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# Differentiation of thymocytes from $CD3^-CD4^-CD8^-$ through $CD3^-CD4^-CD8^+$ into more mature stages induced by a thymic stromal cell clone

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**ABSTRACT** **We have investigated the capacity of our established thymic stromal cell clone (MRL104.8a) or its derived factor(s) to induce the differentiation of immature thymocytes. Culture of purified adult murine double-negative ($CD4^-CD8^-$, indicated here as $CD4^-8^-$) thymocytes on the MRL104.8a thymic stromal cell monolayer for 1 day resulted in the induction of an appreciable percentage of $CD4^-8^+$ thymocytes. A bone marrow-derived stromal cell monolayer or a L929 fibroblast monolayer failed to generate $CD4^-8^+$ cells. This differentiation could also be induced by a semipurified sample of the MRL104.8a culture supernatant, which contained a thymic stroma-derived T-cell growth factor capable of contributing to the growth of double-negative immature thymocytes. $CD4^-8^+$ thymocytes generated 1 day after coculture with the MRL104.8a cells or the sample containing thymic stroma-derived T-cell growth factor were found to be $CD3^-$ and $J11d^+$, excluding the possibility of expansion of mature ($CD3^+4^-8^+$) thymocytes present in the thymus. More importantly, when the culture period was extended to 2 or 3 days, an appreciable number of $CD4^+8^+$ and single-positive ($CD4^+$) cells were generated on the MRL104.8a monolayer. Thus, these results provide the direct demonstration that $CD3^-4^-8^-$ immature thymocytes are promoted to differentiate through a rapidly cycling intermediate ($CD3^-4^-8^+$) into double- and single-positive cells by a specialized thymic stromal component.**

Within the thymus, T-cell precursors derived from bone marrow undergo extensive proliferation and differentiation to acquire the phenotypic markers and functional reactivities characteristic of mature T cells (1–3). Intrathymic T-lineage cells are divided into four major subpopulations based on the expression of CD4 and CD8 antigens (2). Double-negative ($CD4^-CD8^-$, indicated here as $CD4^-8^-$) thymocytes represent immature cells with the capacity to give rise to the other two single-positive ($CD4^-8^+$ and $CD4^+8^-$) mature subsets through the stage of $CD4^+8^+$ cells (4–7). Recent studies have also revealed the existence of a rapidly cycling intermediate ($CD3^-4^-8^+$) in the generation of $CD4^+8^+$ cells (2, 8–12). While these studies helped us to understand the sequence of events occurring during intrathymic T-cell maturation, cellular and molecular mechanisms involved in such maturation remain to be investigated. Since the microenvironment provided by thymic stromal components has a critical role in T-cell maturation as well as repertoire selection in the thymus (1–3), establishing an *in vitro* model in which double-negative thymocytes can differentiate into mature cells could contribute to a better understanding of the thymic microenvironment.

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This laboratory has recently established a thymic stromal cell clone, MRL104.8a (13), that produces a T-cell growth factor designated thymic stroma-derived T-cell growth factor (TSTGF) (14, 15). We demonstrated that this factor is capable of contributing to the proliferation of $CD3^-4^-8^-$ immature thymocytes (16). This study focused on the potential of the TSTGF-producing thymic stromal cells (MRL104.8a) to induce the differentiation of immature thymocytes. The results demonstrate that $CD3^-4^-8^-$ immature thymocytes differentiate through a $CD3^-4^-8^+$ intermediate into more mature stages of cells on the MRL104.8a thymic stromal cells. Thus, our approach provides an *in vitro* model for analyzing the thymic microenvironment required for intrathymic T-cell development.

## MATERIALS AND METHODS

**Thymic Stroma-Derived Cell Clone.** The thymic stroma-derived cell clone utilized (MRL104.8a) was established in our laboratory from long-term liquid-phase cultures of thymic stromal cells from MRL *lpr/lpr* mice (13). As stromal cell controls, a bone marrow stromal cell clone (BSL-35.6) that has been recently established from MRL *lpr/lpr* mouse bone marrow (H. Matsubara and H.F., unpublished data) and a L929 fibroblast cell line were used.

**Reagents and Monoclonal Antibodies.** Recombinant human interleukin 1α (IL-1α), murine IL-2, and murine IL-7 were kindly provided by Dainippon Seiyaku (Osaka), Shionogi Pharmaceutical (Osaka), and DNAX, respectively. The following monoclonal antibodies were used: anti-Lyt-2 (3.155) (obtained from the American Type Culture Collection), anti-L3T4 [GK1.5 (17) and RL172.4 (18)], anti-T3 (2C11) (19), anti-J11d (20), and anti-IL-2 receptor (IL-2R) antibody (7D4) (21). For cell staining, fluorescein isothiocyanate (FITC)-conjugated anti-L3T4 antibody was prepared as described (22). Biotinylated anti-Lyt-2, FITC-conjugated goat anti-hamster immunoglobulin, FITC-conjugated goat anti-rat immunoglobulin, phycoerythrin (PE)-conjugated anti-L3T4 antibodies, and PE-conjugated streptavidin were obtained from Becton Dickinson. FITC-conjugated anti-Thy-1.2 antibody and rat immunoglobulin were also purchased from Biomeda (Foster City, CA) and Cappel Laboratories, respectively.

A semipurified sample of TSTGF was prepared by DEAE-Sephacel chromatography and PBE-94 chromatofocusing as described (15). This semipurified TSTGF sample was free of all activities associated with characterized interleukins and

Abbreviations: FITC, fluorescein isothiocyanate; IL-*x*, interleukin *x*; rIL, recombinant IL; IL-2R, IL-2 receptor; PE, phycoerythrin; TCR, T-cell receptor; TSTGF, thymic stroma-derived T-cell growth factor.

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cytokines, including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, colony-stimulating factors, and interferons (13, 15).

**Culture Medium.** Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum (Armour Pharmaceutical), 50 $\mu$M 2-mercaptoethanol, and gentamycin (50 $\mu$g/ml) was used throughout this study, including the maintenance of the MRL104.8a thymic stromal cell clone.

**Preparation of Double-Negative Thymocyte Population.** C3H/He young adult (3- to 5-week-old) thymocytes were suspended in Hanks' balanced salt solution containing 2% (vol/vol) fetal bovine serum. Antibodies to CD8 (3.155) and CD4 (RL172.4) were added to give predetermined optimum concentrations, and cells were left on ice for 30 min. Cells were pelleted by centrifugation and resuspended at $5 \times 10^7$ cells per ml in rabbit complement (Low Tox-M rabbit complement; Cederlane, Ontario, Canada). Three complement-mediated killing cycles were performed in sequence. Only the double-negative cell preparations that were >98% pure were used in this study. In some experiments, double-negative cells were obtained by sorting cells after two complement-mediated killing cycles by using the FACS IV instrument (Becton Dickinson) according to the procedure described (12). This series of procedures gave >99% purity.

**Immunofluorescence Staining and Flow Microfluorometry.** Cells were stained with FITC-conjugated anti-L3T4 (GK1.5) antibody and with biotinylated anti-Lyt-2 antibody followed by PE-conjugated streptavidin (CD4 vs. CD8). Alternatively, cells were doubly stained by (*i*) anti-T3 (2C11) antibody followed by FITC-conjugated goat anti-hamster immunoglobulin and (*ii*) biotin-conjugated anti-Lyt-2 followed by PE-avidin. Before *ii*, cells were treated with rat immunoglobulin at 1 mg/ml to block cross-reactive antibody sites. Thus *i* and *ii* detect CD3 and CD8, respectively. Cells were also stained with FITC-conjugated anti-Thy-1.2 antibody and then with anti-Lyt-2-biotin, followed by PE-avidin (Thy-1.2 vs. CD8). In the case of staining for CD8 vs. J11d and CD8 vs. IL-2R, the procedure was similar to that performed for CD8 vs. CD3, except that FITC-conjugated goat anti-rat immunoglobulin was used in place of goat anti-hamster immunoglobulin. CD4 was also stained by PE-conjugated anti-L3T4 antibody in combination with staining for CD3 (CD4 vs. CD3). Cells were analyzed for two-color fluorescence with a FACStar (Becton Dickinson) with logarithmic intensity scales. In most cases $10^4$ cells were scored, of which 60–80% were included in the analysis.

## RESULTS

**Generation of a $CD3^-4^-8^+$ Intermediate Thymocyte Population After a 1-Day Culture Period on a MRL104.8a Monolayer.** Double-negative thymocytes were isolated from normal C3H/He thymuses after three killing cycles using anti-L3T4 and anti-Lyt-2 antibodies in the presence of complement. More than 98% of the resulting cells were of the double-negative phenotype. This was also confirmed by staining treated cells with FITC-conjugated goat anti-rat immunoglobulin. These cells were cultured alone or on a monolayer of MRL104.8a thymic stromal cells, BSL-35.6 bone marrow stromal cells, or L929 fibroblasts. The recovery of viable cells was in the range of 4–13% of input, and only a marginal number of cells expressed CD4 and/or CD8 antigens when the cells were cultured alone (Fig. 1*a*). In contrast, a greater percentage of cells (20–35% of input in six experiments) was recovered after culturing for 1 day on the MRL104.8a monolayer, and an appreciable percentage of these cells expressed CD8 but not CD4 antigens (Fig. 1*b*). The induction of CD8 antigens required the MRL104.8a thymic stromal monolayer since the CD8 antigen was not induced on double-negative cells by a bone marrow stromal cell monolayer or an L929 fibroblast monolayer (Fig. 1 *e* and *f*). To determine whether the above $CD8^+$ cells represent a $CD3^-4^-8^+$ intermediate (2, 8–12) or a $CD3^+4^-8^+$ [$\alpha,\beta$ T-cell receptor (TCR)-positive] mature population, the expression of CD3 antigen was also examined. Fig. 1*c* demonstrates that most of $CD4^-8^+$ cells generated expressed no detectable level of CD3. It is also shown that these cells bear the J11d antigen (Fig. 1*d*). These results indicate that the emergence of $CD4^-8^+$ cells is not due to an expansion of mature $CD4^-8^+$ cells present as a contaminant in the double-negative population but is due to the induction of maturing thymocytes not expressing the TCR ($CD3^-4^-8^+$ intermediate).

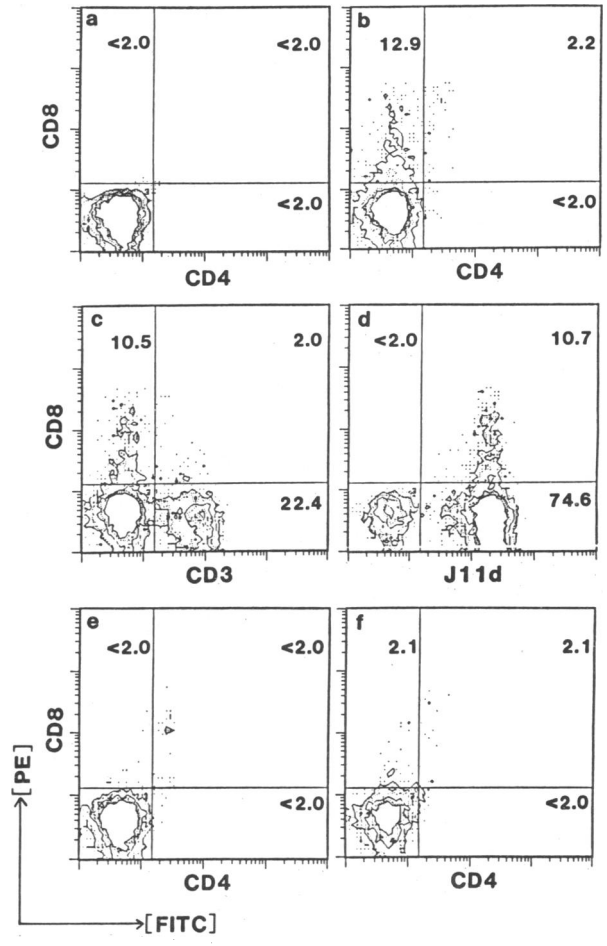


FIG. 1. Generation of a $CD3^-4^-8^+$ intermediate thymocyte population 1 day after culturing on MRL104.8a thymic stromal cells. Double-negative thymocytes ($10^6$ per well) were cultured alone (*a*), on monolayers of MRL104.8a cells (*b*, *c*, and *d*), on monolayers of cells from a bone marrow-derived stromal cell clone (BSL-35.6) (*e*), or on monolayers of L929 cells (*f*) in 24-well culture plates for 24 hr. Thymocytes recovered 1 day after culturing were stained and analyzed for two-color fluorescence. The numbers on the figures are the percentages of cells stained by each of the reagents.

**Induction of a $CD3^-4^-8^+$ Intermediate by MRL104.8a Thymic Stromal Cell-Derived Factor(s).** We next examined whether the expression of CD8 antigen on double-negative thymocytes can be induced by the MRL104.8a cell product, TSTGF. A semipurified TSTGF sample was prepared as described (15). Double-negative thymocytes were cultured with the semipurified TSTGF preparation. Fig. 2 (Exp. 1) shows that culturing double-negative thymocytes with the TSTGF preparation results in the generation of a number of $CD8^+$ cells comparable to that induced by coculture with the MRL104.8a monolayer. Fig. 2 (Exp. 2) also demonstrates that the induction of CD8 antigen was observed when highly purified (>99%) double-negative thymocytes, purified by cell sorting, were used as responding cells.

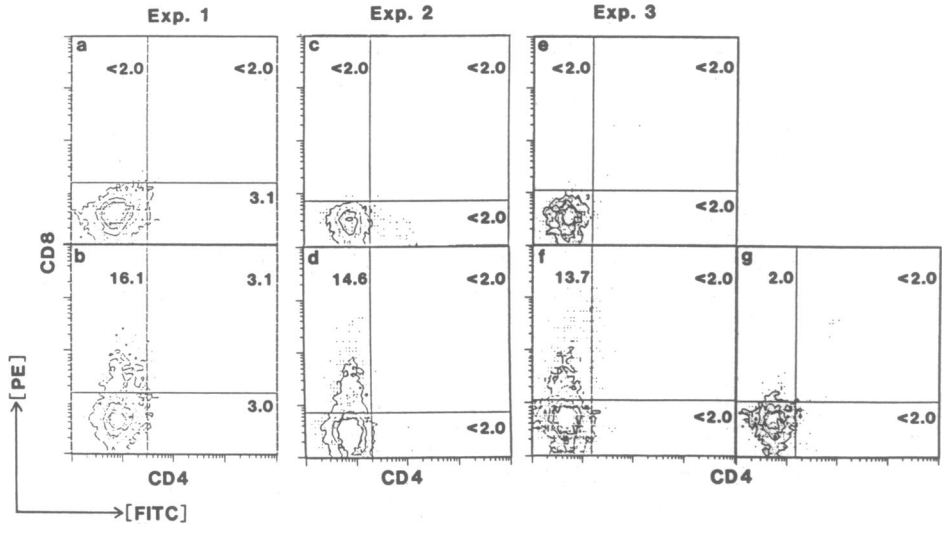


FIG. 2. Generation of a $CD3^-4^-8^+$ intermediate thymocyte population 1 day after culturing in the presence of thymic stroma-derived factor(s). Double-negative thymocytes were obtained after three complement-mediated killing cycles (purity > 98%) (Exps. 1 and 3) or by sorting using a FACS IV (purity > 99%) (Exp. 2) and were cultured for 1 day with either medium (*a*, *c*, and *e*), a TSTGF-containing semipurified MRL104.8a sample (1 unit/ml) (*b*, *d*, and *f*), or rIL-7 (4 units/ml) (*g*). Thymocytes were stained and analyzed for two-color fluorescence.

Previously established lymphokines (IL-1, IL-2, IL-3, IL-4, IL-5, and IL-6) were tested for their capacity to induce CD8 expression on double-negative thymocytes; each cytokine failed to replace the function of TSTGF (data not shown). Since the recently defined IL-7 is produced by bone marrow (23) and thymic stromal cells (24) and contributes to the growth promotion of T- as well as B-lineage cells (25, 26), the capacity of this cytokine had to be carefully examined. The dose of recombinant IL-7 (rIL-7) that produced growth of a T-helper cell clone (15, 24) and a pre-B cell line (24), comparable in magnitude to that obtained by the dose of TSTGF sample used above, was determined. The results of Fig. 2 (Exp. 3) illustrate that IL-7 is unable to induce CD8 expression, in contrast to the capacity of TSTGF to reproducibly induce such expression. These $CD4^-8^+$ cells were also $CD3^-$ and $J11d^+$ (Fig. 3). Thus, taken collectively, the results indicate that the differentiation of $CD3^-4^-8^-$ immature thymocytes toward the CD8 expression is inducible not only by a thymic stromal monolayer but also by its derived soluble factor(s) in the absence of stromal cells.

**Differentiation of Immature Double-Negative Thymocytes Through $CD3^-4^-8^+$ into More Mature Stages.** We extended the culture period of double-negative thymocytes with the TSTGF preparation or with TSTGF-producing MRL104.8a cells. Whereas an insufficient number of viable cells was obtained from >2-day cultures with the TSTGF preparation, an appreciable number of viable lymphocytes (5–15% of input in three experiments) were recovered from 2- or 3-day cultures on the MRL104.8a monolayer. Fig. 4*b* demonstrates that $CD4^+8^+$ cells are present at a high frequency. Fig. 4 *c* and *d* also illustrate that a large proportion but not all of the $CD8^+$ and/or $CD4^+$ cells expresses undetectable levels of CD3 antigens and that some of cells obtained following a 2-day culture express low to moderate levels of CD3 antigens. However, almost all of these cells were "maturing" thymocytes, since they were Thy-$1^{high}$ and IL-$2R^-$, as exemplified by $CD8^+$ populations (Fig. 4 *e* and *f*).

It is known that MRL104.8a cells produce TSTGF, but neither IL-1 nor IL-2 activity is detected in its culture supernatant (13). Since the proliferation of double-negative thymocytes induced by the TSTGF required the presence of IL-1 or IL-2 (16), the costimulatory effect of IL-1 or IL-2 on the differentiation of double-negative cells was investigated. The addition of IL-1 or IL-2 to cultures of MRL104.8a monolayers did not, however, affect the extent of expression of CD8 or CD8 plus CD4 antigens (Fig. 5).

The differentiation of double-negative cells was further promoted when the culture period was extended to 3 days

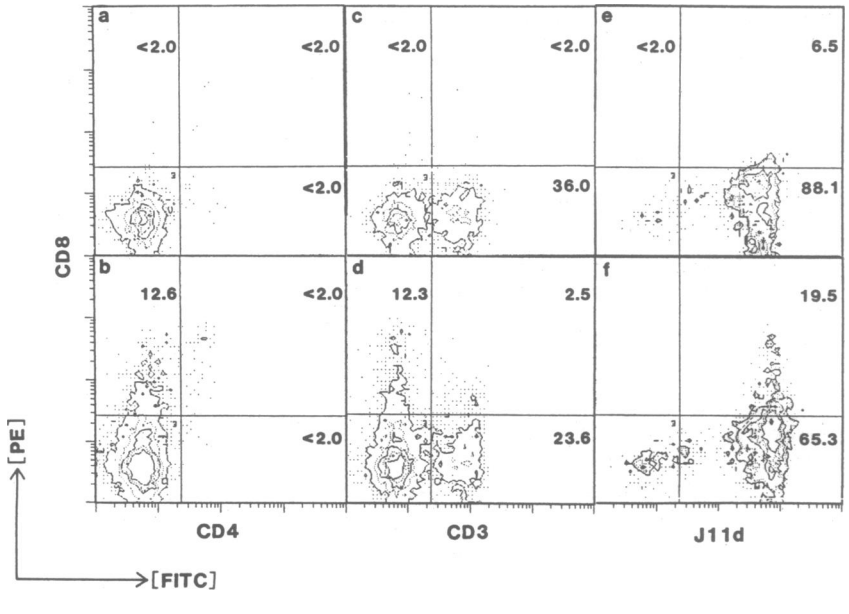


FIG. 3. $CD4^-8^+$ cells induced by thymic stroma-derived factor(s) are $CD3^-$ and $J11d^+$. Double-negative thymocytes were cultured with medium (*a*, *c*, and *e*) or a semipurified TSTGF sample (1 unit/ml) (*b*, *d*, and *f*) for 1 day, stained, and analyzed for two-color fluorescence.

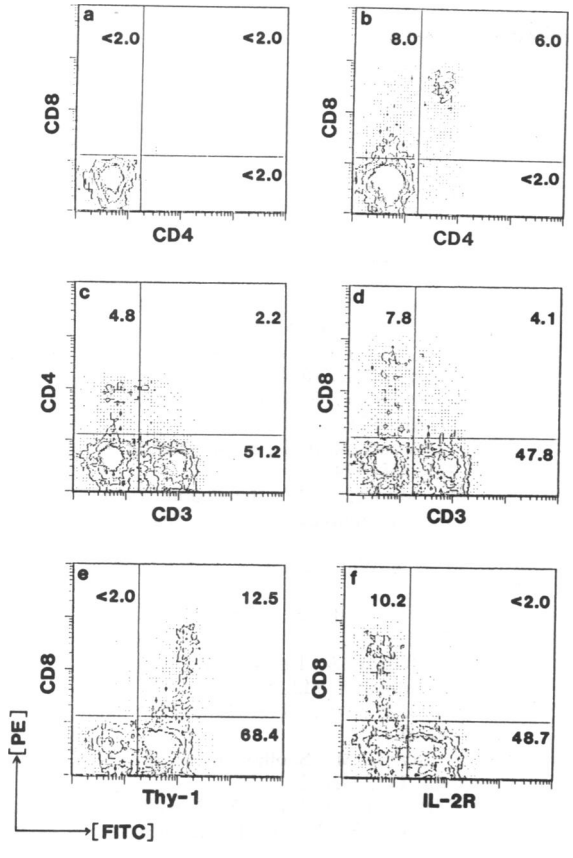


FIG. 4. Induction of $CD3^-4^+8^+$ thymocytes 2 days after culturing on a MRL104.8a monolayer. Double-negative thymocytes were cultured alone (*a*) or on MRL104.8a monolayers (*b–f*) for 2 days, stained, and analyzed for two-color fluorescence.

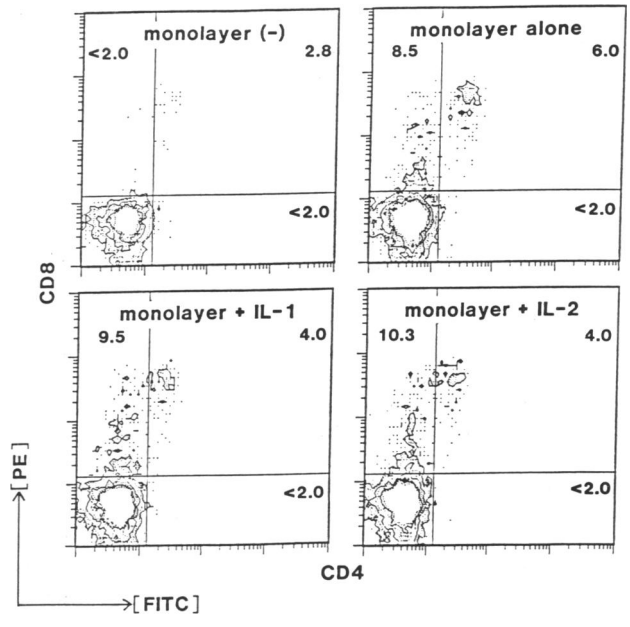


FIG. 5. IL-1 or IL-2 does not influence the induction of CD8 and CD4 antigens. Double-negative thymocytes were cultured on MRL104.8a monolayers in the absence or presence of rIL-1 (10 units/ml) or rIL-2 (10 units/ml).

(Fig. 6). The number of $CD4^-8^+$ cells induced decreased from day 1 to day 3; in contrast, there was an increase in the percentage of $CD4^+8^+$ cells on day 3 relative to the percentage on day 1. Moreover, an appreciable number of $CD4^+8^-$ cells emerged on day 3, and a small number of $CD4^-8^+$ cells still remained at this stage. The differentiation observed on days 2 and 3 was again dependent on the MRL104.8a monolayer; when double-negative cells were cultured for 1 day on bone marrow stromal cells in the presence of the semipurified TSTGF sample, a sufficient number of viable cells were recovered, and comparable percentages (10–14%) of cells to those observed in cultures containing only the TSTGF preparation (Fig. 2) expressed CD8 antigen. However, a small number of viable cells (<2% of input) was obtained from 3-day cultures on the bone marrow stromal cell monolayer, even in the presence of the TSTGF preparation. Moreover, these recovered cells expressed only marginal (<2%) CD8 and/or CD4 antigens. Thus, the MRL104.8a thymic stromal cell monolayer was capable of promoting the differentiation of immature double-negative thymocytes through a $CD3^-4^-8^+$ intermediate into more mature stages.

## DISCUSSION

The process of T-cell development within the thymus is marked by the activation and/or inactivation of a set of genes encoding components of TCRs for antigen as well as other sets of genes whose expression may represent a stage of T-cell differentiation (27). Knowledge has increasingly accumulated to help us understand the elusive lineage pathway between $CD4^-8^-$ thymocytes and their mature progeny (2, 4, 5, 8–12). However, more precise determination of lineage relationships between various immature and mature thymo-

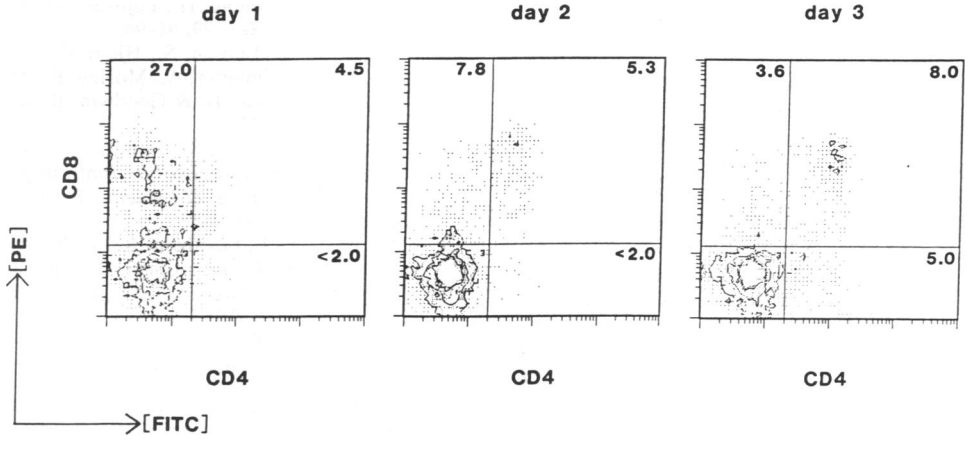


FIG. 6. Differentiation of $CD3^-4^-8^-$ thymocytes through a $CD3^-4^-8^+$ intermediate into more mature stages. Double-negative thymocytes were cultured on MRL104.8a monolayer for 1–3 days.

cyte subsets as well as cellular and molecular mechanisms underlying such an intrathymic maturational process remain to be investigated, especially based on the interaction of maturing thymocytes with thymic stromal components capable of providing the thymic microenvironment.

The major findings to emerge from the present study are that (*i*) the monolayer of our established thymic stromal cell clone is capable of promoting the differentiation of immature double-negative thymocytes through a $CD3^-4^-8^+$ intermediate into more mature stages and (*ii*) the initiation of such differentiation was also inducible by the monolayer-derived soluble factor(s) without requiring direct interaction with stromal cells. In contrast to earlier reports that a proportion of $CD4^-8^-$ cells differentiate into $CD4^+8^+$ cells following overnight culture in the absence of added mitogens or growth factors (28), the differentiation of $CD4^-8^-$ was induced only when double-negative thymocytes were placed on the MRL104.8a thymic stromal cell monolayer. A possibility that this is due to a mere feeder (monolayer) effect was excluded by the fact that the differentiation was not inducible by bone marrow-derived stromal cell or by L929 fibroblast cell monolayers. Thus, this study not only supports the thus far proposed sequence of intrathymic T-cell maturation but also demonstrates the distinct properties of our established thymic stromal cell clone.

The present results should also be discussed in several aspects. First, although the differentiation of double-negative cells into $CD3^-4^-8^+$ cells was reproducibly observed in the presence of the MRL104.8a thymic stromal monolayer or its derived factor(s), some but not all of the double-negative cells were promoted into differentiation. The phenotypic and functional heterogeneity of $CD4^-8^-$ thymocytes have been well established by studies of MacDonald *et al.* (3). Such heterogeneity was demonstrated with respect to differences in cell cycle, phenotypic expression, and *in situ* proliferative status (3). Thus, it is possible to speculate that a subpopulation of double-negative thymocytes is responsible for the differentiation into a $CD3^-4^-8^+$ intermediate.

Second, 3-day culture of double-negative cells (>98% purity) on the MRL104.8a monolayer resulted in the generation of an appreciable percentage of single-positive thymocytes. The emergence of the $CD4^+8^-$ subset subsequent to the appearance of $CD4^+8^+$ cells suggests that some of these $CD4^+8^+$ cells differentiate into the single-positive cells. To exclude a possibility that the generation of this single-positive subset results from the proliferation of single-positive cells contaminating the original responding population, further studies are needed to investigate the maturation of double-negative cells by examining the effects of the addition of anti-class I and/or class II H-2 antibodies to cultures on the generation of double-positive as well as $CD4^+8^-$ cells.

Our results illustrate differentiation of immature thymocytes from young adult mice that is dependent on the specialized thymic stromal monolayer. It has been well accepted that T-cell maturation and thymic selection (positive and negative) require the interaction of maturing thymocytes and thymic stromal components (1–3). In addition to the capacities to promote the growth and differentiation of immature thymocytes, it was shown that the MRL104.8a stromal clone can induce clonal deletion of T cells that is dependent on the stimulation of TCRs with antigen and/or H-2 molecules expressed on its monolayer (29). When combined with the utilization of antibodies against various molecules expressed on thymic stromal cells and defined specificity groups of TCRs, our stromal cells could provide an *in vitro* model for investigating more precisely the maturation and selection events occurring in the thymus.

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