Characterization of c-*kit* Positive Intrathymic Stem Cells That Are Restricted to Lymphoid Differentiation

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Summary

We found that c-*kit*–positive, lineage marker–negative, Thy-1^{lo} cells are present in both bone marrow and thymus ("BM c-*kit*" and "thymus c-*kit*" cells). Although the two cell types are phenotypically similar, only BM c-*kit* cells showed the potential to form colonies in vitro as well as in vivo. However, both of them revealed extensive growth and differentiation potential to T cells after direct transfer into an irradiated adult thymus, or a deoxyguanosine-treated fetal thymus. Time course analysis showed that thymus c-*kit* cells differentiated into CD4CD8 double–positive cells approximately 4 d earlier than BM c-*kit* cells did. In addition, anti-c-*kit* antibody blocked T cell generation of BM c-*kit* cells but not of thymus c-*kit* cells. Intravenous injection of thymus c-*kit* resulted in the generation of not only T cells, but B as well as NK1.1$^+$ cells. These data provide evidence that thymus c-*kit* cells represent common lymphoid progenitors with the differentiation potential to T, B, and possibly NK cells. The c-*kit*–mediated signaling appears to be essential in the transition from BM c-*kit* to thymus c-*kit* cells.

All blood cells, including lymphocytes, originate from the hematopoietic stem cells located in bone marrow (1, 2). With the exception of T lymphocytes that require special thymic environment for maturation and selection (3), most of hematopoiesis takes place in the bone marrow. The existence of a sustained T cell development in the thymus requires a continuous input of precursor cells from bone marrow (4, 5). However, we do not know whether bone marrow–derived pluripotent stem cells come to the thymus and differentiate there into mature T cells, or if they start differentiating in the bone marrow and only immature, T-committed, precursor cells migrate into the thymus for further differentiation. Earliest T cell precursors in the adult murine thymus have recently been identified within the CD4^{lo}CD3$^-$CD8$^-$ population (6). However, the sequence of differentiation events linking these precursor cells to the hematopoietic stem cell in the bone marrow is still unclear.

We have recently developed a mAb that reacts with the murine c-*kit* receptor tyrosine kinase (7). The injection of the mAb into adult mice resulted within 2 d in a marked decrease in the number of peripheral erythroid and myeloid cells (8). However, peripheral B cells were relatively increased in numbers and peripheral T cells and immature thymocytes remained unaffected by the treatment. Animals that received antibody treatment died of severe anemia before the effect on lymphoid cells became obvious. These findings suggested that the stem cells for the erythroid/myeloid lineages behaved differently from stem cells for lymphoid cells in the effect of anti-c-*kit* reagent. When we sorted c-*kit*$^+$, Thy-1^{lo}, lineage marker negative cells (c-*kit*$^+$, Thy-1^{lo}, Lin$^-$) using a FACS® (Becton Dickinson Immunocytometry Systems, San Jose, CA) in adult bone marrow, the population thus highly enriched in stem cells displayed a capacity to reconstitute in the long term, all hematopoietic lineages including B and T lymphocytes (9). Thus, the relative ineffectiveness of the anti-c-*kit* antibody treatment most likely reflects distinctive features of B and T lymphocytes in vivo.

To address the role of c-*kit* and of microenvironment in early lymphoid differentiation, we have attempted to establish an in vitro culture system in which T cells can be generated either from the bone marrow stem cells or from T-committed progenitors. In the present study, we have identified and characterized c-*kit*$^+$ Thy-1^{lo} Lin$^-$ cells from the adult thymus (thymus c-*kit* cells) and compared them with cells

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of similar phenotype isolated from the bone marrow (BM[1] c-*kit* cells). Our results indicate that both thymus c-*kit* and BM c-*kit* cells can differentiate into T cells in the thymic environment and that thymus c-*kit* cells have lost the potential to differentiate into nonlymphoid cells. In addition, we show that, the generation of T cells from BM c-*kit* cells is far more dependent on the c-*kit*–mediated signaling than that from thymus c-*kit* cells.

Materials and Methods

Mice. For the donor of thymocytes and bone marrow cells, congenic mice C57BL/6J-Ly5.1:Pep3[b] (B6-Ly 5.1) were bred and maintained in our animal facility. C57BL/6-N (B6-Ly 5.2) mice were purchased from Clea Japan (Tokyo, Japan) for the recipient of intra-thymic injection and spleen colony assay. Pregnant B6-Ly 5.2 mice, the donor of fetal thymus were purchased from SLC Inc. (Hamamatsu, Japan) at day 9 of pregnancy.

Antibodies. For cell sorting, biotinylated rat mAbs, RA-6B2 (anti-B220) (10), M1/70 (anti-Mac-1) (11), RA3-8C5 (anti-Gr-1) (12), GK1.5 (anti-L3T4) (13), 53-6.7 (anti-Ly 2) (14), and TER119 (15) were used as lineage markers. FITC-labeled 30-H12 (anti-Thy-1.2) (16) and APC-conjugated ACK-2 (anti-c-*kit*) (7) were used as stem cell markers. B220, Mac-1, and GK1.5 were purified by affinity chromatography on immobilized protein A and were derivatized with biotin by standard techniques (17). Ly 2-biotin and Thy-1.2-FITC were purchased from Becton Dickinson Immunocytometry Systems. Gr-1-biotin was purchased from Pharmingen (San Diego, CA). TER119-biotin were obtained from Dr. T. Kina (Kyoto University, Kyoto, Japan). Rat anti-c-*kit* mAb was purified and conjugated with APC by standard method (17). All biotinylated reagents were visualized by using Streptavidin-PE second step reagent (Becton Dickinson Immunocytometry Systems).

For analysis of reconstituted cells, mouse mAbs A20.1 (anti-Ly 5.1) obtained from Dr. Yumiko Saga (RIKEN, Tsukuba, Japan) and 104.2 (anti-Ly 5.2) obtained from Dr. Hidetaka Yakura (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) were derivatized with fluorescein or biotin, respectively. Rat anti–Ly 2 mAb was purified and labeled with allophycocyanin. PE-labeled GK1.5 was purchased from Becton Dickinson. PE-labeled B220 and Gr-1 and Ly 2, and FITC-labeled Mac-1, Gr-1, and anti-NK1.1 (PK136) (18) were purchased from Pharmingen.

Cell Preparation. Thymus glands were surgically excised from 4–6-wk-old female B6-Ly 5.1 mice. Thymus cell suspensions were prepared by pressing the tissue through a 100-gauge stainless steel mesh. Bone marrow cells were flushed from femurs with staining medium (PBS with 3% FCS and 0.1% sodium azide). The suspensions were filtered through a 200-gauge nylon mesh to remove debris.

Staining with Antibodies. 4×10^8 of thymocytes or 4×10^7 of bone marrow cells were reacted with a cocktail of biotinylated rat mAbs specific for mouse differentiation antigens Gr-1, Mac-1, B220, TER119, CD4, and CD8 (see above for antibody designations) for 30 min at 4°C. After washing the cells three times with staining medium, thymocytes were incubated in culture dishes, coated with sheep anti–rat immunoglobulins, and bone marrow cells were treated with magnetic bead-conjugated sheep anti–rat immunoglobulin (Dynabeads, Dynal, A.S., Oslo, Norway), for 30 min at 4°C to remove lineage marker high positive cells. Nonadherent cells were collected and reacted with Thy-1-FITC, c-*kit*-APC, and SAV-PE at 4°C for another 30 min. After a second wash, the cells were resuspended in staining medium at a final concentration of 10^6 cells/ml supplemented with propidium iodide (PI, 1 μg/ml).

FACS® Analysis and Cell Sorting. Stained cells were analyzed by FACStar[plus] (registered trademark of Becton Dickinson) equipped with a 488-nm argon laser and a 599-nm dye laser. Data from 50,000 cells were collected and analyzed. Computer-assisted data analysis of results was done on a MicroVAX computer (Digital Equipment Corp., Maynard, MA) with FACS/DESK software (version 1.8) made available through the FACS development group at Stanford University (Stanford, CA). After analysis, c-*kit*$^+$ Thy-1^{lo} Lin$^-$ cells were sorted. Residual erythrocytes, debris, doublets, and dead cells were excluded by forward scatter, side scatter, and PI gating. Fluorescence intensity of individual cells was measured as relative fluorescence units.

Micro Intra-thymic Injection. Fetal thymus organ cultures and micro injections were done as described previously (19, 20). Briefly, day 14 fetal thymic lobes from B6-Ly 5.2 mice were organ cultured on the surface of filters (0.8-mm pore size; Nucleopore Corporation, Pleasanton, CA) in 3.5 mm petri dishes containing 3 ml of RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Bioproducts, Inc., Walkersville, MD), 1.35 mM 2′-deoxy guanosine (dGuo; Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin for 6 d at 37°C under 5% CO_2 in air. Each thymic lobe was then washed with RPMI and injected with 500–1,000 of sorted c-*kit*$^+$ Thy-1^{lo} Lin$^-$ cells from 4 to 6 wk-old female B6-Ly 5.1 mice by using a micro manipulator (Narishige, Tokyo, Japan). After injection, the lobes were organ cultured for 8–24 d on the surface of 3 ml of 10% FCS supplemented RPMI 1640 medium. The reconstituted thymocytes were stained with anti-Ly 5.1, CD4, and CD8, and analyzed by FACS®.

Intra-thymic Injection. Intra-thymic injections of sorted c-*kit*$^+$ Thy-1^{lo} Lin$^-$ thymocytes or bone marrow cells were performed under nembutal anesthesia (21). 6–8-wk-old female B6-Ly 5.2 mice were irradiated (6.5 Gy) within 6 h before surgery. 10^4 sorted kit$^+$ Thy-1^{lo} Lin$^-$ thymocytes or bone marrow cells from 4–6-wk-old female B6-Ly 5.1 mice were injected into one thymic lobe of each recipient mouse in a volume of 5 μl. Mice were killed by neck dislocation at 8–24 d after injection and a cell suspension was prepared from each thymic lobes. The suspensions were stained and analyzed as described above.

In Vitro Colony Assay (CFU-C and HPP-CFC). Methylcellulose culturing was carried out using a modification of the technique described by Iscove et al. (22). 1 ml of culture medium contained an adequate number of sorted c-*kit*$^+$ Thy-1^{lo} Lin$^-$ thymocytes or bone marrow cells, 1.2% methylcellulose (Fisher Scientific, Norcross, GA), α-medium (Flow Laboratories, North Ryde, Australia), 30% FCS (Flow Laboratories), 1% deionized BSA (Sigma Chemical Company), 0.1 mM 2-ME (Eastman Organic Chemical, Rochester, NY), and IL-3 (100 U/ml) and Epo (1 U/ml). In the case of high proliferative potential colony forming cell (HPP-CFC) assay, IL-1 (2 ng/ml), IL-3 (10 ng/ml), M-CSF (10 U/ml), GM-CSF (10 ng/ml), and stem cell factor (10 ng/ml) were added. The cultures were prepared in 35 mm nontissue culture dishes (Falcon Labware, Oxnard, CA) and incubated at 37°C in a humidified atmosphere of 5% CO_2. The number of colonies was counted after 14 d of culture using an inverted microscope.

In Vivo Colony Assay. Spleen colony assay was performed by using standard method (23). 10–14-wk-old B6-Ly 5.2 female mice were lethally irradiated at a dose of 9.5 Gy total body irradiation.

[1] *Abbreviations used in this paper:* BM, bone marrow; dGuo, 2′-deoxy guanosine; HPSC, hematopoietic stem cells; PI, propidium iodide.

Unfractionated thymocytes or bone marrow cells, sorted c-*kit*$^+$ Thy-1^{lo} Lin$^-$ thymocytes, or bone marrow cells were injected into the irradiated mice intravenously from the retro-orbital plexus. After injection, the spleens were removed at day 10, fixed in Bouin's solution, and macroscopically visible spleen colonies were counted.

Lineage Reconstitution Assay. 10,000 sorted thymus c-*kit* cells from B6-Ly 5.1 mice were injected into the sublethally irradiated B6-Ly 5.2 mice (8.7 Gy). 3 wk after injection, cells from the spleen and thymus were collected and stained in three colors with donor-specific anti-Ly 5.1 (biotinylated) and lineage-specific antibodies (either FITC-conjugated anti-Gr-1, Mac-1, or NK1.1 and either PE-conjugated anti-B220, GK 1.5, or Ly 2), followed by APC-avidin as a second step reagent. Three-color FACS® analysis was performed on the FACStar^{plus}® as described above.

Results

Presence of c-kit$^+$ Thy-1^{lo} Lin$^-$ Cells among Thymocytes and Bone Marrow Cells. It is known that pluripotent hematopoietic stem cells (HPSCs) in bone marrow express low levels of Thy-1 antigens and lack expression of surface markers that characterize cells within defined differentiated hematolymphoid cell lineages (Thy-1^{lo} and Lin$^-$) (2, 24). Our previous studies (9) have shown that pluripotent stem cells express high amount of c-*kit* molecules. We first tested thymocytes for the expression of c-*kit*. Four-color FACS® analysis with CD4 (PE), CD8 (FITC), Thy-1 (TR), and c-*kit* (APC) antibodies was performed with thymocytes from 6-wk-old B6-Ly 5.1 mice. Thymic cells expressing c-*kit* were not visible on a usual contour plot when total thymic population was analyzed without gating. However, when gates were set so that only the c-*kit* and Thy-1 expressions of CD4$^-$CD8$^-$ thymocytes were examined, cells expressing c-*kit* were clearly detectable (Fig. 1). These cells are c-*kit*$^+$ Thy-1^{lo} but negative for Gr-1, Mac-1, B220, and TER119 (data not shown), just as c-*kit*–positive hematopoietic stem cells in bone marrow (9). We assumed that c-*kit*–positive cells in thymus were PHSCs from bone marrow colonized in adult thymus, and the following experiments were performed to test this hypothesis.

Compared Stem Cell Activity of BM c-kit and Thymus c-kit. Fig. 2 demonstrates a staining profile of c-*kit*, Thy-1, and lineage markers among thymocytes and bone marrow cells. There exist a population of cells that are c-*kit*$^+$ Thy-1^{lo} Lin$^-$ both in the bone marrow (BM c-*kit* cells) and in the thymus (thymus c-*kit* cells). Approximately 0.5 ± 0.2% of bone marrow cells and 0.07 ± 0.03% of thymocytes are found to be in this population. To determine whether they possessed stem cell activities, in vitro (CFU-C, HPP-CFC) and in vivo spleen colony assays (CFU-S) of sorted BM c-*kit* and thymus c-*kit* cells were performed. The results are summarized in Table 1. When 10^5 of unfractionated BM cells were injected into lethally irradiated mice, 13.2 ± 1.3 colonies were observed on day 10. Although both of them are phenotypically identical, injection of thymus c-*kit* cells produced no spleen colonies out of 10^4 cells, whereas 1,000 sorted BM c-*kit* cells formed 11.6 ± 0.8 colonies.

In CFU-C assay, sorted BM c-*kit* cells formed 43 ± 4.5 colonies per 200 cells in the presence of IL-3 and EPO. Formed colonies were granulocyte–macrophage colonies, erythroid bursts, and mixed colonies. Thus, approximately one fifth of BM c-*kit* formed colonies, which provided an approximately 35-fold enrichment of CFU-C. On the other hand, sorted thymus c-*kit* cells formed colonies only at a frequency of 1/2,500 in CFU-C assay.

Since these cells express c-*kit*, we added its ligand, stem cell factor, along with IL-1, IL-3, M-CSF, and GM-CSF in the medium for in vitro colony assay. This type of CFU-C (HPP-CFU) assay is known to detect the earliest hematopoietic stem cells (25, 26). 200 sorted BM c-*kit* formed 25.7 ± 0.7 colonies, whereas neither 10^7 unfractionated thymocytes nor 10^4 sorted thymus c-*kit* cells formed colonies.

The data indicated that, in contrast to BM c-*kit*, thymus c-*kit* does not have potential to differentiate into erythroid/myeloid lineages. It is suggested that thymus c-*kit* are the lymphocytes progenitor cells that the cells have already lost their multipotency.

Intra-thymic Injection of BM c-kit and Thymus c-kit Cells. Despite the fact that thymus c-*kit* cells were shown to contain a small number of CFU, cells in this population may still constitute the earliest T precursors in the adult mouse thymus. To investigate the potential of BM and thymus c-*kit* cells to

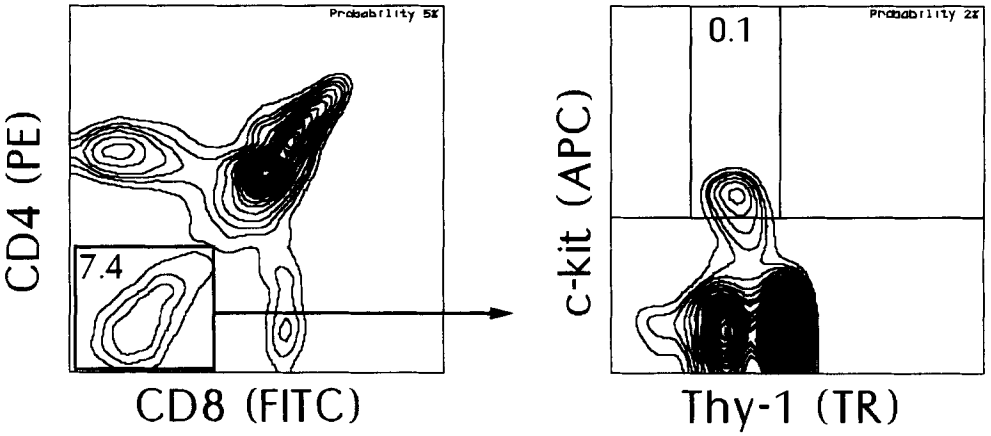

Figure 1. The expression of c-*kit* on Thy-1^{lo} CD4CD8 double negative thymocytes. Total thymocytes obtained from 6-wk-old B6-Ly 5.1 mouse were stained with APC-conjugated c-*kit*, biotinylated Thy-1, PE-conjugated Gk1.5, FITC-conjugated Ly 2 rat mAbs. Biotinylated antibody was visualized by Streptavidin-conjugated Texas red second step reagent. The expression of c-*kit* and Thy-1 in CD4CD8 double negative cells was presented using a 2% probability contour plot (*right*).

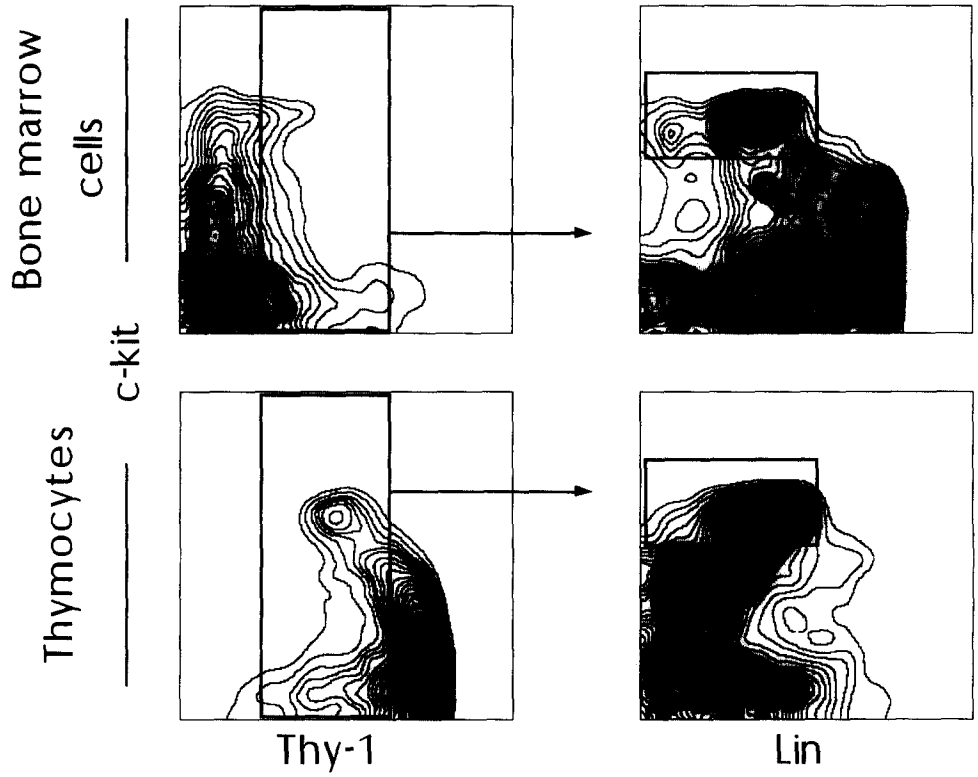

Figure 2. c-*kit*$^+$ Thy-1^{lo} Lin$^-$ cells in adult murine thymus and bone marrow. Both thymocytes and bone marrow cells were depleted of cells bearing lineage markers (Lin markers–Biotin cocktail: B220, Mac-1, Gr-1, TER119, CD4, and CD8) by immunomagnetic beads treatment. The depleted population was stained in three colors with antibodies against c-*kit* (APC), Thy-1 (FITC), and Streptavidin-PE second step reagent. Gate was set for Thy-1^{lo} cells (*left*) and c-*kit*/Lin expression of the gated Thy-1^{lo} cells was shown (*right*).

differentiate into the T cell lineage, we performed organ cultures of fetal thymic lobes microinjected with various sorted cell populations (20). Thus, ~500–1,000 BM or Thymus c-*kit* cells sorted from B6-Ly 5.1 mice were injected into deoxyguanosine-treated thymic lobes (approximately 20 lobes/group) of day 14 B6-Ly 5.2 fetus. Fig. 3 shows the changes with time of CD4/CD8 expression of the donor type (Ly5.1) thymocytes. It is evident that both BM and thymus c-*kit* cells were capable of differentiating into T cells in the fetal thymic microenvironment. At first, the cells proliferated in CD4CD8 double negative stage. And then, they differentiated into CD4CD8 double positive stage via CD8 single positive immature stage as in normal thymocyte development (5, 27, 28).

Of note is the appearance of CD4 single-positive cells from BM c-*kit* but not from thymus c-*kit* cells at day 20. These cells are CD4$^+$ CD8$^-$ but TCR$^-$ immature T cells (data not shown). This may suggest that BM c-*kit* includes T precursor cells that differentiate into CD4CD8 double positive cells via CD4 single positive transitional stage (29). Alternatively, these cells may become CD4$^+$CD8$^-$TCR$^+$ mature T cells by simple upregulation of the TCR (30).

As shown from the results of three independent experiments, both BM and thymus c-*kit* cells differentiated into T cells in organ culture (Fig. 4 *A*). However, CD4CD8 double positive thymocytes differentiated 3–4-d later out of BM c-*kit* cells than out of thymus c-*kit* cells (Fig. 4 *B*). This difference

Table 1. *Colony Formation by BM and Thymus c-kit Cells*

Cells sorted from	Number of CFU-C*	Number of CFU-S‡	Number of HPP-CFC§
Total BM cells	63.0 ± 12 /10^4	13.2 ± 1.3 /10^5	ND
Total thymocytes	7.0 ± 2.8 /10^7	1.8 ± 0.6 /10^7	0 ± 0 /10^7
BM c-*kit*	43.0 ± 4.5 /200	11.6 ± 0.8 /10^3	25.7 ± 0.7 /200
Thymus c-*kit*	4.0 ± 0 /10^4	0 ± 0 /10^4	0 ± 0 /10^4

The c-*kit*$^+$ Thy-1^{lo} Lin$^-$ cells from both bone marrow and thymus were sorted on the basis of staining analysis (Fig. 2). Stem cell assays were performed with both fractions and unfractionated bone marrow cells and thymocytes.

* Numbers of colonies in the presence of IL-3 and Epo at day 14.

‡ Cells were injected into irradiated B6 mice intravenously and the number of spleen colonies were counted at day 10.

§ Number of colonies in the presence of IL-1, IL-3, M-CSF, GM-CSF, and SCF(KL) at day 14. Mean ± SD for quadruplicates.

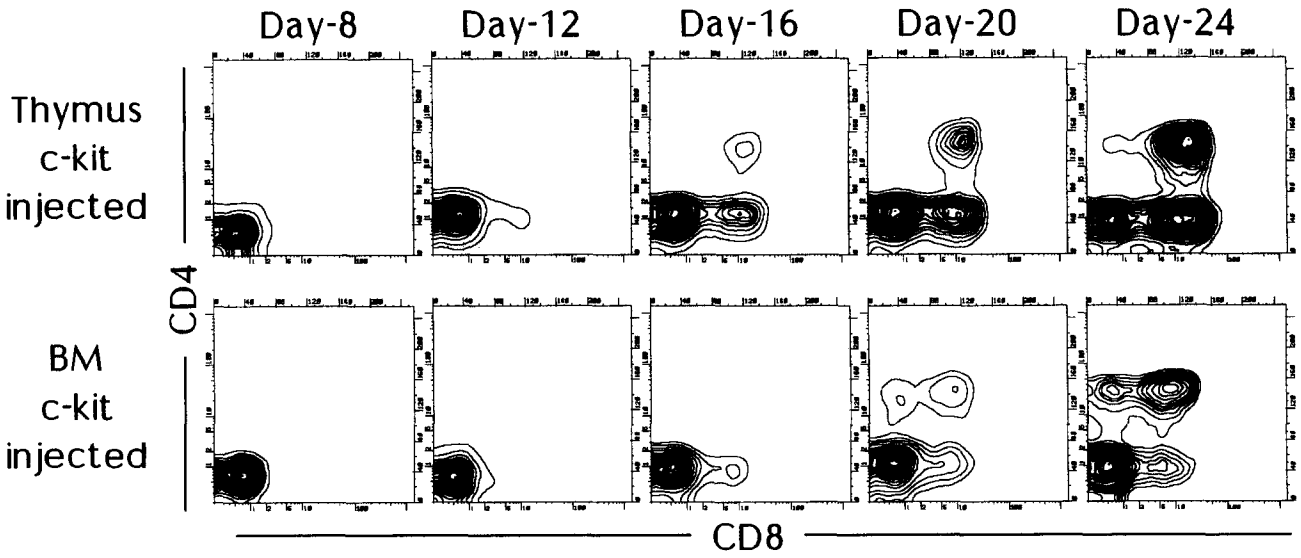

Figure 3. Thymic reconstitution by thymus c-*kit* and BM c-*kit* cells. 500–1,000 purified thymus c-*kit* or BM c-*kit* cells were injected into dGuo-treated fetal thymic lobes by using micro manipulator under the microscope. After the days indicated, the T cell development in donor-derived cells in the repopulated thymic lobes (pooled) were revealed in four-color FACS® analysis with FITC-conjugated anti-donor type Ly 5 (Ly 5.1), PE-conjugated anti-GK1.5 (*CD4*), and APC-conjugated anti-Ly 2 (*CD8*). The expression of CD4 and CD8 on Ly 5.1–positive cells at each time point by either thymus c-*kit* (*top*) or BM c-*kit* (*bottom*) is shown.

in the time course of T cell differentiation of BM c-*kit* cells was confirmed by injection of sorted populations into adult mice thymuses. The results were shown in Fig. 5 and summarized in Fig. 4 C. Both BM c-*kit* cells and thymus c-*kit* cells responded to thymic microenvironment but thymus c-*kit* cells differentiated into T cells earlier than BM c-*kit* cells did, as in the fetal thymus. The maximum proportions of CD4CD8 double positive cells were reached at day 16 when using thymus c-*kit* cells and about day 20 when using BM c-*kit* cells. These data suggest that thymus c-*kit* cells are more advanced in the T cell differentiation pathway than BM-c-*kit* cells.

Blocking Assay by Using c-kit Antibody. To investigate a possible functional role of c-*kit* molecule in the early T cell differentiation process, the micro organ culture of BM c-*kit* cells and thymus c-*kit* cells was performed by using blocking type antibody ACK-2 (anti-c-*kit* mAb) (7). As shown in Fig. 6, addition of anti-c-*kit* antibody to the culture medium at time zero of the organ culture did not change the growth and differentiation of thymocytes from thymus c-*kit* cells, but completely blocked growth and differentiation from BM c-*kit* cells.

As discussed above, we assumed that BM c-*kit* shifted for thymus c-*kit* during first 4 d, and thus, we speculated that c-*kit* is required in this duration. To prove this hypothesis, the antibody was added at day 4 of the culture of thymi injected with BM c-*kit* cells, its effect was less significant and the cells caught up their growth and differentiation. These data suggest that some function associated with c-*kit* is required at least some time during the first 4 d of T cell differentiation from BM c-*kit* cells, and that thymus c-*kit* cells have already passed this c-*kit*–dependent differentiation stage.

Lymphoid and NK Cells Restricted Differentiation Capacity of Thymus c-kit Cells. To define further the developmental potential of the thymus c-*kit* cells, the system of intravenous transfer into mice differing at the Ly 5 locus was used (31). We analyzed cells in the spleen and thymus 3 wk after transfer. The reconstitution was analyzed by comparing donor-derived (Ly 5.2) cells with recipient-derived (Ly 5.1) cells. In the recipient mice, the level of reconstitution by the thymus c-*kit* cells was 0.64 ± 0.46% in spleen and 0.44 ± 0.81 in thymus. These results were much lower than those of BM c-*kit* cells, since injection of 10^4 BM c-*kit* cells usually resulted in more than 95% reconstitution by the donor type cells (data not shown).

As we expected, thymus c-*kit* cells differentiated into T cells but not into myeloid lineage cells. However, we could also find reconstitution of B and NK cells (Fig. 7) after intravenous injection of thymus c-*kit* cells. In contrast, not many lymphoid cells of the recipient type had yet appeared.

Discussion

FACS® multi-color analysis unambiguously revealed the presence of c-*kit*–positive cells in the thymus. These cells, that correspond to 0.05% of total thymocytes, are Thy-1^{lo} and are negative for lineage markers. We named them thymus c-*kit*. On these grounds, they are phenotypically similar to the previously defined pluripotent hematopoietic stem cells in the bone marrow (BM c-*kit* cells).

However, the two cell types appear to be functionally different and thymus c-*kit* cells cannot be considered as mere BM c-*kit* cells having migrated from the bone marrow into

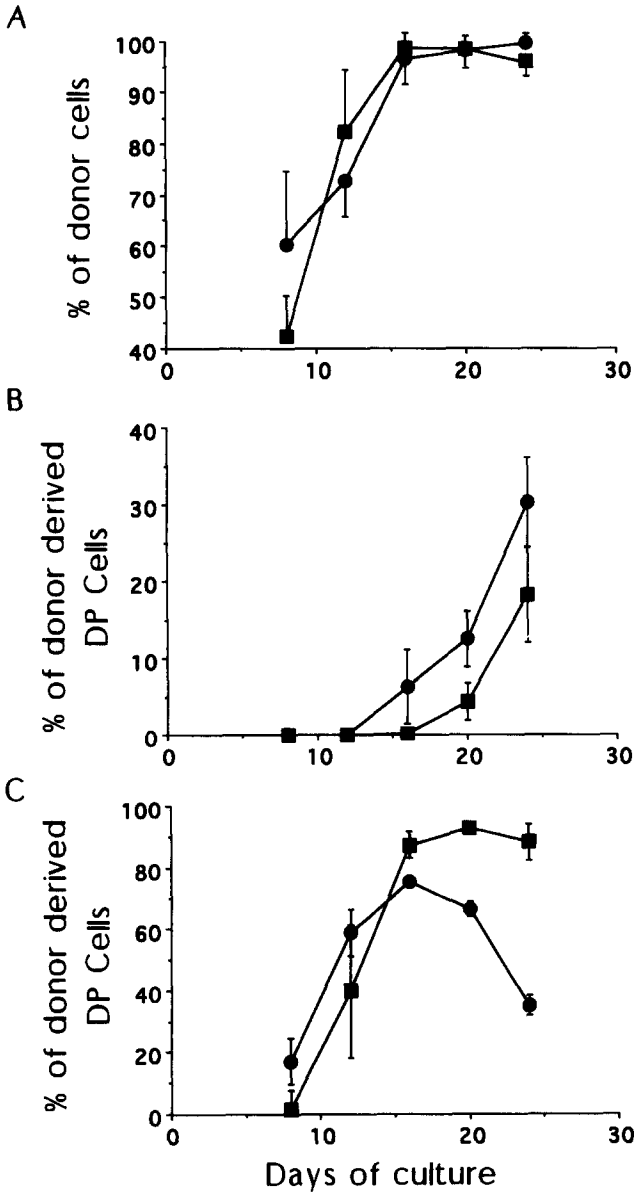

Figure 4. Thymic reconstitution by the BM c-*kit* or thymus c-*kit* cells in the dGuo-treated fetal thymic lobes or irradiated adult thymus. After the times indicated, the relative level of donor-derived cells and the expression of CD4 and CD8 of donor-derived cells in the recipient thymus were revealed by the same staining procedure as in Fig. 3. At each time point, the data represented are the mean values of three independent experiments with BM c-*kit* (■) and thymus c-*kit* (●). (*A*) Overall reconstitution at each time point by the BM or thymus c-*kit* cells, given by the percent of donor-type Ly 5.1^+ cells in the recipient fetal thymic lobes. (*B*) Kinetics of donor-derived CD4CD8 double positive cells in fetal thymic lobes after injection of BM or thymus c-*kit* cells. (*C*) Kinetics of CD4CD8 double positive cells in adult thymus after injection of BM or thymus c-*kit* cells.

the thymus. Indeed, unlike BM c-*kit* cells, thymus c-*kit* cells cannot form myeloid or erythroid colonies either in vivo or in vitro although they have retained the ability to differentiate into cells of the lymphoid lineage. Our data therefore strongly suggest that thymus c-*kit* cells represent committed lymphoid progenitors whereas BM c-*kit* cells are more primitive hematopoietic precursors and include pluripotent stem cells.

In in vivo and in vitro kinetic studies, both BM c-*kit* cells and thymus c-*kit* cells proliferated vigorously into CD4CD8 double negative cells, followed by the appearance of CD4CD8 double positive cells. Interestingly, double positive cells always appeared 3–4 d earlier from thymus c-*kit* than from BM c-*kit* cells. These data support the notion that thymus c-*kit* cell type is a lymphoid committed progenitor whose transition from BM c-*kit* cells takes 3–4 d. Where and how this transition is made is still questionable. Thymus c-*kit* cells may be produced in the bone marrow. In this respect, although the anti-c-*kit* antibody nearly completely blocks the proliferation of BM c-*kit* cells, few cells develop into CD4CD8 double positive thymocytes in the treated thymic lobes. These thymocytes are presumably derived from lymphoid progenitors resistant to the antibody, a conclusion that could be taken as indicative of the presence of a very small number of T cell precursors in the bone marrow, whose differentiation stage is equivalent to that of thymus c-*kit* cells, and would eventually later migrate to the thymus. But thymus c-*kit* cells may also be produced in the thymus from migrating BM c-*kit* cells. The fact that BM c-*kit* cells injected in the thymus developed into T cells clearly indicates that this process can occur in the thymus itself. If BM c-*kit* migrates into the thymus, one expects to find pluripotent hematopoietic stem cells in the organ. Although we could not detect in the thymus cells capable of forming CFU-S or HPP-CFC, the possibility that some BM c-*kit* cells and not the T-committed progenitors, come to the thymus and act as a source of T lymphocyte. Given that a bone marrow stem cell has a large proliferative capacity, only a few cells each a month are necessary to sustain T lymphocyte proliferation in the thymus (32). These few cells could not be detected as such. We presently favor the hypothesis according to which some bone marrow stem cells, rather than T-committed progenitors, migrate to the thymus and differentiate there into T cells.

Irrespective of the place they are formed, are thymus c-*kit* cells the earliest T cell progenitors in the thymus? According to Wu et al. (6, 33), the earliest precursor cells in the adult mouse thymus are CD4 dull positive cells. We have therefore analyzed the surface phenotype of thymus c-*kit* cells using four-color FACS® analysis. We could identify the presence of $CD4^{lo}$ thymocytes among the thymic c-*kit*–positive population. However, as far as we carefully studied, majority of thymus c-*kit* cells that we used in our experiments did not express CD4. Judging from the experimental procedure used by Wu et al., (6, 33) it is likely that the cell population they studied included the thymus c-*kit* cells we describe here. Thus, the characteristics of the $CD4^{lo}$ precursor cells they reported could have been at least in part those of thymus c-*kit* cells. Precise developmental relationship between thymus c-*kit* cells and "low CD4 precursor" remains to be determined. In our opinion, thymus c-*kit* cells are derived from BM c-*kit* cells and the $CD4^{lo}$ lymphoid progenitors found by Wu et al. in the thymus could derive either from Lin^- $Thy\text{-}1^{lo}$ $Sca\text{-}1^+$ (2) or from Lin^- $Thy\text{-}1^{lo}$ c-*kit*$^+$ (9) stem cells. Alternatively, low CD4 precursors may be derived from the $CD4^{lo}$ hema-

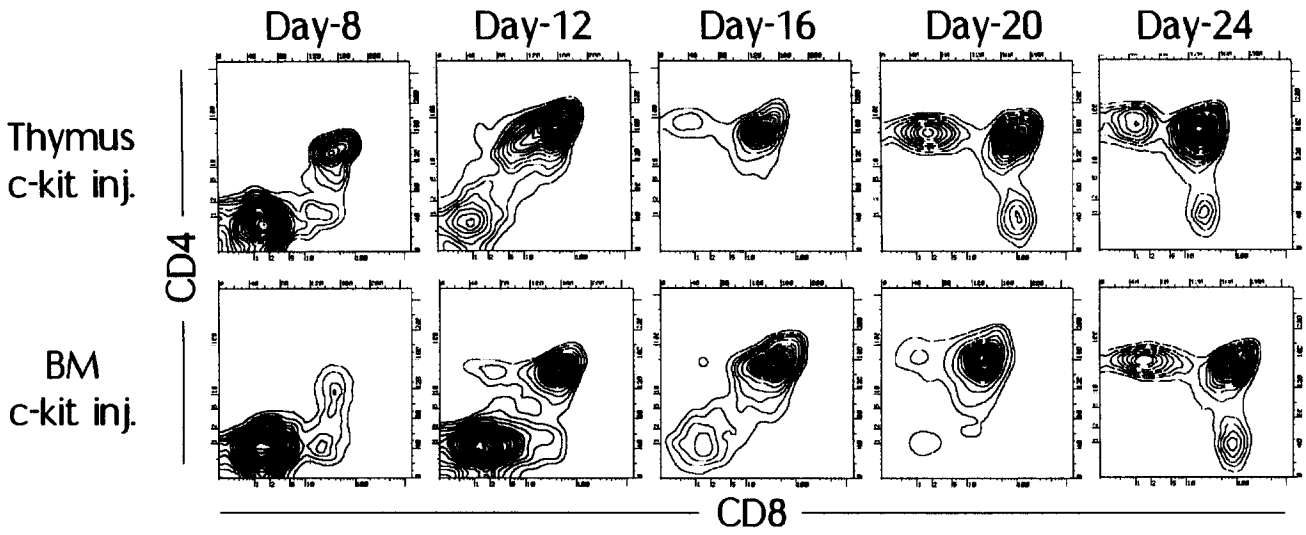

Figure 5. Thymic reconstitution of Thymus c-*kit* (*top*), or BM c-*kit* (*bottom*). 10^4 sorted thymus c-*kit* or BM c-*kit* cells were intra-thymically injected into irradiated (6.5 Gy) recipient mice. After the times indicated, the kinetics of CD4CD8 expression on donor-derived cells were analyzed by the same staining procedure in Fig. 3.

topoietic stem cells in the bone marrow that we and others have reported (34–36) and which appear to be independent of the Lin^- Thy-1^{lo} stem cells. More precise analysis using multi-parameter cell sorting and organ culture, preferably at a single cell level, should aid to clarify the chronological relationships among these cell populations.

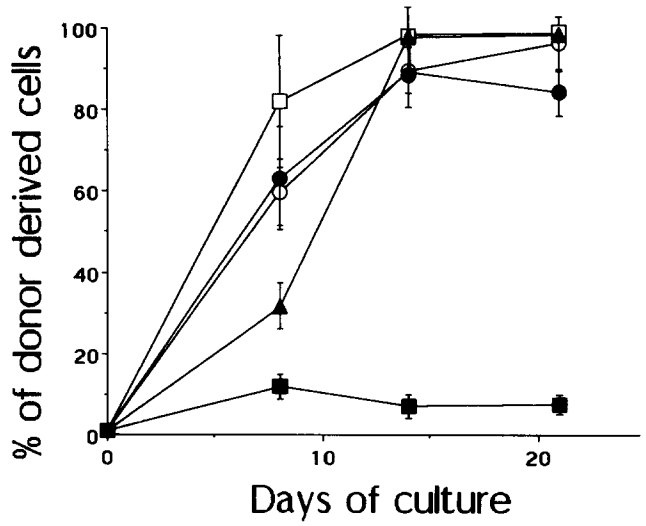

Figure 6. Effect of c-*kit* antibody (ACK-2) on thymic reconstitution of BM c-*kit* and thymus c-*kit* cells. 500–1,000 sorted BM c-*kit* or thymus c-*kit* cells were injected into dGuo-treated fetal thymic lobes. After the days indicated, the T cell development in donor-derived cells in the repopulated thymic lobes were revealed in FACS® analysis. Percentage of donor derived cells in the lobes injected with thymus c-*kit* cells and cultured in the presence of 10 μg/ml of c-*kit* antibody (●), in the lobes injected with BM c-*kit* cells and cultured in the presence of 10 μg/ml of c-*kit* antibody (■), in the lobes injected with BM c-*kit* cells and c-*kit* antibody was added at day 4 of culture (▲), in the lobes injected with thymus c-*kit* cells and cultured in the presence of 10 μg/ml of irrelevant antibody (○), in the lobes injected with BM c-*kit* cells and cultured in the presence of irrelevant antibody (□). The data represented are the mean value and SD of three independent experiments.

Molecular events that take place during these 3 d required for transition from BM c-*kit* to thymus c-*kit* are our main current focus of attention. A study on the role of c-*kit* during early T cell differentiation may constitute a clue to the underlying molecular events. In this respect, the finding that the anti-c-*kit* antibody blocked T cell differentiation of BM c-*kit* cells but not that of thymus c-*kit* cells can be of primary importance. Thymus c-*kit* cells may not be totally independent of c-*kit*, since recent report by Godfrey et al. (37) showed that c-kit^+ thymocytes proliferated in the presence of SCF and IL-7 and that this proliferation was blocked by the anti-c-*kit* antibody at higher concentration (50 μg/ml). However, it is evident from our data that BM c-*kit* and thymus c-*kit* cells are different in terms of dependency on the c-*kit*–mediated signaling. Thymus c-*kit* cells have lost their capacity to differentiate into nonlymphoid cells, whereas on the other hand, they have become less dependent on the c-*kit*–mediated signals. Thus, lymphoid committed progenitors would proliferate and differentiate under the control of other lineage-specific signals such as IL-2 or IL-7 (38–40) and may use c-*kit* in different way than BM c-*kit*. The function of c-*kit* in the T cell progenitor cells can be approached through the fact that the ligand for c-*kit* (KL) is expressed in fetal thymus (41). It is not known whether KL expressed in the thymus is membrane bound, soluble, or sequestered. If the membrane-bound form of KL is expressed on epithelial or stromal cells of the thymus, c-*kit* may play a role as a kind of adhesion molecule ensuring the migration to the proper micro environment within the thymus.

Lastly, we showed that thymus c-*kit* cells had a potential to differentiate into $NK1.1^+$ cells upon intravenous injection. The developmental origin of NK cells and the relationship between T cell and NK cell precursors have been con-

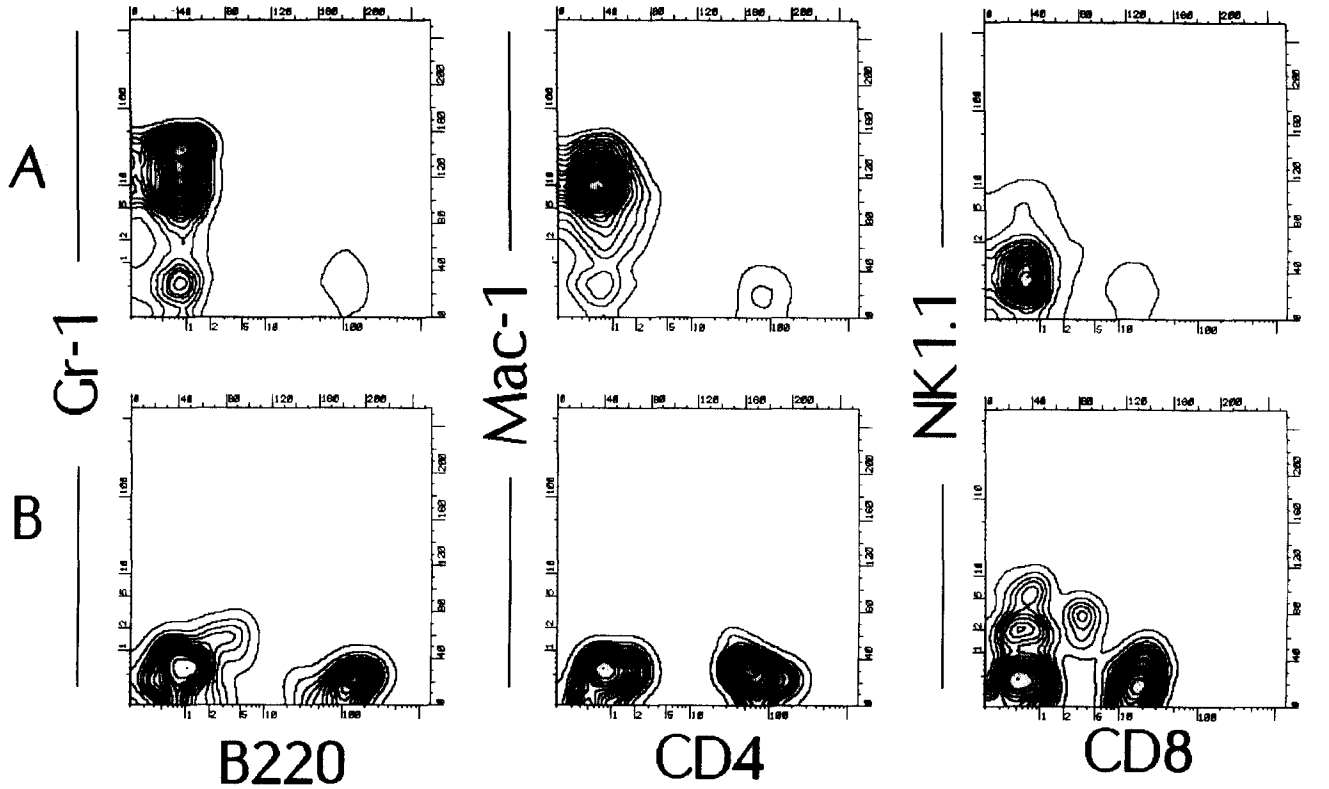

Figure 7. Analysis of hemopoietic lineage reconstitution in spleen by the thymus c-*kit* cells. 10^4 sorted thymus c-*kit* cells were intravenously injected into irradiated mice. 3 wk after injection, the recipient spleen and thymus were analyzed for donor-derived cells by use of three-color flow cytometry with biotinylated anti-Ly 5.1 (donor type Ly 5), FITC-conjugated lineage markers either anti-Gr-1, Mac-1, or NK1.1 and PE-conjugated lineage markers either anti-B220, GK 1.5, or Ly 2 with APC-avidin as second step reagent. In this experiment, donor and host differed in Ly 5 allotypes. After FACS® analysis, gate was set so that the hematopoietic lineage reconstitution of recipient cells (*A*), and that of donor-derived cells (*B*) were shown. The result presented is representative of a series of similar experiments.

troversial. Some investigators have supported an assignment of NK cells to the myeloid or the monocytic lineage (42, 43). On the other hand, a recent report by Rodewald et al. (44) suggested the presence of a common T/NK precursor in the thymus. Although it requires a clonal assay system to draw a definite conclusion, our data support the idea that NK cells and T cells have a common progenitor.

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