Naoko Nakano,
Richard R. Hardy○ and
Tadamitsu Kishimoto

Division of Immunology, Institute for Molecular and Cellular Biology, Osaka University, Osaka

Identification of intrathymic T progenitor cells by expression of Thy-1, IL 2 receptor and CD3*

Immature (L3T4$^-$/Lyt-2$^-$ "double-negative") thymocytes were separated into several functionally distinct fractions based on their expression of IL 2 receptors, Thy-1 and CD3. The majority (60–70%) of double-negative thymocytes in young adult mice lack detectable IL 2 receptor expression, have high levels of Thy-1 and rapidly "progress" to a L3T4$^+$ or L3T4$^+$/Lyt-2$^+$ stage when cultured for 20 h in simple medium. In contrast, the IL 2 receptor-positive fraction retains the double-negative phenotype for as long as it survives in culture and addition of IL 2 has little or no effect on such cells. IL 2 does generate strong proliferation from a fraction of cells expressing low levels of Thy-1, but not detectable IL 2 receptors. Such culture generates an unusual population of double-negative cells that expresses the pan-B cell molecule B220 and which kill both the NK target cell line YAC-1 and the NK-resistant line EL4. This Thy-1-low fraction includes all of the double-negative thymocytes capable of T cell reconstitution. Thy-1-low fraction could be further separated into two populations with regards to CD3 expression. CD3$^-$ but not CD3$^+$ population could reconstitute mature T cells, indicating that Thy-1-low, IL 2R$^-$ and CD3$^-$ cells are the most enriched population of intrathymic T cell progenitors.

1 Introduction

The process of thymocyte maturation and the generation of mature funcitonal T cells has been intensively studied for many years. A variety of criteria have been employed to discriminate thymocytes at different maturational stages [1]. For several years it has been clear that the most immature cells in the thymus lack Lyt-2 (CD8) and L3T4 (CD4) molecules present on mature helper/inducer or suppressor/cytotoxic T cells, and that at least some of these cells can differentiate *in vivo* [2] or *in vitro* [3]. More recently, it has been reported that many of these "double-negative" thymocytes express high levels of the receptor for IL 2 (IL 2R) [4, 5]. The significance of this expression remains unclear as does the relationship of the IL 2R$^+$ and IL 2R$^-$ fractions of double-negative thymocytes.

We have employed multiparameter fluorescence-activated cell sorter (FACS) analysis and sorting to investigate the maturational relationships between subpopulations of double-negative thymocytes based on the expression of Thy-1, the IL 2R and CD3. We find that one fraction (lacking IL 2R and expressing high levels of Thy-1) spontaneously progresses *in vitro* to the Lyt-2$^+$, L3T4$^+$ stage whereas another (expressing IL 2R) does not. Furthermore, a small portion of the fraction defined by lack of detectable IL 2R (and expressing distinctively low levels of Thy-1) proliferates strongly in the presence of IL 2 yielding a relatively unrestricted cytotoxic cell type. The proliferating cells also express a molecule, B220, not found on most typical T cells but rather predominantly restricted to B and natural killer (NK) lineage cells [6]. Finally, reconstitution studies localize all thymic progenitors within this Thy-1-low population and the study with anti-CD3 demonstrates that CD3$^-$ but not CD3$^+$ cells in this Thy-1-low population are T progenitor cells.

[I 6231]

* This study was supported by a Grant-in-Aid to T. K. from the Ministry of Education, Science and Culture.

Correspondence: Tadamitsu Kishimoto, Institute for Molecular and Cellular Biology, Osaka University, 1–3, Yamada-Oka, Suita, Osaka 565, Japan

Abbreviations: **(r)IL 2:** (recombinant) Interleukin 2 **NK:** Natural killer **IL 2R:** IL 2 receptor **FACS:** Fluorescence-activated cell sorter **PE:** Phycoerythrin **PC:** Phycocyanin

2 Materials and methods

2.1 Production of enriched L3T4$^-$/Lyt-2$^-$ (double-negative) thymocyte fractions

Populations of double-negative thymocytes were prepared by treatment of thymocyte cell suspensions from 3–5-week-old C3H mice with a cocktail of anti-thymocyte antibodies (53-6, Lyt-2; GK1.5, L3T4; 53-7, Ly-1), then washing followed by incubation with a facilitating reagent (MAR18.5, IgG_{2a} anti-rat κ), washing again and then a final incubation with rabbit complement (low-tox-M, Cedarlane, Ontario, Canada). Such treatment lysed 97–99% of thymocytes yielding preparations of approximately 90% pure double-negative thymocytes.

2.2 FACS staining reagents, analysis and sorting

Following treatment of adult thymocytes as described above, or just after isolation of day 16 fetal thymocytes, cells were stained with a cocktail of fluoresceinated reagents reactive with Lyt-2, L3T4, Mac-1 (M1/70) and Ia (10-3.6), phycocyanin (PC)-anti-Thy-1.2 (30H12) and Bi-anti-IL 2R (PC61.5) followed by phycoerythrin (PE)-avidin in a second step. Fluorescein, biotin (Bi) and phycobiliprotein labeling have been described elsewhere [7]. Anti-CD3 hybridoma (145-2C11) cell line was provided by Dr. Bluestone (NIH) and the purified monoclonal antibody was reduced and coupled with maleimide group-introduced PC as reported previously [8]. Double-negative fractions (lacking green staining) were then sorted in terms of Thy-1 and IL 2R (see Fig. 1) using a FACS 440 dual-laser dye-laser cell sorter (Becton Dickinson, Mountain View, CA). For the further fractionation of Thy-1-low cells, adult thymocytes after the treatment described above were stained with the cocktail of the fluoresceinated reagents including anti-IL 2R, Bi-anti-Thy-1 and PC-anti-CD3 followed

0014-2980/87/1111-1567$02.50/0

by PE-avidin in a second step. Fractions were also gated to exclude small debris, aggregated cells and granular cells (by forward and large angle scatter). Multiparameter three-color analysis has been described [9].

2.3 Culture conditions

Sorted cells at 10^5/well were deposited directly into wells of microtiter plates, and the plates were centrifuged and suspended in 100 µl of complete medium (with or without IL2). RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2-mercaptoethanol (5×10^{-5} M) and 10% fetal calf serum was used throughout these experiments; in certain instances, 20–50 ng/ml of recombinant (r)IL2 (Ajinomoto, Tokyo, Japan) was added.

2.4 Proliferation and cytotoxicity assays

To measure proliferation, sorted cells were cultured as indicated for 38 h, 1 µCi = 37 kBq [^{3}H]thymidine was added 14 h prior to harvest. To measure cytotoxicity, indicated target cells were loaded with ^{51}Cr by a 1.5-h incubation. Cells at appropriate ratios were incubated for 4 h in wells of a microtiter plate, then centrifuged and supernatant was harvested and counted in a γ counter.

2.5 Cell transfer

After sorting, cells were washed once, resuspended in RPMI 1640 and injected into female 6–7-week-old AKR mice irradiated 24 h earlier with 700 rds from a Cs source. Recipients were maintained with antibiotics in isolation cages equipped with filter tops.

3 Results and discussion

3.1 IL2R expression divides L3T4$^-$/Lyt-2$^-$ (double-negative) thymocytes into populations at different maturational stages

Double-negative thymoctes were separated into 3 distinct subpopulations on the basis of their Thy-1 and IL2R expressions, (I) IL2R$^-$/Thy-1-high, (II) IL2R$^+$/Thy-1-high and (III) IL2R$^-$/Thy-1-low (Fig. 1a). In young mice a relatively consistent fraction of double-negative cells expressed IL2R. As shown in Fig. 1b, most IL2R$^-$/Thy-1-high cells spontaneously progressed to L3T4$^+$/Lyt-2$^+$ (double-positive) or L3T4$^+$ during a 1–2-day culture. On the other hand, IL2R$^+$ cells did not differentiate into double-positive or single-positive cells during culture, suggesting that IL2R$^+$ cells might be more immature than IL2R$^-$ cells. Fetal IL2R$^-$ cells behaved similarly, whereas fetal IL2R$^+$ differed from those in the 3–5-week animal: such cells did spontaneously progress *in vitro*, but to a lesser extent than fetal IL2R$^-$ cells. IL2R expression correlated well with progression: IL2R$^+$ cells from 3–5-week animals retained this expression while IL2R$^+$ cells from 16-day fetal animals rapidly lost such expression and progressed to double-positive cells (data not shown). Therefore, these results suggest that IL2R$^+$/Thy-1-high cells may mature into L3T4$^+$/Lyt-2$^+$ cells through the stage of IL2R$^-$/Thy-1-high cells.

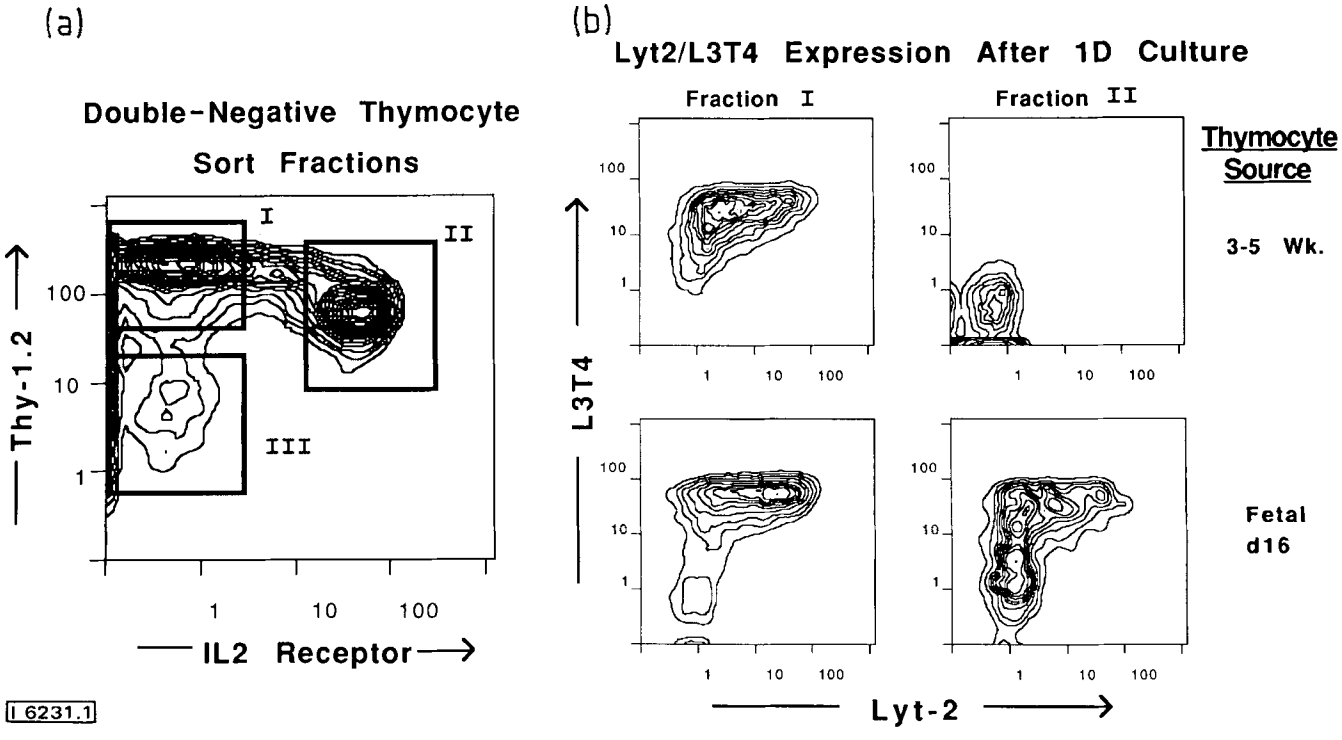

Figure 1. (a) Thy-1 and IL2R expression define 3 major populations of double-negative thymocytes which differ in their capacity to progress *in vitro* to the "double-positive" stage. (b) One × 10^5 cells sorted as IL2R$^-$/Thy-1-high or IL2R$^+$ were cultured for 20 h in complete medium and then analyzed for L3T4/Lyt-2 expression. By employing appropriate combinations of PE and FL reagents, residual staining was eliminated in the analysis (which employed FL/PC staining).

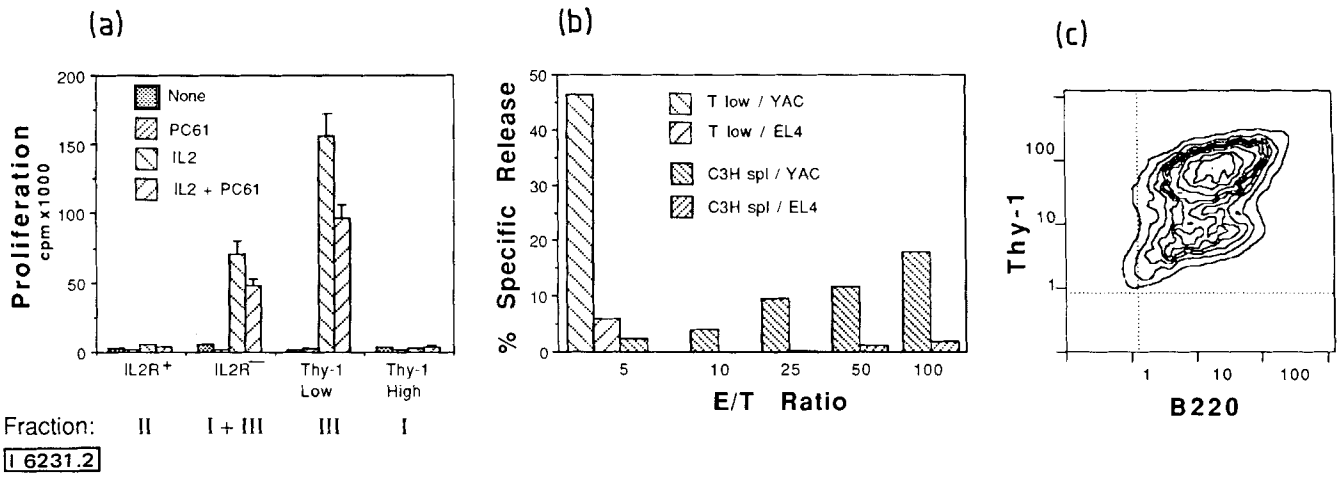

Figure 2. Thy-1-low cells from thymus proliferate in IL2 alone, are cytotoxic effectors and express the B220 surface antigen. (a) For proliferation, 1×10^4 cells were cultured in complete medium with or without 50 ng/ml human rIL2 as described. Twenty μg of purified PC61 antibody was used for blocking of proliferation. (b) For cytotoxicity, Thy-1-low cells were cultured for 6 days in the presence of 50 ng/ml human rIL2. Normal spleen cells were obtained from 1-month-old C3H/He mice and showed NK activity. YAC-1 (NK sensitive) and EL4 (NK resistant) were used at 9×10^3 cells/well. (c) For FACS staining, Thy-1-low cells cultured for 5 days in 50 ng/ml human rIL2 at 1×10^6 cell/ml were analyzed.

3.2 IL2 induces proliferation of Thy-1-low cells in the IL2R$^-$ fraction

Although IL2R was expressed, the IL2R$^+$ fraction (II in Fig. 1a) responded poorly (or not at all) to treatment with IL2 alone, whereas the IL2R$^-$ fraction (I + III in Fig. 1a) responded to treatment with IL2 by rapid cell size increase, failure to express L3T4 or Lyt-2, and eventual (after 2–4 days) proliferation and expression of the B220 cell surface glycoprotein (Fig. 2c). When IL2R$^-$ fraction (II + III in Fig. 1a) was separated into Thy-1-low (III in Fig. 1a) and Thy-1-high (I in Fig. 1a) populations, IL2-induced proliferation was observed only in Thy-1-low cells but not in Thy-1-high cells, and the proliferation was partly blocked by PC61 antibody (Fig. 2a). IL2R expression on this responding fraction remained mostly below detectability by FACS immunofluorescence staining, while IL2R could be readily detected on the nonresponding fraction (data not shown). It was not known whether the responding cells expressed very low levels of IL2R or such cells had only the second 75-kDa polypeptide chain which did not react with anti-IL2R antibody [10]. Partial blocking of proliferation by an antibody to the IL2R (Fig. 2a) suggests that at least some proliferation involves the "Tac" chain, but does not rule out the presence of cells expressing only another 75-kDa IL2R chain.

The unusual cell type derived by culture of IL2R$^-$/Thy-1-low cells with IL2 was characterized by its large size, the expression of Thy-1, B220 and lack of Ly-1. This was a phenotype expressed by at least some NK cells and, interestingly, the cultured Thy-1-low fraction exhibited strong cytotoxic activity against NK target cell line YAC-1 and less activity against NK-resistant line EL4 (Fig. 2b).

3.3 Thy-1-low cells reconstitute T cells in cell transfer

Thymic progenitors are known to be contained within the double-negative population, so we have transferred these 3 cell fracitons from young adult C3H mice (Thy-1.2) into irradiated AKR mice (Thy-1.1). Three-color FACS analysis of the correlated expression of Thy-1.1 (or 1.2) with Lyt-2 and L3T4 demonstrated that most of the reconstitution was derived from cells in the Thy-1-low fraction (Table 1). The reconstitution was very efficient, requiring only 1.5×10^5 cells per animal to give readily detectable repopulation. As has been noted earlier with transfer of Ly-1 dull cells [2], the repopulation was transient, with donor cells gradually accumulating in the more mature populations (Fig. 3).

3.4 Thy-1-low cells without CD3 expression reconstitute T cells

The experiments were carried out in order to study whether Thy-1-low cells express CD3 or not and which population, CD3$^+$ or CD3$^-$, can reconstitute mature L3T4$^+$/Lyt-2$^+$ cells in transfer. As shown in Fig. 4, Thy-1-low cells could be separated into two populations on the basis of CD3 expression; approximately 50% of the cells expressed CD3. Thy-1-low/CD3$^+$ cells or Thy-1-low/CD3$^-$ cells from C3H mice were transferred into irradiated AKR mice. As shown in Table 2, Thy-1-low/CD3$^-$ cells reconstituted the recipients, but Thy-1-low/CD3$^+$ cells could not. This particular experiment was done with a single mouse, but the result was reproducible in repeated experiments.Compared to the result in Table 1, the reconstitution in this experiment was less efficient and the

Table 1. AKR reconstitution by transferred C3H thymocytes

Tissue	Fr. III Thy-1-low	Fr. I Thy-1-high	Fr. II IL2R$^+$
Thymus			
L3T4$^+$Lyt-2$^-$	19.6 (30.1)	16.6 (0.1)	7.9 (0.0)
Lyt-2$^+$L3T4$^-$	6.2 (14.8)	5.7 (0.3)	3.8 (0.0)
L3T4$^+$Lyt-2$^+$	73.3 (45.7)	76.6 (0.1)	87.1 (0.1)
L3T4$^-$Lyt-2$^-$	0.9 (30)	1.0 (0.0)	1.2 (0.0)
Lymph node			
L3T4$^+$Lyt-2$^-$	62.9 (3.6)	64.3 (0.4)	43.8 (0.6)
Lyt-2$^+$L3T4$^-$	15.1 (1.1)	18.3 (0.2)	14.0 (0.2)

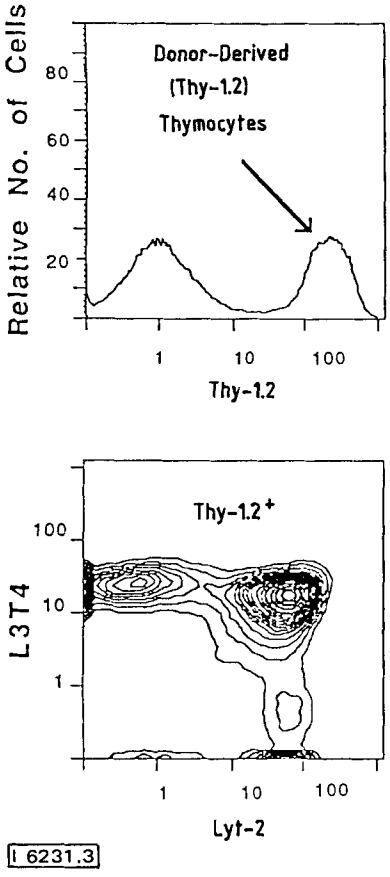

Figure 3. Thy-1-low cells efficiently reconstitute T cells. Double-negative thymocytes (5.5×10^6; prepared as described) from young adult mice were fractionated into the three populations shown in Fig. 1a, and transferred into irradiated mice. Cells (Thy-1-low, 3.3×10^5; IL2R$^+$, 5.5×10^5; IL2R$^-$/Thy-1-high, 8.0×10^5) were injected into 2 mice each and reconstitution was analyzed 3 weeks after sorting. Percentages report total cells for each population and the fraction of this number reconstituted by donor (Thy-1.2) cells is given in parentheses; 10^5 cells were analyzed in each case. Values present representative data which was also found in several different transfers.

Table 2. Reconstitution of AKR thymus with C3H progenitor cells

	Fr. IIIa Thy-1-low CD3$^-$	Fr. IIIb Thy-1-low CD3$^+$
L3T4$^+$Lyt-2$^-$	13.3 (7.3)	13.3 (0.0)
Lyt-2$^+$L3T4$^-$	7.3 (2.5)	10.3 (0.0)
L3T4$^+$Lyt-2$^+$	78.6 (7.9)	75.3 (0.0)
L3T4$^-$Lyt-2$^-$	0.8 (16.3)	1.1 (0.0)

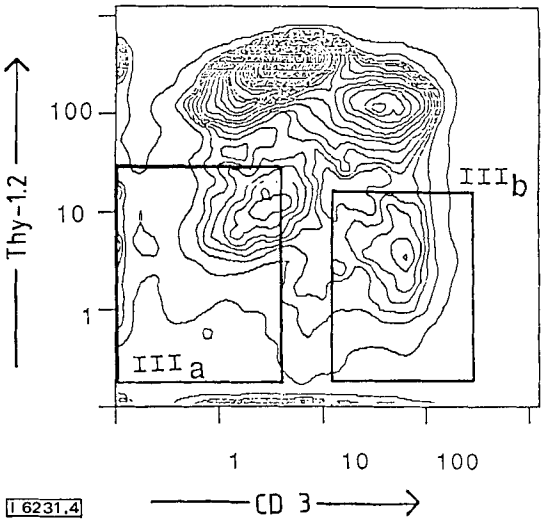

Figure 4. Thy-1-low/CD3$^-$ cells reconstitute T cells. On the basis of the expression of Thy-1 and CD3, double-negative, IL2R$^-$ thymocytes were separated into four subpopulations; Thy-1-high/CD3$^+$, Thy-1-high/CD3$^-$, Thy-1-low/CD3$^+$ and Thy-1-low/CD3$^-$. Thy-1-low/CD3$^-$ (IIIa) and Thy-1-low/CD3$^+$ (IIIb) cells were sorted as shown. Sorted cells (Thy-1-low/CD3$^-$, 2×10^5; Thy-1-low/CD3$^+$, 2×10^5) were injected into 1 mouse each and reconstitution was analyzed 2 weeks after sorting. The method was exactly the same as described in the legend of Fig. 3.

percentage of cells reconstituted in L3T4$^-$/Lyt-2$^-$ fraction was higher than that in L3T4$^+$/Lyt-2$^+$ fraction. This may be due to the experimental condition; the analysis was done 2 weeks after cell transfer in this experiment.

Thus, it appears that Thy-1-low/CD3$^-$ cells represent the most enriched population of intra-thymic T cell progenitors described to date. This population does not contain true pluripotent stem cells, since Thy-1-low cells did not proliferate on an established liver stromal layer that induced strong proliferation of the Thy-1 dull stem cells [11] of fetal liver or bone marrow (data not shown). Human peripheral CD3$^+$ cells that lack both CD4 and CD8, a phenotype also found in thymus [12], possess unrestricted cytotoxic activity and have been shown to express the T cell receptor γ chain in association with another chain, termed "delta" [13]. Murine L3T4$^-$/Lyt-2$^-$ and CD3$^+$ thymocytes have also been demonstrated to express γ chain [14]. It was shown in mice that Thy-1-low immature thymocytes did not have the rearranged β-chain gene of the T cell receptors [15]. Therefore, as reported, Thy-1-low/CD3$^+$ cells in this experiment may express the γ/δ T cell receptor. This could not be confirmed in this study, since enough numbers of the sorted cells were not available for immunoprecipitation. The result that Thy-1-low/CD3$^-$ but not Thy-1-low/CD3$^+$ population reconstituted mature T cells suggests that Thy-1-low/CD3$^+$ population, which presumably express γ/δ receptor and differentiate into NK-like cells with IL2 stimulation, may not be on the maturational process from T progenitors into mature T cells.

Our preliminary study demonstrated that both Thy-1-low/CD3$^+$ and Thy-1-low/CD3$^-$ cells were responsive to IL2 and proliferated into NK-like cells (data not shown). The result may be interpreted in two alternative ways, *i.e.* (a) Thy-1-low/CD3$^-$ T progenitor population still includes two distinct populations or (b) improper growth signals for T progenitors, such as IL2, induce aberrant differentiation of progenitor cells.

We thank Dr. J. Bluestone for providing 145-2C11 cell line and Ms. K. Kubota, J. Mori and M. Kawata for their assistance.

Received June 15, 1987.

4 References

1 Mathieson, B. J. and Fowlkes, B. J., *Immunol. Rev.* 1984. *82:* 141.
2 Fowlkes, B. J., Edison, L., Mathieson, B. J. and Chused, T. M., *J. Exp. Med.* 1985. *162:* 802.
3 Ceredig, R., Sekaly, R. P. and MacDonald, H. R., *Nature* 1983. *303:* 248.
4 Ceredig, R., Lowenthal, J. W., Nabholz, M. and MacDonald, H. R., *Nature* 1985. *314:* 98.
5 Raulet, D. H., *Nature* 1985. *314:* 101.
6 Coffman, R. L., *Immunol. Rev.* 1983. *69:* 5.
7 Hardy, R. R., in Weir, D. M., Herzenberg, L. A., Blackwell, C. C. and Herzenberg, L. A. (Eds.), *Handbook of Experimental Immunology, 4th Edition,* Blackwell Scientific Publication Ltd., Edinburgh 1986, chapter 31.1–31.12.
8 Hardy, R. R., Hayakawa, K., Parks, D. R., Herzenberg, L.A., *Nature* 1983. *302:* 270.
9 Parks, D. R., Hardy, R.R. and Herzenberg, L. A., *Cytometry* 1984. *5:* 159.
10 Teshigawara, K., Wang, H.-M., Kato, K. and Smith, K. A., *J. Exp. Med.* 1987. *165:* 223.
11 Muller-Sieburg, C. E., Whitlock, C. A. and Weissman, I. L., *Cell* 1986. *44:* 653.
12 Bank, I., De Pinho, R. A. Brenner, M. B., Cassimeris, J., Alt, F. W., Chess, L., *Nature* 1986. *322:* 179.
13 Borst, J., Van de Griend, R. J., Van Oostveen, J. W., Ang, S.-L., Melief, C. J., Seidman, J. G. and Bolhuis, R. L. H., *Nature* 1987. *325:* 683.
14 Lew, A. M., Pardoll, D. M., Maloy, W. L., Fowlkes, B. J., Kruisbeek, A., Cheng, S., Germain, R. N., Bluestone, J. A., Schwartz, R. H., Coligan, J. E., *Science* 1986. *234:* 1403.
15 Trowbridge, I. S., Lesley, J., Trotter, J. and Hyman, R., *Nature* 1985. *315:* 666.