Thymic Dendritic Cell Precursors: Relationship to the T Lymphocyte Lineage and Phenotype of the Dendritic Cell Progeny

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Summary
Successive T-precursors isolated from adult mouse thymus were examined for their developmental potential, by transfer to irradiated Ly 5-disparate recipients. The earliest, “low CD4” precursors formed T, B, and dendritic cells (DC), but not myeloid cells, in accordance with earlier studies. Surprisingly, the next downstream CD4^-8^-3^-44^-25+ precursor population still formed DC as well as T cells although it no longer formed B or myeloid cells. Further downstream, the CD4^-8^-3^-44^-25+ population formed only T cells. The thymic and splenic DC progeny of the early thymic precursors all expressed high levels of CD8α, in contrast with normal splenic DC and the splenic DC progeny of bone marrow stem cells, which consisted of both CD8^- and CD8^+ DC. A common precursor of T cells and of a subclass of DC is proposed, with CD8α as a marker of the lymphoid-related DC lineage.

The earliest precursor of T lymphocytes so far identified in the adult mouse thymus resembles bone marrow hematopoietic stem cells (BMSC)^1 in surface phenotype, except for the expression of the antigen Sca-2 and of a low level of CD4: for this reason we have termed it the “low CD4 precursor” (1^-3). This population has TCR β and γ genes in germline configuration. Despite the presence of some enzymes associated with recombination, it still lacks even D-J β gene rearrangements (1, 4). A striking feature of this population is its ability to generate dendritic cells (DC), B, and NK cells, as well as T cells, although it has lost the capacity to generate erythroid and most myeloid cells (2, 5^-9). This suggested the same early intrathymic precursor cell gives rise to both thymic DC and to the thymic T-lineages (5, 6), in contrast with the established myeloid origin for most DC (10, 11). Strict clonal evidence for this hypothesis of a lymphoid origin for thymic DC is still not available. However, some support of the concept has come from the finding that thymic DC express several surface molecules normally considered characteristic of lymphoid cells, in particular the early B cell marker BP-1 (6) and the T cell marker CD8, in the form of an αα homodimer (6, 12).

Another early T-precursor cell was delineated by Godfrey and Zlotnik (13) and Godfrey et al. (14) within the CD4^-8^-3^-CD44^-25+ c-kit^-Thy-1+ (although both CD44 and c-kit should be more precisely defined as low rather than negative). This precursor population represents the stage where TCR β genes are rearranged (4, 13, 15), and on this basis would be considered a T-committed population. Exit from this stage and further development towards α/β T cells involves signals from the newly described pre-T α chain (16).

We now assess the ability of all three of these early T-lineage precursor populations to generate DC, and compare this to their capacity to produce other hematopoietic lineages. The results suggest a sequential rather than simultaneous loss of other developmental potentials en route to T cells, with the ability to form DC only being lost at the stage of TCR β gene rearrangement. Since the artificial transfer of the low CD4 thymic precursor population by intravenous injection produced progeny DC in the spleen as well as in the thymus, we have been able to assess whether the unique surface phenotype of thymic DC is dictated by the environment or by the nature of the precursor. Although some markers varied with the site of development, CD8α was always present and served as a marker of the DC lineage derived from the T cell precursors.

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Materials and Methods

Mice. The mice were bred at The Walter and Eliza Hall Institute under specific pathogen-free conditions. The donors in reconstitution experiments were 4–6-wk-old C57BL/Ka Thy-1.1 (Ly 5.2) or C57BL/6 Thy-1.2, Ly 5.2) mice and the recipients were 7–8-wk-old C57BL/6 Ly 5.1-Pep56 (Thy-1.2) mice.

mAbs and Fluorescent Reagents. The mAbs and hybridoma clones used for depletion and for immunofluorescent labeling, the fluorochromes employed, and the fluorescent reagents used, are all specified elsewhere (4, 6, 12).

Purification of Intrathymic Precursor Populations. The low CD4 precursors were isolated from C57BL/Ka Thy-1.1 or C57BL/6 donor thymuses as described previously (1, 2, 5). This involved complement-mediated cytotoxic depletion, adherence depletion, and then immunomagnetic bead depletion of mature thymocytes, of CD44+ thymocytes, of more developed precursors bearing CD2 or CD25, and of non T-lineage cells. The <1% remaining cells were then stained in three fluorescent colors and the low CD4 precursors (5–10% of the depleted population) sorted as Thy-1+ and heat-stable antigen (HSA)+ c-kit+ cells, was also used with identical results. The triple negative, CD44+25+ and CD44-25+ populations were isolated as described previously (4). This involved cytotoxic and immunomagnetic bead depletion of cells bearing CD4, CD8, and CD3, together with depletion of non T-lineage cells. The depleted cells were then immunofluorescence stained and the precursors sorted on the basis of CD25 and c-kit expression. Purity on reanalysis was 98–99%. In some experiments, an improved procedure (4) involving density separation, adherence, double immunomagnetic bead depletion, then sorting the precursors as Thy-1+ HSA+ c-kit+ cells, was also used with identical results.

Purification of BMSC. The isolation of pure long-term reconstituting BMSC was as described elsewhere (17), with these stem cells being finally sorted as Lin- c-kit+ Ly 6A/E+ Rhodamine 123+ cells. In some experiments, an enriched but not pure lineage marker negative bone marrow (Lin-BM) stem cell preparation was obtained by incubating cells with an antibody mix and then using anti-lg-coated magnetic beads to deplete cells bearing markers of hemopoietic lineages including T cells, B cells, erythrocytes, macrophages, and granulocytes.

Reconstitution of Irradiated Recipients with Precursor Cells. The procedures have been presented in detail elsewhere (2, 5, 6). For intrathymic transfer, the recipient mice were γ-irradiated (7.5 Gy); 10^6 low CD4 precursors, 10^6 CD44+25+ precursors, 10^6 CD44-25+ precursors, or 10^6 BMSC were injected into one thymus lobe. The injected lobes were then analyzed 7–21 d later. For intravenous transfer, the recipient mice received two doses of γ-irradiation (each 5.5 Gy, 3 h apart) and, to ensure survival, received 4 × 10^6 recipient-type bone marrow cells intravenously. The donor cells transferred intravenously were 3 × 10^7 low CD4 precursors, 3 × 10^6 CD44+25+ precursors, 3 × 10^6 CD44-25+ precursors, 10^6 purified BMSC, or 4 × 10^6 Lin-BM. The spleens and thymuses were analyzed 1–4 wk later.

Enrichment of DC. Before analysis for reconstitution by donor-derived cells, it was necessary to enrich the DC by purification from pooled recipient thymuses or spleens. The methods have been described in detail elsewhere (5, 6, 12). The organs were chopped, digested with collagenase, and treated with EDTA. Light density cells were collected by a density centrifugation procedure. Finally, non DC-lineage cells were depleted from the light density cells by coating them with a mixture of mAbs and then depleting the coated cells with anti-lg-coated magnetic beads.

Analysis of the Progeny of Transferred Precursors. The presence of donor-derived (Ly 5.2+) cells bearing markers for T cells (Thy-1), B cells (B220), or granulocytes and macrophages (Gr-1, Mac-1) after intravenous transfer was determined by direct two- or three-color immunofluorescent staining on suspensions of the recipient spleens or thymuses using FITC-conjugated anti donor-type Ly 5.2, PE-conjugated anti-Thy-1, and either biotinylated anti-B220 or biotinylated anti-Mac-1 together with biotinylated anti-Gr-1, followed by Texas red-avidin as the second stage. The presence of donor-derived (Ly 5.2+) cells bearing DC markers (class II MHC, CD11c, DEC-205, CD80, BP-1) was assessed on DC-enriched preparations extracted from pooled recipient thymuses or spleens. The enriched DC were stained in three fluorescent colors with FITC anti-Ly 5.2, allophycocyanin anti-class II MHC and biotinylated antibody against one other marker followed by PE-avidin as the second stage. During flow cytometric analysis, the cells were gated for donor origin (Ly 5.2+) and DC characteristics (high class II MHC and characteristic high forward and side light scatter), then analyzed for expression of a third DC marker. Full details are given elsewhere (5, 6).
**Results**

*Reconstitution Kinetics.* To check that the various thymic T-precursor populations behaved as sequential steps in T cell development, the kinetics of development of T-lineage progeny after intrathymic and intravenous transfer into irradiated recipients differing in Ly5 allotype, was compared (Fig. 1), using criteria previously established in this laboratory (18, 19). Upon intrathymic transfer, the fastest reconstitution was obtained with the CD44⁻⁺⁻ triple negative pre-T precursor, the next fastest with the CD44⁺⁺⁺ triple negative pro-T precursor, and the slowest reconstitution from the low CD4 precursor (Fig. 1). This result is in line with the fetal thymus organ culture repopulation studies of Godfrey et al. (14), assuming that their CD44⁺⁺⁻ population corresponds to our low CD4 precursors. Previously we had established that the low CD4 precursor, in turn, gave faster reconstitution than BMSC (1). Similar reconstitution kinetics were obtained after intravenous transfer (Fig. 1), except that very few progeny were detected with the CD44⁺⁺⁺⁺ precursors population. This population was deficient in its ability to seed the thymus, presumably because of its low expression of CD44, a thymus-homing molecule (20, 21). In addition to this kinetic evidence, we have previously demonstrated that after transfer, the progeny of the low CD4 precursors lose CD4 and gain CD25 (1, 2). Accordingly, all these results support the earlier models (3, 13) of a linear, sequential developmental process with the precursors in the sequence: low CD4 precursor, then CD44⁺⁺⁻ pro-T, then CD44⁺⁺⁺ pro-T.

*Development of Lymphoid and Myeloid Cells from Intrathymic Precursors.* To provide a side-by-side control for the DC precursor studies and to check previous conclusions, the nature of the predominant progeny formed in the spleen and lymph nodes of irradiated recipients after intravenous injection of the intrathymic precursors was determined. Suspensions of pooled recipient spleen and lymph nodes were stained for donor type Ly 5.2, together with anti-Thy-1 (T cells), B220 (B cells), and anti-Mac-1 and Gr-1 (myeloid cells). Three separate experiments, one for each precursor population, were shown; each result was typical of three experiments. The quadrants were set based on the background staining for each experiment. The differences in staining intensity between experiments was due to changes in the reagents used and variations in instrument settings. Note that there is no staining for recipient type Thy-1 + cell in the spleen and lymph nodes reconstituted by Lin⁻BM. This is because in these particular experiments, the Lin⁻BM cells were obtained from C57BL/6 Ka Thy-1.1 (Ly5.2) mice, and in the analysis for donor-derived cells, only anti-Thy-1.1 antibody was used together with anti-Ly 5.2 to reveal the donor type Thy-1 + cell but not the recipient type Thy-1 + (Thy-1.2) cells. In the experiments with thymic precursors, both donor and recipient were Thy-1.2 mice, and the anti-Thy-1.2 antibody revealed both donor and recipient T cells.
Figure 3. The generation of dendritic cells in the spleen and thymus of recipient mice after intravenous transfer of different thymic precursor cells. The purified precursors \((3 \times 10^4)\) were transferred intravenously into irradiated Ly 5 congenic recipient mice. 2 wk after transfer, the recipient thymuses and spleens were collected and DC enriched as described in Materials and Methods. Donor-derived DC were identified by staining cells with antidonor type Ly 5.2 and anti-MHC class II and then gating for Ly 5.2+ MHC class II cells. Note that the relative level of MHC class II fluorescence was high, the sensitivity being reduced to keep all cells on scale. Equivalent results were obtained when CD11c or DEC-205 was used to distinguish DC. The results are typical of five such experiments.

Table 1. The Generation of DC Progeny from Intrathymic Precursor Populations

| Precursor population | No. of cells transferred | Route of transfer | Donor-derived DC per thymus | Donor-derived DC per spleen |
|----------------------|--------------------------|-------------------|-----------------------------|-----------------------------|
| Low CD4              | \(3 \times 10^4\)         | intravenous       | \(1.4 \times 10^3\)         | \(17 \times 10^3\)           |
| CD44+CD25+           | \(3 \times 10^4\)         | intravenous       | \(1.6 \times 10^3\)         | \(14 \times 10^3\)           |
| CD44−CD25+           | \(30 \times 10^4\)        | intravenous       | \(0.3 \times 10^3\)         | \(0.8 \times 10^3\)          |
| CD44+CD25+           | \(10 \times 10^4\)        | intrathymic       | \(0.1 \times 10^3\)         | ND                          |

Results are the means of two experiments, each experiment involving a pool of four to eight recipient mice. In the case of the CD44+CD25+ precursor population, although some cells were counted in the DC progeny quadrants, no clear donor type DC population was delineated after either intravenous or intrathymic transfer.
Development of DC from Intrathymic Precursors. Having confirmed the sequential loss of myeloid and then B lymphocyte potential by these early T-precursor populations, we used the same procedures to assess DC developmental potential. In particular, since the CD44+25+ triple negative pro-T cell was confirmed to have lost the capacity to form B cells despite still being in germline configuration for the TCR genes tested (4, 13, 15), its capacity to form DC in thymus and spleen was compared with that of the low CD4 precursor. Because DC are relatively rare components, it was necessary to pool recipient organs and enrich for DC before analysis. This enabled even low levels of DC to be detected above the background. Examples of the analysis of these enriched preparations for donor type (Ly 5.2+) DC (class II MHC+) after intravenous transfer of precursors is given in Fig. 3; it is important to note that the results were similar when other markers of DC, including CD11c and DEC-205, were used instead or in combination with class II MHC. The total number of DC progeny obtained after transfer of different precursors is given in Table 1.

As we have reported previously (5, 6), the low CD4 precursor population formed DC in both the thymus and the spleen after intravenous transfer. In surprising contrast to the result for B cell progeny, the later CD44+25+ triple negative pro-T cell was confirmed to have the capacity to form DC progeny in thymus and spleen, compared with the low CD4 precursors. However, although 10-fold more cells were transferred, the downstream CD44+25+ triple negative pre-T cells formed very few, if any, DC progeny regardless of the route of transfer (Table 1). Thus, during early thymic precursor cell development, the capacity to form B cells was lost before the capacity to form DC.

Nature of the DC Developing in the Thymus and in the Spleen from Thymic Precursors. To test whether the surface phenotype of thymic DC was determined by the thymic environment or by the nature of the DC precursor, the thymic precursors were transferred intravenously into irradiated Ly5 congenic recipients. 2 wk after transfer, thymic and splenic DC enriched from pooled recipient organs and stained in three colors with anti-donor type Ly 5.2, anti-MHC class II, and anti-BP-1. The progeny DC were detected and gated as Ly 5.2+ MHC class II+, and anti-BP-1. The BP-1 expression then determined using the third fluorescent color. (Dotted line) Iso-type control background staining. The results are compared with the DC present in normal, nonirradiated mice (top). The results are typical of three such experiments.

Figure 4. The expression of BP-1 on the surface of dendritic cell progeny in the spleen and thymus after intravenous transfer of thymic precursor cells or BMSC. Purified low CD4 precursors (3 x 10⁶) or purified BMSC (10⁶) were transferred into irradiated Ly5 congenic recipients. 2 wk after transfer, thymic and splenic DC enriched from pooled recipient organs and stained in three colors with anti-donor type Ly 5.2, anti-MHC class II, and anti-BP-1. The progeny DC were detected and gated as Ly 5.2+ MHC class II+, and the BP-1 expression then determined using the third fluorescent color. (Dotted line) Iso-type control background staining. The results are compared with the DC present in normal, nonirradiated mice (top). The results are typical of three such experiments.
In the third series of experiments, the level of CD8α on the surface of the DC progeny was determined, since thymic DC are almost all CD8α+, whereas a definite population of splenic DC are CD8α− (6, 12). It has also been established that CD8α+ DC contain CD8α mRNA (but not CD8β mRNA), so the CD8α is synthesized by the DC themselves (12). The pure BMSC reconstituted both the CD8− and CD8+ DC populations of spleen, in a proportion almost identical to the normal splenic DC populations (Fig. 5). In contrast, the thymic low CD4 precursor and the thymic CD44+/CD25− precursor both produced only CD8+ DC, in both the thymus and the spleen (Fig. 5). Since the animals injected with the Ly 5.2+ thymic precursors were also injected with recipient type Ly 5.1+ bone marrow cells to ensure survival after irradiation, these experiments also had an internal control. In contrast to the Ly 5.2+ DC progeny of the thymic precursors, the Ly 5.1+ recipient type DC had the normal proportion of CD8−DC, exactly as for the BMSC reconstituted animals (data not shown).

A kinetic study of the DC progeny after intravenous transfer of different precursor populations was performed to ensure these results did not alter with time after reconstitution. It was found that 87% of splenic DC progeny of the low CD4 precursors were strongly CD8+ at day 17, 93% were CD8+ at day 21, and 96% were CD8+ at day 28. At no time point was a discrete CD8− population obtained. Although a small proportion of DC expressing lower levels of CD8 was evident among the progeny, especially early after transfer, these were probably less mature cells in the process of acquiring CD8; we have already demonstrated that the level of CD8 on thymic DC increases with their maturation (6). In comparison, the BMSC gave rise to 76% CD8+ splenic DC progeny at day 17, 78% at day 21, and 60% at day 28, with a discrete CD8− DC population being obtained at all time points.

Our procedure for DC isolation (12) normally includes a step of macrophage depletion, using graded levels of anti-Mac-1 and immunomagnetic beads, which removes cells expressing high levels of Mac-1 but leaves cells expressing...
low levels of Mac-1. We have recently noted that a higher proportion of CD8+ DC can be extracted from spleen if Mac-1 depletion is avoided entirely during DC enrichment. Accordingly, the DC progeny of the thymic precursors were examined using this less stringent enrichment procedure (Fig. 6). Even though this increased the relative level of CD8+ DC detected in normal spleen, no CD8+ DC progeny, only CD8+ DC progeny, were obtained from the thymic precursors.

Discussion

Our kinetic studies on thymus reconstitution reinforce the view (1, 3, 13) that the low CD4 precursors represent the earliest T-precursor population so far defined and isolated from the adult mouse thymus. The rate of thymus reconstitution, as well as the surface phenotype, suggests these precursors are intermediate in development between BMSC and the pro-T CD44+3-44+25+ thymic subset. However, the relatively broad peak of reconstitution from the low CD4 precursors suggests that more than a single wave of reconstitution is involved. This precursor population may therefore include both very early cells and cells almost as mature as the CD44+25+ population. This fits with our concept that the low CD4 precursor population includes cells from some 7 d of development after the initial seeding event (3, 24). This implies that the initial cells seeding from the bone marrow, and their immediate products, might still be minority components of this preparation. These still earlier precursors could even be multipotent, and responsible for the traces of myeloid precursor activity we sometimes see in the low CD4 precursor population. This trace of possible myeloid precursor activity has largely disappeared from the later, CD4+8-3-44+25+ pro-T population.

Even allowing for some heterogeneity in developmental state, the low CD4 early precursor population of the adult mouse thymus displayed some surprising developmental capabilities, apparently differing from those of the early precursor cells in embryonic mouse thymus (25) or blood (26). The capacity to form both T and B cells, but very few erythrocytes, granulocytes, or macrophages (2), suggested the low CD44+25+ precursor shows undiminished DC precursor activity per cell and shows a ratio of DC to T cell progeny similar to that of the low CD4 precursor population. Furthermore, the low CD4 precursor and the CD44+25+ precursor both produce only the CD8α type of DC, even when they seed the spleen, in contrast to BMSC; this is further evidence that they are not contaminated with multipotent stem cells or myeloid precursors. These results indicate there is a coincidence, or at least a very strong linkage, between the thymic DC precursors and the earliest T-lineage precursors. Of course the results with the CD44+25+ precursor do not strengthen the evidence for a common T and B cell precursor potential at the low CD4 precursor stage. However, in support of the view of a common DC/lymphoid precursor, a cell with T, B, and DC generative capacity, but apparently unable to form other lineages, has now been isolated from human bone marrow by Galy et al. (27). Linked pathways of T cell and DC development from human thymus CD34+ precursors have also been described by Marquez et al. (28), although these precursors also had monocyte developmental potential. We favor the concept that the T, B, and DC progenitor activities are developmentally linked, having a common precursor downstream from the BMSC. Since the capacity to form B cells is lost before DC, thymic DC would then be a relatively late branch off the T lineage. This concept still requires direct confirmation, either by a clonal approach or by further evidence that DC bear markers of a lymphoid past.

It is of interest that the c-kit+CD44+25+ pre-T cell, the cell undergoing TCR β gene rearrangement (3, 13, 15), lacks detectable B or DC precursor activity. By this stage this precursor seems fully committed to the T cell lineage, although the issue of α/β versus γ/δ lineage commitment is still under debate. It is not clear whether TCR gene rearrangement itself, or some linked control mechanism, determines this T-lineage commitment.

If thymic DC are a discrete DC lineage, either lymphoid derived or in some other way linked to T cell development, can this lineage be distinguished from other DC on the basis of surface markers? It is now clear that certain molecules on the surface of thymic DC are simply picked up from associated thymocytes and cannot serve as useful markers: Thy-1 is clearly in this category (6, 22) and it is likely that a small amount of CD4 and CD8α/β can also be absorbed onto DC (6). Nor do surface molecules made by the DC themselves in response to a particular inductive environment serve as useful permanent markers of a lineage. BP-1, although formed by thymic DC themselves (6), appears to be induced by the thymic environment, since in

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the spleen the progeny of thymic DC precursors are BP-1-.
However, our evidence suggests that CD8α expression
does mark a distinct thymic type of DC lineage, since the
thymic DC precursors formed only CD8α+ DC even
when allowed to produce DC in the spleen. This was in
contrast to BMSC that formed both CD8- and CD8+ DC
in the spleen. To date, CD8α is the only marker present on
thymic DC that is consistently associated with the progeny
of the thymic precursors regardless of whether they de-
velop in the spleen or the thymus.

If CD8α marks a lymphoid-derived or lymphoid-related
thymic DC population, we must now question the origin
of the CD8α+ DC found in the spleen and other lymphoid
tissues. To date, there is no evidence that the CD8α and
CD8- DC of spleen have a precursor-product relationship,
although a few CD8- DC could be immature cells en
route to CD8+ (6). On overnight incubation these CD8α and
CD8- DC retain their phenotype, the only change be-
ing some increase in the CD8α expression by the CD8α
DC (6). The CD8α+ DC in spleen are unlikely to be thy-
mem derived since they are present at normal levels in athy-
mic “nude” mouse spleen (Vremec, D., and K. Shortman,
unpublished data). We have been unable to detect the exit
from the thymus of either the precursor cells or the DC
progeny after intrathymic transfer (Wu, L., unpublished
data). Our experiment of injecting the thymic precursors
intravenously was therefore artificial, since there is no evi-
dence that such precursors ever exit the thymus. Neverthe-
less this experiment suggests that an equivalent lymphoid-
derived or lymphoid-related precursor may exist outside
the thymus and give rise to a line of splenic CD8α+ DC,
separate from but related to thymic DC. An important
issue is whether these proposed CD8α and CD8- splenic
DC lineages differ in biological function. Our recent stud-
ies indicate that they do differ in function. Although both
CD8α+ and CD8-DC are able to activate CD4 T cells, the
Fas ligand-bearing CD8α+ DC then kill these activated T
cells by Fas-mediated apoptosis (29). The CD8α+ DC may
therefore have a regulatory function distinct from the bet-
ter known stimulatory CD8α- DC.

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