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The Earliest Thymic Progenitors in Adults Are Restricted to T, NK, and Dendritic Cell Lineage and Have a Potential to Form More Diverse TCRβ Chains than Fetal Progenitors[1]

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T cell progenitors in the adult thymus (AT) are not well characterized. In the present study, we show that the earliest progenitors in the murine AT are, like those in fetal thymus (FT), unable to generate B or myeloid cells, but still retain the ability to generate NK cells and dendritic cells. However, AT progenitors are distinct from those in FT or fetal liver, in that they are able to produce ~100 times larger numbers of T cells than progenitors in fetuses. Such a capability to generate a large number of T cells was mainly attributed to their potential to extensively proliferate before the TCRβ chain gene rearrangement. We propose that the AT is colonized by T/NK/dendritic cell tripotential progenitors with much higher potential to form diversity in TCRβ chains than FT progenitors. *The Journal of Immunology,* **2005, 175: 5848–5856.**

It is well known that T cells are mainly produced in the thymus from the progenitors that have immigrated from extrathymic organs (1, 2). However, a long range of controversy exists as to the characteristics of progenitors that immigrate into the thymus. In fetuses, prethymic, as well as intrathymic, progenitors have been extensively characterized at the clonal level. Using the multilineage progenitor (MLP)[3] assay, which can determine the developmental potential of individual progenitors toward T, B, and myeloid lineages, it was shown that progenitors generating only T cells but not B or myeloid cells (p-T) are present in fetal thymus (FT) (3) as well as in fetal liver (FL) (4), aorta-gonad-mesonephros region (5–7), and fetal blood (5, 8) of mice. In these studies, common lymphoid progenitors (CLP), or p-TB in our terminology, have never been detected anywhere, and instead myeloid/T and myeloid/B bipotent-type progenitors were constantly detected in FL and the aorta-gonad-mesonephros region. Because a large majority of progenitors in FT are p-T (3), and the p-T in the earliest population of FT are very similar to those in prethymic tissues in that both retain the potential to generate NK and dendritic cells (DC) (9, 10), we have proposed that the major thymic immigrants are the T cell lineage-restricted progenitors (T cell progenitors) that have not yet shut off the NK and DC potential. We recently showed that the earliest thymic progenitors in ontogeny that reside in the mesenchymal region surrounding the thymic epithelial analage, and thus have not encountered thymic epithelial components, are restricted to the T, NK, and DC lineages, providing direct evidence that the progenitors restricted to the T/NK/DC lineage selectively immigrate into the thymus (11).

In the adult animals, papers published so far suggested some difference in the T cell development process from that in fetuses. It has been shown that the earliest cells in adult thymus (AT) generate T, B, NK cells and DC when they were transferred into irradiated recipients (12, 13). These findings have led us to the idea that common progenitors for T, B, NK, and DC lineage cells migrate to the thymus. In these experiments, however, a large number of cells were transferred, and so it is unclear whether the AT comprised a common progenitor for these cells or a mixture of each unipotential progenitor. In 1997, one paper was published (14) that reported the identification of progenitors in bone marrow (BM) that were able to generate T and B cells, which were called the CLP. Although the authors of this article stated that they failed to detect any CLP in AT, the CLP in BM has become to be considered as the most likely candidate that migrates to the thymus. Recently, a novel population was identified in BM as a candidate for thymic immigrants, which are also bipotent for T and B lineage cells (15).

Findings made by the disruption of the Notch-1 gene (16) were interpreted to have confirmed the CLP immigration model. In this study, it was found that the inactivation of Notch-1 results in complete impairment of T cell development with some accelerated B cell development in the thymus. Mice with a compromised Notch signaling pathway by enforced expression of Deltex or Lunatic Fringe as well as disruption of recombination signal binding protein-J were found to show similar phenotypes (17–19). From these findings, it was proposed that Notch signaling controls the cell fate decision of CLP in the thymus. However, as we have pointed out previously (20), the interpretation of the data for Notch-Delta interaction in T cell development may not necessarily require the presence of a CLP stage. Moreover, in all these studies, no CLP has ever been identified in AT. A recent study by Allman et al. (21)

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[3] Abbreviations used in this paper: MLP, multilineage progenitor; AT, adult thymus; BM, bone marrow; CLP, common lymphoid progenitor; DC, dendritic cell; dGuo, deoxyguanosine; DP, double positive; EGFP, enhanced GFP; FL, fetal liver; FT, fetal thymus; HOS, high oxygen submersion; Lin, lineage marker; rm, recombinant murine; SCF, stem cell factor; Tg, transgenic.

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indicated that T cells in AT are mainly derived from a population in BM that is more restricted to the T cell lineage than the CLP population.

There is a hypothesis in the literature that the process of T cell development differs between the fetus and adult (22), which might explain the discrepancy in that the CLP exists only in adult animals. However, no detailed studies have ever been performed to determine the difference in T cell progenitors between fetuses and adults. In the present study, using limiting dilution analyses and clonal analysis of progenitors in AT, we show that T cell progenitors in AT are restricted to the T cell lineage, in that they are unable to give rise to B or myeloid cells. Like T cell progenitors in FT, they retain NK and DC potential. An interesting difference revealed in the present study is that T cell progenitors in AT produce ~100-times larger numbers of T cells than those in FT or FL.

Materials and Methods

Mice

C57BL/6 (B6), B6Ly5.1, and Rag2$^{-/-}$ (B6 background) mice were maintained in our animal facility. Transgenic mice of B6 background carrying enhanced GFP (EGFP Tg mice) (23) were donated by Dr. M. Okabe (Osaka University, Osaka, Japan). Young adult (4-wk-old) B6Ly5.1 mice, Rag2$^{-/-}$ mice, and EGFP Tg mice were used as the progenitor source. Adult B6 mice were used as the recipients of intrathymus transfer of progenitors, and B6 fetuses were used as the source of FT lobes in organ culture experiments.

Antibodies

The following Abs were used: anti-CD4 (GK1.5; Caltag Laboratories), anti-CD8 (YTS169.4; Caltag Laboratories), anti-CD3ϵ (145-2C11; BD Pharmingen), anti-Thy1.2 (53-2.1; BD Pharmingen), anti-Mac-1 (M1/70; Caltag Laboratories), anti-Gr-1 (RB6-8C5; Caltag Laboratories), anti-B220 (RA-6B2; Caltag Laboratories), anti-NK1.1 (PK136; BD Pharmingen), anti-CD25 (PC61; Caltag Laboratories), anti-FcR (2.4G2; BD Pharmingen), anti-CD44 (IM7; BD Pharmingen), anti-c-*kit* (2B8; BD Pharmingen), TER-119 (established in our laboratory; Ref. 24), and anti-Ly5.1 (A20-1.7; donated by Dr. Y. Saga, Banyu Seiyaku, Tokyo, Japan). Anti-IL-7R (A7R34) and anti-IgM (1B4B1) were purchased from eBioscience.

Growth factors

Recombinant murine (rm) stem cell factor (SCF) (Genzyme), rm IL-3 (Genzyme), rm IL-7 (Genzyme), rm IL-2 (Genzyme), and rm Flt-3L (Genzyme) were used.

Enrichment of progenitor cells

Single-cell suspensions of AT cells were prepared from young adult B6 mice. Cells were then treated twice with rabbit complement (Low-Tox-M rabbit complement; Cedarlane Laboratories) in the presence of anti-CD8 (3.155) and anti-CD3 (145-2C11) mAb for 30 min at 37°C. The cells surviving the complement killing were stained with FITC-anti-lineage markers (Lin; TER-119, anti-Mac-1, anti-Gr-1, anti-B220, and anti-NK1.1), FITC-anti-CD4, FITC-anti-CD8, FITC-anti-CD3ϵ, PE-anti-CD25, and allophycocyanin-anti-c-*kit*. For AT cells from Rag2$^{-/-}$ mice, the step of complement killing was omitted, but stained in the same way as above. In case of staining AT cells of EGFP Tg mice, biotinylated mAb for anti-Lin, anti-CD4, anti-CD8, and anti-CD3 were used as primary reagents, and CyChrome-avidin (BD Pharmingen) was used as secondary reagent in combination with PE-anti-CD25 and allophycocyanin-anti-c-*kit*. The procedure for preparation of progenitor enriched populations from FT and FL has been described elsewhere (8). BM cells were obtained from femurs and tibias of young adult mice and stained with FITC-anti-Lin, allophycocyanin-anti-c-*kit*, and PE-anti-IL-7R. Lin$^-$c-*kit*$^+$IL-7R$^+$ cells were used as the progenitor source. A FACSVantage was used for analysis and sorting.

Coculture with a deoxyguanosine (dGuo)-treated FT lobe under high oxygen submersion (HOS) condition

The basic procedure of FT organ culture under HOS conditions has been described previously (4). Briefly, single dGuo-treated FT lobes were placed into the wells of a 96-well V-bottom plate, to which progenitors were added. The plates were sealed in a plastic bag and the air inside was replaced by a gas mixture (70% O_2, 25% N_2, and 5% CO_2). The plastic bag was incubated at 37°C. The cultures were maintained in RPMI 1640 medium supplemented with 10% FCS and a mixture of growth factors. The combination of growth factors differs depending on the aim of the assay. Half of the medium was replaced with fresh medium every 5 days.

Assessment of the potential of individual progenitors to proliferate before the TCRβ chain gene rearrangement (pre-β-rearrangement proliferation potential)

Two experimental methods were used to evaluate the pre-β-rearrangement proliferation of progenitors, which have been detailed in our previous paper (25). The first one is to culture single progenitor cells from Rag2$^{-/-}$ mice with a dGuo-treated lobe. Cells grown in each lobe were harvested on days 14 and 21 of culture for AT and BM progenitors, respectively, and c-*kit*$^-$CD25$^+$ cells were enumerated.

In determining the pre-β-rearrangement proliferation of progenitors from normal mice, single cells were cultured with a dGuo-treated lobe for 21 days. Genomic DNA extracted from cells generated in each well (1000 cells equivalent) was PCR amplified using primers: Dβ1, 5′-TTATCTG GTGGTTTCTTCCAGC-3′; Dβ2, 5′-GCACCTGTGGGGAAGAAACT-3′; Jβ1.5, 5′-CAGAGTTCCATTTCAGAACCTAGC-3′; Jβ1.6, 5′-GG TAGAAAGGTAGAGGGTTCCAGA-3′; Jβ2.6, 5′- TGAGAGCTGTCTC CTACTATCGATT-3′. The reaction volume was 20 μl containing 5 μl of the cell extract (equivalent to 1000 cells), 1.5 μl of 10× PCR buffer, 0.16 μl of 25 mM dNTPs, 0.4 μl of each primer (10 mM), and 0.6 U of *Taq* polymerase. Thermocycling conditions were as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C, and finally 10 min at 72°C. Resulting products were electrophoresed through an agarose gel and stained with ethidium bromide. The extent of pre-β-rearrangement proliferation was calculated as described previously (25).

Limiting dilution analysis for progenitors having a potential to generate T, B, or myeloid cells

To determine the frequency of progenitors with T cell potential, limiting numbers of cells from the c-*kit*$^+$CD25$^-$ population of AT were cocultured with a dGuo-treated lobe. After 14 days of culture, cells were harvested from each lobe, stained with anti-Ly5.1, anti-Thy-1, and anti-B220, or with anti-Ly5.1, anti-CD4, and anti-CD8, and analyzed with a flow cytometer. The proportions of negative lobes were scored. The inoculum that produces 37% negative recipients is expected to contain one progenitor cell (26).

In the limiting dilution analysis for progenitors with B cell or myeloid potential, a monolayer of the stromal cell lines TSt-4 or PA6, respectively, was used. The culture medium was the same as that used in the HOS culture system except that the FCS concentration was 5%. TSt-4 and PA6 cells were grown as monolayers in a 96-well flat-bottom plate, to which a limiting number of cells was added. After 10 days of culture, cells in wells showing cell growth were harvested by trypsin treatment and examined for the expression of B220 and IgM for B cells and the expression of Mac-1 and Gr-1 for myeloid cells. The frequency of progenitors having B cell and myeloid potential was determined by the same manner as used in the T cell progenitor frequency.

Single-cell cultures for detection of T, B, and myeloid potential, and for detection of T, NK, and DC potential

The procedure of the MLP assay has been described previously (4). Briefly, single cells (B6Ly5.1) were picked up with a micropipet under direct microscopic visualization, and cultured with a dGuo-lobe (B6) in each well of a V-bottom 96-well plate for 14 days. Culture medium is supplemented with rm SCF (10 ng/ml), rm IL-3 (3 ng/ml), and rm IL-7 (5 ng/ml) to support the growth of B and myeloid cells in addition to T cells. After 10 days of culture, cells were harvested from each well. Details for judgment of progenitor types have been described previously (4).

Assessment of NK and DC potential of T cell progenitors was performed as described previously (10). EGFP Tg mice were used as the source of FT progenitors. The culture medium was supplemented with SCF (10 ng/ml), IL-2 (25 U/ml), IL-7 (5 ng/ml), and Flt-3 ligand (10 ng/ml) (10). Cells were recovered on day 10 of the culture, and one-fifth of the recovered cells were stained with anti-CD3 and anti-NK1.1 for flow cytometric analysis to examine T and NK cell generation. The remaining four-fifths of the cells were transferred to the TSt-4 monolayer culture. After 4 days of culture, the plate was examined under a fluorescence microscope. Cells with a dendritic morphology showing green fluorescence were regarded as DC derived from the cultured progenitor cell.

Intrathymus cell transfer experiment

Intrathymic injections of sorted c-*kit*$^+$CD25$^-$ thymocytes were performed as described previously (27). Female B6 (Ly5.2) mice (8 wk old) were

irradiated with 8 Gy and reconstituted with i.v. injection of 10^7 syngeneic BM cells. Lin$^-$c-*kit*$^+$CD25$^-$ AT cells (500 cells) or c-*kit*$^+$CD25$^-$ FT cells (500 cells) were injected into the thymic lobes of recipient mice. Thymocytes were harvested from recipient mice 4–27 days after injection, and were stained with anti-Ly5.1, CD4, CD8, for analysis with a flow cytometer.

Results

AT progenitors produce a larger number of T cells than FT progenitors

For isolating early thymic progenitors, we used c-*kit* vs CD25 staining instead of the commonly used CD44 vs CD25 staining. This is because nearly two-thirds of the Lin$^-$CD44$^+$CD25$^-$ cells in AT are c-*kit*$^-$ (28) and the progenitor activity of such CD44$^+$CD25$^-$c-*kit*$^-$ cells in AT was very low, if any (our unpublished observation). Approximately 2% of AT cells that survived the treatment with anti-CD8 and anti-CD3 plus complement were subsequently three-color stained with FITC-anti-Lin, FITC-anti-CD4, FITC-anti-CD8, FITC-anti-CD3ϵ, PE-anti-CD25, and allophycocyanin-anti-c-*kit*. Proportions of c-*kit*$^+$CD25$^-$ cells and c-*kit*$^+$CD25$^+$ cells, which represent the most immature stage and the next stage, were 0.007% and 0.014% of whole AT cells, respectively (Fig. 1*A*). These early cells exhibit a germline configuration for both the TCRβ and TCRγ chain genes (Fig. 1*B*), confirming the previous finding (28).

We first compared the time course of T cell generation from AT and FT progenitors. c-*kit*$^+$CD25$^-$ cells from AT or FT (100 cells each per lobe) of Ly5.1 mice were cocultured with a dGuo-treated FT lobe (Ly5.2), and the cells grown in the wells were harvested periodically for analysis with a flow cytometer. The numbers of CD4$^+$CD8$^+$ (double-positive (DP)) cells are plotted in Fig. 1*C*. For comparison, generation of DP cells from FL Lin$^-$c-*kit*$^+$IL-7R$^+$ cells and BM Lin$^-$c-*kit*$^+$IL-7R$^+$ cells was also examined. It is indicated that a 3- to 4-day longer period is required for AT progenitors than FT progenitors to produce T cells. Moreover, the latent period for AT progenitors was longer than that for FL progenitors, which evidently represent the prethymic T cell progenitors (4–6).

Because it is possible that the longer latent period required for AT progenitors is due to the inappropriateness of the FT environment for AT progenitors, we next examined the T cell producing ability of these progenitors in the AT environment by directly injecting them (500 cells per mouse) into the thymus of irradiated recipients (Ly5.2). The numbers of Ly5.1$^+$ DP cells generated in the thymus are plotted in Fig. 1*D*. Again the latent period for the T cell generation from AT progenitors is longer than that from FT progenitors, although the difference seems slightly smaller than that seen in the FT organ culture system. In contrast, it is of particular interest that the number of T cells produced by AT progenitors is much larger (~100 times) than that by FT progenitors.

Extensive proliferation of AT progenitors before the TCRβ chain gene rearrangement

The ability of AT progenitors for producing a large number of T cells could be attributable to the higher capability in proliferation of AT progenitors than FT progenitors. As we have reported previously, the extent of proliferation of T cell progenitors can be assessed by enumerating the CD44$^-$CD25$^+$ cells generated in single-cell cultures of progenitors from Rag2$^{-/-}$ mice with a dGuo-lobe, which show a growth arrest at the stage of TCRβ chain gene rearrangement (29).

Virtually all Rag2$^{-/-}$ thymocytes are CD3$^-$CD4$^-$CD8$^-$ (28), and the c-*kit* vs CD25 profiles are as shown in Fig. 2*A*. A total of 40 c-*kit*$^+$CD25$^-$ cells were individually cultured with a dGuo-treated FT lobe, and cell growth was seen in 15 wells. Fig. 2*B* shows representative flow cytometric profiles of cells generated from a single c-*kit*$^+$CD25$^-$ progenitor. The numbers of CD25$^+$ cells per clone are scored in Fig. 2*C* (filled columns), together with

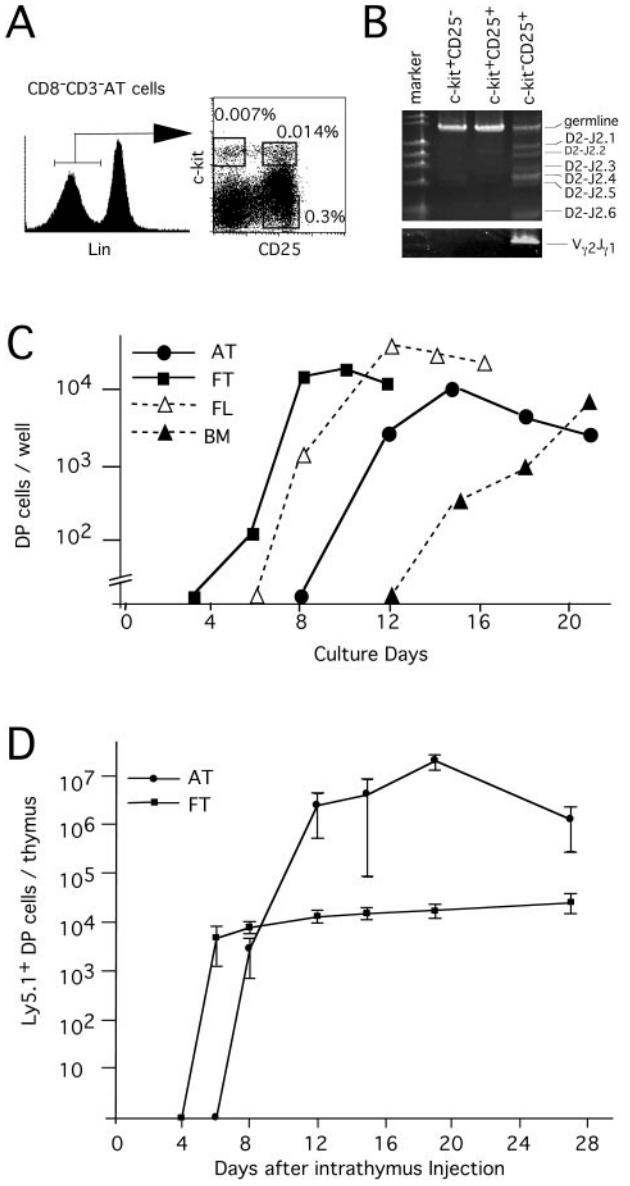

FIGURE 1. AT progenitors require a longer latent period in T cell generation and produce a larger number of T cells than FT progenitors. *A*, Subpopulations of triple-negative cells in AT determined by c-*kit* and CD25. Percentages shown in the figure represent those of cells in the gated areas against whole AT cells. *B*, Absence of rearrangement for TCRβ or TCRγ gene in c-*kit*$^+$CD25$^-$ as well as c-*kit*$^+$CD25$^+$ AT cells. Genomic DNA from 1000 cells was analyzed by PCR. *C*, c-*kit*$^+$CD25$^-$ cells from AT and FT (Ly5.1 mice) were cocultured (100 cells per well) with a dGuo-treated lobe (B6 mice). Cells were recovered various days after cultivation, counted, and examined for the expression of Ly5.1, CD4, and CD8. The geometric mean numbers of Ly5.1$^+$CD4$^+$CD8$^+$ (DP) cells from five wells were scored. SD are not shown, but in most cases they were within the range of symbols. For comparison, T cell generation from FL Lin$^-$c-*kit*$^+$IL-7R$^+$ cells (100 cells) and BM Lin$^-$c-*kit*$^+$IL-7R$^+$ cells (100 cells) was examined similarly. Accumulated data from three independent experiments are shown. *D*, c-*kit*$^+$CD25$^-$ cells from AT and FT of B6Ly5.1 mice were injected (500 cells per mouse) into the thymus of irradiated mice (Ly5.2). Various days later, mice were killed and the recovered thymus cells were examined for the expression of Ly5.1, CD4, and CD8. The geometric mean number ± SD of Ly5.1$^+$ DP cells from three to five mice are scored. Accumulated data from four independent experiments are shown.

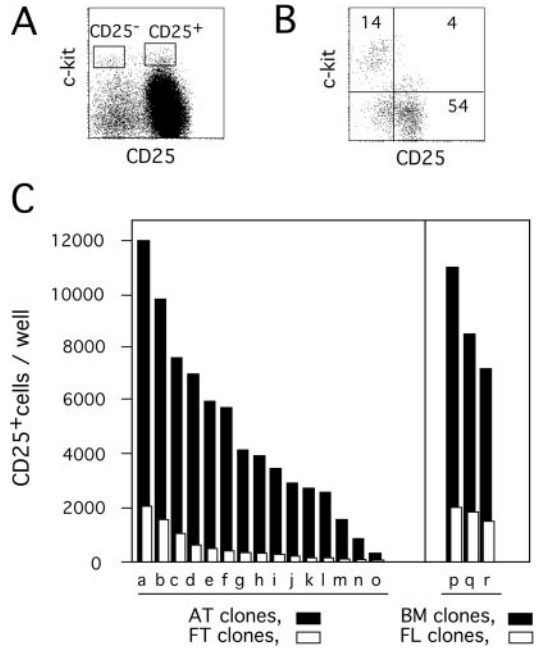

FIGURE 2. Proliferation of c-*kit*$^+$CD25$^-$ AT progenitors from Rag2$^{-/-}$ mice in dGuo-treated FT lobes. *A*, c-*kit* vs CD25 expression profiles on Rag2$^{-/-}$ AT cells. *B*, A representative sample of cells generated from a single c-*kit*$^+$CD25$^-$Rag2$^{-/-}$ AT progenitor in an FT organ culture. The culture period was 14 days. *C*, Numbers of CD25$^+$ cells generated from individual Rag2$^{-/-}$ AT progenitors. A total of 40 c-*kit*$^+$CD25$^-$ AT cells from Rag2$^{-/-}$ mice (4-wk-old) were individually cultured with a dGuo-treated FT lobe for 14 days. Recovered cells were counted and examined for c-*kit* vs CD25 expression. Numbers of CD25$^+$ cells generated from each AT progenitor are shown with filled columns. Open columns represent the numbers of CD25$^+$ cells generated from single FT and FL progenitors from 12-day postcoitus fetuses (data from Ref. 25). For comparison, numbers of CD25$^+$ cells generated from single BM as well as FL T cell progenitors are also shown. In the case of BM progenitors, the culture period was 21 days. Representative results of three experiments are shown.

the data of three individual progenitors in c-*kit*$^+$IL-7R$^+$ adult BM cells, which are able to give rise to T cells, from Rag2$^{-/-}$ mice. For comparison, previous data on the size of clones derived from single CD44$^+$CD25$^-$ FT and c-*kit*$^+$IL-7R$^+$ FL progenitors (25) are also shown with open columns. The results indicated that the large majority of c-*kit*$^+$CD25$^-$ AT cells produced >2000 CD25$^+$ cells, and that the levels of expansion of AT progenitors were much higher than that of FT progenitors. Expansion of some AT progenitors (clones a–d) is comparable to that of prethymic progenitors in BM. The heterogeneity of proliferative potential within the c-*kit*$^+$CD25$^-$ population of AT may represent the hierarchy in developmental stages of these progenitors. The mean number of CD25$^+$ cells generated from a single Rag2$^{-/-}$ DN2 cell was estimated to be 30 (Table I), which did not differ so much from that of a single FT cell (25), indicating that the difference in expansion size of a c-*kit*$^+$CD25$^-$ progenitor between AT and FT is mainly attributed to the expansion rate of progenitors at the c-*kit*$^+$CD25$^-$ stage.

The extent of the pre-β-rearrangement proliferation can also be estimated by examining the D-J rearrangement profiles in T cells derived from single progenitors of normal mice (25). The procedure of single-cell culture and locations of primers used for PCR are illustrated in Fig. 3, *A* and *B*, respectively. c-*kit*$^+$CD25$^-$, c-*kit*$^+$CD25$^+$, and c-*kit*$^-$CD25$^+$ populations of AT were used as the progenitor source. Profiles of DJ-rearranged constructs visualized by PCR are shown in Fig. 3, *C–E*. Virtually all possible DJ constructs are detected in c-*kit*$^+$CD25$^-$ clones (Fig. 3, *lanes a–g*), as in the case of BM clones (single-cell cultures of c-*kit*$^+$IL-7R$^+$ BM cells). The number of bands is significantly reduced in c-*kit*$^+$CD25$^+$ clones (Fig. 3, *lanes h–t*), and only a few bands (zero to two bands) are seen in c-*kit*$^-$CD25$^+$ clones (*lanes u–x*). The extent of pre-β-rearrangement proliferation was evaluated by using these DJ rearrangement data based on the simulation of progenitor proliferation and TCRβ gene rearrangement (25). The mean pre-β-rearrangement proliferation of progenitors in c-*kit*$^+$CD25$^-$, c-*kit*$^+$CD25$^+$, and c-*kit*$^-$CD25$^+$ populations was estimated as >400, 10.8, and 3.6, respectively (Table I).

Table I. *Pre-β-rearrangement proliferation of T cell progenitors in subpopulations of early AT cells*

Progenitor Source	Number of CD25$^+$ Cells Generated from a Single Rag2$^{-/-}$ Thymic Progenitor	Expansion of Progenitors (estimated by D-J signature analysis)
c-kit$^+$CD25$^-$	4000 (200–12000)[a]	>400[b]
c-kit$^+$CD25$^+$	30[c]	10.8
c-kit$^-$CD25$^+$	ND	3.6

[a] The data are from Fig. 2. Numbers in parentheses represent the range of values.
[b] The expansion of progenitors was estimated from the data shown in Fig. 3, based on the calculation detailed in our previous paper (25).
[c] Mean number of progeny cells was estimated by culturing 10, 30, and 100 c-kit$^+$CD25$^+$Rag2$^{-/-}$ AT cells with a dGuo-treated FT lobe.

Failure of AT progenitors to give rise to B cells

The aforementioned findings for AT progenitors that they require a longer latent period in T cell production and they produce a larger number of T cells suggested that AT progenitors are more immature than FT progenitors. Thus a possibility may exist that they retain multipotent nature. We examined the individual cells in the c-*kit*$^+$CD25$^-$ population of AT with the MLP assay, with which progenitors can be classified into those generating all T, B, and myeloid lineage cells (p-MTB), those generating two lineage cells (p-MT, p-MB, p-TB), and those generating only one lineage cell (p-M, p-T, p-B). We found that 57 cells among 300 cells were p-T, 2 cells were p-M, but no other type of progenitors were detected (Fig. 4*A, right panel*). As control groups, we examined the Lin$^-$c-*kit*$^+$FcR$^{-/low}$ BM cells, in which the most immature progenitors are enriched, and Lin$^-$c-*kit*^{low}IL-7R$^+$ BM cells, which were reported to contain the CLP. Among Lin$^-$c-*kit*$^+$FcR$^{-/low}$ cells, various types of progenitors except for p-TB were seen. In contrast, the majority of Lin$^-$c-*kit*^{low}IL-7R$^+$ cells are p-T and p-B, with p-B being very dominant. Small numbers of p-MTB, p-MT, p-TB, and p-M were also detected in this population. Of note, one p-TB was detected in this population, which has never been detected in fetal progenitors. However, as we have discussed previously (6), the detection of progenitors with limited potentials does not necessarily mean that such a commitment status really exists, because some progenitors may fail to fully express their original potential. So, we speculate that this p-TB has actually been a multipotent progenitor for T, B, and myeloid lineages.

It previously has been shown that myeloid and B cell progenitors exist in AT (12, 13, 30, 31). The failure in detecting p-B in the above experiments may indicate that their frequency is too low to be included in the 300 cells examined. Therefore, we investigated the frequency of myeloid, B, and also T cell progenitors by limiting dilution analysis, where we cultured serially reduced numbers of c-*kit*$^+$CD25$^-$ AT cells from 4-wk-old mice on PA6 and TSt-4 monolayers. Limiting dilution analyses for T cell progenitors was

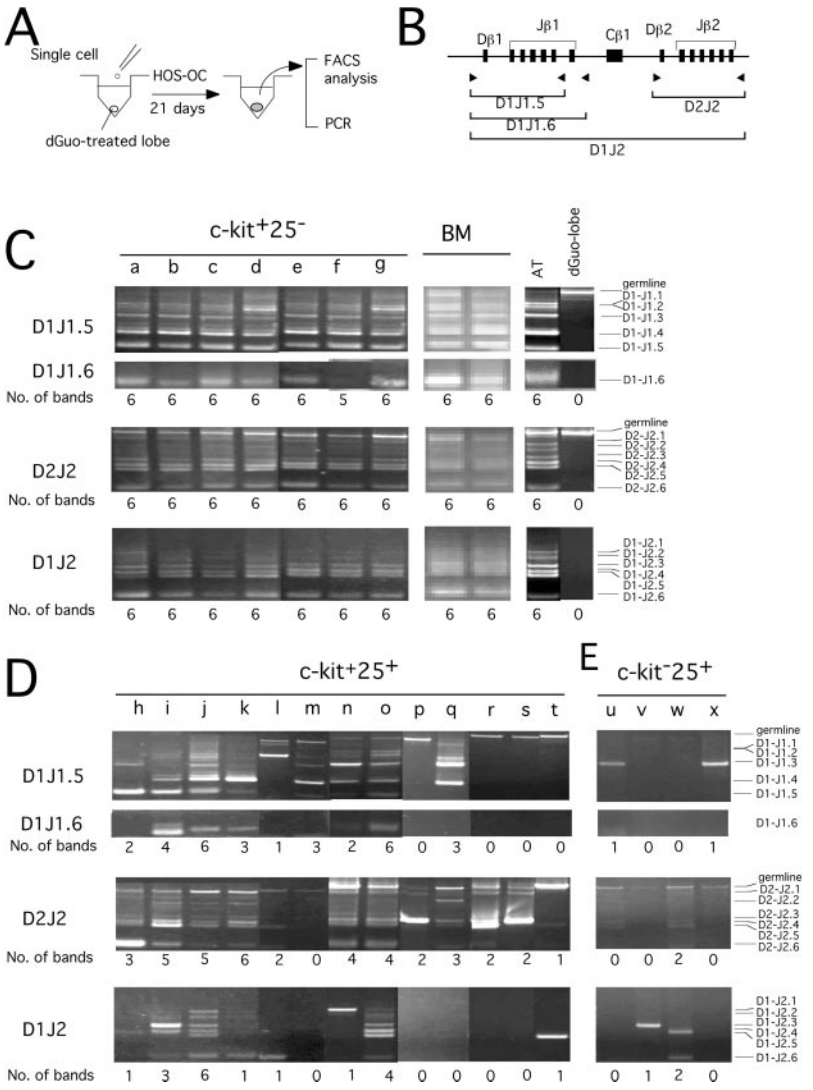

FIGURE 3. Pre-β-rearrangement proliferation of individual progenitors in three subsets of $CD4^-CD8^-CD3^-$ AT cells. *A*, Procedure of the clonal culture. Single cells of c-*kit*$^+$CD25$^-$, c-*kit*$^+$CD25$^+$, and c-*kit*$^-$CD25$^+$ populations were cultured under HOS conditions for 21 days together with a dGuo-treated FT lobe. *B*, Locations of primers used for PCR amplification are indicated by arrowheads. *C–E*, D-J rearrangement profiles in T cells derived from single AT progenitors. Data of prethymic progenitors in BM (Lin$^-$c-*kit*$^+$IL-7R$^+$ cells) are also shown. PCR products amplified covering D1–J1, D2–J2, and D1–J2 regions of TCRβ gene were electrophoresed through an agarose gel and stained with ethidium bromide. As negative and positive controls, a dGuo-treated lobe cultured without seeding progenitors and unfractionated AT cells, respectively, were used. The expansion levels of T cell progenitors estimated from these data are shown in Table I.

also performed. The frequency of T cell progenitors was approximately one of six (Fig. 4*B*), conforming to the results of the MLP assay. In contrast, the B cell progenitor frequency was only 1 of 450 (Fig. 4*C*). The B cell progenitor frequency in AT decreases along with the age, the frequency being <1 of 1000 in c-*kit*$^+$CD25$^-$ AT cells of 10-wk-old mice (data not shown). These results indicate that an overwhelming majority of progenitors with T cell potential in AT have no B cell potential. The frequency of myeloid progenitors among c-*kit*$^+$CD25$^-$ cells was $\sim$1 of 60 (Fig. 4*D*).

NK and DC potentials of AT progenitors

We have previously shown that the early T cell progenitors in FT retain NK and DC potentials, and they shut off these potentials with the progression of their thymic stage (10). The potential of progenitors for NK and DC lineages in addition to the T cell lineage was examined by culturing individual progenitors from EGFP Tg mice in a modified FT organ culture system as shown in Fig. 5*A*. Representative flow cytometric profiles for T/NK markers of cells generated from three types of progenitors are shown in Fig. 5*B*. A photomicrograph of DC grown on a stromal cell monolayer is shown in Fig. 5*A*. Among 42 c-*kit*$^+$CD25$^-$ AT cells examined, 13 generated both T and NK cells (Fig. 5*C*). Six clones generated only T cells, and two clones generated only NK cells. DC generation was observed in 6 of 13 clones showing T/NK potential and 4 of 6 clones showing T but not NK cell potential. Two NK-restricted clones do not show any DC potential. A considerable number of T/NK-type progenitors still exist in the c-*kit*$^+$CD25$^+$ population, although T cell lineage-restricted ones represent the majority. Some of these T/NK as well as T progenitors were found to retain DC potential. These results indicated that the process of commitment for T, NK, and DC lineages of the earliest progenitors in AT are quite similar to that in FT (10).

Discussion

The possibility has been proposed that the process of T cell development differs between adults and fetuses (22). However, no exact comparative studies on the characteristics of thymic progenitors had ever been performed. The present study is the first to characterize the T cell progenitors in AT in comparison with those in FT as well as in adult BM. Results of the limiting dilution analysis for progenitors with T, B, or myeloid potential and the MLP assay indicated that a large majority of AT progenitors are restricted to the T cell lineage in that they are unable to give rise to B or myeloid cells (Fig. 4).

With a new clonal assay system effective in determining the potential of a progenitor toward T, NK, and DC, it was further shown that a majority of AT progenitors are tripotential for these lineages, and that the NK and DC potentials are shut off with the

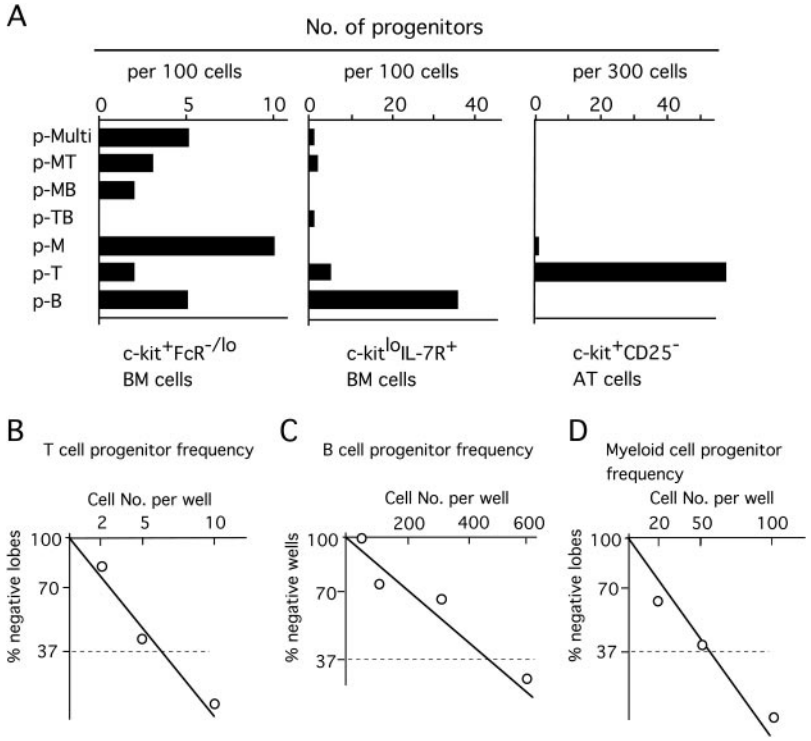

FIGURE 4. Cells in the earliest population of AT are restricted to the T cell lineage. *A*, A total of 300 cells in the c-*kit*$^+$CD25$^-$ population of AT were individually examined with the MLP assay, with which progenitors can be classified into those generating all T, B, and myeloid lineage cells (p-MTB), those generating two lineage cells (p-MT, p-MB, p-TB), and those generating only one lineage cells (p-M, p-T, p-B). As controls, cells in the Lin$^-$c-*kit*$^+$FcR$^{-/low}$ (100 cells) and Lin$^-$c-*kit*$^+$IL-7R$^+$ (100 cells) populations were examined. *B*, Limiting dilution analysis in FT organ culture. Serially reduced numbers of c-*kit*$^+$CD25$^-$ AT cells (Ly5.1) were cocultured with a dGuo-lobe (Ly5.2). After 14 days of culture, cells were recovered from each lobe for analysis with a flow cytometer. Ten to 15 lobes were used for each point, and the proportions of negative lobes are scored. The frequency of progenitors having T cell potential was determined to be one of six. *C*, Limiting dilution analysis for progenitors with B cell potential. Serially reduced numbers of c-*kit*$^+$CD25$^-$ AT cells were cultured on a monolayer of the TSt-4 stromal cell line. After 10 days of culture, floating cells were harvested and examined for expression of B220 with a flow cytometer. Ten to 15 wells were used for each point, and the proportions of negative wells are scored. The frequency of progenitors with B cell potential was determined to be 1 of 450. *D*, Limiting dilution analysis for progenitors with myeloid potential. The experiment was performed as in *B*, except that the stromal cell line PA6 was used. Cells were harvested on the 10th day of culture for detection of myeloid cells. The frequency was determined to be 1 of 57.

progression of their thymic stage (Fig. 5). The T/NK/DC tripotentiality of the earliest AT progenitors suggests that they are the immediate progeny of immigrants from BM, because virtually all prethymic T cell progenitors are T/NK/DC tripotential (our unpublished data). The finding that the pre-β proliferation potential of such T cell lineage-restricted progenitors in BM was similar to those of the earliest intrathymic progenitors (Fig. 2) strongly suggested that such T cell lineage-restricted progenitors produced in BM colonize the thymus. A recent study by Allman et al. (21), done with a population-level analysis, showed that the putative earliest thymic progenitors are more restricted to the T cell lineage than the CLP in BM. Very recently, Porritt et al. (30) and Balciunaite et al. (31) showed that progenitors generating T cells and those generating B cells have already been segregated in the earliest thymic population. These findings seem to be consistent with our present results.

However, the possibility remains that the earliest thymic progenitors are multipotent or T/B bipotent and lose the B cell potential immediately after they migrate into the thymus. Martin et al. (15) have suggested that the B220$^+$ CLP in BM may represent progenitors bound for the thymus. Schwarz et al. (32) have proposed that the circulating multipotent progenitors colonize the thymus, based on the findings that other types of progenitors with T cell potential were not detected in the circulation. Several very recent studies on the earliest thymic progenitors are in line with this idea. Sambandam et al. (33) and Tan et al. (34) suggested that the earliest thymic population contains progenitors having both T and B cell potentials. Furthermore, using a clonal analysis, Benz and Bleul (35) demonstrated the presence of such progenitors with T and B cell potentials in the thymus. Thus, it is possible that the progenitors at various stages can migrate into the thymus (36). In the case of fetuses, progenitors just before colonizing thymic epithelial anlage can be isolated, and analysis of such progenitors provided evidence that the majority of thymus-colonizing progenitors in early fetal period are committed to the T cell lineage in that they have lost B cell potential (11, 37). However, in the case of adults, no methods are available at present to isolate such progenitors just colonizing the thymus. Further studies are required to determine which type of progenitors represent the major pathway of thymic colonization in adults. We have previously proposed a model of hemopoiesis in which myeloid potential is retained in the lineage restriction processes toward lymphoid lineages, even after the divergence into T and B cell lineages (6, 20, 38). In contrast, several studies showed the presence of T/B lineage-restricted progenitors, so called CLP, in adult BM (14, 39, 40). Because our findings were based on the findings on fetal progenitors, it has been argued that our model is valid only in fetal hemopoiesis. In the present study, by using the MLP assay, we assessed cells in the earliest adult thymic population, in comparison with the most primitive progenitors and lymphoid progenitors in adult BM. The

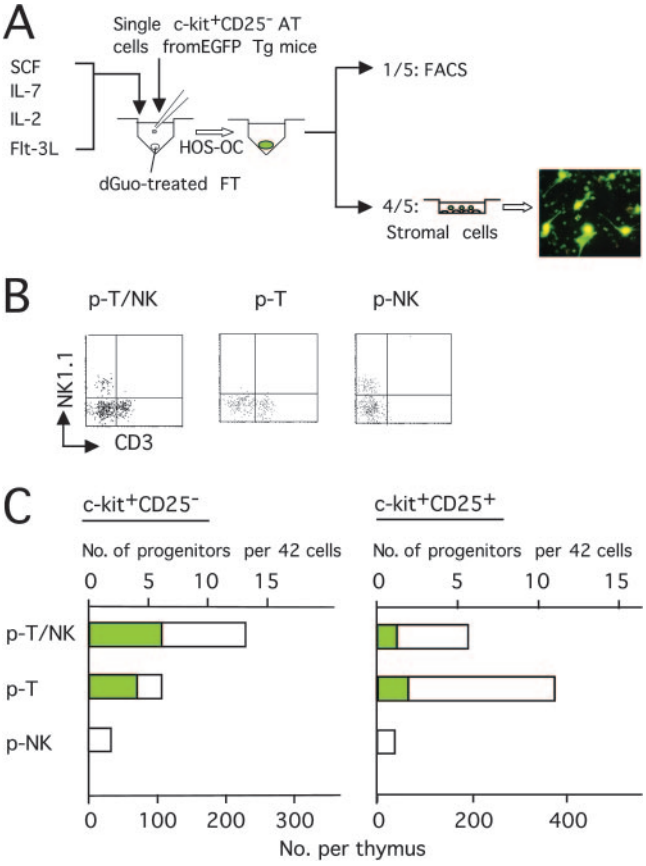

FIGURE 5. Determination of the T, NK, and DC potential of individual progenitors in early AT populations. *A*, The procedure of the clonal assay culture is shown. Single dGuo-treated lobes were placed into the wells of a 96-well V-bottom plate, to which single c-*kit*$^+$CD25$^-$ AT cells from EGFP mice were added. Generation of T cells, NK cells, and DC was determined as described in *Materials and Methods*. *B*, Representative flow cytometric profiles of cells generated from AT progenitors. Based on the flow cytometric profiles, progenitors were classified into three types, which are those generating both T and NK cells (p-T/NK), only T cells (p-T), and only NK cells (p-NK). *C*, Distribution of DC potential in different types of progenitors in c-*kit*$^+$CD25$^-$ and c-*kit*$^+$CD25$^+$ populations of AT. Progenitors showing DC potential in each group are scored as a green bar.

results were largely similar to what we found in fetuses; p-MTB, p-MT, and p-MB in addition to p-M, p-T, and p-B were detected in the FcR^{low} population, and the IL-7R$^+$ population mainly comprised of a mixture of p-T and p-B, with p-B being the most dominant. Detection of p-MT and p-MB in BM suggests that the lineage restriction process in adults is basically the same as in fetuses. We also detected, although only one, p-TB in the CLP population. However, the failure of detecting substantial numbers of p-TB may not be due to the limitation of our assay system, as has been discussed previously (6). Rather, this result may reflect that the BM CLP population mainly contains a mixture of progenitors committed to either the B or T cell lineage, just like the Lin$^-$c-*kit*$^+$IL-7R$^+$ population in FL (6). In addition, we interpreted that the p-TB detected in the CLP population could be a common progenitor for myeloid, T, and B cell lineage that accidentally failed to express its myeloid potential in the MLP assay, because there is a tendency that BM multipotent progenitors do not fully express their potential (our unpublished data) and because several p-MTB were also detected in the same population.

We could not detect substantial myeloid potential in the intrathymic T cell progenitor population. However, in limiting dilution analysis, the macrophage potential was detected with a frequency of ~1 of 60. This result is comparable to the very recent findings by Balciunaite et al. (31), in which they showed that cells in the earliest intrathymic population, which contains virtually no B cell-generating progenitors, contains a substantial number of progenitors with macrophage potential. Therefore, they stated that our model could be applied to adult hemopoiesis. To unambiguously prove that the T cell progenitors retain macrophage potential, clonal evidence is required. We are currently setting up the experimental system to address this issue.

Experimental findings made by using mice deficient for genes that play an important role in T cell development are sometimes interpreted on the assumption that the CLP stage exists in the process of T and B cell development. Especially, the data of Notch-1 knockout mice tend to be adopted as the most important evidence for the existence of the CLP stage, not only in prethymic organs but also in the thymus. However, because the CLP are undetectable in the thymus, findings with the Notch-1 knockout mouse should be explained from a different point of view. An alternative interpretation is that when T cell progenitors meet Delta expressing cells, they are induced to generate T cells, whereas when B cell progenitors meet Delta expressing cells, they may be arrested in differentiation and/or proliferation (20).

Like in FT, a small number of B cell progenitors were detected in AT, although the frequency was much lower than in FT. It is probable that these B cell progenitors differentiate into mature B cells in the thymus as a very minor component (41, 42). However, a recent study by Dorshkind and colleagues (43) demonstrated that the thymic environment inhibits B cell development. Thus, it remains to be determined whether B cell generation takes place from these thymic B cell progenitors in the normal thymic environment.

Several studies have focused on the dynamics of proliferation during intrathymic T cell development, but the proliferation rate at early stages has not been well studied because earlier progenitors form a very minor population in the AT. By assuming that the number of thymus-colonizing cells per day is very low, Shortman et al. (44) has presumed that extensive intrathymic expansion of progenitors occurs before the TCRβ gene rearrangement. However, with an assessment by BrdU cell cycle analysis, it was shown that the earliest CD44$^+$CD25$^-$ cells in adult thymocytes are mostly dormant (45). Although such a finding did not necessarily preclude the possibility that the early progenitors are very slowly growing, it was proposed that only two to three cell divisions occur in total before the TCRβ rearrangement. Previous findings that T cells with a broad range of repertoire for Ag specificity were generated from a single progenitor that has colonized the thymus may support the idea that the extensive proliferation of progenitors takes place at early stages in the thymus (46, 47). Recently, a unique case of X-linked severe combined immunodeficiency was reported, in which a reverse mutation was assumed to have occurred in a T cell progenitor (48). The authors demonstrated that thousands of T cell clones with distinctly rearranged TCRβ chain genes appeared in the peripheral T cell pool of the patient. However, in these studies, the frequency of progenitors having a high proliferative potential was not assessed. Moreover, it remained to be clarified whether the progenitors they examined were T cell lineage restricted. We have succeeded in direct assessment of the expansion size before TCRβ gene rearrangement at a single-cell level, using fetal thymic progenitors (25). In that study, we showed that a single T cell lineage-restricted progenitor in the earliest thymic population is mostly able to produce >1000 precursors before TCRβ gene rearrangement.

In the present study, we showed that the AT progenitors produce about a 100-times larger number of T cells than FT progenitors

when transferred into irradiated recipients. Such characteristics of AT progenitors may partially be attributable to their 10-fold higher levels of pre-β-rearrangement proliferation than that of FT progenitors (Fig. 2). However, the 10-fold higher level in pre-β-rearrangement proliferation may not be sufficient to explain the 100-times higher number of T cells produced by AT progenitors. In this context, it is of special interest to examine whether the proliferation potential of AT progenitors after the β-selection is also higher than that of FT progenitors. When DN3 cells of normal mice were individually cultured in fetal thymic organ culture, no difference was seen in numbers of generated DP cells between fetus and adult (data not shown). However, this could be due to the limitation of space allowed for progenitor expansion in the fetal thymic organ culture system. Development of a new experimental system is required to address this issue.

The extent of pre-β-rearrangement proliferation of the earliest AT progenitors was much higher than those of FT or FL (Fig. 2*C*), and only prethymic progenitors for T cells in BM show a comparable level of proliferation to that of the earliest T cell progenitors in AT. These results may indicate that the T cell progenitors in AT are not the descendents of those that have migrated during the fetal period, but are derived from BM. Considering also that the young adult mice used as the source of thymic and BM cells are only 4 wk of age, it is strongly suggested that the progenitors in the thymus are completely replaced by BM progenitors within 4 wk, confirming the early findings by Jotereau et al. (49).

It is well known that different types of T cells are produced sequentially in ontogeny in the thymus. Early studies have clarified that $\gamma\delta$ T cells showing different tissue distribution were sequentially generated during the fetal age (50, 51). "Natural suppressor" cells are well known to be generated after mice have become 3 days old (52, 53). Such a sequential production of different types of T cells may be attributable to either or both the sequential change in progenitors or the thymic environment, as has been pointed out previously (54, 55). Our present work provided a strategy to compare the progenitors for $\alpha\beta$ T cells at a single-cell level. A larger potential in proliferation of AT progenitors before the TCRβ chain gene rearrangement may contribute to the production of a broader or more elaborate antigenic repertoire of T cells.

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Disclosures

The authors have no financial conflict of interest.

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