In Vitro Proliferation and Cloning of CD3$^-$CD16$^+$ Cells from Human Thymocyte Precursors

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Summary

Purified CD3$^-$4$^-$ thymocytes were obtained by depletion of CD3$^+$ and CD4$^+$ cells from fresh thymocyte suspensions. 5–15% of these cells were found to express CD16 antigen, while other natural killer (NK) cell markers were virtually absent. Double fluorescence analysis revealed that 20–40% of thymic CD16$^+$ cells coexpressed CD1, while approximately half were cyCD3$^+$. When cultured in the presence of peripheral blood lymphocytes and H9 leukemia cell line as a source of irradiated feeder cells and interleukin 2 (IL-2), CD3$^-$4$^-$ thymocytes underwent extensive proliferation. In addition, after 1–2 wk of culture, 30–50% of these cells were found to express CD16 surface antigen. Cloning under limiting dilution conditions of either CD3$^-$4$^-$ or CD3$^-$4$^-$16$^-$ thymocytes in the presence of irradiated H9 cells resulted in large proportions (~50%) of CD16$^+$ clones. On the basis of the expression of surface CD16 and/or cyCD3 antigen, clones could be grouped in the following subsets: CD16$^+$cyCD3$^+$; CD16$^+$cyCD3$^-$; CD16$^-$cyCD3$^+$; and CD16$^-$cyCD3$^-$. All clones expressed CD56 surface antigen, displayed a strong cytolytic activity against NK sensitive (K562) and NK-resistant (M14) target cells, and produced IFN-γ and tumor necrosis factor, but not IL-2. Similar to peripheral NK cells, thymic CD16$^+$ cells expressed transcripts for CD16 and for CD3 ϵ (Biassoni, R., S. Ferrini, I. Prigione, A. Moretta, and E.O. Long. 1988. *J. Immunol.* 140:1685.) and ζ chains (Anderson, P., M. Caligiuri, J. Ritz, and S.F. Schlossman. 1989. *Nature [Lond.].* 341:159). Therefore, it appears that cells that are phenotypically and functionally similar to CD3$^-$CD16$^+$ NK cells may arise from immature thymocytes.

It is a common notion that the thymus represents the primary site of T cell maturation while the CD3$^-$CD16$^+$ NK cells derive from bone marrow precursors and do not require the thymic environment for their maturation (1, 2). Recent reports have suggested that the CD7$^+$1$^-$3$^-$4$^-$8$^-$ thymocyte subset (containing the most immature thymocytes) may not be uniquely composed of cells already committed to the T cell lineage. For example, under appropriate culture conditions, CD7$^+$1$^-$3$^-$4$^-$8$^-$ thymocytes were shown to be capable of maturation into hematopoietic cells (1, 3). In the present study, we demonstrate that CD16$^+$ cells coexpressing cytoplasmic CD3 (cyCD3) or surface CD1 antigen (typical thymocyte markers) are found within the CD7$^+$ CD3$^-$4$^-$ thymocyte population. Moreover, under appropriate culture conditions, a large fraction of clones derived from CD3$^-$4$^-$ fresh thymocytes were characterized by the CD3$^-$CD16$^+$CD56$^+$ surface phenotype and a strong non-MHC-restricted cytolytic activity.

Materials and Methods

Isolation of CD3$^-$4$^-$ and CD3$^-$CD4$^-$CD16$^-$ Human Thymocytes. Normal human thymocytes were obtained from thymus fragments removed during cardiac surgery of patients (2 mo to 4 yr old), as described (4, 5). CD3$^-$4$^-$ thymocyte populations were obtained by depletion of CD3$^+$4$^+$ cells by the use of immunocoated ox RBC and immunocoated magnetic beads as described (4). Both cell purification steps were performed at 4°C. Cells recovered were >99.9% CD3$^-$4$^-$. In some experiments, CD16$^+$ cells were further removed by using immunomagnetic beads. The resulting cells were >99.9% CD3$^-$4$^-$16$^-$.

Generation of Cell Lines Enriched in CD16$^+$ Cells from Human Thymocytes. Highly purified CD3$^-$4$^-$ or CD3$^-$4$^-$16$^-$ fresh

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thymocytes were cultured in 0.2 ml of RPMI 1640 supplemented with 10% FCS and 100 U/ml of rIL-2 (Cetus Corp., Emeryville, CA) in 96-well U-bottomed plates (Grenier, Nurtingen, FRG) containing 10^5 irradiated PBL either in the presence or in the absence of 2×10^4 irradiated H9 or CEMK human T cell lines. In some experiments, PHA (0.5%) or PMA (0.5 ng/ml) were added to the culture. Cell growth was evident after 5–7 d of culture. High percentages (40–60%) of $CD16^+$ cells were found only in cell cultures in which H9 tumor cells were present. Cells proliferated in the presence of rIL-2 for at least 4–6 wk.

Double Fluorescence Cytofluorometric Analysis. The techniques used have been described in detail elsewhere (6). The mAbs used included KD1 (anti-CD16, γ2A), OKT6 (anti-CD1, γ1), and Leu-4 (anti-CD3, γ1). An anti-γ2A FITC-conjugated goat anti-mouse Ig and an anti-γ1 PE-conjugated goat anti-mouse Ig (Southern Biotechnology, Birmingham, AL) were used as second reagent. Analysis of the expression of cytoplasmic CD3 antigen was performed as described in detail elsewhere (4). Samples were run on a FACStar® (Becton Dickinson & Co., Mountain View, CA), and results are expressed as log green fluorescence intensity (*x*-axis) vs. log red fluorescence intensity (*y*-axis).

Cloning of $CD3^-4^-$ or $CD3^-4^-16^-$ Thymocytes. Cloning of highly purified $CD3^-4^-$ or $CD3^-4^-16^-$ thymocytes was performed under limiting dilution conditions as previously described (7) in RPMI 1640 (Biochron, Berlin, FRG) plus 10% FCS supplemented with 100 U/ml of rIL-2. Microcultures contained, as feeder cells, 10^5 irradiated PBL either in the presence or in the absence of irradiated H9 tumor cell line (2×10^4 cells/well). Evident cell growth could be detected after 12–18 d of culture. Clones were further maintained in culture for 2–4 wk.

Functional Analysis. Cytolytic activity of thymocyte clones against NK-sensitive (K562) and NK-resistant (M14) target cells was tested in a 4-h ^{51}Cr release assay, as previously described in detail (6). In the redirected killing assay, we used P815 ($Fc\gamma R^+$) as target cells and anti-CD3 (JT3A) or anti-CD16 (KD1) mAbs and PHA, as described (4, 6). The production of TNF-α, IFN-γ, and IL-2 by thymocyte clones was tested by enzyme immunoassays, as previously described (4).

Northern Blot and Slot Blot Analysis. RNA for Northern blot analysis was prepared, size fractionated by electrophoresis in agarose gels containing formaldehyde, blotted, and hybridized as described (8). Cellular cytoplasmic RNA was prepared starting from 10^6 cells. Cells were washed once in cold PBS and resuspended in 45 μl of 10 mM cold Tris, pH 7.5, 1 mM EDTA, and then lysed by adding 5 μl of 5% NP-40, and mixed on ice for 5 min. The nuclei were then pelleted, and 50 μl of a freshly prepared solution composed of 30 μl of 20× SSC and 20 μl of 37% (wt/vol) formaldehyde were added to the supernatant. The mixture was incubated at 60°C for 15 min, and 10^5 cell aliquots were spotted using a 15× SSC solution to a charged nylon membrane (Gene Screen Plus; New England Nuclear, Boston, MA) using a microfiltration apparatus (Bio Dot SF; BioRad Chemical Division, Richmond, CA). The nylon membranes were UV exposed (Stratalinker 1800; Stratagene, La Jolla, CA). The slot-blots were prehybridized and hybridized as described (8). The probes used were a 0.7-kb EcoRI fragment of CD3-γ cDNA clone pJ6T3γ-2 (9), a 0.9-kb OxaNI/BamHI fragment of the CD3-ε cDNA clone pDJ4 (10), a 0.8-kb PstI fragment of CD3-δ cDNA (derived from clone 25 kindly provided by Dr. Tak Mak, Ontario Cancer Institute, Toronto, Canada), a 1.7-kb XbaI/EcoRI fragment of the CD3-ζ cDNA (11) and a 1.0-kb EcoRI/HindIII fragment of the CD16 cDNA FcγIII-2/NA-2 (12). Blots were exposed to X-ray film using intensifying screens at −70°C.

Results and Discussion

Presence of $CD16^+$ Cells in the $CD3^-4^-$ Thymocyte Population. Immature thymocytes were obtained by depletion of $CD3^+$ and $CD4^+$ cells from fresh thymocyte suspensions. The resulting cell populations represented 1–5% of the total thymocytes and were composed of $CD3^-4^-8^-$ cells. In addition, these cells expressed CD7 (90–100%), CD1 (20–30%), CD2 (60–80%), CD5 (50–70%), HLA class I (100%), and HLA class II (5–15%) antigens. Analysis of NK cell surface markers indicated that 5–15% of these cells expressed surface CD16 antigen, whereas other NK cell markers (including CD57, GL183, and EB6) (6) were absent or present in very low amounts ($CD56^+$ cells were 0–5%). Finally, cytoplasmic staining with anti-CD3 mAbs (specific for CD3-ε chain) (13) revealed that 40–60% of these cells were $cyCD3^+$.

Thymic $CD16^+$ Cells Can Coexpress CD1 or cyCD3 Antigens. We next analyzed whether the $CD16^+$ cells present in $CD3^-4^-$ thymocytes coexpressed markers of immature T cells such as surface CD1 and cyCD3 antigens. Double fluorescence and FACS® analysis revealed that 20–40% of $CD16^+$ cells coexpressed surface CD1 (see Fig. 1 *A*). Moreover, ~50% of $CD16^+$ cells expressed cyCD3 antigen (Fig. 1 *B*). It should be noted that, in the same series of experiments, no $cyCD3^+$ cells could be detected in peripheral blood–derived $CD3^-CD16^+$ NK cells used as control (Fig. 1 *C*). Thus, the coexpression of CD1 and/or cyCD3 antigen indicates that (at least some) $CD16^+$ NK cells present in fresh thymus are phenotypically different from typical peripheral blood–derived NK cells, and suggests that they may belong to immature thymocytes.

Growth of $CD16^+$ Cells from $CD3^-4^-$ Thymocytes Is Induced in the Presence of H9 Cell Line. We further investigated whether $CD16^+$ thymic cells could be expanded in vitro under suitable culture conditions. To this end, we used as starting population $CD3^-4^-$ thymocytes and tested their ability to respond to different stimuli including PMA, PHA, and IL-2. In addition, $CD3^-4^-$ thymocytes were cultured in the presence of different (irradiated) feeder cells, including normal PBL or tumor cell lines (alone or in various combinations). Strong proliferative responses were observed when $CD3^-4^-$ thymocytes were cultured in the presence of an exogenous source of IL-2 and a mixture of irradiated H9 tumor cells and PBL as feeder cells. Under these culture conditions, the cell number recovered after 3–4 wk was up to 100-fold higher than the initial cell input. It is of note that already after 1–2 wk in culture, cells were homogeneously $CD56^+$, and 30–50% expressed CD16 surface antigen (data not shown). Moreover, double fluorescence analysis revealed that, at day 10 of culture, 10–20% of $CD16^+$ cells coexpressed cytoplasmic CD3 antigen, while no $CD1^+$ cells could be detected (data not shown). It should be noted that in all culture conditions in which feeder cells did not include H9 cells, $CD16^+$ cells were virtually absent.

Growth of $CD16^+$ Cells from $CD3^-4^-16^-$ Thymocyte Precursors. We next investigated whether $CD16^+$ cells could be also obtained starting from $CD3^-4^-$ thymocytes that

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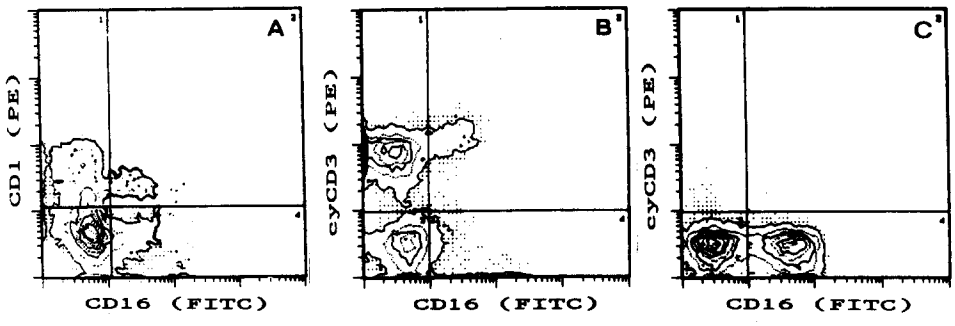

Figure 1. Expression of surface CD1 or cytoplasmic CD3 antigens by thymic CD16$^+$ cells. In these experiments, the surface expression of CD16 antigen has been evaluated in combination with surface CD1 (*A*) or cytoplasmic CD3 antigen (*B*) in fresh CD3$^-$CD4$^-$ thymocytes. In *C*, peripheral blood CD3$^-$ cells are shown for comparison. Cells were stained with KD1 (anti-CD16) followed by anti-γ2A FITC-conjugated goat anti-mouse Ig, and with either OKT6 (anti-CD1) or Leu-4 (anti-CD3) mAb followed by anti-γ1 PE-conjugated goat anti-mouse Ig. For cytoplasmic staining (*B* and *C*), cells were fixed and permeabilized before staining with anti-CD3 mAb, as described (4). The contour plot was divided into quadrants representing unstained cells (*lower left*), cells with only red fluorescence (*upper left*), cells with both red and green fluorescence (*upper right*), and cells with only green fluorescence (*lower right*). Samples were analyzed on a FACStar®, and results are expressed as log green fluorescence intensity vs. log red fluorescence.

had been further depleted of CD16$^+$ cells. The starting population did not contain detectable CD16$^+$ cells, as assessed by FACS® analysis. However, after 1–2 wk of culture, under the culture conditions indicated above, 30–40% of cells expressed surface CD16 molecules (data not shown).

Cloning of CD16$^+$ Cells from Thymus. Freshly isolated CD3$^-$CD4$^-$ thymocytes were cloned under limiting dilution in the presence of rIL-2 and irradiated PBL, either in the presence or in the absence of irradiated H9 cells. The maximal clonal efficiency (~5% in several experiments), and optimal cell proliferation was detectable when irradiated H9 cells were added to the culture. More importantly, in all six experiments performed in the presence of H9 cells, >50% of clones were CD3$^-$CD16$^+$, whereas CD3$^+$ clones were <10% and the remaining (30–40%) were CD3$^-$CD16$^-$. In contrast, low proportions (0–5%) of CD16$^+$ clones were obtained from CD3$^-$4$^-$ thymocytes cultured in the absence of H9 cells (under these conditions, >80% of clones were CD3$^-$CD16$^-$). Importantly, also when CD16$^+$ cells had been removed from the starting CD3$^-$4$^-$ thymocyte populations, approximately half of the resulting clones were CD3$^-$CD16$^+$. These data provide further evidence that proliferating CD16$^+$ thymic cells may derive from CD16$^-$ thymocyte precursors. In these experiments, >300 clones have been analyzed for CD3 and CD16 surface antigen expression. 28 of such clones have been further characterized for the expression of cyCD3 and surface CD56 antigens.

On the basis of the expression of surface CD16 and/or of cytoplasmic CD3 antigens, clones derived from CD3$^-$4$^-$ thymocytes (depleted or not of CD16$^+$ cells) can be grouped in four different subsets (Fig. 2). Thus, ~25% of all clones coexpress surface CD16 and cyCD3 antigen (CD16$^+$cyCD3$^+$). The remaining CD16$^+$ clones lack detectable cyCD3 antigen (CD16$^+$cyCD3$^-$). On the other hand, among CD16$^-$ clones, those displaying cyCD3 represent 10% of all clones (CD16$^-$cyCD3$^+$), while the remaining are "double negative" (CD16$^-$cyCD3$^-$). It should be noted that, independent on their surface phenotype, all clones expressed high levels of CD56 antigen (Fig. 2).

Thymic CD16$^+$ Cells Express Transcripts for CD16 and for CD3 ε and ζ Chains. We next analyzed the expression of CD3 and CD16 transcripts both in CD3$^-$16$^+$ and CD3$^-$16$^-$ cultured thymocyte populations (these populations contained 10% and 20%, respectively, of cyCD3$^+$ cells). CD3$^-$

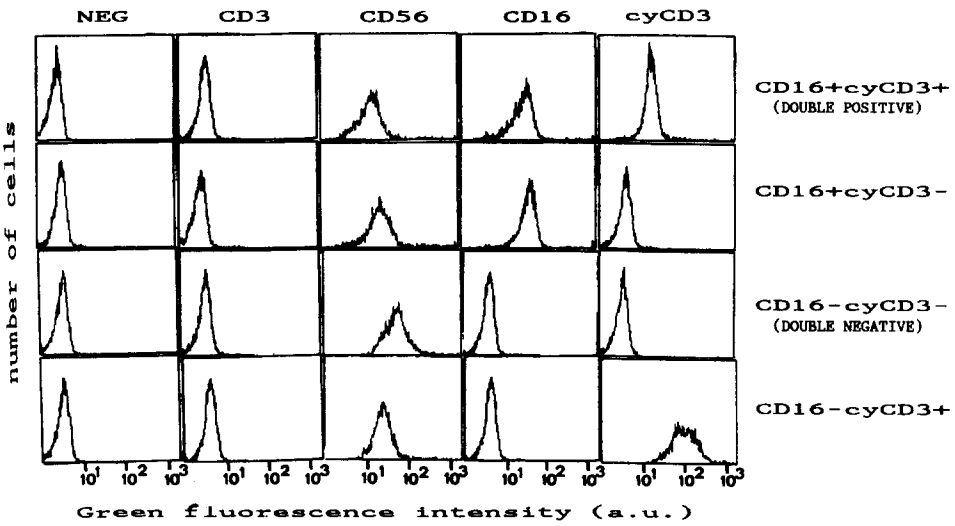

Figure 2. Surface phenotype of clones derived from CD3$^-$4$^-$ thymocytes. Cells were stained with either one of the following mAbs: Leu-4 (anti-CD3), Leu-19 (anti-CD56) and KD1 (anti-CD16). Fluorescein-conjugated goat anti-mouse Ig was used as a second reagent. For cytoplasmic staining, cells were fixed and permeabilized before incubation with anti-CD3 mAb, as described (4). Note that, on the basis of surface CD16 and cytoplasmic CD3 antigen expression, four distinct types of clones can be distinguished. The four clones shown are RO.3/25 (double positive), RO.6/50 (CD16$^+$cyCD3$^-$), RO.12/50 (double negative), and RO.6/100 (CD16$^-$cyCD3$^+$). They are representative of 28 clones that have been analyzed for all the phenotypic markers indicated.

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16^+ thymocyte populations expressed the transcripts for CD16 or CD3 ϵ and ζ, but not for CD3-γ and -δ chains (14). On the other hand, the $CD3^-16^-$ populations expressed a similar pattern of CD3 transcripts but did not express the CD16 mRNA (Fig. 3 *A*). The $CD3^-16^+$ thymocyte clone (termed 12H7), (homogeneously $cyCD3^+$) was further analyzed by slot-blot analysis. A pattern similar to that of polyclonal $CD3^-16^+$ populations was observed for the expression of transcripts for both CD16 and CD3 chains (Fig. 3 *B*).

Functional Analysis of Thymic $CD16^+$ Cells. Analysis of cytolytic functions indicated that all clones derived from $CD3^-4^-$ thymocytes efficiently lysed both NK-sensitive (K562) and NK-resistant (M14) tumor target cells (Table 1). In a redirected killing assay, using P815 target cells, both $CD16^+cyCD3^+$ and $CD16^+cyCD3^-$ clones (but not $CD16^-$ clones) displayed a strong cytolytic activity in the presence of anti-CD16 mAbs (Table 1). This indicates that, similar to peripheral $CD3^-CD16^+$ NK cells, $CD16^+$ thymic cells also have a functional CD16-mediated activation pathway. Further analysis of lymphokine production in response to either PHA alone or a combination of PHA and

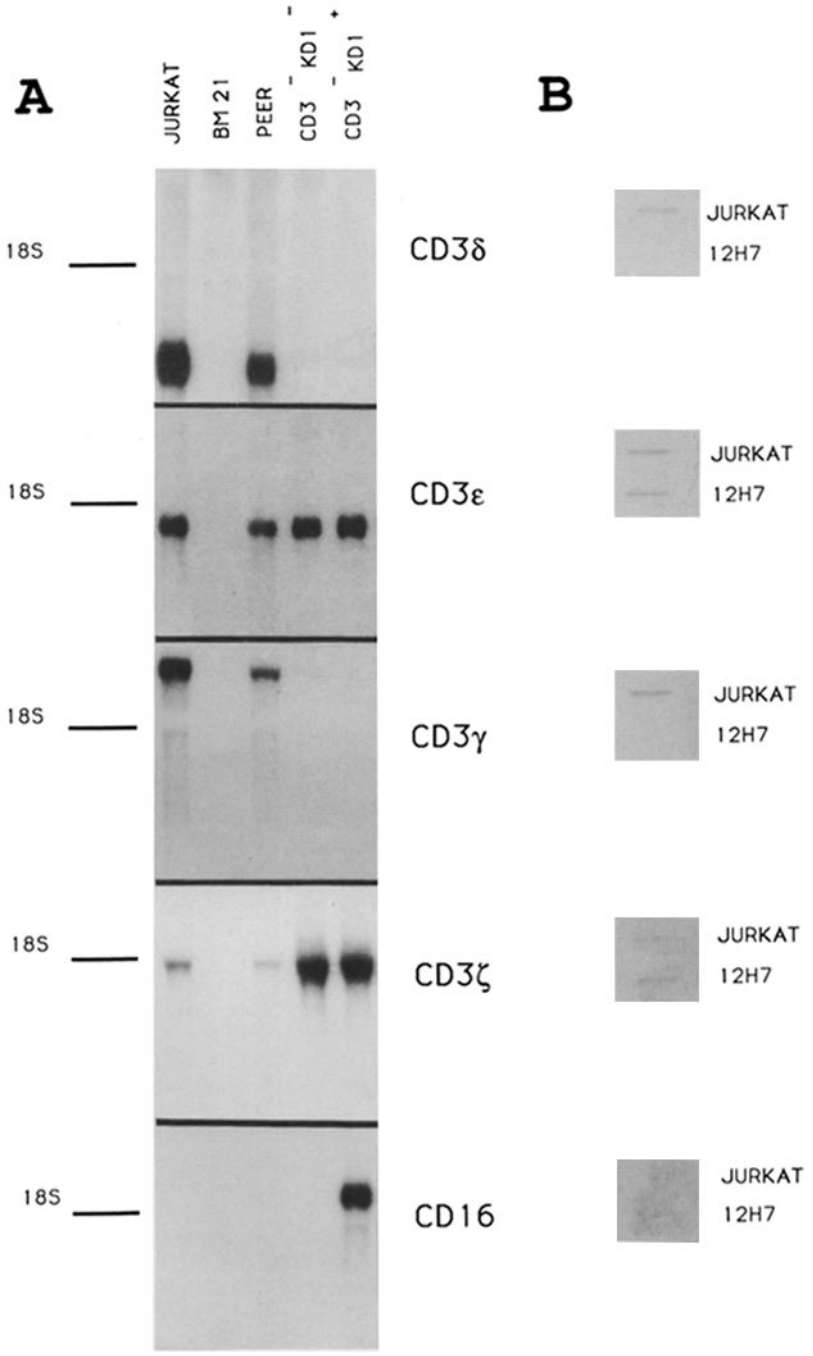

Figure 3. Transcripts coding for CD3 and CD16 molecules in cultured $CD3^-$ thymocytes populations. In *A*, total RNA (5 μg) was isolated from the following cell populations: $CD3^-CD16^+$ or $CD3^-CD16^-$ thymocytes; the TCR-α/β^+ Jurkat T cell line; the TCR-γ/δ^+ Peer T cell line; and the BM21 lymphoblastoid B cell line. Cells were treated as described in Materials and Methods and hybridized with the indicated probes. The 18S rRNA subunit, used as marker, is indicated. In *B*, aliquots of cytoplasmic RNA corresponding to 10^5 cells were blotted on Gene Screen Plus membranes. 12H7 is a $CD3^-CD16^+$ $cyCD3^+$ thymic clone.

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Table 1. *Cytolytic Activity of Clones Derived from CD3⁻4⁻ Fresh Thomocytes*

Clone* (phenotype)	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	P815 at E/T ratio of 1:1; Stimuli added to the cytolytic test‡: None	PHA	Anti-CD16 mAb	Anti-CD3 mAb
RO53 cyCD3⁻CD16⁺	100§	90	70	90	40	20	4	42	50	0
AL4/50 cyCD3⁻CD16⁻	95	70	50	100	70	50	35	60	30	1
RO 1/25 cyCD3⁺CD16⁺	100	90	60	100	80	40	0	46	62	2
AL 2/25 cyCD3⁺CD16⁻	100	90	70	90	70	50	4	43	2	0

* Clones are representative of four different subsets, as defined by the expression of surface CD16 and cytoplasmic CD3 antigen.
‡ In the redirected killing assay, using the FcγR⁺ P815 murine mastocitoma cell line, the KD1 (anti-CD16) and the JT3A (anti-CD3) mAbs were added to the cytolytic test at the final concentration of 20 ng/ml. PHA was used at 1 μg/ml.
§ Results are expressed as percent of specific ^{51}Cr release.

PMA showed that CD16⁺ thymic clones produced IFN-γ and TNF-α, but not IL-2 (data not shown).

In conclusion, our data demonstrate that CD16⁺ cells are present in human thymus. At least a fraction of these cells belong to a novel CD3⁻ thymocyte subset also expressing cyCD3 molecules. It is possible that these cells represent the thymic precursors of the minor peripheral blood CD3⁺ T cell subset expressing CD16 antigen (2). On the other hand, they may represent cell precursors capable of differentiation into either T or CD3⁻CD16⁺ cells. In addition, in vitro experiments demonstrated that CD3⁻16⁺ proliferating cells (phenotypically similar to peripheral blood–derived NK cells) could be obtained from CD3⁻4⁻ thymocyte precursors. Moreover, cloning experiments showed that the precursors of CD16⁺ cells are present in the CD16⁻ fraction of CD3⁻4⁻ thymocytes. Whether these precursors require the thymus environment for their normal differentiation in vivo, or rather are unable to undergo substantial differentiation towards CD16⁺ cells within the thymus, remains to be defined. Previous studies indicated that culture of different thymocyte populations in the presence of IL-2 resulted in the generation of effector cells displaying non-MHC-restricted cytolytic activity. However, in most instances, these cells were CD3⁺ and expressed a TCR-α/β or -γ/δ (15–17).

Interestingly, culture of CD3⁻4⁻ thymocytes in the absence of H9 cells or, as shown previously, in the presence of PMA or PHA, resulted in the growth of cells with different phenotypic characteristics (including CD3⁻16⁻, CD3⁺ TCR-α/β⁺, or CD3⁺TCR-γ/δ⁺ cells) (1, 4). These data suggest that different stimuli may induce maturation of thymic precursors towards different lineages. However, they do not allow to define whether CD3⁻CD16⁺ cells and CD3⁺ TCR⁺ mature T lymphocytes may derive, at least in part, from the same or different cell precursors.

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