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Delineation of human thymocytes with or without functional potential by CD1-specific antibodies

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Abstract

Hematopoietic precursors lacking T cell antigen receptors (TCR – CD3$^-$) and CD4 and CD8 surface markers (i.e. double-negative thymocytes) give rise to functionally mature T lymphocytes. Yet their major progeny are immunologically unresponsive thymocytes in spite of having acquired TCR – CD3 and CD4 – CD8. Because only mature thymocytes migrate to peripheral lymphoid organs and most thymocytes die *in situ*, the knowledge of the events associated with functional maturation in the double-negative thymocyte progeny is a fundamental question in T cell development. We reasoned that a clue to trace the fate of early human thymocytes may perhaps come from the study of the developmental acquisition of CD1 antigen, currently used to define better the functionally inert CD4$^+$8$^+$ (double-positive) stage and absent in mature, medullary thymocytes and peripheral T cells. By using antibodies specific for CD1 (HTA 1/T6) we show here that a large fraction of double-negative thymocytes also express CD1. CD1$^+$3$^-$, CD1$^+$3$^+$, CD1$^-$3$^+$, and CD1$^-$3$^-$ subsets all exist. The CD1$^+$3$^-$ subset generates CD1$^+$3$^-$4$^-$8$^+$ precursors of CD1$^+$ double-positive cells. A large portion of the CD1$^+$3$^+$ subset bears TCR$\gamma\delta$ – CD3 complexes. The CD1$^-$ subsets are responsive in assays of function, in which they can be stimulated to use the interleukin 2 pathway of proliferation and to mediate cytotoxicity. In contrast, all CD1$^+$ thymocytes behave as functionally inert cells. Thus, the CD1 surface marker delineates human thymocyte precursors and their products which lack, or possess, functional potential *in vitro*, on both $\alpha\beta$ and $\gamma\delta$ lineages.

Introduction

Triggering of resting peripheral T cells occurs through occupancy of the antigen receptors, $\alpha\beta$ or $\gamma\delta$ heterodimers associated with CD3 (TCR – CD3 complex) and CD4 or CD8 co-receptors. TCR ligands stimulate T cell proliferation via the antigen-non-specific, interleukin 2 receptor (IL2-R)/IL2 growth pathway. Such activated, mature T cells can mediate helper or cytotoxic effector functions in an antigen-specific fashion upon a later occupancy of the TCR (1).

Acquisition of TCR – CD3, CD4, and CD8, as well as T cell associated function programs, occurs during T cell development in the thymus (2,3). Three sub-populations of thymocytes have been defined on the basis of their antigen receptor phenotype and their functional competence: early precursors and either immunologically unresponsive (inert) or mature thymocytes (4,5). The early thymocytes, which lack surface expression of TCR – CD3, CD4, and CD8 receptors, give rise to some phenotypically and functionally mature thymocytes which can migrate to the periphery (6). Yet their major progeny are functionally unresponsive thymocytes, despite the fact that about half of them co-express TCR – CD3, CD4, and CD8 on the membrane (5 – 9). The events signaling the functional fate of early thymocyte progeny are thus a fundamental question in T cell development because inert thymocytes do not migrate to the periphery (7).

The most striking finding of the functional studies performed with thymocytes is their lack of correlation with antigen receptor expression (9 – 11). We reasoned that a clue to trace the functional fate of early thymocytes may perhaps come from developmental analyses of a third type of cell-surface marker that, unlike TCR – CD3 complex or CD4 and CD8, were selectively expressed on inert thymocytes. It has been previously shown that CD1 (HTA 1/T6) occurs on functionally inert human CD4$^+$8$^+$ double-positive thymocytes but not in immunocompetent thymocytes or peripheral T cells (4,12 – 14). We have therefore studied the expression of CD1 on human early

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thymocytes using CD1-specific antibodies. We show here that subsets of CD3⁻ and CD3+ double-negative thymocytes also express CD1 antigen on their surfaces. CD1⁻ subsets contain mature αβ and γδ T cells, whereas CD1+ subsets behave like inert thymocytes belonging to αβ and γδ lineages.

Methods

Isolation of cells

Single cell suspensions were obtained from normal pediatric thymus that had been removed during corrective cardiac surgery of patients 2 weeks – 5 years old. Viable thymocytes were isolated by Ficoll – Hypaque density centrifugation (15) Double-negative thymocytes were isolated by immunoselection using cytotoxic antibodies to CD4 (HP2/6) and CD8 (B9.4) plus a 1:4 dilution of selected rabbit complement (Beringwerke, Marburg, FRG) (3,15). The surviving cells (99% CD4⁻8⁻) were incubated with either anti-CD1 (OKT6, γ_1 antibody) (CD1+3⁻4⁻8⁻ cells), anti-CD3 (T3b, γ_{2a}) antibody (CD1⁻3+4⁻8⁻ cells), or both (CD1+3+4⁻8⁻ and CD1⁻3⁻4⁻8⁻ cells), and the respective population positively or negatively selected after incubation with affinity-purified rabbit anti-mouse immunoglobulin for 1 h at 4°C. The CD1+3+ subset behaves as total CD1+ cells For accuracy in some experiments, including ones in this report, the minor CD1+3+ subset within the CD1+ or CD3+ selected population was depleted by incubation with either cytotoxic CD3 (T3b) or CD1 (Na1/34) antibodies plus complement. CD1⁻3⁻4⁻8⁻ thymocytes express CD7 and CD2 T cell differentiation antigens *in vivo* (15 and data not shown). CD3⁻4⁻8+ thymocytes were isolated after two rounds of cytotoxic elimination with monoclonal antibody (mAb) CD3 (T3b) and CD4 (OKT4A) plus complement followed by positive selection with mAb CD8 (B9 4, γ_{2b}) in flasks coated with goat anti-mouse γ_{2b} antibodies (Southern Biotechnology, Birmingham, AL, USA). Most recovered cells expressed CD1. All purified populations were >98% pure by FACS analyses An intracardiac blood sample was obtained before establishment of the extracorporeal bypass circulation. Mononuclear cells were separated by Ficoll – Hypaque density centrifugation.

mAbs

CD1 [OKT6, γ_1, American Type Culture Collection, Rockville, MD, USA, Na1/34 (12), γ_{2a}, or Coulter Clone T6, Coulter], CD2 [MAR 206.1 (16), γ_1], CD3 [T3b (17), γ_{2a}, and Coulter Clone T3], CD4 [HP2/6 γ_2 (16), Leu3a, Becton-Dickinson, γ_1, or OKT4A, Ortho, Raritan, NJ, USA, γ_{2a}], CD7 [3A1, American Type Culture Collection, Rockville, MD], CD8 [B9.4 (18), γ_{2b}], TCRαβ [WT31 (19), γ_1], TCRγδ [TCRδ1 (20), γ_1], TCR-$V_{\delta 1}$ [TCSδ1, T-cell Sciences, Boston, MA, USA], TCR-$V_{\gamma 9}$ [Ti-γA (21), γ_{2a}], and CD25 [Tac chain of the IL2R, H108 (16)] were used

Quantitative flow cytometry

Two-color immunofluorescence studies were carried out as previously described (22). Cells were stained in sequential steps.

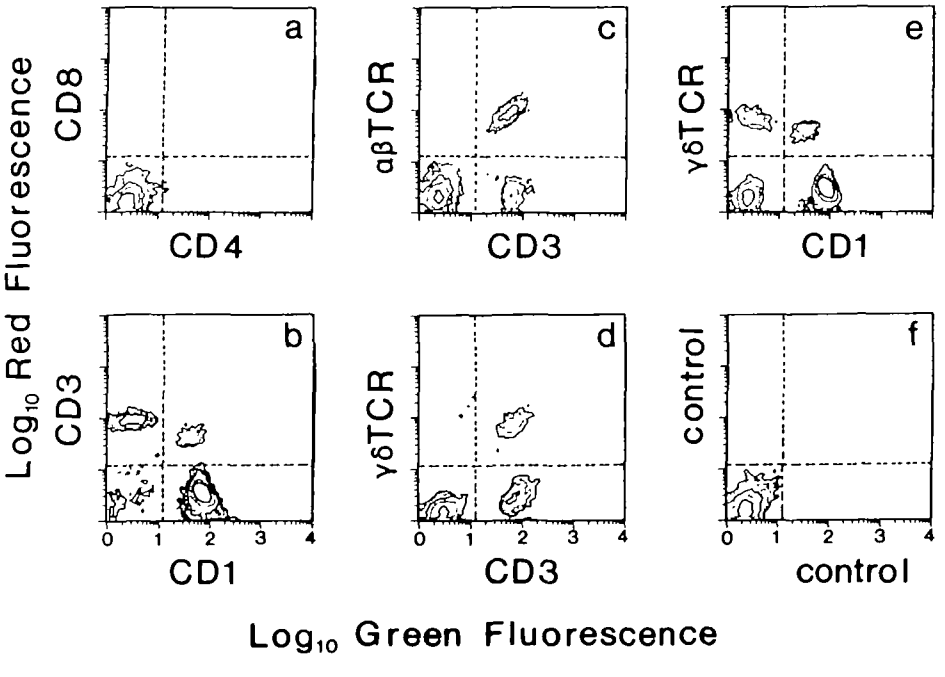

Fig. 1. Two-color immunofluorescence FC analyses for co-ordinate expression of CD1, CD3, αβ, and γδ TCRs in CD4⁻8⁻ thymocytes. Double-negative thymocytes were isolated using cytotoxic antibodies to CD4 (HP2/6) and CD8 (B9.4) plus rabbit complement (Beringwerke, Marburg, FRG), and two-color FC studies were carried out as described in Methods Briefly, thymocytes were stained by indirect immunofluorescence Reagents included mAbs of predefined isotype CD1 [(b) OKT6, γ1, or (e) Na1/34, γ_{2a}], CD3 [T3b, γ_{2a}], CD4 [Leu3a, γ_1], CD8 [B9 4, γ_{2b}], αβ TCR [WT31, γ_1], and γδ TCR [TCRδ1, γ_1], followed by either FITC (green)-conjugated or PE (red)-labeled goat anti-mouse isotype-specific second step reagents. Preliminary studies showed that each anti-isotype antibody did react with one but not the other primary antibody Experiments using directly labeled antibodies yield similar results (Table 1). FC analyses were performed in a FACScan analyzer equipped with a four decade log amplifier and 10^4 viable cells were accumulated in each contour plot Background values are indicated by dotted lines. Percentages of positive cells in each quadrant are indicated in the text for clarity Results are representative of six independent experiments.

Reagents included mAbs of predefined isotype, as indicated, followed by either fluorescein isothiocyanate (FITC; green)-conjugated or phycoerythrin (PE; red)-labeled goat anti-mouse isotype-specific second step reagents (Southern Biotechnology). Background values are indicated by dotted lines and were obtained using isotype-matched, irrelevant antibodies and their respective second antibodies. These procedures rule out any artifice due to carry over of mAb from the cytotoxic incubation (23). Percentages of positive cells in each quadrant are indicated in the text for clarity. Two-colour flow fluorocytometry (FC) analyses were performed in a FACScan analyzer interfaced to a Consort 30 (Beckton-Dickinson).

Assays for differentiation

CD1+3-4-8- or CD3-4-8+ cells were isolated from thymocyte populations as indicated above. They were stained, either fresh or after their culture in the presence of 50 U/ml rIL2 (3) for 36 h, with mAbs specific for the indicated combinations of T cell differentiation antigens and analyzed for two color immunofluorescence and FC.

Assays for function

(i) To quantitate IL2 secretion, 2×10^6 cells in each double-negative subset were stimulated with 5 μg/ml phytohemagglutinin (PHA) and 25 U rIL1β (Hoffmann-LaRoche, Inc , Nutley, NJ, USA) for 36 h. Supernatants were collected and IL2 activity measured using the IL2-dependent CTLL2 line in triplicate cultures, and the sensitive hexoseaminidase read-out assay (15). Units were calculated as indicated (15) and compared to a rIL2 control (Hoffmann-LaRoche, no. R023-6019). (ii) The expression of the p55 chain of the IL2R (Tac or CD25) was measured either on freshly isolated thymocytes or after their stimulation (2×10^6 cells) with 5 μg PHA for 24 h, using biotin-conjugated anti-Tac (H108) antibody followed by PE-labeled streptavidin (Becton-Dickinson). (iii) Proliferative cell responses of 2×10^5 cells cultured with either 50 U rIL2 or 5 μg/ml PHA and 25 U rIL1β were carried out at 37°C for 80 h in triplicate cultures in 96-well microtiter plates. [^{3}H]Methylthymidine uptake (1 μCi/well) was measured during the last 11 h of culture (15) (iv) For quantitation of cytotoxic activity, effector cells were cultured for 4 days at 10^6 cells/ml in 50 U/ml rIL2. Their cytotoxic activity was assessed against ^{51}Cr-labeled P-815 cells either uncoated or coated (24) with T3b (CD3)-, WT31 (TCRαβ)-, or TCRδ1 (TCRγδ)-specific antibodies. MAR206.1 antibody (CD2) gave similar results to those obtained with uncoated target cells (not shown) The 4 h ^{51}Cr-release assay was performed as described elsewhere (24). The results are expressed as the mean ^{51}Cr specific release of triplicate cultures at 25/1 effector-to-target ratio. Spontaneous release never exceeded 18%.

Table 1. Detection of CD1+ TCRγδ – CD3+ cells in the thymus but not in peripheral blood

Cells	CD1+	CD3+	CD1+ CD3+	CD1+ TCRγδ+	CD1+ $TCRV_{\gamma 9}$+	CD1+ $TCRV_{\delta 1}$+	CD3+ $TCRV_{\gamma 9}$+	$TCRV_{\delta 1}$+ CD3+	CD1+ cells in the TCRγδ+ subset
Total thymocytes	83	69	47	<1	<1	<1	<1	1	ND
Double-negative thymocytes	61	38	17	13	2	9	5	23	44
Peripheral blood lymphocytes	<1	73	<1	<1	<1	<1	4.7	1	<1

The indicated cell populations were isolated and two-color FC studies were carried out as described in Fig 1 Here direct immunofluorescence was used. Antibodies in the upper row were conjugated with PE (red) and those in the lower row were FITC (green). The proportion of CD1+ cells in the γδ population was calculated by two-color FC measuring the number of CD1+ cells (red) after electronically gating in the TCRγδ+ subset (green). Reagents were Coulter Clone T6 (CD1), Coulter Clone T3 (CD3), TCRδ1 (TCRγδ), Ti-γA (TCR-$V_{\gamma 9}$), and TCSδ1 (TCR-$V_{\delta 1}$) Results show the percentages of positive cells in a child representative of four similar cases studied, ND, not done

Table 2. Immunological unresponsiveness of the CD1+ subset of CD4-8- double-negative thymocytes

Cell populations[a]	(a) IL2 secretion (U/ml) PHA + IL1β	(b) Tac expression (% positive cells)		(c) Proliferation (cpm/min x 10-3)		(d) Antibody retargeted cytotoxicity (% specific ^{51}Cr release)			
		fresh	PHA activated	rIL2	PHA + rIL1β	None	CD3	TCRαβ	TCRγδ
Total thymocytes	5.2	1	38	3	109	3	37	30	11
CD1-3-4-8-	6 3	46	76	87	189	5	55[a]	38	33
CD1+3-4-8-	0 1	1	1	0.9	3	1	2	1	0
CD1-3+4-8-	3 3	8	52	15	161	2	68	49	23
CD1+3+4-8-	0.2	2	6	1 8	7	3	3	2	1

Function assays were (see Methods for details), (a) IL2 secretion promoted by PHA plus rIL1β was estimated using IL2-dependent mouse CTLL2 cells, which respond to human rIL2 but not rIL4, (b) Tac expression in fresh or PHA activated cells was evaluated using H108 antibody and flow cytometry, (c) proliferation was measured by [^{3}H]thymidine incorporation after 80 h of culture with rIL2 or PHA + rIL1β; and (d) cytolytic potential was assessed against ^{51}Cr-labeled P-815 cells either uncoated or coated (24) with T3b (CD3)-, WT31 (TCRαβ)-, or TCRδ1 (TCRγδ)-specific antibodies CD2 antibody (MAR206 1, γ_1) gave similar results to those obtained with uncoated target cells (not shown) The results are expressed as the mean ^{51}Cr specific release of triplicate cultures at a 25/1 effector-to-target ratio Spontaneous release never exceeded 18%.

[a]See Methods. The data presented here were obtained in a simultaneous experiment; similar results were obtained in five experiments

[b]CD1-3-4-8- thymocytes express CD7 and CD2 T cell differentiation antigens *in vivo* (15, and data not shown), and acquire TCR – CD3 complex and cytotoxic activity upon culture with rIL2 (3,22)

Results

Isolation of early T cell precursors from postnatal thymus requires their separation from inert and mature thymocytes (3,4). In the human system we have used anti-CD1 antibodies to aid in the depletion of inert cells. Combined with anti-CD4 and anti-CD8 antibodies, we obtained a high proportion of TCR−CD3+ mature thymocytes in the double-negative isolate (2,15). In the mouse system others have used anti-CD5 antibody to aid in the depletion of mature cells when preparing CD4-8- thymocytes (6). Unexpectedly, anti-CD5 antibody treatment depletes the above TCR−CD3+ mature subset of double-negative thymocytes (26). We thought that perhaps the depletion of CD1+ cells in our previous studies could have eliminated an inert CD1+4-8- precursor subset (23)

We have now tested this possibility by using two-color fluorescence staining and FC and assessing the co-ordinate expression of CD1, TCRαβ−CD3, and TCRγδ−CD3 on total CD4-8- thymocytes. Figure 1a shows that only CD4-8- thymocytes are left after treatment of thymocytes with anti-CD4 and -CD8 cytotoxic antibodies and complement. Figure 1b shows the distribution of CD1 and CD3 surface molecules on double-negative thymocytes. We found that 44% of double-negative thymocytes were CD1+3-, 13% were CD1+3+, 14% were CD1-3-, and 29% were CD1-3+. We also studied the presence of TCRαβ−CD3+ and TCRγδ−CD3+ thymocytes among the double-negative thymocyte subset by using the pan-TCRαβ monoclonal antibody WT31 and anti-TCRδ1 antibody specific for a framework determinant of the human TCRδ chain We found among the population of double-negative thymocytes 29% TCRδ1+CD3+, 13% WT31+CD3+, and 58% TCRδ1WT31-CD3- cells (Fig. 1c and d). We also found a minor population CD3+TCRδ1+ (Fig. 1d) among the CD1+ double-negatives (8−13%, Fig 1e). Similarly a CD1+3+WT31+ minor subset also exists (1−6%, data not shown) As shown in Table 1, a subset of γδ T cells in thymus but not in autologous peripheral blood co-expresses TCRγδ and CD1. Moreover, studies using antibodies specific for TCRs using $V_{\gamma 9}$ and $V_{\delta 1}$ regions (21,27) showed a preferential use of $V_{\delta 1}$ in the thymus and $V_{\gamma 9}$ in the periphery

Mature single-positive thymocytes respond to polyclonal T cell activators (i e. PHA, anti-CD3 antibody) with IL2R expression, IL2 secretion, proliferation, and subsequent expression of effector functions (i.e. cytotoxicity) (1,5). We isolated the CD1+3+, CD1+3-, CD1-3+, and CD1-3- double-negative subpopulations by panning and carried out a comparative study of these functions with the purified double-negative thymocyte subsets. Table 2a shows that the CD1+3- and CD1+3+ populations, the CD1-positive subsets, did not secrete IL2 upon stimulation with PHA plus rIL1β, while the CD1-negative subsets, either CD3- or CD3+, did so. Approximately 46% unstimulated CD1-3- double-negative thymocytes expressed IL2Rα (Tac,p55); unstimulated CD1-3+ double-negative thymocytes had barely detectable levels of IL2Rα but both the number of positive cells and the levels of IL2Rα increased after their stimulation with PHA. In contrast, CD1+ double-negative thymocytes did not express IL2Rα under any of the conditions studied (Table 2b). The ability to express IL2Rα correlated well with the proliferative responses driven by rIL2 alone or by PHA plus rIL1β (Table 2c). The CD1-3- double-negative population exhibited the best responses to rIL2, the CD1-3+ population responded to a lesser extent and the CD1+ subsets were unresponsive. The CD1-3+, which can express IL2Rα after

Table 3. Mitogenic combination of either anti-CD2 or anti-CD3 antibodies stimulate proliferation in IL2 in CD1-3+ but not CD1+3+ double-negative thymocytes

Thymocyte subset[a]	[^{3}H]Thymidine incorporation (cpm × 10^{-3})							
	CD2 + rIL2			CD3 + rIL2			None	
	d2	d3	d4	d2	d3	d4	d2	d3
CD1+3+4-8-	2 2	3.7	5 1[b]	1 8	3 2	4.8	2.3	0.7
CD1-3+4-8-	17	33	81	24	47	107	1 2	0 4
Total	5	13	29	8	19	40	0 8	0 2

Thymocytes, 2×10^5 per microtiter tray well, were incubated for 2, 3, or 4 days under the indicated culture conditions, rIL2 was added at 50 U/ml, the mitogenic pair of CD2 antibodies was D66 1 (38) + MAR 206 1, and anti-CD3 antibody was in solid phase (plate-bound, 1 μg/ml T3b) (39) Results represent the average counts of triplicate determinations

[a]Thymocyte subsets were either unfractionated or fractionated as indicated in Table 2

[b]It is noteworthy that a few proliferating cells occur in cultures of the poorly responsive CD1+3+ double-negative subset. Phenotypic analyses of these cells showed that they were CD1- T cells (data not shown)

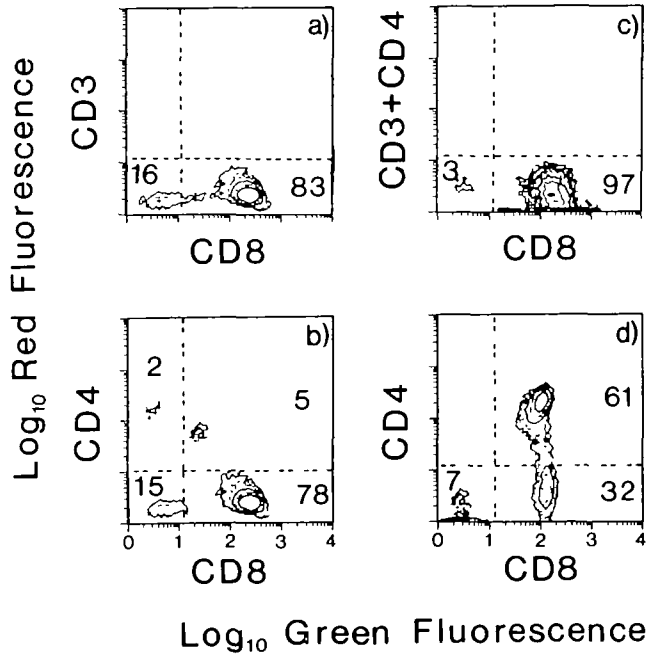

Fig. 2. Development *in vitro* of human double-positive thymocytes CD1+3-4-8- (a and b) or CD3-4-8+ (c and d) cells were isolated and stained, either fresh (c) or after their culture in the presence of 50 U/ml rIL2 for 36 h (a, b, and d), with CD8 (B9.4, γ_{2b}), (a) CD3 (T3b, γ_{2a}), or (b) CD4 (OKT4A, γ_{2a}) in the indicated combinations, followed by the respective isotype-specific, fluorochrome-conjugated second antibody. Numbers in each contour-plot panel (a−d) represent the percentage of positive cells in every quadrant Note the CD3 + CD4 legend in panel (c) indicates that cells were simultaneously incubated with CD3 and CD4 γ_{2a} mAb followed by PE conjugated γ_{2a}-specific second antibody Similar results were obtained with four independent thymuses

activation (Table 2b), exhibited proliferative responses to PHA plus rIL1β, while the CD1+ thymocytes neither expressed IL2R nor proliferated. The lack of proliferative responses in CD1+ subsets is not due to the absence of CD1− cells or factors released by them in culture. In complementation experiments the CD1+ subset failed to proliferate in the presence of irradiated CD1− double-negative thymocytes (data not shown). CD1−3− and CD1−3+ double-negative thymocytes generated CD3+ cytotoxic cells (3,22) that killed anti-TCR/CD3-coated targets while the CD1+ populations did not (Table 2d). It is still possible that differences in the ability of CD1+ and CD1− subsets to use IL1 or bind PHA account for the CD1+ cells' unresponsiveness. We therefore analyzed the proliferative responses of CD1+3+ and CD1−3+ double-negative cells to antibodies binding to well-established membrane ligands. We found that CD1−3+ but not CD1+3+ double-negative cells proliferated in response to mitogenic combinations of anti-CD2 or anti-CD3 antibodies in the presence of IL2 (Table 3).

The findings of non-functional CD1+ double-negative thymocytes and that ~95% of double-positive thymocytes express CD1 on the cell membrane raised the possibility that CD1+ double-negative thymocytes would contain precursors for CD1+ double-positive thymocytes. That this is indeed the case is shown in the results depicted in Fig. 2. We found that CD1+3−4−8+ thymocytes give rise to CD1+3−4−8− thymocytes in culture (Fig. 2a and b). Differentiation was achieved in medium only, was not modified by the addition of exogenous IL2, and was readily evident after 24 h of culture. Some CD4+ and double-positive cells started to accumulate in the culture after 36 h (Fig. 2b), and the frequencies of double-positive cells quickly increased thereafter (data not shown). Because the recoveries of viable cells began to drop by ~50% every 24 h, after 2 days in culture it was difficult to discriminate whether CD1+3−4−8+ cells acted as intermediate precursors of such CD4+8+ cells. To study this possibility, a population of CD1+3−4−8+ thymocytes found in normal human thymus was isolated *ex vivo* (Fig. 2c). We found that after short-term culture these cells gave rise to CD1+4+8+ double-positive thymocytes (Fig 2d).

Discussion

We have studied the correlated distribution of CD1 antigen and αβ and γδ TCR−CD3 complexes on the surface of double-negative thymocytes and found that CD1 is acquired at early stages of development, i.e. on some TCR−CD3− and TCR−CD3+ double-negative thymocytes. A subset of TCRγδ−CD3+ cells co-expresses CD1 in the thymus but not in the periphery. Others have previously shown expression of CD1 on TCRαβ−CD3$^{low\ or\ -}$ double-positive cortical thymocytes but not on peripheral T cells (4,5,9,13). CD1 expression is therefore restricted in αβ and γδ linkages to an intrathymic phase of development.

The *in vitro* assays for function show that the CD1+ double-negative subsets behave as inert cells whereas some cells among either CD1−3− precursors or their progeny (CD1−3+ double-negative thymocytes) are functionally competent. A striking aspect of this finding is that cell responsiveness does not appear to be associated with cell-surface TCR−CD3 expression. Previous studies have shown that CD1 occurs on most double-positive thymocytes which are functionally incompetent cells despite the cell-surface expression of TCRαβ−CD3 on half of them (4,5,9,13,28) The functional competence of some CD1−, TCR−CD3−4−8− thymocytes has been definitively established with the development of several clones with such phenotype that use the IL2 pathway of proliferation *in vitro* (P.Aparicio *et al*, unpublished observations) Our results also suggest that not all γδ T cells are born immunocompetent.

It could be argued that the binding of anti-CD1 antibody to the cells might itself cause CD1+ thymocyte unresponsiveness prior to assays of function However, this is ruled out by the following (i) separation of CD1+ thymocytes by negative selection of CD3^{bright} or CD44(Pgp-1)+ thymocytes yields also functionally inert CD1+ cells (28,29); and (ii) the addition of saturating amounts of anti-CD1 antibodies does not inhibit cell proliferation, IL2 secretion, or IL2-R acquisition in the Jurkat thymoma, cells which co-expresses TCRαβ and CD1 (A. de la Hera, unpublished observations). We conclude that there is a tight linkage between CD1 expression and incompetence in assays of function *in vitro* among human thymocytes. Indeed, the precursor frequencies for CD1+ cells responding to PHA plus IL2 are always very low (<1/100 cells), and all growing T cells thus far studied are CD1− (28). Also, mature αβ and γδ T cells, of double-negative, double-positive, or single-positive phenotypes, can be cloned from CD1− thymocytes after stimulation with PHA plus IL2, with high plating efficiencies *in vitro* (~1/2 cells; 28 and our unpublished results).

We have studied the differentiation potential of the CD1+3− double-negatives. We show that they pass through a CD3−4−8+ intermediate *en route* to the major (CD1+) double-positive thymocyte population. The precursors are IL2-R− and do not require the addition of exogenous IL2 for their differentiation. In our previous studies we removed the CD1+ population from isolated CD4−8− thymocytes prior to culture and hence did not find CD1+4+8+ thymocytes (3). Similarly, in the mouse it was shown that only the IL2-R−, Thy-1^{hi} subset of CD4−8− thymocytes generates immature CD4+8+ cells *in vitro* (30,31). Most CD1+ double-positive thymocytes generated *in vitro* bear cytoplasmic TCRβ chain, indicating that they are αβ pre-T cells, and some may express low levels of TCR−CD3 on the cell membrane. CD1+ TCR−CD3+ thymocytes cannot be expanded *in vitro*, nor can normal peripheral mature CD1+ T cells be detected *ex vivo* (Table 1; unpublished observations).

Interestingly enough, a minor subset (<5%) of CD1−3+ double-positives occurs *in vivo* We have previously shown that polyclonal activators such as PHA and IL2 stimulate the generation of such CD1−3+ double-positives from CD1− double-negatives *in vitro* (32). CD1−3+4+8+ thymocytes are functionally competent and can proliferate in the presence of IL2 (32,33). We therefore conclude that CD1+ and CD1−, CD4−8− cells can act as precursors for two distinct subsets of double-positive thymocytes *in vitro*. Experiments are underway to define at the molecular genetic level whether the mature CD1− double-positives are the intermediate stage in the generation of CD4+8− and CD4−8+ αβ T cells (33).

In summary, CD1 antigen is expressed early in ontogeny by double-negative thymocytes including TCR−CD3+ T cells. CD1+ double-negative thymocytes are precursors of double-positive thymocytes which show no functional competence *in vitro*. Inducibility of IL2 pathway responsiveness *in vitro* occurs

in some T cell precursors before CD1, TCR – CD3, CD4, and CD8 expression. At later stages of development, there is a good correlation between cell unresponsiveness *in vitro* and CD1 expression, both on cell-surface positive and negative αβ and γδ TCR – CD3 cells

Such studies have been performed *in vitro*. What can be the putative implications of these findings to the situation *in vivo*? It has been estimated that ~95% of thymocytes die intrathymically, most likely at the inert double-positive thymocyte stage (7,8) It is generally thought that the cells that are eliminated have been tolerized or would never terminally differentiate (7,8). The reason that receptor occupancy leads to clonal deletion in the immature thymocytes, instead of the clonal expansion observed in mature T cells, is unknown, but the occupancy of CD4/CD8 has been implicated in this process (30,34,35) We and others have previously shown that CD1 is covalently associated with CD8 on the surface of double-positive but not mature thymocytes, that functionally competent CD1+ thymomas exist but do not assemble CD1 multimolecular complexes, and that functional CD1- double-positive thymocytes do exist (13,32,33,36,37). In light of these findings, we shall test the possibility that such CD1 multimolecular complex expression is, perhaps, related to the mechanisms that lead to a selective elimination of immunologically unresponsive cells in the thymus.

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Abbreviations

CD	cluster of antibodies directed to differentiation antigens
FC	flow fluorocytometry
FITC	fluorescein isothiocyanate
mAb	monoclonal antibody
MHC	major histocompatibility complex
PE	phycoerythrin
PHA	phytohemagglutinin
rIL	recombinant interleukin
TCR	T cell receptor

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