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T/NK Bipotent Progenitors in the Thymus Retain the Potential to Generate Dendritic Cells

Hui Qing Shen,* Min Lu,*† Tomokatsu Ikawa,* Kyoko Masuda,‡ Koichiro Ohmura,* Nagahiro Minato,‡ Yoshimoto Katsura,§ and Hiroshi Kawamoto*‡‡

We have previously shown that the earliest thymic progenitors retain the potential to generate T and NK cells and that they lose the bipotentiality to give rise to unipotent T and NK progenitors during the progression of intrathymic developmental stages. The present study examines the ability of these thymic progenitors for generation of dendritic cells (DC) with a new clonal assay that is capable of determining the developmental potential for DC in addition to T cells and NK cells. We found that the large majority of the T/NK bipotent progenitors in the earliest population of fetal thymus was able to generate DC. Although the DC potential is lost with the progression of the differentiation stage, some of the T/NK bipotent progenitors still retain their DC potential even at the CD44+CD25+ stage. The Journal of Immunology, 2003, 171: 3401–3406.

Dendritic cells (DC) have been recognized by their dendritic morphology and a distinguished capability of presenting Ags to T cells. It is well known that DC are essential for initiation of the primary T cell response as well as for negative selection during T cell development in the thymus (1). DC family members are heterogeneous in tissue distribution, surface phenotype, and morphology, although it is unclear whether such heterogeneity represents the difference in their origin. DC have originally been regarded to belong to the myeloid family, since their major function is similar to what is played by macrophages. Such an idea was confirmed by the findings that monocytes and even mature macrophages are able to differentiate into DC (2–4). On the other hand, it has been shown that nearly 70% of thymic cells in the earliest CD3−CD4−CD8−CD44+CD25− (CD44+CD25−) population were able to give rise to DC (5). These thymocyte-derived DC were thought to belong to the T cell lineage, because CD44+CD25+ cells, which do not contain any myeloid or B cell progenitors (6), were also found to generate DC (5, 7).

Previous studies using population level analysis suggested that the earliest thymic T cell progenitors retain the potential to generate B cells and NK cells in addition to DC (6, 8–10). A substantial macrophage potential is seen in the early thymic cells of fetuses (11, 12). It remained unclear, however, whether the multipotentiality of cells in the earliest thymic population is attributable to multipotent progenitors or to a mixture of lineage-committed progenitors. With a clonal assay, named the multilineage progenitor assay (13), which is effective in discriminating the individual progenitors based on the potential for generating myeloid, T, and B cells, we have previously shown that progenitors in the earliest population in fetal thymus (FT) are restricted to the T cell lineage in that they are unable to give rise to B or myeloid cells (14). We have subsequently modified the assay system to examine the potential of individual progenitors toward T and NK cells and showed that all of the earliest progenitors in FT retain NK potential (15). We further showed that T-specific and NK-specific progenitors are produced in the thymus from bipotential T/NK progenitors (15). In the present study, by further modifying this assay system, it becomes possible to examine whether these progenitors retain any DC potential. We showed that nearly all of the earliest thymic progenitors are tripotential for T, NK, and DC. The NK and DC potentials were found to be gradually lost with the progression of the stage in thymic T cell differentiation.

Materials and Methods

Mice

C57BL/6 (B6) and BALB/c mice were purchased from SLC (Shizuoka, Japan). B6Ly5.1 mice are maintained in our laboratory. Transgenic (Tg) mice of the B6 background carrying enhanced green fluorescent protein (EGFP Tg mice) (16) are maintained in our animal facility. B6 fetuses at 15 days postcoitum (dpc) were used in organ culture experiments as the source of FT lobes. Fetuses of B6 mice, B6Ly5.1 mice, and EGFP Tg mice were used as the progenitor source.

Antibodies

The following Abs were used: anti-Ly5.1 (A20), anti-CD4 (H129.19), anti-CD8 (53-6.7), anti-CD3ε (145-2C11), anti-Thy1.2 (53-2.1), anti-NK1.1 (PK136), anti-FcγRII (FcR) (2.4G2), anti-CD44 (IM7), anti-κ (2B8), anti-CD25 (PC61), anti-CD11c (HL3), anti-class II (MS/114), and anti-DEC205 (NLC145), anti-CD80 (16-10A1), and anti-CD86 (GL1). All Ab were purchased from BD PharMingen (San Jose, CA) except for anti-DEC205 (Cosmo Bio, Tokyo, Japan).

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Abbreviations used in this paper: DC, dendritic cell; dGuo, deoxyguanosine; dpc, days postcoitum; EGFP, enhanced green fluorescent protein; FT, fetal thymus; p-NK, unipotent progenitors generating NK cells; p-T, unipotent progenitors generating T cells; p-T/NK, progenitors generating T and NK cells; Tg, transgenic; rm, recombinant murine; SCF, stem cell factor; L, ligand.
Growth factors
Recombinant murine (rm) stem cell factor (SCF), rmIL-2, rmIL-3, rmIL-7, rmGM-CSF, rmFlt3 ligand (Flt3L), rmIL-1α, and rmTNF-α were used. All factors were purchased from Genzyme (Cambridge, MA).

Culture conditions for DC generation
RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FCS (BioWhittaker, Walkersville, MD), 1-glutamine (2 mM), sodium pyruvate (1 mM), sodium bicarbonate (2 mg/ml), nonessential amino acid solution (0.1 mg/ml; Life Technologies), 2-ME (5 × 10⁻⁶ M), streptomycin (100 μg/ml), and penicillin (100 U/ml) was used. A mixture of the following cytokines, SCF (10 ng/ml), IL-3 (10 ng/ml), IL-7 (10 ng/ml), GM-CSF (10 ng/ml), Flt3L (10 ng/ml), IL-1α (10 ng/ml), TNF-α (10 ng/ml), was used as the cytokine mixture. In the experiments shown in Fig. 1, 100 FT cells were cultured in wells of 96-well plates (Costar, Cambridge, MA). In the experiments shown in Fig. 2, individual FT cells were picked up with a micropipette under microscopic visualization and cultured in wells of Terasaki plates (Nunc, Naperville, IL) at one cell per well.

Paired daughter analysis for determining the potential of a progenitor for T cells, NK cells, and DC
Individual FT cells from B6Ly5.1 mice were picked up with a micropipette under microscopic visualization and cultured in wells of a Terasaki plate (Nunc) at one cell per well. Medium and added cytokines were the same as described above. After 8 h of cultivation, wells containing paired daughter cells were screened by a microscope. Usually 60–80% of wells have paired daughter cells. One of the daughter cells in each well was picked up under microscopic visualization using a micropipette and transferred to coculture with a deoxyguanosine (dGuo)-treated FT lobe to induce T and NK generation. The basic procedure of cultivation of single cells with a dGuo-treated FT lobe in the presence of exogenous cytokines has been described previously (15). Briefly, single dGuo-treated lobes were placed into the wells of a 96-well V-bottom plate. The RPMI 1640 medium containing 10% FCS supplemented with rmSCF (10 ng/ml), rmIL-7 (10 ng/ml), and rmIL-2 (10 ng/ml) was used. The plates were placed into a plastic bag (Ohmi Oder Air Service, Hikone, Japan), the air inside was replaced by a gas mixture (70% O₂, 25% N₂, and 5% CO₂), and the plastic bag was incubated at 37°C. Cells were recovered 10 days thereafter from the lobe and analyzed for the expression of Thy-1, CD3, NK1.1, and Ly5.1. The remaining one of the daughter cells was cultured in the same well of a Terasaki plate to induce DC generation for 5 days, which was microscopically determined.

Single-cell cultures for determining the potential of progenitors for generating T cells, NK cells, and DC
The basic procedure of cultivation of single cells with a dGuo-treated FT lobe in the presence of exogenous cytokines has been described previously (13, 15). The culture medium and cytokine conditions were the same as those used in the paired daughter analysis except that rmFlt3L (20 ng/ml) was added to the culture medium. In this experiment, EGFP Tg mice were used as progenitor sources. After 7 days of culture, cells inside the FT lobe were harvested from each well and every sample was divided into three batches (½ × 2 and ½). The first batch was stained with PE-anti-NK1.1 and allophycocyanin anti-CD3, the second batch was stained with PE-anti-CD4 and allophycocyanin anti-CD8, and these cells were served for flow cytometric analysis. The remaining batch (containing three-fiveths of recovered cells) was cultured with the stromal cell line TS-1 (17) monolayered onto a 16-well glass slide (Nunc). After 5 days of culture, cells in each well were examined with a fluorescence microscope. Cells with a dendritic morphology showing green fluorescence were regarded as DC derived from the seeded progenitors.

PCR analysis of rearrangement in TCRβ and TCRγ chain genes
Genomic DNA extracted from cells (equivalent to 1000 cells) was PCR amplified. The procedures for PCR have been described previously (15). Primers were: Dβ2, 5'-GAATCCGTGGGGAAAAGAATT-3'; Jβ1, 5'-TGAGACGGTTGCCTCTACTATGATT-3'; Vy4, 5'-ATGTGGCCAAAGGCCCAGATGCA-3'; and Jγ1, 5'-AGGGAGGATTACTATGAC-3'.

MLR for assessing DC function
Various numbers of DC generated in vitro from B6 FT progenitors and freshly isolated DC from the spleen of adult B6 mice were cultured with 2 × 10⁴ CD4⁺ splenic T cells from BALB/c mice purified by a cell sorter. A 16-h pulse of [³H]Thi or was given at day 3 of culture. Cells were harvested onto glass fiber filters and incorporated [³H]Thi or was measured by a liquid scintillation counter. Splenic DC were prepared basically as described previously (18).

Results
Generation of DC from the most immature progenitors in FT
CD44⁺ CD25⁻ FcR⁻ (FcR⁻) cells from 12 dpc FT (Fig. 1A), which have previously been shown to contain the earliest thymic T cell progenitors (12), were cultured in a 96-well plate at 100 cells/well for 7 days in the presence of a cytokine mixture (5). Approximate cell recovery was 1000 cells/well and > 90% of these cells were morphologically regarded as DC (Fig. 1B). Flow cytometric analysis of the recovered cells indicates that the large majority of

FIGURE 1. DC generated from the earliest thymic progenitors. A. FT cells from 12 dpc B6 fetuses were two-color stained with anti-CD44 and anti-CD25 and with anti-FcR and anti-c-kit. B. Photographs of cells generated from FcR⁺ FT cells. FcR⁻ cells were cultured at 100 cells/well for 7 days in the presence of SCF, IL-3, IL-7, IL-1α, TNF-α, and Flt3L. C. Flow cytometric profiles of cells generated from FcR⁺ FT progenitors in the cultures performed as in B. Shaded area represents negative control of staining. D. Absence of TCR gene rearrangement in DC generated from the FcR⁺ progenitors. Noncultured FcR⁺ FT cells and adult thymus (AT) cells were used as controls. Genomic DNA from each sample was PCR amplified using primers shown under the figures. PCR products were electrophoresed through a 1.2% agarose gel, and stained with ethidium bromide. E. The T cell stimulatory activity of DC generated from FcR⁺ FT progenitors. Various numbers of DC from B6 mice were cultured with 2 × 10⁴ CD4⁺ splenic T cells of BALB/c mice.
these cells are class II "CD11c+", and they also express the activated DC markers CD80, CD86, and DEC205 (Fig. 1C). The 12 dpc FT cells before the culture do not express these markers (data not shown). These cells were CD8− as is the case of the DC produced in vitro from immature adult thymic cells (5). Rearrangement of the TCRβ or TCRγ genes was not observed in generated DC (Fig. 1D), indicating that the branching point for the production of the DC progenitors is at a stage before the TCR gene rearrangement. We next examined whether the DC derived from early FT cells retained Ag-presenting ability. Fig. 1E indicates that the DC generated in vitro from FT cells are functional as the stimulator in a MLR, although the stimulating activity is somehow lower than that of freshly isolated splenic DC.

To examine the frequency of DC precursors and also the number of DC generated from each precursor, a total of 30 FcR− 12 dpc FT cells were individually cultured in a Terasaki plate in the presence of the cytokine mixture. Generation of DC was observed in 10 wells. This DC progenitor frequency is very similar to the T cell progenitor frequency in FcR− FT, but much higher than the frequency of B cell progenitors or myeloid progenitors in the same fraction (14, 15). Cells grown in each well were counted on days 2, 4, and 7 (Fig. 2). The maximum cell number was attained around day 4 of culture and the average number of DC generated from a single precursor is ~40. This value is much higher than that (~7/clone) reported for adult thymic progenitors (5).

We next examined whether the thymocytes at more advanced stages retain the potential to generate DC. Cells from CD44−CD25−, CD44−CD25+, and CD44+CD25+ populations (40 cells each) of 14 dpc FT were individually cultured with the cytokine mixture on a Terasaki plate. The data shown in Table I indicate that 12 among 40 CD44−CD25− cells generated DC and that a substantial proportion of CD44−CD25− FT cells still retain potential to generate DC. It is also seen that the number of DC generated from CD44−CD25− cells are almost comparable to those generated from CD44−CD25− cells. Cells of the CD44−CD25− stage, at which the TCRβ chain gene is rearranged, exhibited no DC potential. These results are basically in line with the findings obtained with adult thymic progenitors (5, 7).

**The earliest thymic T/NK progenitors retain DC potential**

To examine whether the DC precursors detected in liquid culture retain T cell potential, we performed paired daughter analysis, in which one of the daughter cells generated in the cytokine mixture culture condition was picked up for FT organ culture, while the other cell was examined for DC generation. Among a total of 60 FcR− cells examined, 47 cells generated paired daughters in 8 h. Fig. 3A shows a representative flow cytometric profile of cells generated from a single daughter cell in a FT organ culture that generated T and NK cells. An example of DC derived from one of the paired daughter cells, the other one of which was found to generate both T and NK cells, is shown in Fig. 3B. The results of this experiment are summarized in Table II. It was found that about one-half of the progenitors that showed DC potential also generated T and NK cells, thus demonstrating the T/NK/DC tripotentiality of the thymic early progenitors. In other words, no less than one-half of the cells that showed DC potential in the cytokine mixture condition are common T/NK/DC progenitors. Some of the pairs exhibiting DC potential but not NK nor T cell potential (seven pairs) may represent DC-committed progenitors, although the possibility cannot be ruled out that these progenitors simply failed to express their full potentials.

We next tried to examine the DC potential simultaneously in the same culture for the detection of T and NK cell generation in FT organ culture. As shown in the above experiments, the developmental potential of a single progenitor toward T and NK lineages can be investigated by analyzing the cells generated in a cytokine-supplemented FT organ culture with a flow cytometer. It is not easy to use the flow cytometric analysis in identifying DC generated in such FT organ cultures, because the number of DC generated from a single thymic progenitor is very small (Fig. 2). To tangibly detect a small number of DC among a large number of T and/or NK cells generated in the same culture, we used EGFP Tg mice as the progenitor source, in which almost all somatic cells are EGFP+ (16). Cells generated in FT organ cultures were transferred onto a monolayer of stromal cells, where most of the T and NK cells will die within several days so that remaining DC might be easily identified with a fluorescence microscope.

FcR− 12 dpc FT cells (total 50 cells), CD44+CD25− 14 dpc FT cells (total 50 cells) and CD44+CD25+ 14 dpc FT cells (total 50 cells) were individually cultured with a dGuo-FT lobe from normal mice in the presence of SCF, IL-7, IL-2, and Flt3L (Fig. 4A). On day 7 of culture, cells were harvested from each well and two-thirds of the recovered cells were served for flow cytometric analysis. Based on the flow cytometric analysis, progenitors can be 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) (Fig. 4B; see also Ref. 15). The remaining three-fifths of the cells in the wells were transferred onto a monolayer of TSti-4 cells and cultured for an additional 5 days. Cells surviving on stromal cells were screened with a fluorescence microscope for generation of DC. Representative photographs of DC derived from p-T/NK are shown in Fig. 4C. These cells showed dendritic morphology and were found to express CD11c and class II (Fig. 4D). Collectively, these cells can be regarded as DC.

Among 50 FcR− cells examined, 15 exhibited generation of both T and NK cells and thus were determined as p-T/NK (Fig. 4E). Seven p-NK were also detected. p-T were not seen in this population, confirming our previous finding (15). Generation of DC was observed in 14 of 15 p-T/NK clones, the number of DC per clone ranging from 10 to 50. These results indicated that the earliest thymic p-T/NK retain DC potential. Such tripotential progenitors can be termed p-T/NK/DC. On the other hand, five of six p-NK did not generate any DC.

Cells in the CD44+CD25− and CD44+CD25+ populations from 14 dpc FT were also examined, and the frequencies of p-T/NK, p-T, and p-NK in these populations (Fig. 4E) was largely comparable to that in our previous report (15). In the CD44+CD25− population of 14 dpc FT, all p-T/NK, p-T, and p-NK were detected. The DC generation was observed in six of nine p-T/NK clones and two of six p-T clones. None of five p-NK
showed DC generation. The proportion of p-T dramatically increased at the CD44⁺CD25⁺ stage, although small numbers of p-T/NK and p-NK still exist. It was found that some of p-T/NK (two-thirds) and p-T (one-twentieth) still retain DC potential at the CD44⁺CD25⁺ stage. These results indicated that some of the CD44⁺CD25⁺ progenitors still remain tripotential for T, NK, and DC, whereas they immediately lose NK and DC potential to become fully T cell lineage restricted within the CD44⁺CD25⁺ stage.

To investigate whether or not the p-T/NK/DC represent the primitive progenitors on the thymic pathway of T cell development, we characterized the T cells generated from single p-T/NK/DC. In these experiments, two-fifths of the generated cells were transferred onto a monolayer of TSt-4 cells for detection of DC potential, and the remaining cells were used for FACS analysis (two-fifths) and PCR analysis (one-fifth). All four p-T/NK/DC clones examined exhibited the generation of CD4/CD8 double-positive cells as well as CD4 and CD8 single-positive cells (Fig. 5A). yS T are also generated in these clones. We next inquired whether these p-T/NK/DC have a potential to proliferate before the TCR chain gene rearrangement. Genomic DNA was extracted from cells of these clones and rearrangement status of the Dβ2-Jβ2 region was examined by PCR using primers shown in Fig. 1D (19). If a seeded progenitor has proliferated before the TCRβ chain gene rearrangement, the T cells generated from this progenitor may exhibit diversification in the rearrangement profiles of the TCRβ gene. It was seen that all four clones exhibited all possible six bands (Fig. 5B), providing evidence that the p-T/NK/DC retain the potential to extensively proliferate for the production of TCRβ chain diversity. Together, these results indicate that the p-T/NK/DC represent the authentic progenitors generating thymic T cells.

**Discussion**

In the present study, using a newly devised clonal culture system, we showed that virtually all of the earliest FT progenitors are able to generate DC as well as T and NK cells. Similar results were obtained with prethymic progenitors from fetal liver, fetal blood, and from adult thymus (our unpublished observations). Thus, the tripotential p-T/NK/DC may represent the key stage for these three lineage cells.

We showed that some progenitors maintain the T/NK/DC tripotentially at the CD44⁺CD25⁺ stage, where the majority of progenitors are fully T lineage committed, suggesting that the differentiation stages at which the progenitors lose NK potential and DC potential are very close to each other. This finding is in contrast with the findings obtained from the rat thymus, where T/DC-type progenitors are most frequently found while T/NK/DC types are very rare (20). Our data also disclose a tendency that the DC potential does not accompany the NK progenitors (Fig. 4E). On the other hand, it remains unclear whether DC-committed progenitors also exist in FT, because our system is not designed to detect such DC-specific progenitors. As we have previously reported, GFP Tg mice, in which the expression of GFP is controlled under the lck proximal promoter, are useful in flow cytometrically subdividing these early thymic populations (21). More precise staging of T cell development regarding the point where T cell progenitors shut off the DC or NK potential is ongoing by using the thymic progenitors from these mice.

Although the determination of the DC potential was performed by culturing the cells generated in a cytokine-supplemented FT organ culture on a stromal cell monolayer, the majority of DC observed on the stromal cells must have been generated inside the FT lobe, because the DC were observed even 1 day after transferring cells to the coculture with stromal cells (data not shown). It should also be noted that DC can be generated in FT organ culture without adding exogenous cytokines, although the number of DC generated per clone as well as the frequency of progenitors showing DC generation were slightly smaller in this case (data not shown). Therefore, we propose that thymic T cell progenitors produce DC in the thymic microenvironment. However, elucidation of the DC potential of thymic T cell progenitors does not necessarily indicate that T cell progenitors are the major source of DC in the thymus. It is probable that some of the DC in the thymus are derived from thymic macrophage precursors, since a small number of myeloid progenitors exist in the thymus (11, 12, 14). Whereas CD8α have often been used as a marker of “lymphoid lineage-associated DC” (22), recent studies showed that CD8α⁺ DC are not necessarily derived from lymphoid lineage progenitors (23, 24). It has been proposed that the thymic DC are mainly derived from progenitors other than T cell progenitors based on the findings that DC are present at normal levels in a thymus that contains no or very few T lineage cells in c-kit⁻γc⁻ mice or conditionally Notch1-knockout mice (25, 26). However, these findings do not necessarily preclude the possibility that the inactivation of c-kit and the common γ-chain or Notch1 selectively impaired the growth or differentiation of T cells but not of DC after they have branched off from their common progenitor. On the other hand, it

**FIGURE 3.** Generation of T cells, NK cells, and DC from a single thymic progenitor in a two-step culture system. Individual FT cells were placed in a well of a Terasaki plate and cultured for 8 h in the presence of a cytokine mixture. One of the paired daughter cells was transferred to a FT organ culture to examine T/NK cell generation, while the other was left to examine DC generation. A, A representative flow cytometric profile of T cells and NK cells generated from one of the paired daughter cells. B, A representative photograph of DC generated from one of the paired daughter cells.
was reported that normal numbers of DC are seen in the thymus of PU.1-deficient mice, which lack DC in other tissues in addition to macrophages. It seems to be an irreversible specification step. The identification of lineage restrictions during hemopoiesis. This may imply that the myeloid program represents a common basic program of hemopoietic cells on which a specification program is added. In this context, it can be presumed that the DC potential retained in T cell progenitors has been developed in phylogeny by modifying the basic myeloid program retained in T cell progenitors. During ontogeny, the transition from T/myeloid progenitors to T/DC progenitors through losing the ability to give rise to myeloid cells seems to be an irreversible specification step. The identification of

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*A total of 60 individual 12 dpc FT FcRα cells were placed in a well of a Terasaki plate. After 8 h of cultivation in the presence of a cytokine mixture, wells were screened with a microscope, and 47 wells were found to contain paired daughter cells. One of the daughter cells was transferred to a FT organ culture to examine T and NK cell generation, while the other was left to examine DC generation.*

**FIGURE 4.** Generation of T cells, NK cells, and DC from a single thymic progenitor in FT organ culture. *A,* A modified FT organ culture system used for the determination of T, NK, and DC potential of a single progenitor. *B,* Representative flow cytometric profiles of cells generated from a single progenitor of the 14 dpc FT CD44+CD25− population. Cell recoveries from these p-T/NK, p-T, and p-NK clones are 1.2 × 103, 5.0 × 102, and 3.6 × 102, respectively. *C,* Representative photographs of DC derived from a single FcRγc FT progenitor. Cells with dendritic morphology showing green fluorescence were regarded as DC. Scale bar in each photograph indicates 50 μm. *D,* Expression of CD11c and class II on DC generated in FT organ culture. DC surviving on stromal cells were recovered by trypsin treatment, stained with anti-CD11c or anti-class II, and analyzed with a flow cytometer. *E,* DC potential of progenitors in various subpopulations of FT cells. The number of p-T/NK, p-T, and p-NK determined by flow cytometric analysis of progeny cells per 50 cells examined individually per each population is plotted. The green portion represents the number of progenitors that showed DC potential. The results are representative of three independent experiments.
genes that are involved at this transition stage will facilitate clarification of the molecular mechanism underlying the specification of a progenitor to the T cell lineage.

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