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A Common Pathway for Dendritic Cell and Early B Cell Development

David Izon, Kristina Rudd, William DeMuth, Warren S. Pear, Cynthia Clendenin, R. Coleman Lindsley, and David Allman

B cells and dendritic cells (DCs) each develop from poorly described progenitor cells in the bone marrow (BM). Although a subset of DCs has been proposed to arise from lymphoid progenitors, a common developmental pathway for B cells and BM-derived DCs has not been clearly identified. To address this possibility, we performed a comprehensive analysis of DC differentiative potential among lymphoid and B lymphoid progenitor populations in adult mouse BM. We found that both the common lymphoid progenitors (CLPs), shown here and elsewhere to give rise exclusively to lymphocytes, and a downstream early B-lineage precursor population devoid of T and NK cell precursor potential each give rise to DCs when exposed to the appropriate cytokines. This result contrasts with more mature B-lineage precursors, all of which failed to give rise to detectable numbers of DCs. Significantly, both CLP and early B-lineage-derived DCs acquired several surface markers associated with functional DCs. Together, these data demonstrate that loss of DC developmental potential is the final step in B-lineage commitment and thus reveals a previously unrecognized link between early B cell and DC ontogeny. *The Journal of Immunology*, 2001, 167: 1387–1392.

In adult mice B cells develop ultimately from hemopoietic stem cells (HSCs) in the bone marrow (BM). Although it is generally thought that the earliest B-lineage-committed precursors derive from semirestricted progenitors characterized by their ability to give rise to two or more lymphoid lineages, the precise identities of these pivotal developmental branch points and the signals promoting their differentiation toward the B cell vs alternative lymphoid lineages are not well characterized.

The signals and cellular compartments underlying dendritic cell (DC) development are also unclear (reviewed by Banchereau et al. (1)). Although controversial, several studies suggest that functionally unique subsets of DCs arise from distinct pools of lineage-restricted progenitors. For instance, putative lymphoid DCs, defined by a CD8α⁺CD11b⁻ surface phenotype, are selectively reduced following removal or inhibition of certain lymphoid-specific transcription factors (2, 3). This contrasts with mice lacking RelB or PU.1, which exhibit specific defects in myeloid development and selective reductions in CD8α⁺CD11b⁻ DCs (4, 5). Furthermore, early thymocyte precursors lacking detectable myeloid potential, including a B/T/NK precursor population and a downstream progenitor pool lacking B and NK cell potential, each give rise to CD8α⁺ DCs (6–11). However, recent studies indicate that different DC surface phenotypes are not determined by ontogeny. For instance, Martin et al. report that thymic CD4⁺ DC progenitors give rise to both CD8α⁺ and CD8α⁻ DCs (12), and Traver et al. recently demonstrated that a myeloid-restricted progenitor population gives rise to CD8α⁻ DCs (13). These observations suggest that DC expression of CD11b and CD8α may be determined by signals unique to particular microenvironments.

In this report we take advantage of a culture system previously described to support DC differentiation from early thymocytes (9) to assess DC precursor potential among a wide spectrum of B-lineage progenitor populations representing varying degrees of B-lineage commitment. These include pluripotent HSCs, common lymphoid progenitors (CLPs) and pre-pro- and pro-B cells previously shown to give rise to B cells, but not T or NK cells. First, we confirm and extend previous reports by Kondo et al. (14, 15) by showing that CLPs can be defined among lineage marker (Lin)⁻ BM cells via coexpression of IL-7Rα and the C1q receptor (C1qR/AA4). Second, we show that both CLPs and a subset of pre-pro-B cells, defined previously by their expression of sterile transcripts derived from the IgH locus and a CD4⁺B220⁺CD19⁻ surface phenotype, rapidly differentiate into DCs in culture. Furthermore, among B-lineage precursor populations, the capacity to give rise to DCs was clearly restricted to CD4⁺B220⁺ pre-pro-B cells, as CD4⁺ pre-pro-B cells as well as more mature B-lineage cells failed to differentiate into DCs. Together, these findings reveal a previously unrecognized common developmental pathway for early B and DC ontogeny and demonstrate that loss of DC differentiative potential in the final step in the onset of B-lineage commitment.
Materials and Methods

Mice and cell preparation

Six- to 10-wk-old C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and B6.Ly5.1 (referred to herein as B6.Ly5<sup>flk-1</sup>) mice were purchased from the National Cancer Institute animal facility (Frederick, MD). BM cells were flushed from tibias and femurs of 8- to 10-wk-old C57BL/6 mice with FACS buffer (PBS containing 3% FCS, 1 mM EDTA and 0.05% sodium azide). Following lysis of RBCs with 0.165 M NH<sub>4</sub>Cl, cells were washed, then stained with the appropriate Abs before sorting.

CD4<sup>+</sup> T cells were prepared by perfusing spleens from C57BL/6 and BALB/c mice with cold PBS containing 3% FCS. Following RBC lysis, cell suspensions were incubated with ascites containing anti-B220 Abs (RA3-6B2) and purified CD8, CD11b, and CD14 (PharMingen, La Jolla, CA) before washing and depletion with sheep anti-rat Ig-coated magnetic microspheres (Dynal, Lake Success, NY) according to the manufacturer’s instructions.

Abs, cell sorting, and analytical flow cytometry

For cell sorting experiments BM suspensions were stained with optimal dilutions of directly conjugated fluorescent Abs for 30 min on ice, then washed twice in FACS buffer. For HSCs, cells were stained with fluorescein (FL)-antiscIa/ly6 A/E (E13-167.1), PE-labeled lineage markers (B220 (RA3-6B2), CD11b/Mac-1 (M1/70), Gr-1 (8C5), Ter-119, and CD3 (2C11)), and allophycocyanin anti-CD11ckit (2B8). For CLPs, cells were stained with FL-antiscIa-1, PE-anti-Lin, PE-Cy5-anti-IL-7Rα (A7R34) (16), and allophycocyanin-anti-C1qRαA4A1.4 (17, 18). For pre-pro-B cell subsets, cells were stained with FL-anti-B220, PE-anti-AA4.1, biotin (BI)-anti-CD24/HSA (30F1), and allophycocyanin-anti-CD4 (GK1.5). For pro- and pre-B cells, cells were stained with FL-anti-CD43 (S7), PE-anti-CD19, BI-anti-CD11b (revealed with PE-Cy5 streptavidin), and allophycocyanin-anti-CD3, and CLPs or HSC-derived cells were identified by uploading files into FlowJo (Tree Star, San Carlos, CA).

Adoptive transfers

For each recipient, 3000 sorted CLPs or HSCs from C57BL/6 mice were mixed with 10<sup>5</sup> unfractionated BM cells from B6.Ly5<sup>flk-1</sup> mice, then transferred i.v. into B6.Ly5<sup>flk-1</sup> recipients given 900 rad 20 h previously. Twenty 10 mM glutamine, 10 mM HEPES, 0.5 mg/ml gentamicin, and 5 × 10<sup>-5</sup> M 2-ME) supplemented with 100 ng/ml rIL-7 (R&D Systems, Minneapolis, MN) containing pre-established monolayers of S17 stromal cells and 200 μl complete medium. Six days later cultures were pulsed with 1 μCi [3H]thymidine for 18 h before scintillation counting.

Identification of CLPs in adult BM via coexpression of IL-7Rα and AA4

Several lines of evidence suggest that the Lin<sup>−</sup> IL-7Rα<sup>+</sup> C1qR/AA4<sup>+</sup> Sca-1<sup>−</sup>low population represents 0.04% of total BM, which we will show below to correspond to CLPs (21) (Fig. 1A), and two subsets of early B-lineage cells (pre-pro-B cells, fractions (Fr.) A<sub>1</sub> and A<sub>2</sub>). Significantly, each pre-pro-B cell subset represents 0.1% of total BM (Fig. 1B) and is characterized by the expression of sterile IgH locus transcripts but lack of the pan-B cell marker CD19 (15), and was shown previously to give rise to pro-B cells, but not T or NK cells or cells of the myeloid or erythroid lineage (14).

Results

DC precursor cultures

Progenitor cells were sorted directly into complete medium containing 2 ng/ml IL-1β, 400 ng/ml IL-3, 10 ng/ml IL-7, 10 ng/ml SCF, 100 ng/ml Flt3L, and 1 ng/ml TNF-α (R&D Systems) as previously described (20). After sorting, 1000 cells/well in 200 μl were added to V-bottom 96-well plates and incubated for 4–6 days before harvesting and determination of viable cell numbers and expression of indicated cell surface molecules by flow cytometry.

MLR

DC precursor cultures were initiated with 5000 cells/well and extended to 9 days to generate sufficient numbers of DCs. Fifty percent of the medium was exchanged with fresh cytokine-containing medium every 3 days. On day 9 viability was determined by trypan blue exclusion and graded doses of DCs mixed in quadruplicate at a final volume of 200 μl with 100,000 purified CD4<sup>+</sup> splenic T cells in 96-well round-bottom plates containing complete medium. Six days later cultures were pulsed with 1 μCi [3H]thymidine for 18 h before scintillation counting.

Sequences of evidence suggest that the Lin<sup>−</sup> IL-7Rα<sup>+</sup> C1qR/AA4<sup>+</sup> Sca-1<sup>−</sup>low population illustrated in Fig. 1A corresponds to the

FIGURE 1. Resolution of CLPs and pre-pro-B cells in adult mouse BM. A. Unfractionated BM cells from an 8-wk C57BL/6 mouse were stained with FL-anti-Ly5<sup>+</sup>, PE-anti-lineage markers (see Materials and Methods), PE-Cy5-anti-IL-7Rα, and allophycocyanin-AA4.1. B. BM cells from the same mouse were stained with FL-anti-B220, PE-AA4.1, BI-anti-CD24/ HSA, and allophycocyanin-anti-CD4. For A and B, data were generated by analyzing 200,000 events and are representative of at least five separate experiments.
tal stimuli can drive CLPs and perhaps early B-lineage cells to confirm the existence of a CLP population in adult mouse BM and described for BM CLPs (data not shown) (21). Together, these data HSA intermediate Thy-1.2 were readily detected. Second, single Lin2 low cells gave rise to B cells, but not macrophages, when cultured CD11b17R and CD192.

FIGURE 2. Generation of B and T cells, but not myeloid lineage cells, following adoptive transfer of CLPs. Three thousand sorted BM HSCs (Lin IL-7Rα Sca-1 CD117/c-kit+) or CLPs (Lin IL-7Rα+AA4 Sca-1low) from C57BL/6 (Ly5.6) mice were mixed with 105 unfractionated BM cells from B6.Ly5.2 congenics before transfer to lethally irradiated B6.Ly5.2 recipients. Data show analyses of PBL 21 days post-transfer, and are representative of five mice per group at 17–40 days post-transfer. CLP- and HSC-derived cells are identified as Ly5.2+.

BM CLP population described by Kondo et al. (21). First, in competitive adoptive transfer experiments Lin IL-7Rα+ C1qR/AA4+ Sca-1low cells gave rise to donor-derived B and T cells, but not CD11b+ myeloid lineage cells (Fig. 2). This finding contrasted with recipients of Lin c-kit+Sca-1+ HSCs, in which CD11b+ cells were readily detected. Second, single Lin IL-7Rα+ AA4+Sca-1low cells gave rise to B cells, but not macrophages, when cultured on S17 stromal cells, and this also contrasted with cultures seeded with HSCs that typically contained both CD19+ CD11b+ B cells and CD19+ CD11b+ macrophages (Table I). Third, because S17 cultures do not support T cell development, we injected single HSCs vs CLPs into the MLP assay previously shown to support B cell, T cell, and myeloid development from HSCs (19). As shown in Fig. 3, donor-derived (Ly5.2+) CD11b+ myeloid as well as TCRγδ+ T cells and CD19+ B cells were detected when fetal thymi were seeded with single HSCs. In contrast, seven of seven thymi seeded with BM CLPs (Lin IL-7Rα+ AA4+Sca-1low) contained detectable numbers of donor-derived TCRγδ+ T cells and CD19+ B cells, but not CD11b+ myeloid cells. Finally, Lin IL-7Rα+AA4+Sca-1low cells also expressed a c-kitlow CD24/ HSAintermediate Thy-1.2+ surface phenotype as previously described for BM CLPs (data not shown) (21). Together, these data confirm the existence of a CLP population in adult mouse BM and support the possibility that particular combinations of environmental stimuli can drive CLPs and perhaps early B-lineage cells to adopt different cell fates.

Table I. Lin IL-7Rα+AA4+Sca-1low progenitors yield pro-B cells but not macrophages in stromal cultures

<table>
<thead>
<tr>
<th>Input Cells</th>
<th>No. Wells Containing CD19+CD11b+ Cells Only</th>
<th>No. Wells Containing CD19+CD11b+ Cells Only</th>
<th>No. Wells Containing Both CD19+CD11b+ and CD19+CD11b+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSCs</td>
<td>5</td>
<td>7</td>
<td>45</td>
</tr>
<tr>
<td>CLPs</td>
<td>62</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Single Lin IL-7Rα− c-kit+Sca-1+ (HSCs) Lin IL-7Rα+ AA4+1+Sca-1low (CLPs) were each sorted into two 96-well plates containing pre-established S17 stromal cells in complete medium supplemented with 10 ng/ml IL-7. Wells containing cell growth were enumerated then analyzed by flow cytometry 14 days later.

CLPs and CD4+ B220+ (Fr. Aγ) pre-pro-B cells selectively differentiate into DCs in culture

We assessed the degree to which several lymphoid precursor populations differentiated into DCs in culture by exposing 1000 sorted cells from each population to a cytokine combination previously shown to promote lymphoid DC development from thymic progenitors (9). The progenitor populations tested include HSCs, CLPs, CD4+ B220− (Fr. Aγ), and CD4+ B220+(Fr. Aδ) pre-pro-B cells and pro-B cells (Fr. B/C) defined by a CD19+ CD43+sIgM+ surface phenotype and readily detectable heavy chain rearrangements (20, 22). Significantly, pre-pro-B cells and pro-B cells each lack detectable T-lineage progenitor activity when derived from wild-type mice (14) and thus serve as a test of whether DC progenitor activity is maintained after loss of T-lineage potential during BM lymphopoiesis. We chose this in vitro DC progenitor assay over in vivo adoptive transfer experiments for several reasons. First, these cultures allow a direct and immediate assessment of DC differentiative potential and thus avoid complications arising from the potential maturation of multipotent progenitors into more immediate DC precursors. Second, unlike adoptive transfer of CLPs, we have found that detection of donor cells derived from Fr. Aγ requires the transfer of at least 20,000 cells/recipient, making it difficult to control for small numbers of contaminating HSCs. Therefore, we controlled for potential contamination by directly comparing parallel short term cultures seeded with as few as 1000 cells derived from a wide array of progenitor populations. Finally, with this system we can readily assess differences in proliferation, surface phenotype, and function that result from exposure of different progenitors to the combination of cytokines used.

Although all DC subsets in mice can be identified via surface coexpression of MHC class II and CD11c (23), we did not find detectable class II surface expression in any of the progenitor populations examined (data not shown). However, as shown in Fig. 4, among HSCs and the lymphoid-restricted progenitor populations tested, both CLPs and CD4+ B220− (Fr. Aδ) pre-pro-B cells gave rise to class II+ CD11c+ cells 4 days in culture. Significantly, these cells also exhibited a high forward and side light scatter profile typical of DCs (data not shown), and cultures seeded with CLPs and CD4+ B220− (Fr. Aδ) pre-pro-B cells contained 60–70% and nearly 100% class II+ CD11c+ cells, respectively (Fig. 4). These data clearly contrasted with those for cultures seeded with equivalent numbers of CD4+ B220− pre-pro-B cells (Fr. Aγ) and CD19+ CD43+ pro-B cells (Fr. B/C), in which significant numbers of DCs were not detected (Figs. 4 and 5). There were also significant differences in numbers of viable cells and DCs recovered from cultures seeded with Fr. Aγ vs CLPs, with 4-fold increases in numbers of CLP-derived DCs over input cell numbers compared with no more than 2-fold increases in cultures seeded with Fr. Aγ pre-pro-B cells (Fig. 5). Although CD19+ CD43−
pro-B cells readily expanded in response to these culture conditions on day 4, and we were unable to detect class II+ CD11c+ cells in these cultures. Furthermore, most of these cells remained CD19+ and also failed to up-regulate expression of surface molecules associated with DC function such as CD80 and CD86 (Fig. 5 and data not shown). Thus, among these early B-lineage progenitor populations, we found that only CLPs and CD4+ B220+ pre-pro-B cells contained significant numbers of cells susceptible to cytokine-induced DC differentiation.

**Function and surface phenotype of CLP and pre-pro-B-derived DCs**

To examine DC function, we tested whether graded doses of CLP- and Fr. A1-derived DCs could stimulate the proliferation of resting allogeneic T cells and tested for up-regulation of CD80 and CD86. Because Fr. A1 yields limiting numbers of DCs in these cultures (Fig. 5), only three doses of Fr. A1-derived DCs were used in these experiments compared with six doses of CLP-derived DCs. As shown in Fig. 6, C57BL/6 (H-2b) CLP-derived (Fig. 6a) and Fr. A1-derived (Fig. 6b) class II+ CD11c+ cells readily stimulated the proliferation of CD4+ T cells purified from BALB/c (H-2d), but not C57BL/6 splenocytes. Second, class II+ cells induced from both CLPs and Fr. A1 also expressed the CD28 costimulatory ligands CD80 and CD86 (Fig. 7). Together, these data demonstrate that exposure of CLPs and CD4+ B220+ (Fr. A1) pre-pro-B cells to cytokines known to induce DC differentiation resulted in the generation of functional DCs.

Whether functionally and phenotypically distinct subsets of DCs arise from separate progenitor populations is currently unclear. Although it was originally proposed that CD8α+ CD11b− peripheral DCs were a functionally unique DC subset derived exclusively from the lymphoid lineage, Traver et al. recently reported that CD8α+ DCs can also develop from common myeloid progenitors (13), raising the possibility that different DC phenotypes, and perhaps functions, are a consequence of unique environmental stimuli as opposed to ontogeny. Indeed, while CD8α+ splenic DCs are reportedly poor stimulators in MLRs, we found that CLP and Fr. A1-derived DCs readily induce proliferation of allogeneic CD4+ T cells (Fig. 6). Thus, to gain insights into the propensity of lymphoid-derived DCs to adopt particular cell surface phenotypes associated with unique DC functions, we also assessed the expression of CD8α and CD11b on CLP- and Fr. A1-derived DCs. As shown in Fig. 7, CLP and Fr. A1-derived DCs expressed low levels of CD8α relatively to what is typically found on splenic CD8α+ DCs (23) and surprisingly high levels of CD11b. These data, together with the capacity of CLP-derived DCs to stimulate alloreactive T cells, indicate that different DC phenotypes and functions may result from exposure to unique combinations of cytokines.

**Discussion**

Our data demonstrate a previously unrecognized link between B and DC development and allow three additional novel conclusions regarding early B and DC ontogeny. First, both CLPs and CD4+ B220+ pre-pro-B cells (Fr. A1), the latter of which lacks detectable T and NK cell precursor activity (14), gave rise to DCs in culture. Thus, we conclude that the loss of DC precursor potential is the final step in B-lineage commitment. Second, among lymphoid progenitors, the capacity to give rise to DCs is not limited to a single population, but, in fact, appears to be restricted to early B-lymphoid progenitors that remain susceptible to signals that promote adoption of alternative cell fates. Third, although lymphoid progenitors were previously thought to yield only CD8α+ CD11b− DCs with a marginal ability to stimulate alloreactive T cells, we found that CLP and CD4+ B220+ pre-pro-B cell-derived DCs each...
exhibited a CD8αlowCD11b− surface phenotype and readily stimulated the proliferation of allogeneic CD4+ T cells. Thus, our data further support the idea that DC phenotype and function are dictated, in part or in whole, by unique environmental stimuli rather than ontogeny (13).

We also confirm and extend previous reports characterizing CLPs in adult BM (21). In particular, we show that B/T-restricted progenitors can be identified among Lin− BM cells based on an IL-7Rα+ C1qR/AA4+ Sca-1low surface phenotype and suggest that these cells correspond to the BM CLP population described by Kondo et al. (21). Supporting this conclusion, each population expressed a Lin−IL-7Rα− Sca-1−CD117−/c-kitlow Thy-1− CD24+ HSA− intermediate surface phenotype (Fig. 1 and data not shown) (21), and both populations gave rise to B and T cells, but not myeloid lineage cells, in a competitive adoptive transfer assay (Fig. 2) and in fetal thymic organ culture under conditions previously shown to support multilineage progenitor differentiation (Fig. 3) (19). In addition, single CLPs, as defined here, exhibited highly efficient cloning efficiencies and failed to give rise to detectable numbers of myeloid lineage cells on S17 stromal cells (Table I), yet gave rise to clonal populations of B and T cells in fetal thymic organ culture. Therefore, these cells coupled with each pre-pro-B cell subset constitute pivotal stages of early B cell development and B-lineage commitment.

Our data support the idea that loss of DC differentiative potential is the final step in B-lineage commitment, because CD4+ B220+ pre-pro-B cells (Fr. A1) yield DCs (Figs. 4 and 5), but not T or NK cells (14). Interestingly, a potentially analogous relationship has been reported for T-lineage commitment in the adult thymus, because both an early B/T progenitor and a more restricted downstream progenitor lacking B-lineage potential reportedly give rise to DCs (10). Regarding DC precursors among early B-lineage cells, it is noteworthy that cells within Fr. A1 were previously shown to express transcripts for several B-lineage-restricted genes, including Pax5 (15), a zinc finger transcription factor associated with maintaining and perhaps inducing B cell commitment (24, 25). Although this finding might appear to be at odds with the capacity of Fr. A1 to give rise to DCs, we would point out that the precise activity of Pax5 in CLPs and early B-lineage precursors remains to be determined.

DC differentiation potential of several B-lineage precursors clearly correlated with developmental maturity, with the least mature of two subsets of pre-pro-B cells (Fr. A1) defined by low CD4 expression clearly giving rise to DCs, while later stages did not (Figs. 4 and 5). Indeed, we did not observe measurable numbers of DCs when later B-lineage subpopulations, including CD4+− pre-pro-B cells (Fr. A2) and CD19+CD43+− pre-pro-B cells, were introduced into this system. At present it is difficult to reconcile these data with a previous report using this culture system in which low frequencies of DC precursors were detected among CD19+CD43+− pre-pro-B cells (20). However, because disparate results might result from differences in sorting gates, cytokine concentrations, and/or duration of cultures, we were particularly careful to use consistent sorting gates, single-use aliquots of each cytokine, and to restrict our phenotypic analyses to 4- to 5-day cultures. In fact, we have been able to detect small numbers of class IIbright CD11c−/− cells by extending pro-B cultures to 8 days (data not shown), suggesting that small numbers of contaminating cells may be responsible for this discrepancy. We would also point out that while early CD4+− pre-pro-B cells readily differentiated into DCs, more mature CD4− pre-pro-B cells, which have been shown to readily differentiate into pro-B cells in culture (14), failed to either proliferate or adopt a DC phenotype (Figs. 4 and 5). Therefore, these data suggest that loss of DC precursor potential accompanies the development of late (CD4−) pre-pro-B cells and their CD19+ progeny and should therefore serve as a baseline for studies exploring the signals that induce and prevent B-lineage progenitors from adopting a DC fate.

Certain previous studies suggest that lymphoid-restricted progenitors yield exclusively CD8α+CD11b+ DCs. However, both CLP and Fr. A1-derived DCs were CD8αlow CD11b+ (Fig. 7), suggesting that environmental stimuli can dictate or modulate DC
phenotype and perhaps function. Supporting this model, two recent studies demonstrate that BM common myeloid progenitors and thymic CLPs but not CD19+ DCs do not develop simultaneously in the thymus from a common precursor population. Nature 362:761.


