Differentiation of CD3⁻4⁻8⁻ Thymocytes in Short-Term Thymic Stromal Cell Culture

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Summary

We have investigated the ability of a heterogeneous thymic stromal cell (HTSC) culture system to promote in vitro differentiation of CD3⁻4⁻8⁻ thymocytes. Culture of purified murine CD3⁻4⁻8⁻ thymocytes on HTSC for 1 d resulted in the appearance of CD4⁺8⁺ cells, which did not occur when the sorted cells were maintained in medium alone. It is remarkable that when the culture period was extended to 2 d, CD3⁻4⁻8⁻ progenitors differentiated further to CD4⁺8⁻ and CD4⁻8⁺ cells, which also expressed high levels of TCR-CD3. This rapid differentiation on stroma in vitro appears to outpace parallel development in vivo. The differentiation potential of a subset of CD3⁻4⁻8⁻ thymocytes that express high levels of a marker of normal and neoplastic thymic progenitors, the 1C11 antigen, was examined next. 1C11ʰⁱCD3⁻4⁻8⁻ cells also gave rise to CD4⁻8⁺ and CD4⁺8⁺ populations after 1 d of culture on HTSC. Extending the culture period to 2 d resulted in a significant percentage of CD3-expressing cells that were CD4⁺8⁺, CD4⁺8⁻ and CD4⁻8⁺ cells. These results suggest that in the in vitro HTSC culture system, various subsets of immature thymocytes can differentiate into all the mature phenotypes of cells normally found in the adult mouse thymus. This may provide a novel and rapid assay for thymic progenitors.

The thymus is the major site of differentiation of T lymphocytes. Bone marrow- or fetal liver-derived thymic progenitors migrate to the thymus where they undergo rapid proliferation and differentiation, a process known as thymic maturation (1, 2). In the thymus, under the influence of the thymic stromal microenvironment, immature thymocytes acquire various cell surface molecules including the MHC coreceptors CD4 (MHC class II-restricted) and CD8 (MHC class I-restricted), and the TCR $\alpha\beta$ or $\gamma\delta$ heterodimers associated with the invariant CD3 polypeptides (3-6). Based on their CD4 and CD8 expression, thymocytes are divided into four categories: CD4⁻8⁻, CD4⁺8⁺, CD4⁺8⁻, and CD4⁻8⁺ (1). The immature CD3⁻4⁻8⁻ cells contain a subpopulation that proceeds through sequential CD3⁻4⁻8⁺ large cells (a rapidly cycling population) and CD3ˡᵒ4⁺8⁺ large thymocyte intermediates, before giving rise to the phenotypically and functionally mature CD3ʰⁱ4⁺8⁻ and CD3ʰⁱ4⁻8⁺ T cells, as well as CD3ˡᵒ4⁺8⁺ small thymocytes that die in situ (7-9). During the process of thymic education, maturing T cells acquire the property of responsiveness to foreign antigens presented in association with self-MHC molecules (10) by the APCs, and of tolerance or nonrespon-

siveness to self-antigens (10-13). Recently, it has been suggested (14) that interaction of CD3ˡᵒ4⁺8⁺ cells with affinity for self-MHC complexed with unknown self-peptides may be required for positive selection. Negative selection to Mls- and Vβ17-detected antigens occurs at a later stage when CD3ᵐᵉᵈ4⁺8⁺ cells are committed to either the CD4 or the CD8 lineage (14).

Although the lineage relationships between CD3⁻4⁻8⁻ cells and the phenotypically and functionally mature T cell subsets have been established, the precise nature of the cell interactions and the role of soluble factors involved in this process is poorly understood. It seems likely that epithelial cells present within the thymic stroma play an important role in thymocyte development (15). For example, it has been proposed, but not directly shown, that one important function of these epithelial cells is the presentation of MHC proteins to maturing T cells, which contributes to T cell-repertoire selection.

To be able to manipulate experimentally various aspects of T cell development, one approach is to establish thymic stromal cell cultures in which immature thymocytes can undergo differentiation. The mouse thymic stromal microen-

vironment is comprised of various types of epithelial cells (cortical and medullary types), mesenchymal derivatives, dendritic cells, and macrophages (15, 16). Most of these cells have been grown in tissue culture and characterized on the basis of their expression of specific differentiation antigens (17, 18). Some epithelial cell cultures or epithelial cell lines derived hitherto have been shown to be effective in promoting in vitro growth and, at least, partial differentiation of either murine (19) or human (20) $CD3^-4^-8^-$ thymocytes, or putative prothymocytic murine T cell clones (21).

We have developed methods for growing heterogenous thymic stromal cell (HTSC)[1] cultures that are comprised of both critical and medullary epithelial cells, as well as of dendritic cells and macrophages (16, 17). In this communication, we describe the ability of these cultures to promote in vitro maturation of $CD3^-4^-8^-$ murine adult thymocytes in a short-term differentiation assay. We have also examined the ability of a subset of $CD3^-4^-8^-$ cells, identified by the expression of a heterodimeric cell surface antigen 1C11 (22) to differentiate in vitro. We present evidence that in this assay system, both the $CD3^-4^-8^-$ and the $1C11^{hi}CD3^-4^-8^-$ subsets can differentiate into phenotypically mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes. In a separate study (M. Small, A. Sen-Majumdar, and I. L. Weissman, manuscript in preparation), we describe a second $CD3^-4^-8^-$ subset which, upon coculture with HTSC, matures predominantly to $CD3^+4^+8^-$ cells.

Materials and Methods

Mice. 4–6-wk-old C57BL/Ka (Thy-1.2, Ly5.1) and C57BL/Ka BA (Thy-1.1, Ly-5.1), and C57BL6/J-Ly5.2 (Thy 1.2, Ly-5.2) mice were bred and maintained in the central animal facility in the Department of Comparative Medicine, Stanford University.

mAbs and Fluorescent Reagents. The sources and fluorochrome modifications of mAbs specific for CD4, CD8, Thy-1.1 and Thy-1.2, Ly5.1, and Ly-5.2 have been described (23). Sources and specificities of other Abs were as follows: FITC-145-2C11 (anti-CD3 ε; Boehringer Mannheim Corp., Indianapolis, IN); PE-conjugated GK1.5 (anti-CD4; Becton Dickinson & Co., Mountain View, CA) biotinylated anti-TCR/αβ (Pharmingen, San Diego, CA). Avidin-conjugated Texas red (TR), -FITC, -PE and allophycocyanin (AP) were purchased from Caltag Laboratories (South San Francisco, CA).

Rat hybridoma cell lines producing antibodies to IL-2R (mAb 7D4, [24]) and heat stable antigen (HSA, mAb M-169, [25]) were obtained from American Type Culture Collection (Bethesda, MD). In all cell sorting experiments, mAb 1C11 (22) was used as the hybridoma culture supernatant, and its reactivity was monitored with TR-conjugated goat anti–rat Ig (Caltag Laboratories). In some experiments, purified 1C11 antibodies were biotinylated using N-hydroxysuccinimide biotin (Sigma Chemical Co., St. Louis, MO) after standard conjugation procedures. The reactivity of biotinylated reagents was monitored with avidin-conjugated fluorochromes.

HTSC Culture System. Thymic stromal cells from 4–6-wk-old

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[1] *Abbreviations used in this paper:* AP, allophycocyanin; HSA, heat stable antigen; HTSC, heterogeneous thymic stromal cell; PI, propidium iodide; TR, Texas red.

C57/BL/Ka (either Thy-1.1 or Thy-1.2) or C57BL/6J-Ly 5.2 (Thy 1.2, Ly5.2) mice were cultured as described previously in detail (16, 17). Briefly, after removing the thymocytes, stromal cells were grown in MEM containing D-valine for 3–5 wk. In some cases, adherent cells from primary culture dishes were removed by trypsin-EDTA (Gibco Laboratories, Grand Island, NY) treatment and placed into 24-well (Costar, Cambridge, MA) plates. D-valine containing medium was withdrawn just before sorted thymocytes (see below) were added to the epithelial cell cultures. Sorted thymocytes were resuspended in IMDM or RPMI-1640 supplemented with 5–10% FCS, L-glutamine, 50 μM 2-ME, and penicillin-streptomycin. Thymocytes removed after 1 (18–20 h) or 2 (40–43 h) d of culture by gentle pipetting were centrifuged, and viability was determined before flow cytometric analysis.

Cell Sorting and Flow Cytometer Analyses. Freshly isolated thymocytes were stained for two-, three-, or four-color analysis, and the fluorescence was analyzed using a highly modified dual laser FACS® IV (Becton Dickinson & Co.) with four-decade logarithmic amplifiers as described (9, 22). Dead cells were detected by adding 1 μg/ml of propidium iodide (PI), and gated out by setting an electronic gate to exclude PI-positive cells (22). For four-color analysis, dead cells were eliminated by the usual scatter gating method (22), and the excitation wavelength of the dye laser was raised from 590–605 nm. Contaminating stromal cells present in the thymocyte populations were eliminated by setting electronic gates on forward angle light scatter as well as on obtuse scatter (granularity measuring index). Fluorescence data were analyzed by using the FACS/DESK® program and presented either in the form of histograms or two-parameter probability plots (5%). The histograms and the contour plots are labeled according to the established nomenclature of the molecules detected by particular antibodies.

The sorting procedure for $CD3^-4^-8^-$ and $1C11^{hi}CD3^-4^-8^-$ thymocytes has been described elsewhere in detail (9, and Sen Majumdar, A. et al., manuscript submitted for publication). Briefly, normal thymocytes were stained with biotinylated anti-CD4 and CD8, and thymocytes expressing CD4 and CD8 molecules were depleted by the use of avidin-conjugated paramagnetic beads (usually 10 μl of bead/5 × 10⁶ thymocytes) as described (9). The procedure was repeated, although in some experiments one round of bead separation was found to be sufficient. Finally, $CD4^-8^-$ thymocytes were stained with a mixture of AV-PE (to detect cells expressing low levels of CD4 and CD8) and FITC-conjugated anti-CD3 antibody. PE- and FITC-positive cells were gated out in the cell sorter and $CD3^-4^-8^-$ cells were collected. To select the $1C11^{hi}$ subsets of $CD3^-4^-8^-$ cells, thymocytes were stained with mAb 1C11 followed by goat anti–rat Ig-conjugated TR. After blocking the remaining reactive sites of the secondary antibody with normal rat serum, the thymocytes were treated as described above for total $CD3^-4^-8^-$ cell sorting. Electronic gates were set so that $1C11^{hi}$ cells (expressing high levels of TR) within the $CD3^-4^-8^-$ population were collected. A small aliquot of the sorted cells was reanalyzed to calculate the purity of the preparation, and in all experiments described here, the purity of sorted cell populations was >98%.

Results

Differentiation of $CD3^-4^-8^-$ Thymocytes after 1 d Culture on HTSC. To analyze the ability of HTSC to promote thymocyte differentiation, $CD3^-4^-8^-$ thymocytes were isolated from normal adult C57BL/Ka or C57BL/Ka/Thy-1.1 mice as described. After 1 d of culture, the viability of the

input cells was 40–60% in the presence of HTSC, but only 20–25% of the cells survived in the medium alone. About 15–20% of the thymocytes recovered from the medium alone acquired low levels of CD8 antigen, but only a marginal percentage of cells were CD4$^+$8$^+$ (Fig. 1 c). In contrast, in the presence of the stromal cells, about 7–14% (in three experiments) of the cells were CD4$^+$8$^+$ after 1 d of culture (Fig. 1 d). The thymocytes recovered from the stromal layer had a lower percentage of CD4$^-$8$^+$ cells (6–9%) than those which were kept in medium, or that were found after intrathymic injection in vivo (9). Inspection of the two-color probability plots reveals that the shift towards higher expression of CD8 reflect an overall increase in staining in the FITC (CD8) channel, rather than emergence of a bimodality of CD8 staining.

The CD3$^-$4$^-$8$^-$ cells were cultured alone or on a monolayer of HTSC for 1 d (usually 18–20 h). The stromal cell cultures used in this study had been in culture for at least 3–5 wk before use. Hence, it is unlikely that there would be any thymocytes left in the primary or secondary epithelial cell culture. Still, to rule out the possibility of minor thymocyte contaminants, thymocyte donors were either Thy-1 or Ly-5 congenic to the donors of the HTSC cultures. Almost all cells analyzed after 1 or 2 d of in vitro culture were found

to be positive for the congenic marker of the input thymocytes (Fig. 1, c and d, insets).

Differentiation of CD3$^-$4$^-$8$^-$ Thymocytes into Mature Cell Subsets. It was possible that longer culture periods would enable further maturation of the CD3$^-$4$^-$8$^-$ cells in HTSC. Thus, CD3$^-$4$^-$8$^-$ thymocytes were cultured on HTSC for 2 d and phenotypic analysis was performed to assess their differentiation pattern in the presence of epithelial cells. Although the general pattern of differentiation in different experiments was similar, we observed that the percentages of different cell subsets vary from one experiment to another. Therefore, results from a representative experiment are presented in Fig. 1. The CD4 vs. CD8 profile of normal adult thymocytes is shown for comparison (Fig. 1 a). The in vitro differentiation assay (1 e) shows that >80% of the input cells have differentiated into predominantly CD4$^-$8$^+$ (57.4%) cells, as well as into CD4$^+$8$^+$ (23.8%) and CD4$^+$8$^-$ (2.9%) thymocytes. CD3$^-$4$^-$8$^-$ thymocytes from Ly-5.1 donors were grown on HTSC of Ly-5.2 origin. The emerging differentiated thymocytes expressed the Ly-5.1 marker (Fig. 1 e, inset) demonstrating their origin from the input thymocytes.

In the second experiment, we examined the percentage of differentiated thymocytes expressing the CD3 antigen after a 2-d culture period, and the results are presented in Fig. 2.
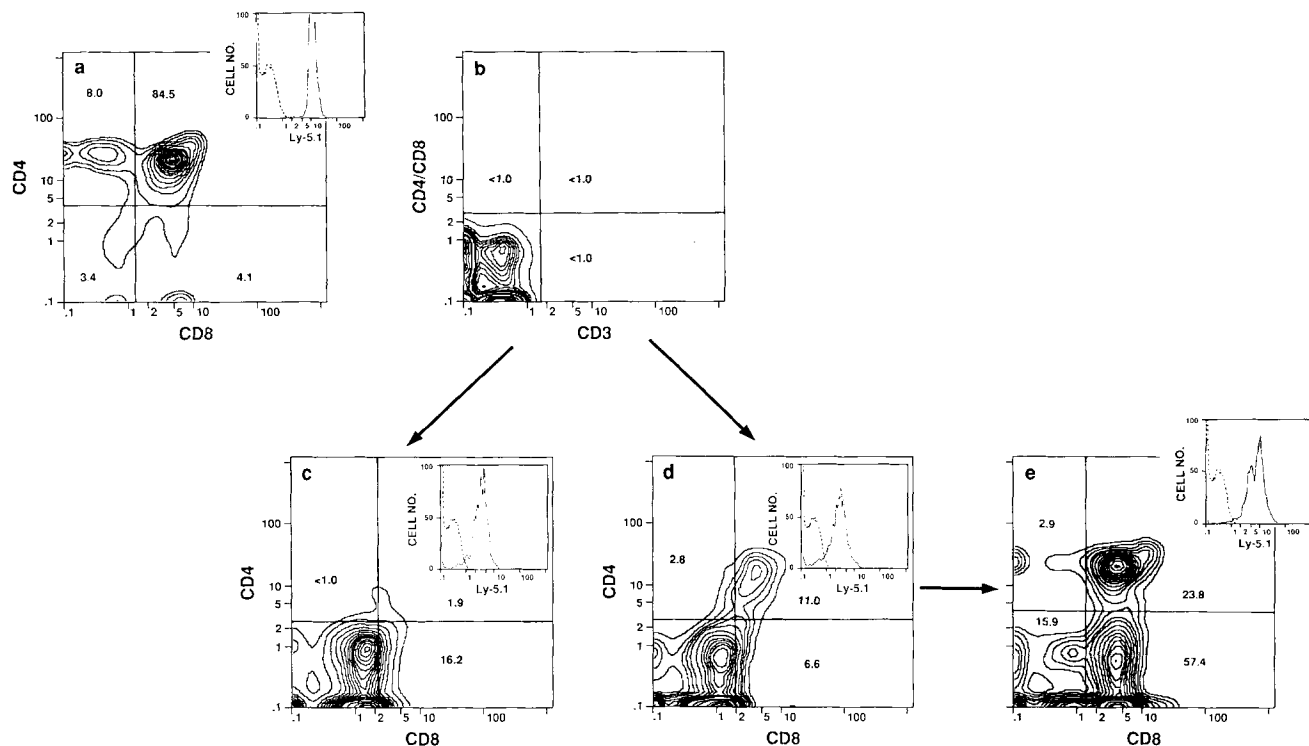

Figure 1. In vitro differentiation of CD3$^-$4$^-$8$^-$ thymocytes. (a) CD4 vs. CD8 of normal thymocytes (for comparison). CD3$^-$4$^-$8$^-$ thymocytes were sorted as described in Materials and Methods. Sorted cells were reanalyzed (b), and allowed to differentiate for 1 d either in medium alone (c) or on HTSC (d). (e) Differentiation of CD3$^-$4$^-$8$^-$ thymocytes on HTSC after 2 d of culture. Numbers in each quadrant show percentages of CD4$^-$8$^-$ (lower left), CD4$^-$8$^+$ (lower right), CD4$^+$8$^+$ (upper right), and CD4$^+$8$^-$ (upper left) cells. (Insets) Depict the Ly-5.1 expression of normal and cultured thymocytes, respectively. Thymocytes were stained with anti-CD4 conjugated to PE, FITC-conjugated anti-CD8, and biotinylated anti-Ly-5.1 followed by AV-TR. Representative CD4 vs. CD8 profiles are shown. (Dashed lines) Background fluorescence.
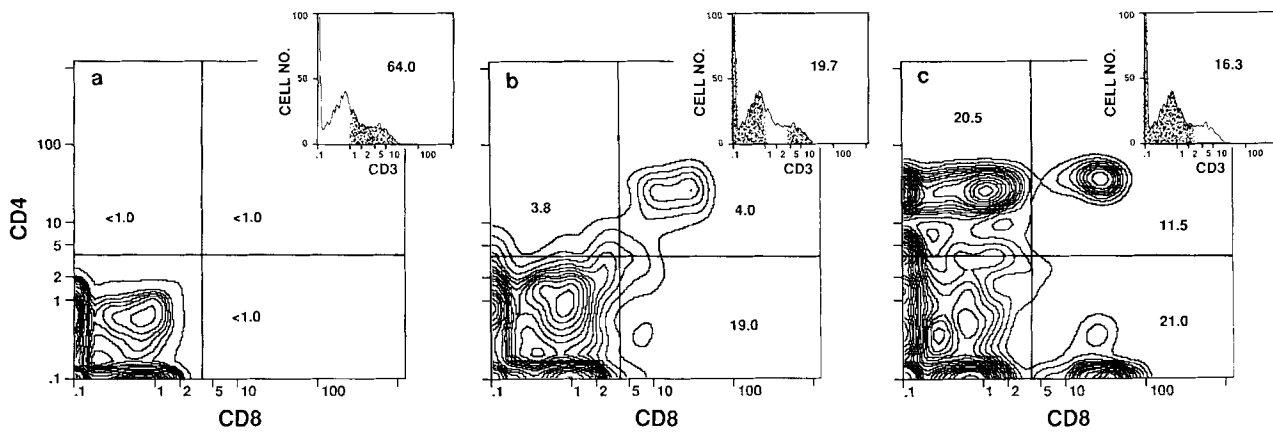
Figure 2. Generation of CD3⁺ T cells when CD3⁻4⁻8⁻ thymocytes are grown in HTSC for 2 d. Each panel represents CD4 vs. CD8 contour plots of CD3⁻ (*a*), CD3⁺ (*b*), and CD3²⁺ (*c*), respectively. The open areas in each of the CD3 profiles (*insets*) represent the gated cells and their percentages. For this experiment, cultured thymocytes were stained with PE-conjugated anti-CD4, biotin-conjugated anti-CD8, and FITC-conjugated anti-CD3.

As compared with day 1 when 3–6% cells were CD3⁺ (data not shown), a significantly higher percentage (36%) of the cultured thymocytes on day 2 expressed CD3/TCR marker. The level of expression of CD3/TCR antigen receptor complex on thymocytes relates to their state of maturation in vivo. Based on the relative density of CD3, we have subdivided the in vitro differentiated thymocytes into three categories: cells expressing little or no CD3 (Fig. 2 *a*, inset, open area); cells that are low- to medium- positive (Fig. 2 *b*, inset, open area) and medium- to highly positive (Fig. 2 *c*, inset, open area). CD4 vs. CD8 profiles of each of these CD3 subsets are presented in Fig. 2, *a–c*. About 25% of the CD3⁻4⁻8⁻ were found to have differentiated in this experiment and acquired either CD4 or CD8 antigen. Most of the cells in the CD3⁻ category were CD4⁻8⁻, whereas CD3⁺ cells included a significant subset that coexpressed both CD4 and CD8 antigens. However, when we examined the CD4/CD8 phenotype of the CD3²⁺ cells (Fig. 2 *c*) a significant number of CD4⁺8⁻ and CD4⁻8⁺ mature, single-positive cells were found. In addition, CD4⁻8⁻ and CD4⁺8⁺ subsets were also present in the CD3²⁺ population. These results suggest that the heterogenous stromal cell culture in vitro was capable of inducing differentiation of immature CD3⁻4⁻8⁻ thymocytes into mature subsets of T cells normally present in the mouse thymus (1).

Although this differentiation of immature thymocytes was consistently observed in the HTSC culture system, the percentage of mature thymocytes varied between experiments. In this context, it is important to mention that primary or secondary cultures of HTSC contained various types of adherent cells, with significant variations among different culture dishes, not only in terms of presence or absence of a stromal cell type, but also between the proportions of various kinds of stromal cells. It is conceivable that the survival and ability to differentiate of CD3⁻4⁻8⁻ thymocytes could depend to a large extent on the stromal cell types, and also on the relative ratio of these cells to input thymocytes in a

given culture dish. Indeed, we found differences in the viability (∼33% of input cells survived in expt. 1, and 21% of input cells survived in expt. 2), as well as in the extent to which the input cells would differentiate in the in vitro HTSC culture system.

To show that the observed in vitro differentiation of thymocytes is not due to preferential proliferation of mature cell contaminants, we performed the following experiment. To the thymic stromal cell cultures (C57BL, Thy-1.1/Ly-5.1), we added cell sorter-purified CD3⁻4⁻8⁻ (C57BL, Thy-1.2/Ly-5.1) plus 2% mature CD3²⁺ contaminants obtained from Ly-5.2 (C57BL, Thy-1.2/Ly-5.2) thymocytes. In control cultures, no contaminants were added. 2 d later, in vitro cultured thymocytes were analyzed, and the results are presented in Fig. 3. It is clear from the data that although
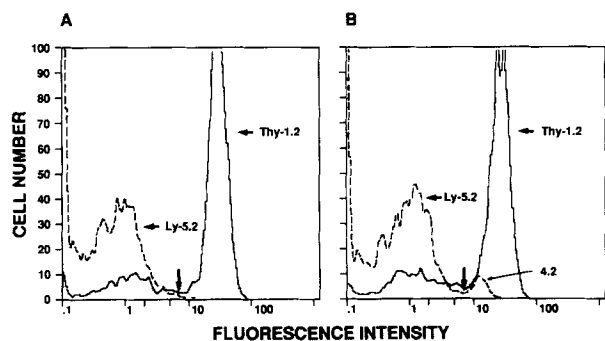

Figure 3. In vitro maturation of thymocytes is not due to preferential proliferation of mature contaminants. CD3⁻4⁻8⁻ thymocytes (Ly-5.1/Thy-1.1) were sorted and added on the HTSC culture with 2% CD3²⁺ contaminants (Ly-5.2/Thy-1.2) or without contaminants. Fluorescence profiles of 2-d cultured thymocytes without (*A*) or with (*B*) contaminants were determined for Thy-1.2 (*solid lines*) and Ly-5.2 (*dashed lines*). Numbers in *B* represent percentages of contaminants remaining after 2 d of culture.
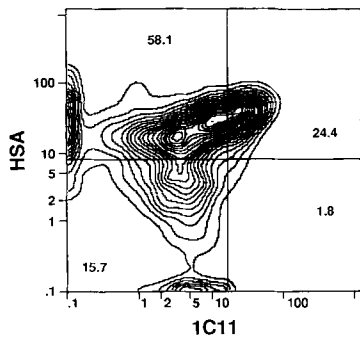
Figure 4. A distinct subset of CD3⁻4⁻8⁻ thymocytes express high levels of 1C11 and HSA. Four-color analysis was performed as described in Materials and Methods. Normal thymocytes were stained with a mixture of anti-CD4 plus anti-CD8 (PE), anti-CD3 (FITC), 1C11 (AP), and anti-HSA (TR). The fluorescence signal for CD4/CD8 and CD3 were gated out so that the contour plots represent 1C11 vs. HSA profile of thymocytes, which were negative for CD4, CD8, and CD3. Numbers in each quadrant indicate percentages.

>80% cells were positive for Thy-1.2, only 4.2% of these cells expressed Ly-5.2 in the well where contaminants were deliberately added (Fig. 3 *B*). Obviously, no Ly-5.2 expressing cells were found where contaminants were not added (Fig. 3 *A*). Thus, it appears that the thymocyte maturation observed in the presence of HTSC was due to differentiation of the immature precursor populations and not to preferential proliferation of mature contaminants present in the sorted cell preparation.

A Distinct Subset of CD3⁻4⁻8⁻ Thymocytes Express High Levels of 1C11 Antigen. R. C. Howe and H. R. MacDonald (26) have shown the heterogeneity within the CD4⁻8⁻

double-negative thymocytes, with respect to their phenotypes and distinct potentials for differentiation. We have recently described a mAb, 1C11, that preferentially marks thymic progenitor cells, including the CD3⁻4⁻8⁻ subsets (22). To determine whether the 1C11ʰⁱ CD3⁻4⁻8⁻ thymocytes, which constitute about 26% of the CD3⁻4⁻8⁻ population, coexpress other T cell differentiation antigens present on the CD3⁻4⁻8⁻ cells, we compared the expression of 1C11 with that of HSA (Fig. 4). Almost all 1C11ʰⁱ CD3⁻4⁻8⁻ exhibited high levels of HSA expression, although many additional HSAʰⁱ cells failed to express 1C11. Thus, a distinct subset of cells within the total CD3⁻4⁻8⁻ population is identified by the high expression of 1C11 and HSA.

Differentiation of 1C11ʰⁱCD3⁻4⁻8⁻ Thymocytes to CD4⁻8⁺ and CD4⁺8⁺ Cells after 1-d Culture on HTSC. A representative set of data (out of four experiments) illustrates the differentiation potential of 1C11ʰⁱ CD3⁻4⁻8⁻ thymocytes in the presence of HTSC vs. medium alone (Fig. 5). By 18–20 h these cells could generate 8% of CD4⁻8⁺, and about 9% of CD4⁺8⁺ cells when cultured in the presence of HTSC (Fig. 5 *C*). Again, a significant percentage (8%) of cells, representing the shoulder of the modal negative population, expressed CD8 antigen when sorted cells were cultured in medium only (Fig. 5 *B*). Whether this represents a background staining problem, or true differentiation is unclear, although a small subset of CD3⁻4⁻8⁻ blast thymocytes have been reported to differentiate in vitro to CD4⁻8⁺ and CD4⁺8⁺ in the absence of thymic epithelial cells (2). Occasionally we have also found that a small percentage (2–4%) of cells recovered from medium only on day 1 expressed both CD4 and CD8 antigens (data not shown). A small percentage (3–4%) of thymocytes recovered from HTSC
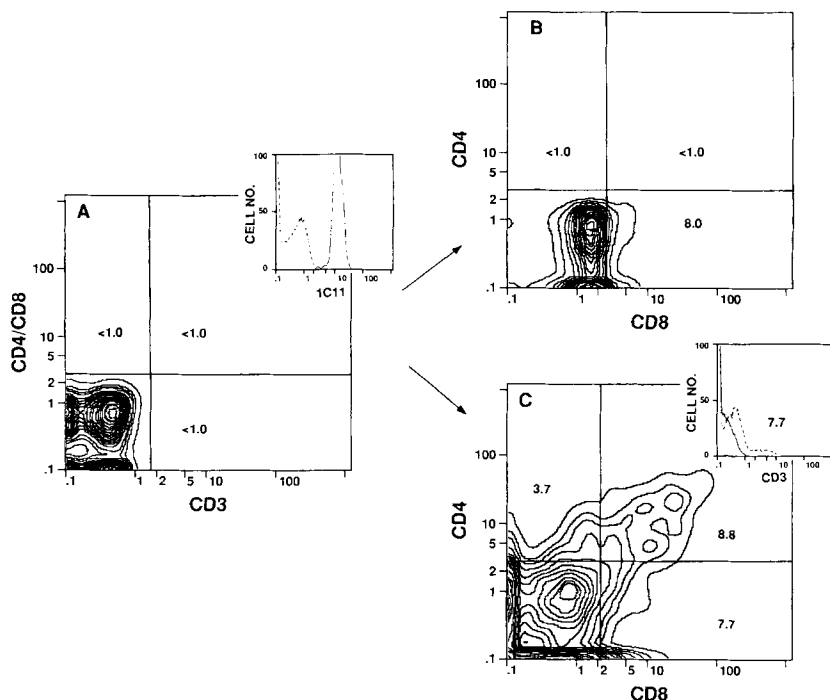

Figure 5. In vitro differentiation of 1C11ʰⁱ CD3⁻4⁻8⁻ thymocytes. 1C11ʰⁱCD3⁻4⁻8⁻ were sorted as described and reanalyzed for CD4/CD8 and CD3 expression (*A*), as well as for 1C11 expression (*A, inset*). Sorted cells were grown in medium (*B*) or on HTSC culture (*C*) for 1 d, and subsequently analyzed for CD4 (PE) and CD8 (TR). (*C, inset*) CD3 (FITC) profile of thymocytes cultured on the stroma. (*Dashed line* in *insets*) Background fluorescence.
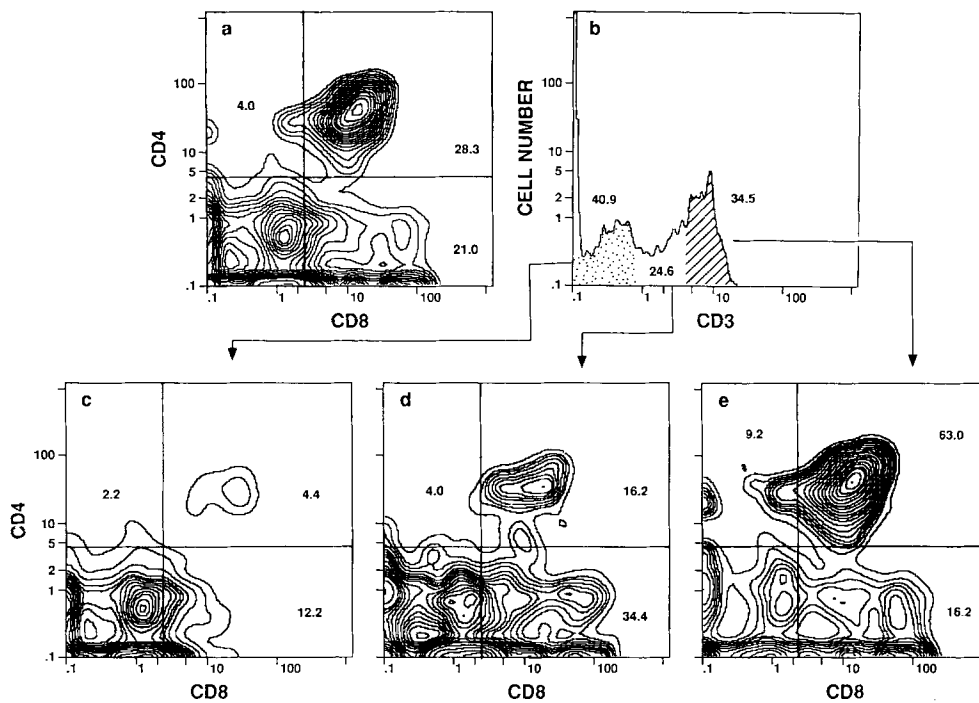
Figure 6. Coculture of 1C11^{hi}-CD3$^-$4$^-$8$^-$ thymocytes on HTSC for 2 d generate phenotypically mature T cells. Results from two representative experiments are presented. (*a*) CD4 vs. CD8 contour plots (*a*), and (*b*) CD3 histogram of total thymocytes recovered after 2 d of culture. (*b*) CD3 profile was subdivided into CD3$^-$ (*dotted area*), CD3$^+$ (*open area*), and CD3^{2+} (*hatched area*), and their corresponding CD4 vs. CD8 profiles are shown (*c*, *d*, and *e*, respectively).

cultures on day 1 expressed low levels of CD4 antigen (CD4$^+$8$^-$), the significance of which is not clear at this time. Similar to the total CD3$^-$4$^-$8$^-$ thymocytes, 1C11^{hi} cells in the presence of HTSC, generated 7–10% of cells that were CD3$^+$ (Fig. 5 *C*, inset), and about 70% of these cells were CD4$^+$8$^+$ (data not shown).

Generation of Mature Cell Subsets from 1C11^{hi}CD3$^-$4$^-$8$^-$ Thymocytes. In the presence of thymic epithelial cells, the differentiation of 1C11^{hi} thymocytes was further advanced when the culture period was extended to 2 d (40–43 h). Although 20–32% of the 1C11^{hi}CD3$^-$4$^-$8$^-$ input cells survived after 2 d of culture in HTSC, most (>98%) of these cells were dead when cultured in the absence of the stromal cells. Phenotypic analysis of thymocytes recovered after 2 d of culture was performed, and representative results are

presented in Fig. 6. More than 50% of the surviving thymocytes were found to have differentiated to CD4$^-$8$^+$ (21.0%), CD4$^+$8$^+$ (28.3%), and CD4$^+$8$^-$ (4.0%) cells in the presence of the stroma (Fig. 6 *a*). Of the differentiated thymocytes, >50% expressed varying levels of the CD3 antigen (Fig. 6, *b*). Although CD4$^-$8$^+$ thymocytes were found to be distributed among CD3$^-$, CD3$^+$ and CD3^{2+} subsets (Fig. 6, *c–e*), most of the CD4$^+$8$^-$ cells were CD3^{2+} (Fig. 6 *e*). The majority of the CD4$^+$8$^+$ cells belonged to the CD3^{2+} category (Fig. 6, *e*), but only a small percentage of CD4$^+$8$^+$ thymocytes belonged to the CD3$^+$ subset (Fig. 6, *d*). The significance of such high numbers of CD4$^+$8$^+$ cells in the CD3^{2+} subset is not clear, but the existence of such cells in the normal thymus has been reported (27). We next examined the relative level of CD3 among the differentiated subsets
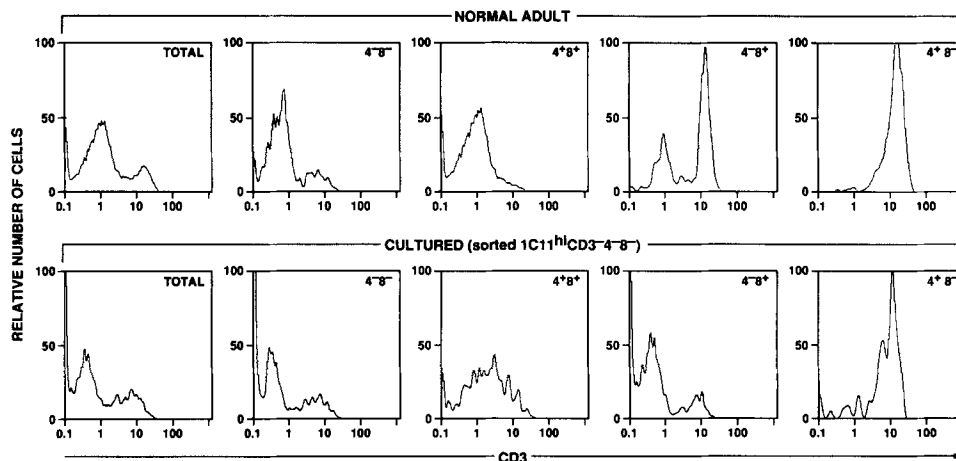

Figure 7. Relative levels of CD3 expression among various CD4/CD8 subsets of cultured and normal adult thymocytes. 1C11^{hi}CD3$^-$4$^-$8$^-$ were cultured for 2 d on HTSC and their CD4 (PE), CD8 (TR), and CD3 (FITC) phenotypes were determined by three-color FACS® analysis. (*Left, x axis*) CD3 profile of total thymocytes after 2 d of culture. The fluorescence signals were computer-gated to display the CD3 fluorescence in each CD4/CD8 subpopulations (*top right*). For comparison, the CD3 profiles of normal adult thymocytes are also presented.
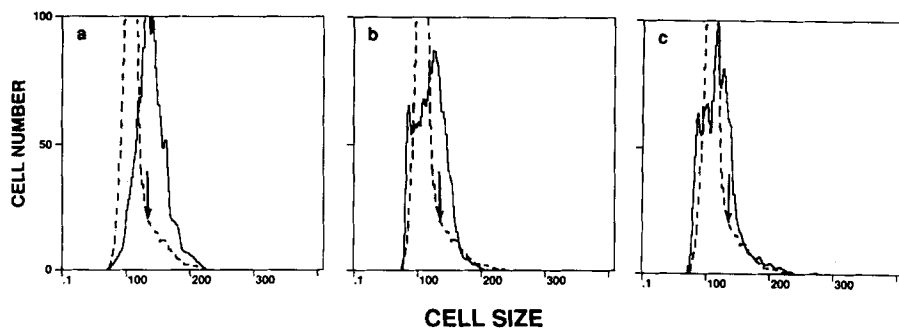
Figure 8. Gradual decrease in the percentages of blast cells when 1C11hiCD3-4-8- thymocytes were cultured in vitro on HTSC. Forward angle light scatter of sorted 1C11hiCD3-4-8- thymocytes (a, solid line), of 1-day cultured thymocytes (b, solid line), and of 2-day cultured thymocytes (c, solid line) are shown. For comparison, forward angle light scatter of total normal adult thymocytes (dashed lines) is depicted in each panel. Cells to the right of the arrows indicate blast type.

obtained from 1C11hiCD3-4-8- thymocytes (Fig. 7) Although the CD4+8- cells obtained after 2 d of culture expressed the highest levels of CD3, the CD4-8+ and CD4-8- cells could be clearly subdivided into two categories: CD3- and CD3^{2+}. All of these populations are present in the normal thymus, which suggests that thymic stromal cells in vitro can facilitate the differentiation of immature thymocytes into various types of phenotypically mature T cells.

Immature blast cells within the thymus can be detected by their distinct forward scatter characteristics (28). About 50% of these blast cells express the 1C11 antigen. We compared the cell size of 1C11hiCD3-4-8- thymocytes after cell sorting and 1 and 2 d of culture of culture on HTSC with that of total normal thymocytes (Fig. 8). About 54% of the sorted cells were found to be blasts, and the cell size gradually decreased when the thymocytes were cultured for 1 (34.9% blasts) and 2 d (19.6% blasts). These results, along with the phenotypic profiles of the T cells recovered after 2 d of culture of 1C11hiCD3-4-8- cells on HTSC suggest that immature thymocytes were capable of responding to the differentiation-transmitting signals delivered from the stromal cells in much the same way as observed with total CD3-4-8- thymocytes.

Discussion

In the present communication, we have demonstrated the capacity of CD3-4-8- immature thymocytes from adult mice to differentiate rapidly in vitro in the presence of thymic stromal cells. In this system, total CD3-4-8- thymocytes, as well as a subset of these cells, marked by the high expression of the 1C11 antigen, were found to give rise to CD4+8+, CD4+8- and CD4-8+ T cells.

The thymic stromal microenvironment is believed to govern the development and antigen repertoire selection of T cells (15). Thymic stromal cells not only produce growth factors to which thymocytes respond, they also express MHC proteins that are recognized by the $\alpha\beta$-TCR of developing T cells, and can induce either positive or negative selection (29–31). The direct transfer of various types of immature thymocytes intravenously (7) or into the thymus (9) of unirradiated or irradiated hosts has established to a large extent the lineage relationships between immature and mature T cell subsets. On administration to irradiated hosts, CD4-8-

thymocytes generated CD4+8+, as well as mature, single-positive cells (CD3hi4+8- and CD3hi4-8+) within 5–10 d (7, 32). In similar studies, C. J. Guidos et al. (9) demonstrated that upon injection of CD4-8- thymocytes into unirradiated recipients, donor cells could become CD4+8+ within 3.5 d. Furthermore, CD3-4-8+ thymocytes were shown to be the transitional intermediate between the CD3-4-8- and CD3lo4+8+ stages, and to give rise to mature, single-positives on intrathymic transfer (9). These in vivo studies indicate that CD3-4-8- immature thymocytes differentiate to CD4+8+ intermediates and, to a certain extent, to mature T cells within 4–7 d of intrathymic transfer. In comparison with the in vivo experiments, the maturation of thymic precursors in the presence of HTSC is remarkably rapid (2 d). At present, we cannot explain these kinetic discrepancies between in vivo and in vitro T cell differentiation. A plausible explanation might be that the ratio of thymocytes to stromal cells is lower in vitro than in vivo. To verify this, different ratios of thymocytes to stromal cells will be used in future experiments.

The composition of the thymic stroma may also be responsible for the more rapid maturation in vitro than in vivo. In vivo, many thymic microenvironments may not be conducive to maturation, thus the time needed to accumulate identifiably differentiated cells may be lengthened. It is also conceivable that HTSC culture is selective, and the cell types that proliferate in short-term stromal cultures favor thymocyte differentiation. In this context, we have observed variability, both in the composition of stromal cultures (preponderance of cells with epithelial, dendritic, or macrophage morphology), and in the relative proportions of CD4+8+, CD4+8-, and CD4-8+ thymocytes generated on these cultures. Attempts are now being made to identify and isolate normal stromal components to determine their role in thymocyte differentiation.

The data presented here may be interpreted to show that CD3-4-8- cells and their 1C11hi subset differentiate to CD3-4-8+ and, to a much smaller extent, to CD4+8+ cells after 1 d in culture in the absence of feeder cells. The emergence of CD4+CD8+ cells has been previously reported (33, 34). J. Nikolic-Zugic and M. W. Moore (37) have demonstrated that the CD4-8- thymocytes which differentiate to CD 4+8+ cells in vitro, in the absence of a feeder layer, express only low levels of CD3. The published reports

on this subject are somewhat controversial. For example, Y. Tatsumi and coworkers (36) failed to demonstrate such autonomous in vitro differentiation of CD4⁻8⁻ cell after 1 d in culture. We did not observe a distinct population of CD4⁺CD8⁺ cells, but rather saw an increase in the CD8 staining of the whole population. Similar observations were reported by other investigators for both fetal (35) and adult thymocytes (2). It is possible that the discrepancies in the published reports result from differences in methodology, as well as in the stringency of the electronic gates used for cell sorting. By day 2, we found that most of the thymocytes had died in the medium only.

In contrast, coculture of thymocytes in the presence of HTSC not only results in a 3–5-fold increase in the percentage of CD4⁺8⁺ cells at day 1, but allows continued survival and differentiation for a second day, to more CD4⁺8⁺ (a two-fold or greater increase is observed between day 1 and day 2), and to mature, single-positive cells. These observations suggest that the input CD3⁻4⁻8⁻ cells were sorted at a time when some of them have already received the signal in situ to turn on CD8 gene expression. Therefore by day 1, these cells express CD8 in medium, whereas in the presence of HTSC, they further differentiate to CD4⁺8⁺, and subsequently to mature single positives. The majority of the input

CD3⁻4⁻8⁻ thymocytes first differentiate to CD4⁻8⁺ transitional intermediates in HTSC and, subsequently, to generate CD4⁺8⁺ cells. Alternatively, it is conceivable that some CD3⁻4⁻8⁻ cells can differentiate directly to CD4⁺8⁺ without going through the CD4⁻8⁺ intermediate stage. At present, we cannot distinguish between these two possibilities.

Expression of the 1C11 determinant defines a novel subset of immature CD3⁻4⁻8⁻ (HSAʰⁱ) thymocytes. The 1C11ʰⁱCD3⁻4⁻8⁻ cells show a similar pattern of differentiation in vitro, as observed with total CD3⁻4⁻8⁻ thymocytes. The level of maturation of the 1C11ʰⁱ subset of the CD3⁻4⁻8⁻ populations, and its significance in the overall scheme of T cell development, are yet to be elucidated.

Finally, results presented in this communication show that under the influence of HTSC, thymocyte precursors can differentiate in vitro to give rise to all mature phenotypes of T cells, present within the normal adult murine thymus. Identification and characterization of particular cell types in the stroma that have this function may permit us to begin to understand the nature of molecular mediators that are essential to T cell development. Furthermore, use of stromal cell cultures or clones derived from Mls-antigen positive strains of mice may eventually unravel the biochemical processes underlying positive and negative selection of T cells.

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