Bifurcated Dendritic Cell Differentiation In Vitro From Murine Lineage Phenotype-Negative c-kit+ Bone Marrow Hematopoietic Progenitor Cells

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We have recently established the culture system to generate dendritic cells (DCs) from murine Lin−c-kit+ bone marrow hematopoietic progenitor cells (HPCs) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) + stem cell factor (SCF) + tumor necrosis factor-α (TNF-α). We present here the identification of two DC precursor subsets originated from HPCs with the phenotype of CD11b+CD11c− and CD11b−CD11c+ that develop independently at early time points (days 4 to 6) in the same culture conditions. Both of CD11b+/−CD11c− and CD11b+/−CD11c+ precursors could differentiate at day 10 to 14 into CD11b+/−CD11c+ mature DCs with typical morphology, phenotype, and the ability to stimulate allogeneic mixed leukocyte reaction (MLR). However, the endocytic capacity of fluorescein isothiocyanate-dextran was markedly reduced during the differentiation. CD11b+/−CD11c− precursors expressed high levels of Ia, CD86, CD40, and E-cadherin molecules, but not c-fms transcript, and mature DCs derived from this precursor subset continue to express abundant E-cadherin antigen, a discernable marker for Langerhans cells. In contrast, CD11b+/−CD11c+ precursors expressed c-fms mRNA, but low levels of Ia, CD86, and E-cadherin, whereas CD40 was undetectable. CD11b+/−CD11c+ mature DCs differentiated from these precursors displayed abundant c-fms mRNA and nonspecific esterase activity. Interestingly, CD11b+/−CD11c+ precursors, but not CD11b+/−CD11c− precursors, may be bipotent cells that can be induced by M-CSF to differentiate into macrophages. All of these results suggest that CD11b+/−CD11c− and CD11b+/−CD11c+ cells are distinct DC precursors derived from Lin−c-kit+ HPCs, which differentiate into mature DCs through bifurcated and independent DC differentiation pathways.

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precursors represent two distinct intermediate-stage cell subsets with the capacity to differentiate into mature DCs.

MATERIALS AND METHODS

Cytokines and antibodies. Recombinant murine SCF and GM-CSF were kindly provided by Kirin Brewery Co (Tokyo, Japan) and Dr T. Sudo (Basic Research Institute of Toray Co, Kanagawa, Japan), respectively. M-CSF was kindly provided by Morinaga Milk Industry Cooperation (Morinaga, Japan). Mouse TNF-α was produced as described previously.15 Endotoxin was not detectable in these cytokine preparations using a Toxicolor assay kit (Seikagaku-Kogyo, Tokyo, Japan). These cytokines were used at the optimal concentrations as previously described.7 An anti-c-kit antibody (ACK-2) was kindly provided by Dr T. Sudo (Toray, Kanagawa, Japan) and conjugated with biotin by using an NHS-Biotin kit (Pharmacia-Biotech, Uppsala, Sweden) according to the manufacturer’s instructions.19 A rat monoclonal antibody (MoAb) to murine dendritic cell marker, DEC-205 (NLDC145), was a generous gift of Dr R.M. Steinman (Rockefeller University, New York, NY).20,21 MoAb to mouse E-cadherin was purchased from Dainippon Pharmaceutical Co (Tokyo, Japan) and maintained under pathogen-free conditions in the Animal Facility of Department of Molecular Preventive Medicine, the University of Tokyo (Tokyo, Japan). All animal experiments complied with the standards set out in the Guideline for Care and Use of Laboratory Animals of the University of Tokyo.

Suspension culture of Lin−c-kit+ HPCs. BM cells were obtained by aspirating femurs and tibiae of 8- to 10-week-old female mice. Lin−c-kit+ HPCs were isolated from nonadherent BM mononuclear cells (MNCs) using an EPICS ELITE cell sorter (Coulter Electronics, Hialeah, FL) as previously described.22,23 In brief, nonadherent MNCs were stained with an indirect staining composed of biotin-conjugated anti-c-kit MoAb and phycoerythrin (PE)-labeled streptavidin followed by a set of fluorescein isothiocyanate (FITC)-labeled MoAbs to CD3 (145-2C11), CD4 (H129.19), CD8 (53-6.7), B220 (RA3-6B2), Gr-1 (Ly-6G), CD11a (2D7), and CD11b (M1/70). The contamination by other types of cells in these preparations was consistently less than 0.5% as shown by an immunofluorescence analysis.

Purified Lin−c-kit+ HPCs were incubated as previously described7 at a cell concentration of 1 × 10^6 cells/mL in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), 5 × 10^{-5} mol/L 2-mercaptoethanol, penicillin G (100 U/mL), and streptomycin (100 µg/mL) in the presence of GM-CSF + SCF + TNF-α. Optimal conditions were maintained by splitting these cultures at day 4 with medium exchange containing fresh cytokines. For most experiments cells were collected after 6 days of culture for cell sorting.

Isolation of CD11b−hiCD11c− and CD11b−dullCD11c− DC precursors by a cell sorter. After 6 days of culture in the presence of GM-CSF, SCF, and TNF-α, cells were collected, labeled with FITC-conjugated anti-CD11b and PE-conjugated anti-CD11c (HL3), and sorted into CD11b−hiCD11c− and CD11b−dullCD11c− cell subsets. In some experiments, the CD11b−CD11c− cell fraction was also sorted. All the staining and sorting procedures were performed in the presence
of 1 mmol/L EDTA to avoid cell aggregation. Reanalysis of the sorted populations showed a purity higher than 98%. Sorted cells were routinely incubated in medium containing GM-CSF + TNF-α or M-CSF for an additional 5 to 8 days. The cultured cells were collected between days 10 and 14.

Immunofluorescence analysis. Immunofluorescence analyses were performed as previously described. In three-color analyses, 4 × 10⁶ cells were incubated with biotinylated hamster anti-CD11c MoAbs and rat anti-CD11b MoAbs, followed by staining with Cy-Chrome (CY)-labeled streptavidin and PE-conjugated goat anti-rat IgG (Fab')₂ antibody. The cells were then stained with various FITC-conjugated MoAbs. In some experiments, the cells were first stained with rat anti-ß2 integrin MoAb and biotinylated anti-CD11c, followed by staining with PE-conjugated goat anti-rat IgG(Fab')₂ antibody and CY-labeled streptavidin, and then stained with FITC-conjugated anti-CD11b. In other experiments, the cells were first stained with biotinylated antibodies and revealed by CY-conjugated streptavidin, followed by staining with PE-conjugated anti-CD11c and FITC-conjugated anti-CD11b. The instrument compensation was set in each experiment using single-color and/or two-color stained samples.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs were extracted from 1 × 10⁵ indicated cells using RNeasy columns (Biotex Laboratories Inc, Houston, TX), according to the manufacturer’s instructions. First-strand cDNA was synthesized in a 25-µL reaction volume using an RT-PCR kit (Takara Shuzo, Kyoto, Japan) with random primers. Thereafter, cDNA was amplified for 25 cycles consisting of 94°C for 30 seconds, 57°C for 1 minute, and 72°C for 1.5 minutes with the c-fms-specific oligonucleotide primers (5'-CCGAGAAGCGTTGGTAGAGCC-3' and 5'-CAGCTTTGCTAGGCTCAATT-3'), which specifically result in a 500-bp cDNA encoding c-fms. As a control, mouse ß-actin transcript was amplified in parallel as previously described. The PCR products were fractionated on 1.5% agarose gel and visualized by ethidium bromide staining.

Electron microscopy. Cultured cells sampled after 7 and 14 days of culture were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon 812 (E. Fullan, Inc, Latham, NY).
Ultrathin sections were then cut and stained with uranyl acetate and lead citrate, and examined with an electron microscope, HITACHI H-800 (HITACHI, Tokyo, Japan).

Statistical analysis. Differences were evaluated using the Student’s t-test. P values of <.05 were considered to be statistically significant.

RESULTS

Differentiation of two DC precursor subsets from Lin- c-kit+ HPCs. To elucidate the cellular basis of the development of DC, we followed the kinetics of CD11b and CD11c expression during differentiation of murine Lin- c-kit+ HPCs stimulated with GM-CSF + SCF + TNF-α. At day 0, murine Lin- c-kit+ HPCs did not express CD11b or CD11c (Fig 1A). However, two cell populations characterized by the expression of CD11b-CD11c+ and CD11b+CD11c+ emerged independently in the cultures at day 4. At day 6, a distinct population of CD11b+CD11c+ cells could be identified (7.2% ± 1.2%) which increased rapidly thereafter and reached the maximum levels by days 10 to 14. Although CD11b+CD11c+ cells (22.9% ± 3.5%) were usually dominant over the CD11b-CD11c+ cells in the cultures at day 6, they did not increase thereafter during more prolonged culture periods (Fig 1A), implying that CD11b+CD11c+ cells may contribute to the later generation of CD11b-CD11c+ cells in the cultures. To examine this possibility, the three cell populations CD11b-CD11c+, CD11b+CD11c+, and CD11b-CD11c+ were routinely sorted at day 6 (Fig 1B) and cultured in the presence of GM-CSF + TNF-α for an additional 5 to 8 days. Most of the CD11b-CD11c+ cells could differentiate into cells with the CD11b-CD11c+ phenotype at days 10 to 12 (81.7 ± 11.2%, n = 15) and did not revert into a CD11b+ phenotype. Interestingly, the CD11b-CD11c- cell population generated both of CD11b-CD11c+ (8.4% ± 2.1%, n = 4) and CD11b+CD11c+ (25.1% ± 3.4%, n = 4) cells at a similar rate and extent as Lin- c-kit+ HPC cultures at day 6 (Fig 1B). These results suggest that Lin- c-kit+ HPC may generate CD11b-CD11c+ cells through at least two independent differentiation pathways.
one may directly differentiate from Lin<sup>−</sup>-c-kit<sup>+</sup> HPCs at the early stage, while another one is an intermediate stage for CD11b<sup>+</sup>/CD11c<sup>−</sup> cells at the latter stage of culture.

To further characterize the phenotype of CD11b<sup>+</sup>/dull CD11c<sup>−</sup> and CD11b<sup>+</sup>hi CD11c<sup>−</sup> cells generated in the cultures at day 6, three-color immunofluorescence analyses were performed. These showed that both of the subsets expressed comparable levels of Thy-1, CD11a, and CD32/16, CD11b<sup>+</sup>/dull CD11c<sup>−</sup> cells expressed much higher levels of Ia, CD86, E-cadherin, and DEC-205 than CD11b<sup>+</sup>/dull CD11c<sup>−</sup> cells. Interestingly, CD11b<sup>+</sup>/dull CD11c<sup>−</sup> cells also expressed high levels of CD40 that were undetectable on CD11b<sup>+</sup>/dull CD11c<sup>−</sup> cells at this time point (Fig 2). Taken together, these data would indicate that the two populations differ phenotypically from each other in the expression of a number of surface markers.

Because both CD11b<sup>+</sup>/dull CD11c<sup>−</sup> and CD11b<sup>+</sup>hi CD11c<sup>−</sup> cells can differentiate into cells of the CD11b<sup>+</sup>/dull CD11c<sup>−</sup> phenotype, a previously demonstrated DC specific phenotype, we therefore designated these two cell populations sorted from Lin<sup>−</sup>-c-kit<sup>+</sup> HPC cultures at day 6 as CD11b<sup>+</sup>/dull CD11c<sup>−</sup> and CD11b<sup>+</sup>hi CD11c<sup>−</sup> DC precursors, respectively.

Both CD11b<sup>+</sup>/dull CD11c<sup>−</sup> and CD11b<sup>+</sup>hi CD11c<sup>−</sup> DC precursors can differentiate into mature phenotypically distinct DC-like cells. To better understand the phenotypic differences between the two DC precursors and their derived mature DCs, we therefore performed three-color immunofluorescence analyses on day 6. Although both subsets expressed comparable levels of Thy-1, CD11a, and CD32/16, CD11b<sup>+</sup>/dull CD11c<sup>−</sup> cells expressed much higher levels of Ia, CD86, E-cadherin, and DEC-205 than CD11b<sup>+</sup>/dull CD11c<sup>−</sup> cells. Interestingly, CD11b<sup>+</sup>/dull CD11c<sup>−</sup> cells also expressed high levels of CD40 that were undetectable on CD11b<sup>+</sup>/dull CD11c<sup>−</sup> cells at this time point (Fig 2). Taken together, these data would indicate that the two populations differ phenotypically from each other in the expression of a number of surface markers.

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same culture conditions (Fig 3D and E). These cells could be easily detached and had the morphological characteristics of DC-like cells (Fig 3F).

During the differentiation of these two DC precursors, c-fms was exclusively expressed by CD11b<sup>hi</sup>CD11c<sup>+</sup> DC precursors and their mature offspring, but not by CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors and their mature CD11b<sup>+</sup>CD11c<sup>+</sup> offspring (Fig 4A). Interestingly, most of the CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors (Fig 4D) and their mature offspring (Fig 4E) also displayed nonspecific esterase activity in contrast with the CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors (Fig 4B) and their mature offspring (Fig 4C) in which the nonspecific esterase activity was undetectable.

Ultrastructural observation showed that both CD11b<sup>+</sup>CD11c<sup>+</sup> (Fig 5A) and CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors (Fig 5B) were small and mostly round in shape. They contained mitochondria and rough endoplasmic in the cytoplasm and projected a few short cytoplasmic processes. After culture for an additional 5 to 8 days, the cells became larger and showed well-developed Golgi apparatus, mitochondria, rough endoplasmic reticulum (RER), and a few small lysosomes. The cells also developed abundant long cytoplasmic processes, a tubulovesicular system, vesicles, and multivesicular bodies near the nucleus, particularly adjacent to the Golgi apparatus in the cytoplasmic (Fig 5C and D). There were no significant morphological differences between CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursor-derived mature DCs and CD11b<sup>+</sup>CD11c<sup>+</sup> precursor-derived ones.

To characterize the immunophenotype of the mature DCs differentiated from the two DC precursor subsets, three-color immunofluorescence analyses were performed. As shown in Fig 6, CD11b<sup>+</sup>CD11c<sup>+</sup> mature DCs derived from either CD11b<sup>+</sup>CD11c<sup>+</sup> or CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors expressed higher levels of Ia, CD86, CD40, and DEC-205, characteristic of active mature DC. However, E-cadherin antigen, a discernible marker for LCs,<sup>28</sup> was exclusively expressed only on CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursor-derived mature DCs but not by CD11b<sup>+</sup>CD11c<sup>+</sup> precursor-derived ones. All these results indicate that CD11b<sup>+</sup>CD11c<sup>+</sup> and CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors and their derived mature DCs share a combination of different phenotypes and cannot be converted by each other, even though their mature offspring have the common phenotype of CD11b<sup>+</sup>CD11c<sup>+</sup>.

Mature DCs derived from CD11b<sup>+</sup>CD11c<sup>+</sup> and CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors each stimulate allogeneic MLR. As examined by allogenic MLR, CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors, but not CD11b<sup>+</sup>CD11c<sup>+</sup> ones, could effectively enhance allogeneic MLR, suggesting that CD11b<sup>+</sup>CD11c<sup>+</sup> cells may be functionally active in presenting antigen as well as stimulating T-cell proliferation (Fig 7A).

Decrease in endocytic ability during CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursor maturation. During maturation, DCs gradually lose their endocytic ability.<sup>29,30</sup> As shown in Fig 8, either CD11b<sup>+</sup>CD11c<sup>+</sup> or CD11b<sup>+</sup>CD11c<sup>+</sup> precursors could efficiently take up FITC-DX at 37°C, but this was blocked by incubating them at 0°C. This capacity was significantly reduced when these cells differentiated into CD11b<sup>+</sup>CD11c<sup>+</sup> mature DCs induced by GM-CSF + TNF-α at days 12 to 14, while CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors are still functionally immature at day 6, even though they express active markers of DC.

Decrease in endocytic ability during CD11b<sup>+</sup>CD11c<sup>+</sup> and CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursor maturation. During maturation, DCs gradually lose their endocytic ability. CD11b<sup>+</sup>CD11c<sup>+</sup> DC precursors differentiate into macrophage when stimulated with M-CSF. Upon stimulating with M-CSF for an additional 5 to 8 days, all of the CD11b<sup>+</sup>CD11c<sup>+</sup> precursors differentiated uniformly into macrophages with numerous vacuoles and nonspecific esterase activity (Fig 3H and I, Fig 4F). These M-CSF–induced cells expressed moderate to high levels of CD11b but not Ia, CD11c, CD86, or DEC-205 molecules (Fig 9A) and were incapable of stimulating allogeneic MLR (Fig 7B). In contrast,
M-CSF did not induce CD11b<sup>−/−</sup>/CD11c<sup>+</sup> DC precursors to develop into macrophages and all of them died within 3 days in the cultures (Fig 3G). This is consistent with the fact that c-fms transcripts were selectively expressed in CD11b<sup>+</sup>/CD11c<sup>+</sup> DC precursors but not in CD11b<sup>−/−</sup>/CD11c<sup>+</sup> precursors (Fig 4A). These results indicate that CD11b<sup>+</sup>/CD11c<sup>+</sup> precursors may have dual potential to differentiate into either mature DCs or macrophages depending on the supplemented growth factors, whereas CD11b<sup>−/−</sup>/CD11c<sup>+</sup> precursors have already committed to DC lineage, as illustrated in Fig 9B.

**DISCUSSION**

Previous studies have shown the generation of heterogeneous mature DCs in the cultures of mouse BM hematopoietic cells. However, it remains to be established whether the heterogeneous DC subsets may differentiate from a distinct precursor and/or the same precursor committed to DC at a different maturation stage. The present investigation shows that Lin<sup>−</sup>c-kit<sup>+</sup> HPCs generate mature DCs in vitro in response to GM-CSF + TNF-α through the bifurcated DC differentiation pathways CD11b<sup>−/−</sup>/CD11c<sup>+</sup> and CD11b<sup>+</sup>/CD11c<sup>+</sup> DC precursors, respectively (Fig 9B). The CD11b<sup>−/−</sup>/CD11c<sup>+</sup> DC precursors expressed the high levels of CD40, Ia, and CD86. Morphologically, the CD11b<sup>−/−</sup>/CD11c<sup>+</sup> DC precursors were small and mostly round in shape with less cytoplasmic projections than mature DCs as shown by electron microscopy observation. These cells differentiated into mature DCs, but not other myeloid cells, and became large in size with abundant long cytoplasmic processes and multivesicles. During maturation, the endocytic capacity was reduced while the capability of stimulating the proliferation of allogeneic T cells was significantly enhanced. All these features suggest that CD11b<sup>−/−</sup>/CD11c<sup>+</sup> DC precursors are committed DC precursors at a functionally immature stage, which makes them distinguishable from CD11b<sup>−/−</sup>/CD11c<sup>+</sup> mature DCs.

In contrast, CD11b<sup>+</sup>/CD11c<sup>+</sup> DC precursors expressed high levels of myeloid antigens CD11b and c-fms, but low to moderate levels of Ia and CD86, implying that they are myeloid precursor-derived cells. In response to GM-CSF and TNF-α, they differentiated into mature DCs with the phenotype of CD11b<sup>+</sup>/CD11c<sup>+</sup> and obtained the expression of high levels of CD40 and other markers of mature DC. As with their precursors, these mature DCs continuously displayed abundant c-fms mRNA and nonspecific esterase activity. Interestingly, CD11b<sup>+</sup>/CD11c<sup>+</sup> DC precursors could differentiate into macrophages in response to M-CSF, indicating that CD11b<sup>+</sup>/CD11c<sup>+</sup> DC precursors may be an intermediate-stage cell population of myeloid origin rather than being restricted to DC commitment cells at the immature stage.

The observations by electron microscopy indicate that both CD11b<sup>−/−</sup>/CD11c<sup>+</sup> and CD11b<sup>+</sup>/CD11c<sup>+</sup> DC precursors contained mitochondria and rough endoplasmic reticulum in the cytoplasm, while their mature DC offspring had well-developed Golgi apparatus, mitochondria, RER, and tubulovesicular system, particularly vesicles and multivesicular bodies near the Golgi apparatus. As previously described, the tubulovesicular system and vesicles in DCs may be an intracellular storage compartment of MHC class II molecules which has connections to the lysosomal apparatus in the same region within the cells. For successful expression of antigenic peptide-MHC class II complexes on the cell surface, the endocytosed antigen needs to be in proximity to newly synthesized class II MHC molecules in the specialized compartments of the endosome/lysosome system. Most recently it has been shown that DCs can modulate these parameters to control antigen presentation, and maturation of DC engages the appropriate cellular component to stimulate.
FURTHERMORE, it remains unclear whether the DC populations described here correspond to those reported by Maraskovsky et al.16 and Pulendran et al.17 who characterized several in vivo DC subsets including CD11b⁺CD11c⁺, CD11b⁺CD11c⁻, and CD11b⁺CD11c⁺ cell populations in the spleen, but only CD11b⁻CD11c⁺ DC subset in the thymus, of Flt3L-treated mice.16,17 It is noted that in Flt3L-treated mice CD11b⁺CD11c⁺-derived DCs, which have been considered to be of myeloid precursor origin, cannot be induced to differentiate into CD11b⁻CD11c⁺ and CD11b⁺CD11c⁺ cells in vitro by overnight culture.17 CD11b⁻CD11c⁻ DC precursors in our cultures can consistently not differentiate into the same mature DCs with CD11b⁻CD11c⁻ DC precursor-derived mature DCs in the expression of E-cadherin, c-fms, and nonspecific esterase activity; this substantially supports the findings observed by Maraskovsky et al. and Pulendran et al that various DC subsets may develop in vivo along distinct differentiation pathways.16,17

the trafficking of MHC class II molecules onto the cell surface.29,30,34 However, several questions remain to be clarified: (1) the molecular basis of the changes in MHC class II trafficking after forming the complexes with loaded antigens; (2) the signals responsible for constitutive membrane ruffling and endocytosis in immature DCs; and (3) the mechanisms for downregulating this response. All of these questions may be critical for designing DC-based immunotherapy.29,30,34,35 The existence of distinct differentiation pathways mediated by CD11b⁻CD11c⁺ and CD11b⁺CD11c⁺ DC precursors implies that heterogeneous DCs might develop the capacity for driving MHC class II and antigenic peptide complexes to the cell surface in a distinct manner or at a distinct rate. Our findings may provide an important tool for elucidating these questions by using purified and well-defined differentiation or maturation stage of DC precursors based on immunophenotypings; eg, the expression of CD11b and CD11c in our system.

Based on the phenotype and differentiation potential, the DC precursor subpopulations described here likely correspond to those generated from human cord blood CD34⁺ HPCs as described by Caux et al.14,15 The phenotype of CD11b⁻CD11c⁺ precursor-derived DCs appears identical to that of CD14⁺c-fms⁻CD1a⁻ precursor-derived DCs. CD11b⁻CD11c⁺ precursor-derived DC populations appear similar to CD14⁺c-fms⁻CD1a⁻ precursor-derived DCs.14,15 Since it has been shown that CD14⁺c-fms⁻CD1a⁺ and CD14⁺c-fms⁻CD1a⁻ precursor cell–derived mature DCs play in vitro differential roles in regulating cellular and humoral immune responses, respectively,14,15 our findings might be helpful for elucidating their individual biological function in vivo by using various murine models.
However, the DC and DC precursor subsets described here were generated in the culture system in vitro, which are apparently different from the DC subpopulations in Flt3L-treated mice in vivo.\textsuperscript{16,17} It will be difficult to directly compare at this time the DC and DC precursor subsets generated in vitro with those DC subsets developed in vivo in Flt3L-treated mice. Moreover, previous investigations have shown that CD4\textsuperscript{low} thymic precursor cells can differentiate in vivo into CD8\textsuperscript{a1} lymphoid mature DCs in mice. In contrast, CD8\textsuperscript{a} antigen cannot be detected on mature DCs derived in vitro from the same CD4\textsuperscript{low} thymic precursor cells stimulated with various combinations of cytokines,\textsuperscript{36,37} indicating that some other unknown factor might control DC differentiation and its phenotype in vivo. It will be necessary to directly show the differentiation pathways of DC subsets from Lin\textsuperscript{c-kit}\textsuperscript{1} HPCs in vivo by taking the advantage of animal models and/or by establishing in vitro a culture system for generating lymphoid mature DCs, which may help to elucidate the relationship of DCs and their precursor subsets generated in vitro and those in vivo.

Several lines of evidence indicate that murine DCs bearing CD8\textalpha antigen are of lymphoid-precursor origin, whereas DCs expressing high levels of CD11b antigen are derived from myeloid-precursors.\textsuperscript{3,6,36,37} Furthermore, early murine thymic precursors can only differentiate into lymphoid DCs and DCs, but not myeloid cells,\textsuperscript{3,6,37} suggesting that the topographic organization of heterogeneous DC may have been already determined, at least in part, at the progenitor levels.\textsuperscript{14,38-46} Therefore, further characterization of the phenotype of murine DC precursor or DC-committed progenitor cells, which account for the generation of CD11b\textsuperscript{high}CD11c\textsuperscript{+} and CD11b\textsuperscript{high}CD11c\textsuperscript{+} precursors, respectively, will prove to be valuable for investigating the cellular and molecular mechanisms of DC differentiation from HPCs in vivo and in vitro.

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\textbf{REFERENCES}

cells from circulating CD34+ hematopoietic progenitor cells. Blood 87:1292, 1996


Bifurcated Dendritic Cell Differentiation In Vitro From Murine Lineage Phenotype-Negative c- kit + Bone Marrow Hematopoietic Progenitor Cells

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