic machinery of $CD3^-$ thymocytes by interaction with still undefined triggering surface molecules.

Functional analysis of cultured $CD3^-$ thymocytes revealed, in addition to cytolytic activity, the production of lymphokines such as IFN-γ and TNF-α, but not IL-2. In addition, clonal analysis of $CD3^-$ thymocytes indicated that both functions are expressed by the same cells.

Analysis of the transcripts for CD3 or TCR chains, allowed us to further characterize cultured $CD3^-$ thymocytes and to distinguish these cells from either $CD3^+$ mature T lymphocytes or $CD3^-CD16^+$ peripheral NK cells. Thus, a major finding was the presence of a transcript for CD3γ chain that is known to be absent in $CD3^-CD16^+$ NK cells (36). On the other hand, they differed from $CD3^+$ cells because of the lack of CD3δ transcripts. The presence of transcripts for both TCR-γ and -δ chains (displaying a size similar to the functional mRNA present in TCR-γ/δ^+ cells), together with the lack of TCR-α transcripts, could suggest that these cells may represent a maturational step towards mature $CD3^+$ TCR-γ/δ^+ cells. However, this concept is not further supported by the finding that TCR-δ gene is in a germline configuration (Fig. 6). Our data provide further evidence that genes coding for CD3ϵ, γ, and δ chains (although they are all closely located on chromosome 11) can be expressed asynchronally in different cell types and/or at different stages of T cell maturation.

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