Isolation of the Most Immature Population of Murine Fetal Thymocytes That Includes Progenitors Capable of Generating T, B, and Myeloid Cells

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Summary

Thymus cells of murine fetuses at day 12 of gestation are exclusively of the $CD3^-CD4^-CD8^-CD44^+CD25^-$ phenotype, which is known as a hallmark of the most immature subset of thymus cells. In the present study, we show that day 12 fetal thymus (FT) cells express FcγRII/III (FcR) at a broad range of levels on their surface. The FcR^+ FT cells seem to represent T lineage cells, because a large majority of them express the T lineage specific transcription factors TCF-1 and GATA-3 as well as CD3ε in the cytoplasm. Also shown is that the FcR^- population contains progenitors capable of developing into not only T cells but also B and myeloid cells, whereas FcR^+ progenitors are mostly committed to the T lineage. These findings indicate that thymic T lineage cells express FcR on their surface at the earliest stage of differentiation, and thus FcR is a useful marker in isolating the most immature population of murine FT cells.

Whereas T cell development occurs in the thymus, the progenitor cells are of extrathymic origin. It is still controversial whether or not the progenitors immigrating into the thymus are precommitted to the T or lymphoid lineage (1–3). Neither the prethymic progenitor migrating to the thymus nor the most primitive progenitor in the thymus has been identified. Recent advances in flow cytometric technology in combination with the establishment of a variety of mAb have enabled us to identify and isolate very minor subpopulations of immature thymocytes (4–6). Immature T lineage cells in murine fetal as well as adult thymuses that are $CD4^-$, $CD8^-$, and $CD3^-$ (triple negative, [TN][1]) can be divided into $CD44^+CD25^-$, $CD44^+CD25^+$, $CD44^-CD25^+$, and $CD44^-CD25^-$ subpopulations, with differentiation proceeding in this order (7, 8). All thymus cells of fetuses at day 12 of gestation (d12 FT) belong to the most immature $CD44^+CD25^-$ population, which rapidly decreases as gestation proceeds (9, and our unpublished data). In addition to T cells, B and myeloid cells have been shown to be generated from d12 FT cells (10), although it is still to be determined whether d12 FT contains multipotent progenitors or comprises a mixture of monopotent progenitors that give rise to a separate lineage. On the other hand, the finding that mRNA for CD3ε and pre-TCRα (pTα) are expressed in $CD44^+CD25^-$ cells (11, 12) strongly suggests that cells already initiating the differentiation toward T cells are included in this population.

Our recent flow cytometric investigation disclosed that ~30% of $CD44^+CD25^-$ cells expressed T lineage–specific transcription factors TCF-1 and GATA-3 (13), indicating that a large proportion of these cells are committed to the T cell lineage or even proceeding the differentiation toward T cells. To explore the earliest events in T cell development, it is necessary to identify and isolate a population devoid of differentiating cells. However, so far no surface marker has been found effective in subdividing the $CD44^+CD25^-$ population. In the present work, we show that d12 FT cells express FcγII/III (FcR) at a fairly broad range of levels and that the expression of TCF-1, GATA-3, and intracellular CD3ε (IC-CD3ε) is exclusively seen in FcR^+ cells. We further demonstrate that FcR^- cells include progenitors capable of generating not only T cells but also B and myeloid cells, whereas FcR^+ progenitors are committed to the T cell lineage. Characteristics of the thymic FcR^- progenitors are discussed in reference to the FcR^- progenitors in the fetal liver (FL).

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from SLC (Shizuoka, Japan) and maintained in our animal facility. FT and FL were obtained from time-mated pregnant mice on various days of gesta-

[1] *Abbreviations used in this paper:* BM, bone marrow; Cy5, Cyanine 5; d12 FT, day 12 fetal thymus; dGuo, deoxyguanosine; FcR, FcγRII/III; FL, fetal liver; FT, fetal thymus; HOS, high oxygen submersion, IC-CD3ε, intracellular CD3ε; PI, propidium iodide; pTα, pre-TCRα; RT, reverse transcription; SP, single positive, TN, triple negative

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tion. The date of finding the vaginal plug was taken as day 0. B6Ly5.1 mice, maintained in our animal facility, were used in organ culture experiments as the source of FT lobes. CD4 or CD8 single positive cells were obtained from 4-wk-old B6 mice.

Antibodies

The following antibodies were used: FITC–anti-Ly5.1 (A20-1.7, donated by Dr. Y. Saga, Banyu Seiyaku, Tokyo, Japan), biotinylated anti-Ly5.2 (ALI-4A2, donated by Dr. I.L. Weissman, Stanford University, Stanford, CA), FITC–anti-Mac-1 (M1/70, Caltag Labs, South San Francisco, CA), FITC–anti-Gr-1 (RA3-8C5; PharMingen, San Diego, CA), PE–anti-B220 and FITC–anti-B220 (RA-6B2; Caltag Labs.), FITC–anti-IgM (Organon Teknika, West Chester, PA), APC–anti-Thy1.2 and FITC–anti-Thy1.2 (5a-8; Caltag Labs.), FITC–anti-CD4 (GK1.5; Caltag Labs.), FITC–anti-CD8 (YTS169.4; Caltag Labs.), FITC–anti-CD44 (IM.7.8.1; PharMingen), PE–anti-CD25 (PC61; PharMingen), APC–anti-CD3ε (145-2C11; PharMingen), anti-human GATA-3 that cross-reacts with mouse GATA-3 (HG3-31; Santa Cruz Biotechnology, Santa Cruz, CA), anti-FcR (2.4G2) (14), anti–c-kit (ACK-2; donated by Dr. S.-I. Nishikawa, Kyoto University, Kyoto, Japan) (15), anti-erythroid lineage cells (TER119, produced by T. Kina in our laboratory). Rabbit polyclonal antibody to TCF-1 (anti–TCF-1) were raised in our laboratory (13). Anti-FcR and TER119 were labeled with FITC as described (16). Anti–c-kit, anti–TCF-1, and anti–GATA-3 were labeled with Cyanine 5 (Cy5; Cy5™ labeling kit; Biological Detection Systems, Pittsburgh, PA) whose fluorescence characteristics are similar to those of APC.

Flow Cytometric Analysis

Surface staining and flow cytometric analysis of FT and FL cells were performed as previously described (17). For the simultaneous detection of surface FcR and intracellular proteins (TCF-1, GATA-3, and CD3ε), d12 FT cells were reacted with FITC–anti-FcR, washed in PBS, and fixed in 100 μl of reagent A (FIX & PERM cell permealization kit; Caltag Labs.) for 15 min at room temperature (18). The fixed cells were washed and resuspended in 50 μl of permealization reagent B (FIX & PERM) containing Cy5–anti-TCF-1, APC–anti-CD3ε, or biotinylated anti–GATA-3. In the last case, cells were washed again and reacted with APC-conjugated streptavidin. These stained cells were analyzed by a FACS® Vantage (Becton Dickinson & Co., San Jose, CA).

Reverse Transcription–PCR

RNA Isolation. Total RNA was isolated from FcR^- and FcR^+ populations of d12 FT cells, $CD25^+$ population of d13 FT cells and nonfractionated d15 FL cells using a modification of the acid guanidinium thiocyanate-phenol-chloroform method (19) with addition of carrier ribosomal RNA. The cells (10^6) were washed in PBS, resuspended into Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosine, and 0.1 M 2-ME), and sheared by passage through a 21-gauge needle. The sheared lysate was mixed with 10 μg of *Escherichia coli* ribosomal RNA (Boehringer Mannheim, Mannheim, Germany), 0.1 vol of 2 M sodium acetate, pH 4.0, an equal volume of water-saturated phenol, and 0.2 vol of phenol-chloroform-isoamyl alcohol. After centrifugation, RNA in the supernatant was precipitated with isopropanol. Pelleted RNA was lysed again in Solution D, precipitated with isopropanol, followed by a wash with 70% ethanol, and then dried. The extracted RNA was dissolved in 25 μl water.

Reverse Transcription. A mixture of RNA solution (25 μl) and 1 μl of 3 mg/ml random primers (GIBCO BRL, Gaithersburg, MD) was incubated at 65°C for 5 min. Samples were placed on ice and the following was added: 8 μl of 5× RT buffer (0.25 M Tris-HCl, pH 8.3, 0.37 M KCl, and 15 mM $MgCl_2$), 4 μl of 0.1 M DTT, 1 μl of 100 mM dNTPs, and 1 μl of M-MLV reverse transcriptase (200 U/ml; GIBCO BRL). The reaction samples were incubated at 37°C for 60 min and heated to 95°C for 5 min, then chilled on ice. To check for contamination of genomic DNA, reactions were also performed without reverse transcriptase.

PCR. cDNA was amplified by PCR using various primers chosen on the basis of GENETYX-MAC program (Software Development Co., Tokyo, Japan). Primers used: pTα sense, 5′-CTGCAACTGGGTCATGCTTC-3′; pTα antisense, 5′-TCAGAGGGGTGGGTAAGATC-3′; lck sense, 5′-CATTCCCTTCAACTTCGTGG-3′; lck antisense, 5′-TAATGGCGGACTAGATCGTG-3′; mb-1 sense, 5′-TGCCAGG-GGGTCTAGAAGCC-3′; mb-1 antisense, 5′-TCATTGGCACCCAGTACAA-3′; c-fms sense, 5′-TCAGAAGCCCTTCGACAAAG-3′; c-fms antisense, 5′-TGGTAC-TTCGGCTTCTGCTT-3′; btk sense, 5′-TGCTAAATCTACTGG GGAGC-3′; btk antisense, 5′-CCCATATTTCACGACACCGA-3′; β-actin sense, 5′-TCCTGTGGCATCCATGAACT-3′; β-actin antisense, 5′-GAAGCACTTGCGGTGCACGAT-3′. The reaction volume was 10 μl containing 2 μl of cDNA sample, 1 μl of 10× PCR buffer (500 mM Tris-HCl, pH 8.3, 2.5 mg/ml BSA, and 30 mM $MgCl_2$), 200 mM dNTPs, 0.5 U of Taq polymerase (Wako Pure Chemical Industries, Osaka, Japan), and 100 pM of each primer. PCR amplification was performed using the Air Thermo-Cycler™ (Idaho Technology Inc., Idaho Falls, ID) under optimal rapid-cycle PCR condition (20, 21). Cycling times and temperatures were as follows: denaturation at 94°C for 5 s, annealing at 57°C for 5 s, and elongation at 75°C for 30 s. Amplification was performed for 35 cycles. Whole PCR product was electrophoresed through a gel containing 4% NuSieve GTG agarose (FMC Corp. BioProducts, Rockland, ME) and 1% agarose and stained with ethidium bromide.

Cell Cycle Analysis

The amount of nuclear DNA was determined by propidium iodide (PI) staining as follows. FcR^- and FcR^+ d12 FT cells and a mixture $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) young adult (4-wk-old) thymus cells were fixed in 50% ethanol at 4°C for 30 min, washed, and incubated in PBS containing 1 mg/ml RNase at 37°C for 20 min. The cells were washed in PBS, resuspended in PBS containing 100 μg/ml of PI, and analyzed by a FACScan® (Becton Dickinson & Co. Inc.).

High Oxygen Submersion Culture

The basic procedure for high oxygen submersion (HOS) culture has been described previously (22, 23). RPMI 1640 medium supplemented with 10% FCS, sodium pyruvate (1 mM), sodium bicarbonate (2 mg/ml), nonessential amino acids solution (1 mM), 2-ME (5×10^{-5} M), streptomycin (100 μg/ml), and penicillin (100 U/ml) was used as complete medium. FT lobes obtained from day 15 fetuses of B6Ly5.1 mice were cultured with deoxyguanosine (dGuo; 1.35 mM) for 6 d to deplete all the hematopoietic cells in the lobe. Single dGuo-treated lobes were submerged in 0.2 ml of complete medium in a well of a 96-well U-bottom plate (Costar Corporation, Cambridge, MA). Cells from the FT or FL of B6 mice were put into each well. Plates were placed into a plastic bag (Ohmi Oder Air Service, Hikone, Japan), and the air

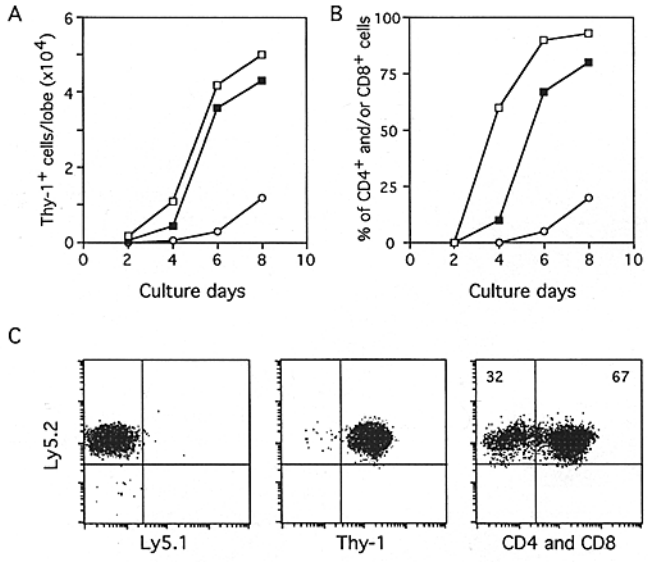

Figure 1. Comparison of the time course of T cell generation from early FT and FL cells. d12 FT cells (10^3), d14 FT cells (10^3), or $Lin^-c\text{-}kit^+$ d12 FL cells (10^3) from B6 fetuses were cultured together with dGuo-treated lobes (B6Ly5.1). Various days later, cells were harvested from the lobes, counted, and analyzed by a flow cytometer. Numbers of $Thy\text{-}1^+$ cells per lobe are plotted in A, and the percentages of cells expressing CD4 and/or CD8 are shown in *B*. ■, d12 FT; □, d14 FT; ○, $Lin^-c\text{-}kit^+$ d12 FL. Surface profiles of the cells recovered from the lobes seeded 6 d earlier with d12 FT cells are shown in three panels in *C*. It is indicated that the recovered cells are exclusively of progenitor type. Nearly 100% of recovered cells express Thy-1, whereas 67% express CD4 and/or CD8. Data are representative of three independent experiments.

was exchanged with a gas mixture of 5% CO_2, 70% O_2, and 25% N_2. The plastic bag was incubated in a 37°C incubator.

Analyses for Development into B and Myeloid Lineages

Bone marrow–derived stromal cell lines ST2 and PA6 were used to determine the ability of FT or FL cells to develop into B and myeloid lineages, respectively (24). Monolayers of ST2 and PA6 were prepared in a six-well plate (Costar Corporation). The culture medium was RPMI 1640 supplemented with 5% FCS, 2-ME (5×10^{-5} M), streptomycin (100 μg/ml), and penicillin (100 U/ml). 500 cells from various sources were placed into each well. After 7 and 14 d of coculture, cells from each well were harvested, counted, and phenotypically analyzed using a FACS® Vantage.

Results

Comparison of the T Cell Precursor Activity between d12 FT and d14 FT Cells. Using the organ culture system, we investigated the time course of T cell generation from d12 FT and d14 FT cells. d12 FT cells, d14 FT cells, and for comparison, $c\text{-}kit^+$ d12 FL cells (B6) were cultured together with dGuo-treated FT lobes (B6Ly5.1 strain) in HOS conditions. At days 2, 4, 6, and 8, cells were removed from the lobes, counted, and analyzed with a flow cytometer. The number of recovered $Thy\text{-}1^+$ cells and the percentage of cells expressing CD4 and/or CD8 are shown in Fig. 1, *A* and *B*. In Fig. 1 *C*, flow cytometric profiles of cells recovered from lobes cocultured with d12 FT cells for 6 d are shown. All cells recovered were $Ly5.2^+$ (B6 precursor type) and $Thy\text{-}1^+$, and nearly 70% of them express CD4 and/or CD8. Virtually no difference in the generation of $Thy\text{-}1^+$ cells was seen between d12 FT and d14 FT progenitors. However, generation of $CD4^+$ and/or $CD8^+$ cells proceeds 2 d earlier with d14 FT cells than d12 FT cells. Similar results have also been obtained when FT cells were injected into the lobes with a microinjector (25).

The observed difference in the time course of T cell development may reflect the difference in the developmental stage of progenitors in d12 FT and d14 FT. Surface phenotypes of these FT cells were analyzed with various early differentiation markers, and the results are shown in Fig. 2. d12 FT and d14 FT cells belong to the TN population as indicated by the absence of expression of CD4, CD8, and CD3. However, in accordance with previous findings (9), d12 FT cells are exclusively $c\text{-}kit^+Thy\text{-}1^-CD44^+CD25^-$, whereas d14 FT cells are heterogeneous with respect to all these surface markers. Because the phenotype of d12 FT cells represents the most immature subset of FT cells, we used d12 FT cells hereafter as the youngest thymocytes.

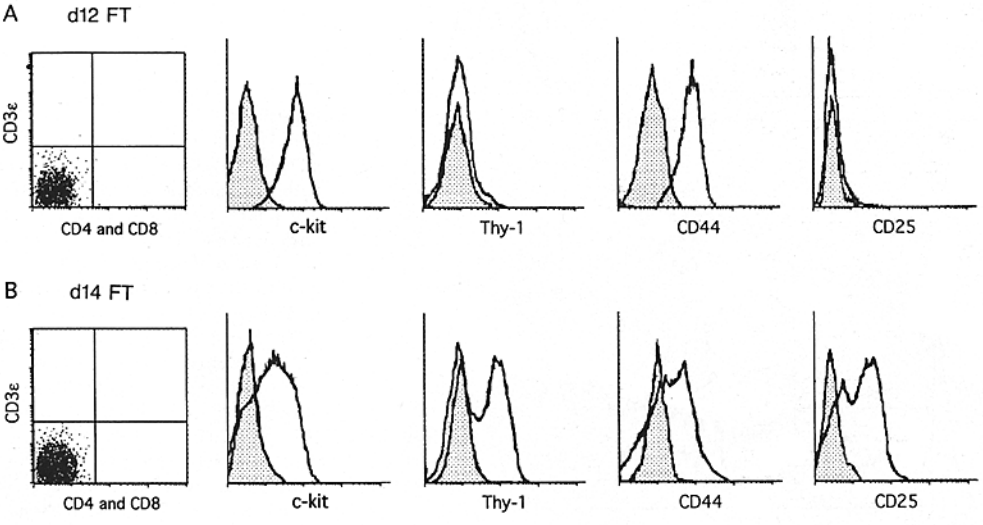

Figure 2. Flow cytometric analysis of d12 FT and d14 FT cells. d12 FT (*A*) and d14 FT (*B*) cells were stained with Cy5–anti-c-kit, FITC–anti-CD44, FITC–anti-Thy1, or PE–anti-CD25, or with a combination of APC–anti-CD3ε, FITC–anti-CD4, and FITC–anti-CD8. Shaded areas in the histograms represent background staining of FT cells with APC-, FITC-, or PE-streptavidin. APC-streptavidin is used as a control for Cy5 staining, because the background staining with Cy5 is very similar to that with APC.

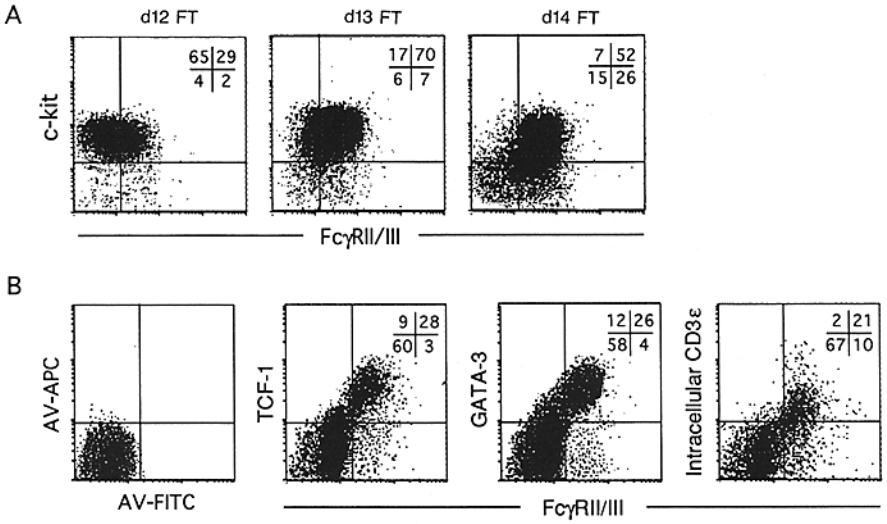

Figure 3. Flow cytometric analysis of the intracellular expression of transcription factors and CD3ε in early FT cells. (*A*) Two-color analysis for surface expression of FcR on FT cells. d12, d13, and d14 FT cells were stained with Cy5–anti-c-kit and FITC–anti-FcR. (*B*) Two-color analysis of expression of FcR and TCF-1, GATA-3, or IC-CD3ε. d12 FT cells were stained with FITC–anti-FcR, fixed, permealized, and reacted with Cy5–anti-TCF-1, Cy5–anti-GATA-3, or APC–anti-CD3ε. The extreme left panel shows the profile stained with APC-conjugated streptavidin and FITC-conjugated streptavidin.

Correlation of Surface FcR Expression with Expression of T Lineage–Specific Transcription Factors. d12 FT cells, which are exclusively of the c-kit$^-$CD44$^+$CD25$^-$ phenotype, have never been subdivided with any surface marker. Since it has been shown that TN cells from d15 FT express FcR (26), in this section, we investigated the expression of FcR on thymocytes from younger fetuses. Two-color flow cytometric profiles for c-kit and FcR of d12 FT, d13 FT, and d14 FT cells are shown in Fig. 3 *A*. It was found that ~30% of d12 FT cells express FcR, whereas the remaining cells do not express or only faintly express this molecule. FcR$^-$ and FcR low positive cells tend to disappear with the advance of age, and the expression of FcR proceeds the loss of c-kit expression.

Because we have previously found that ~30% of d12 FT cells produce T lineage–specific transcription factors TCF-1 and GATA-3 (13), we investigated whether there was a correlation between surface expression of FcR and the production of these factors. d12 FT cells were stained for surface FcR and intracellular TCF-1, GATA-3, or CD3ε. The results shown in Fig. 3 *B* indicate that an increase in the expression levels of TCF-1 and GATA-3 correlates with the increase in FcR expression. Furthermore, almost all the FcR$^+$ cells express TCF-1 and GATA-3 at high levels. It is also shown that a majority of FcR$^+$ cells express CD3ε intracellularly.

Further Characterization of FcR$^-$ and FcR$^+$ d12 FT Cells. d12 FT cells were stained with Cy-5–anti-c-kit and FITC–

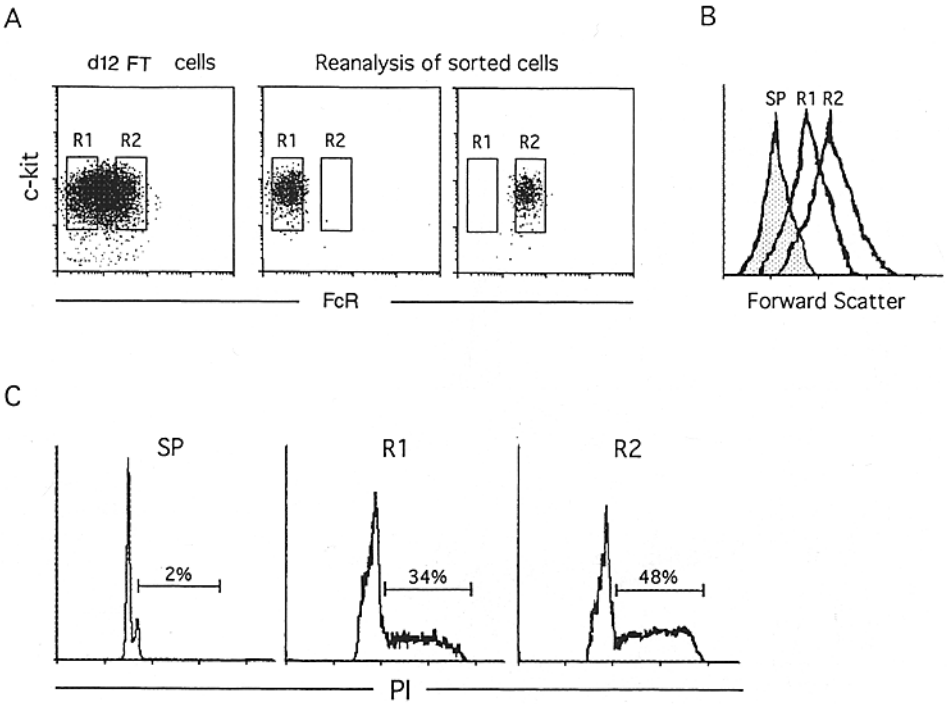

Figure 4. Cell cycle analysis of FcR$^-$ and FcR$^+$ populations of d12 FT cells. (*A*) d12 FT cells were stained with Cy5–anti-c-kit and FITC–anti-FcR and separated according to the sorting gates (*R1* and *R2*). The purity of sorted populations is >98% (*B*) Forward scatter analysis of sorted FcR$^-$ and FcR$^+$ fractions of d12 FT cells. Shaded histogram represents the forward scatter profile of CD4 and CD8 single positive cells (*SP*) sorted from young adult (4-wk-old) thymus cells. (*C*) Cell cycle analysis of sorted FcR$^-$ and FcR$^+$ fractions of d12 FT cells. Sorted cells were stained with PI, and flow cytometrically analyzed

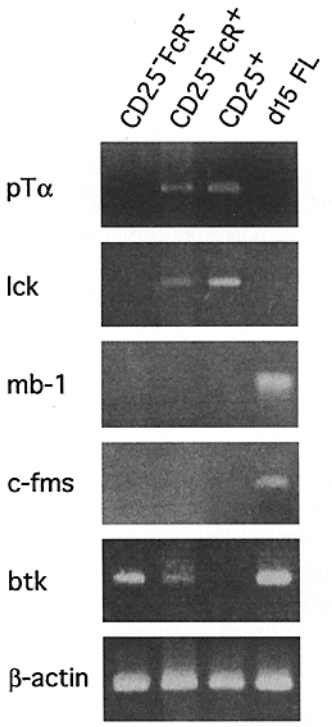

Figure 5. RT-PCR analysis of the expression of hematopoietic lineage associated genes in the subpopulations of FT cells and nonfractionated FL cells. RNA was prepared from sorted FcR^- and FcR^+ d12 FT cells, $CD25^+$ d13 FT cells, and d15 FL cells and reverse-transcribed. The cDNA was amplified with specific primers for each gene indicated in the figure. PCR products were electrophoresed and stained with ethidium bromide.

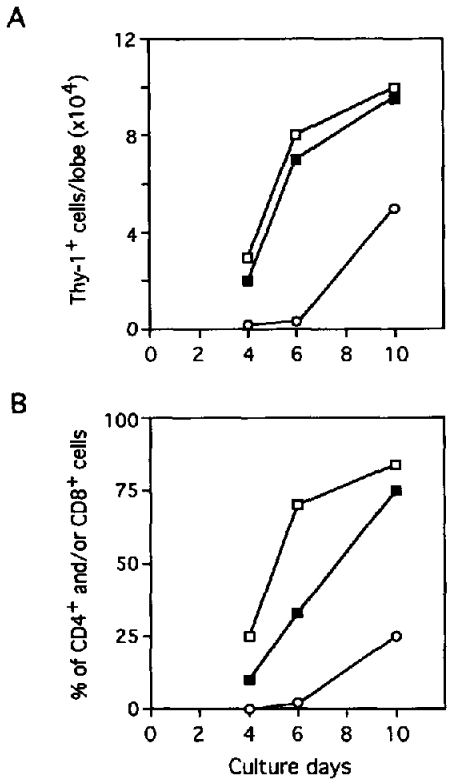

Figure 6. A longer period is required for FcR^- population than FcR^+ population of d12 FT cells to mature into CD4 and/or CD8 expressing cells. c-kit$^+$ FcR^- and c-kit$^+$$FcR^+$ fractions of d12 FT cells (B6) and Lin^-c-kit$^+$ d12 FL cells (B6) were isolated, and these cells (10^3) were cultured under HOS conditions together with a dGuo-treated lobe (B6Ly5.1). On days 4, 6, and 10 after culture, the cells in the lobes were harvested, counted, and analyzed by a flow cytometer. Numbers of Thy-1$^+$ cells (*A*) and the proportion of CD4 and/or CD8 expressing cells (*B*) are shown. Recipient-type T cells were undetectable in any groups (data not shown). Data are representative of two independent experiments. ■, c-kit$^+$$FcR^-$ d12 FT; □, c-kit$^+$$FcR^+$ d12 FT, ○, Lin^-c-kit$^-$ d12 FL.

anti-FcR. The staining profiles of whole FT cells as well as sorted FcR^- (R_1) and FcR^+ (R_2) cells are shown in Fig. 4 *A*. Forward light scatter analysis indicates that FcR^+ cells are larger than FcR^- cells, whereas both FcR^- and FcR^+ cells are much larger than thymic SP cells (Fig. 4 *B*). FcR^- (R_1) and FcR^+ (R_2) cells were isolated and stained with PI to determine the proportion of cycling cells. The results (Fig. 4 *C*) indicate that a large proportion of both FcR^- and FcR^+ cells are cycling, and that the proportion of cycling cells in the FcR^+ population is higher than that in the FcR^- population.

We then investigated the expression of several genes related to differentiation toward T, B, or myeloid lineage: pTα (12) and lck (27) specific for T lineage, mb-1 (28) specific for B lineage, and c-fms (29) specific for macrophages. The expression of btk that has been shown in humans to be expressed by early B and myeloid cells (30) was also examined. RNA was extracted from sorted FcR^- and FcR^+ d12 FT cells, and for comparison, from $CD25^+$ d13 FT cells and d15 FL cells. The RNA was subjected to reverse transcription (RT)–PCR analysis, and the results are shown in Fig. 5. With the exception of btk expression, FcR^- d12 FT cells expressed none of the genes specific for T, B, or myeloid lineage. In contrast, FcR^+CD25^- d12 FT cells resembled $CD25^+$ d13 FT cells in that both these populations expressed pTα and lck but not mb-1 or c-fms. However, the fact that btk was expressed, albeit at low amounts, in FcR^- $CD25^-$ cells but not in $CD25^+$ cells strongly suggests that cells were committed to the T cell lineage after the expression of CD25. Neither pTα nor lck was expressed by d15 FL cells, indicating that virtually no T lineage cells exist in the FL.

Comparison of FCR^- and FcR^+ Progenitors for Their Kinetics of T Cell Generation. FcR^- and FcR^+ cells were isolated as described in the preceding section (Fig. 4 *A*), and these cells were cultured together with dGuo-treated lobes in the HOS conditions. The protocols of organ culture and analysis of cultured cells are the same as those in Fig. 1. The number of Thy-1$^+$ cells recovered from the cultured lobes and the proportion of CD4 and/or CD8 expressing cells are shown in Fig. 6. Whereas FcR^- and FcR^+ progenitors are comparable to each other with reference to the time course of the generation of Thy-1$^+$ cells, a delay by ~2 d was seen in FcR^- progenitors regarding the differentiation into CD4 and/or CD8 expressing cells. These results strongly suggest that FcR^- progenitors are in an earlier stage of T lineage than FcR^+ progenitors. It should be stressed, however, that FcR^- progenitors were able to give rise to T cells much more quickly than FL progenitors.

Capability of FcR^- and FcR^+ Cells to Develop into B and Myeloid Lineages. FcR^- and FcR^+ cells were sorted from d12 FT cells and seeded (500 cells/well) onto a monolayer

Table 1. *The Activity of FcR$^-$ and FcR$^+$ d12 FT Cells to Generate B and Myeloid Lineage Cells*

Cell population* cultured	Culture period	Cultured on the monolayer of: ST2 B220$^+$ cells per well ($\times 10^3$)	ST2 sIgM$^+$ cells	PA6 Mac-1$^+$ and/or Gr-1$^+$ cells per well ($\times 10^3$)
	d		%	
d12FT	7	15	2	6
	14	42	9	22
d12 FT FcR$^-$	7	38	3	17
	14	98	10	52
d12 FT FcR$^+$	7	0	0	1
	14	0.1	0	8
d15 FT	7	2	1	0.5
	14	6	4	2
d12 FL Lin$^-$c-kit$^+$	7	256	5	155
	14	620	16	1,420

*500 cells were seeded onto the monolayer of stromal cell line, ST2 or PA6, in six-well plate

of stromal cell lines, ST2 and PA6, to examine their potential for developing into B and myeloid cells, respectively. As controls, d15 FT cells and c-kit$^+$ d12 FL cells were also seeded onto the same monolayers. 7 and 14 d later, cells were harvested, enumerated, and assayed for surface phenotypes.

The results shown in Table 1 indicate that B cell progenitors are exclusively in the FcR$^-$ fraction and that a large majority of myeloid progenitors are found also in the FcR$^-$ fraction. Generation of myeloid, but not B cells, from FcR$^+$ population strongly suggests that these myeloid cells are derived from myeloid lineage committed progenitors present in this population. On the other hand, it is still unclear whether or not the progenitors in the FcR$^-$ population are multipotent. In Table 1, it is also shown that the progenitor activity of d15 FT cells to generate B and myeloid cells is low, if there is any, whereas the progenitor activity of c-kit$^+$ FL cells is much higher than that of d12 FT cells.

Discussion

It has been shown that fetal as well as adult thymuses contain progenitors capable of developing into B and myeloid cells (10, 31, 32), although progenitors generating myeloid cells are not abundant in the murine adult thymus (4, 5). Such non–T lineage progenitor activity is found in a very immature subpopulation of thymocytes, suggesting that the earliest thymic progenitors retain a multipotent developmental capacity. However, it has not been shown whether multipotent progenitors are present in the thymus or whether the progenitors generating B or myeloid cells are identical with the earliest thymic T cell progenitors. CD44$^+$CD25$^-$ cells, known as the most immature subset in fetal and adult thymocytes, have recently been shown to express T lineage–specific genes, including the components of CD3 at the RNA level (11). This is consistent with our previous work (13), which showed that 20–30% of cells in this population express IC-CD3ε as well as TCF-1 and GATA-3 proteins. These findings indicate that the CD44$^+$CD25$^-$ cell population is heterogenous and does not represent the earliest nondifferentiated cells. No conventional surface marker, however, had been found to subdivide CD44$^+$CD25$^-$ cells into progenitor cell enriched and T lineage cell populations.

In the present study, we have found that CD44$^+$CD25$^-$ cells express various levels of FcR and that the expression levels of FcR correlate with those of TCF-1, GATA-3, and IC-CD3ε. Moreover, RT-PCR analysis indicates that FcR$^+$, but not FcR$^-$ cells, produce mRNA for pTα and lck. Investigation of the progenitor activity of isolated FcR$^-$ and FcR$^+$ cells shows that the FcR$^-$ population contains progenitors capable of generating T, B, and myeloid cells. On the other hand, the FcR$^+$ population comprises T cell progenitors almost exclusively, although a very small number of myeloid progenitors are also included in this population. The finding that FcR$^+$ cells are unable to generate B cells indicates that no multipotent stem cells exist in this population. On the other hand, it is possible that multipotent progenitor cells exist in the FcR$^-$ population, although it should still be clarified whether the apparently multipotent progenitor activity of FcR$^-$ cell population is attributable to a mixture of progenitors committed to different lineages.

The anti-FcR mAb (14) used in the present study reacts with FcγRII and FcγRIII expressed on lymphocytes. FcγRIII and also FcγRI have the γ chain mediating the transmission of a signal into the nucleus (33), whereas no such chain has been discovered in FcγRII. Recent studies by Sandor and coworkers (33, 34) indicated that early FT cells express FcγRII but not FcγRI or FcγRIII. It was suggested that FT cells expressed a ligand for FcγRII to ensure the interaction between FT cells (34). No concrete evidence, however, has been obtained to suggest a role of FcR in T cell development. Knockout of the FcR γ chain does not result in any disruption of T cell development (35).

Stem cells or T cell progenitors in FL are Lin$^-$c-kit$^+$. The Lin$^-$c-kit$^+$ cells in FL are phenotypically similar to those in FT in that both populations express various adhesion molecules at similar levels (17). We have recently found that FL Lin$^-$ c-kit$^+$ cells also express FcR at a broad range of levels. Detailed investigation of the progenitor activity of FL Lin$^-$ c-kit$^+$ cells indicated that multipotent progenitor activity was seen only in the FcR$^-$ population (Kawamoto, H., N. Hattori, and Y. Katsura, submitted for publication). T cell progenitor activity in FL was also exclusively found in FcR$^-$ population. FcR$^+$ cells were able to give rise only to myeloid cells. Comparing c-kit$^+$ FT and

c-kit$^+$ FL cells for the developmental capacity and FcR expression, it is clear that the FcR expression does not necessarily correlate with commitment to or differentiation into a specific lineage but seemed to be associated with the progress of differentiation and/or growth of progenitors regardless of the lineage. In FT, FcR$^-$ progenitors represent an earlier stage in T lineage than FcR$^+$ progenitors (Fig. 6). Because FcR$^-$ but not FcR$^+$ cells in FL retain T cell progenitor activity (36), the prethymic progenitors that immigrate into the thymus may be FcR$^-$.

It is probable that the FcR$^+$ T cell progenitors are generated in FL, but such FcR$^+$ T cell progenitors would not be able to survive in FL. On the other hand, FcR$^+$ progenitors for all lymphoid and myeloid lineages would be generated in FT from FcR$^-$ multipotent progenitors immigrated from FL or other hematopoietic organs. The thymic microenvironment, however, preferentially supports the differentiation and growth of T cell progenitors, and thus B and myeloid cell progenitors may not be supported to give rise to FcR$^+$ stage in FT. The origin of a small number of FcR$^+$ myeloid progenitors in FT is unclear. It is likely that they emigrate from FL or other hematopoietic organs after expression of FcR.

An important outcome of the present study useful for further investigations is that it became possible by using the surface marker FcR to isolate a subpopulation from CD44$^+$ CD25$^-$ cells expressing no T lineage–specific molecule. This provides a means to investigate the earliest events in the thymic T cell development as well as the relationship between prethymic and intrathymic progenitors. Kinetic experiments indicated that FcR$^-$ T cell progenitors were the most immature cells among the thymic progenitors so far detected (Fig. 6). However, these earliest thymic T cell progenitors are at a much more advanced stage than those in FL. We have not succeeded in detecting any thymus-type progenitors in FL, yolk sac, and other organs of the fetus, strongly suggesting that thymic FcR$^-$ progenitors have progressed in the FT or during the transit from FL to FT at least one step of differentiation from FL progenitors. Rodewald et al. (37) isolated T lineage committed progenitors as a Thy-1$^+$c-kit^{low} population from the blood of 15.5 dpc fetus. Comparison of the time course of T cell production in the irradiated thymus (37) suggests that these fetal blood progenitors lie between FL type and FT type progenitors. No direct evidence has been obtained proving that FL progenitors are committed to the T cell lineage. However, we have recently found that FL Lin$^-$c-kit$^+$ cells are distinct from adult bone marrow (BM) Lin$^-$c-kit$^+$ cells in that the former population includes progenitors capable of rapidly generating T cells (Kawamoto, H., N. Hattori, K. Ohmura, and Y. Katsura, manuscript submitted for publication). No difference was observed between FL and BM progenitors in their ability to generate B and myeloid cells. These results suggest that FL includes T lineage committed progenitors, and such progenitors may emigrate to FT via the blood stream. Although Thy-1$^+$ cells do not exist in d12 FT, a large proportion of FT cells at day 14 or later of gestation are Thy-1$^+$. It is probable that these thymic Thy-1$^+$ cells are the progeny of FB Thy-1$^+$c-kit^{low} progenitors. If the progenitors immigrating into FT are T lineage committed, the progenitors capable of generating B or myeloid cells present in FT should have immigrated from FL or other hematopoietic organs independently of T progenitors. More detailed studies are in progress to elucidate the process of lineage commitment of hematopoietic stem cells.

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