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Characterization of Distinct Conventional and Plasmacytoid Dendritic Cell-Committed Precursors in Murine Bone Marrow

Jun Diao,*‡ Erin Winter,*‡ Wenhai Chen,*‡ Claude Cantin,†‡ and Mark S. Cattral2*‡

The developmental pathways and differentiation relationship of dendritic cell (DC) subsets remain unclear. We report that murine CD11c+MHC II+ bone marrow cells, which are immediate DC precursors of CD8α+, CD8α−, and B220− DC in vivo, can be separated into B220+ and B220− DC precursor subpopulations. Purified B220− DC precursors expand, and generate exclusively mature CD11c+CD11b+B220+ DC in vitro and after adoptive transfer. B220+ DC precursors, which resemble plasmacytoid pre-DC, have a lower proliferative potential than B220− DC precursors and generate both CD11b+B220+ and CD11b+B220− DC populations. Both DC precursor populations can give rise to CD8α+ and CD8α− DC subtypes. Our findings indicate that CD11c+MHC II+B220+ and CD11c+MHC II+B220− bone marrow cells are distinct DC lineage-restricted precursors. The Journal of Immunology, 2004, 173: 1826–1833.

Dendritic cells (DCs) are specialized APCs that play a critical role in the initiation and regulation of immune responses. Although well recognized for their potent stimulatory capacity of naive T cells, recent studies indicate that DC are also involved in the induction and maintenance of tolerance (1). In peripheral organs and tissues, DC are heterogeneous and can be distinguished by a variety of cell surface markers and functional attributes (2). Whether differential immune responses are mediated by distinct subsets or simply reflect a functional stage of all DC remains controversial. There is therefore great interest in delineating more precisely the developmental pathways between immediate DC precursors (DCp) and their progeny.

Most DC, with the possible exception of Langerhans cells and brain microglial cells, have very short lifespans in vivo and are continuously replenished by migrating precursor cells (3–6). Evidence suggests that these precursors arise predominately from proliferating Flt3+ hemopoietic progenitor cells in bone marrow (7, 8). A longstanding issue in DC ontogeny is whether there is a distinct or common immediate precursor for the various DC subpopulations. Support for the concept of a common DCp pathway was provided by a recent study in which purified murine CD11c+ MHC class II+ (MHC II+) bone cells were shown to differentiate exclusively into various DC subpopulations in spleen, including CD8α+ DC, CD8α− DC, and the recently recognized B220+ DC, which produce IFNs in response to viral infection (9).

Circulating monocytes can also become DC, but the relative importance of this source as compared with DC-committed precursors is not known (10–12). We identified a CD11c−MHC II− DCp population in mouse bone marrow that, like CD11c+MHC II+ blood cells, possesses the capacity to generate various mature DC populations in vivo. Further phenotypic analysis of this common DCp population revealed heterogeneous expression of several cell surface markers, however. It therefore became crucial to determine whether these DC populations arising in vivo were derived from an identical common precursor or from separate precursors. Our results show that CD11c−MHC II− DCp can be subdivided into B220+ precursor (B220+ DCp) and B220− precursor (B220− DCp) subpopulations, which have distinct functional and proliferative potentials in vitro and distinct capacities to generate mature DC populations in vivo.

Materials and Methods

Mice

Male and female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Montreal, Canada). C57BL/6.SJL congenic mice were originally purchased from Taconic Farms (Germantown, NY) and bred in our animal facility. Mice were maintained in pathogen-free conditions in accordance with institutional guidelines, and used at 6–8 wk, unless indicated otherwise.

Abs and cytokines

Anti-CD11c (clone HL3), CD11b (M1/70), B220 (RA3 6B2), CD19 (ID3), Ly-6C (AL-21), CD117 (2B8), CD115 (AFS98), CD127 (SB/199), Fli3 (A2F10.1), GR-1 (RB6-8C5), CD44 (IM7), CD45 (30-F11), CD3 (17A2), CD4 (CT-CD4), CD8α (53-6.7), I-AK (KH74, 25-9-17), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), CD54 (3E2), H-2Kd (AF6-88.5), CD62L (MEL-14), pan-NK (DX5), and CD16/52 (2.4G2) Abs were purchased from BD Pharmingen (San Diego, CA). Anti-F4/80 (A3-1) was purchased from Serotec (Oxford, U.K.). These Abs were either unlabeled or conjugated to FITC, PE, or biotin, as indicated. Biotinylated Abs were revealed with allophycocyanin or CyChrome. Recombinant GM-CSF, IL-3, and TNF-α were purchased from R&D Systems (Minneapolis, MN).

Isolation of CD11c−MHC II− B220− and CD11c−MHC II− B220+ subsets

Mononuclear cells were isolated from the bone marrow of 8–10 mice by Lympholyte-M (Cedarlane Laboratories, Hornby, Canada) density gradient centrifugation and enriched for CD11c− cells by positive selection using MACS (Miltenyi Biotec, Auburn, CA) and CD11c+ immunomagnetic...
beads. Cells retained in the column were eluted and labeled with anti-I-A<sup>d</sup>, anti-CD4, anti-CD8<sup>a</sup>, anti-CD19, and anti-DX5 biotin allophycocyanin; anti-CD11c PE; and anti-B220 FITC mAbs. CD11c<sup>+</sup>MHC II<sup>+</sup> (B220<sup>+</sup> DCp) and CD11c<sup>+</sup>MHC II<sup>+</sup> B220<sup>−</sup> (B220<sup>−</sup> DCp) cells were sorted on a MoFlo high-performance cell sorter using Summit acquisition and analysis software (DakoCytomation, Fort Collins, CO). Auto-fluorescent cells were electronically gated out during sorting. The purity of the cell populations used was routinely >99% based on reanalyzed samples.

**Flow cytometry**

Flow cytometry was performed on an EPICS XL-MCL cell analysis system (Corixa, Miami, FL) using EXPO 32 software (Corixa), as described previously (13). Briefly, cell suspensions were preincubated with anti-CD16/32 to block Fc receptors, then washed and incubated with the indicated conjugates for 30 min at room temperature in a final volume of 100 μl of PBS containing 0.5% BSA and 2 mM EDTA. In all experiments, appropriate control isotype-matched mAbs were included to determine the level of background staining. Dead cells were excluded by propidium iodide staining.

**Electron microscopy**

Freshly isolated B220<sup>−</sup> and B220<sup>+</sup> DCp were fixed in 2.5% glutaraldehyde in PBS at 4°F for 2 h, washed in PBS, postfixed in 1% osmium tetroxide in PBS for 1 h, dehydrated in acetone, and embedded in epon. Ultrathin sections were stained with lead citrate and uranyl acetate and analyzed with a Philips 400 electron microscope (Eindhoven, The Netherlands).

**In vitro culture of DCp**

Sorted B220<sup>−</sup> DCp and B220<sup>+</sup> DCp were cultured for 36 h in 96-well U-bottom culture plates at a cell density of 5 x 10<sup>3</sup> well per 200 μl of RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 1 mM sodium pyruvate, 10 mM nonessential amino acids, 50 μM penicillin, and 50 μg/ml streptomycin (complete medium) in the presence of IL-3 (10 ng/ml) in triplicate; in some wells, CpG (0.2 μM; Hospital for Sick Children DNA Synthesis Laboratory, Toronto, Canada) or LPS (1 μg/ml; Sigma-Aldrich, St. Louis, MO) was added to the medium. At the end of the culture period, supernatants were collected and analyzed for IFN-γ, TNF-α, and IL-12 p70 by ELISA, as described previously (14), and the cells in each treatment group were pooled and analyzed by flow cytometry. The properties of DCp were evaluated at 36 h of culture because at this time point most of the cells had acquired a mature DC phenotype and cell viability was relatively high.

In other experiments, B220<sup>−</sup> DCp and B220<sup>+</sup> DCp were cocultured on a supportive stromal monolayer of S17 cells (kindly provided by K. Dorsch, Department of Pathology and Laboratory Medicine, University of California, Los Angeles, CA) (15). S17 cells were plated at a density of 5 x 10<sup>3</sup>/ml in 60-mm tissue culture petri dishes (BD Biosciences, Franklin Lakes, NJ) and γ irradiated (25 Gy) 24 h later. Sorted cells (5 x 10<sup>5</sup>) were placed on the monolayer in 10 ml of RPMI 1640 complete medium in the presence of GM-CSF (1000 U/ml). Cells were cultured for 12–14 days, with twice weekly refreshment of the medium half-life replaced with fresh medium every 3 days. In some experiments, TNF-α (100 U/ml; PeproTech, Rocky Hill, NJ) or LPS (1 μg/ml) was added for the final 24–48 h of culture. At the end of the culture period, nonadherent cells were collected by gentle pipetting for further analyses.

**CFSE labeling**

Freshly sorted B220<sup>−</sup> DCp and B220<sup>+</sup> DCp were washed and resuspended in RPMI 1640 solution at 10<sup>7</sup> cells/ml, and incubated with 1 μM CFSE (Molecular Probes, Eugene, OR) for 15 min at 37°C. Cells were then washed with RPMI 1640 three times, resuspended in complete medium, and cultured on S17 stromal monolayer for 3 days. To harvest cells, the monolayer was disrupted with 0.25% trypsin/1 mM EDTA and repeated pipetting. The recovered cells were washed; stained with anti-CD11c, anti-CD45.2, and anti-B220 mAbs, and analyzed by flow cytometry. Dead cells were excluded by propidium iodide staining. Analysis of cell division (CFSE fluorescence) was limited to CD45.2<sup>−</sup> cells.

**Adoptive transfer studies of DCp**

To determine the capacity for DC development in vivo, 2–5 x 10<sup>5</sup> B220<sup>−</sup> DCp and B220<sup>+</sup> DCp from CD45.2<sup>−</sup> mice, along with 5 x 10<sup>4</sup> bone marrow cells from CD45.1<sup>−</sup> congenic mice, were injected i.v. into sublethally irradiated (6 Gy) CD45.1<sup>−</sup> recipients, as described (16). The spleen was removed from recipients 3 or 7 days later; mononuclear cells were isolated by Lymphocyte-M (Cedarlane Laboratories) density gradient cen-

trifugation, stained with fluorochrome-conjugated Abs, and analyzed by flow cytometry.

**Mixed leukocyte reaction**

Graded numbers of irradiated DC were seeded in triplicate in U-bottom 96-well culture plates (BD Biosciences) for use as stimulator cells. Responder allogeneic splenic lymphocytes (1 x 10<sup>7</sup>/well) from BALB/c mice were added to the wells in a total volume of 200 μl of RPMI 1640 complete medium, and cultured for 3 days in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. The culture was pulsed with 1 μCi of [3H]thymidine (American Biosciences, Arlington Heights, IL) 16 h before harvest, and collected onto glass filter filters (Millipore, Etobicoke, Canada); [3H]thymidine incorporation was quantified using a Beckman scintillation counter.

**FIGURE 1. Isolation and characterization of CD11c<sup>+</sup>MHC II<sup>+</sup> bone marrow cells.**

*a, CD11c<sup>−</sup> bone marrow cells were enriched by anti-CD11c magnetic beads, then labeled with anti-I-A<sup>d</sup> FITC, anticlell line markers (anti-CD4, anti-CD8<sup>a</sup>, anti-CD19, and anti-DX5 biotin allophycocyanin), and anti-CD11c PE mAbs. Lineage-negative cells were subdivided according to CD11c and MHC class II expression and purified by FACS. b, Reanalysis of purified population. Data are representative of >10 experiments. c, Expression of other surface markers. Purified CD11c<sup>+</sup>MHC class II<sup>−</sup> cells were stained with the indicated FITC-labeled mAb (filled histograms) or isotype control (open histograms). Data are representative of 3–10 experiments. d, CD11c<sup>+</sup>MHC II<sup>−</sup> bone marrow cells generate DC in vivo. CD11c<sup>+</sup>MHC class II<sup>−</sup> bone marrow cells from CD45.2<sup>−</sup> mice were transplanted into sublethally irradiated CD45.1 congenic mice, along with CD45.1 bone marrow cells. Mice transplanted with CD45.1 bone marrow only were used as a negative control. At day 7, spleenocytes were isolated, and expression of CD45.2 was used to identify donor-type cells. Gated cell populations used was routinely >99% based on reanalyzed samples.
(Beckman Coulter, Fullerton, CA). Background controls with spleen cells or DC only were included in all experiments and were <1000 cpm. Results are expressed as the mean cpm of triplicate cultures.

Statistical analysis
Continuous variables are expressed as mean ± SD and analyzed with Student t tests. A value of \( p < 0.05 \) was considered statistically significant.

Results
Characterization of CD11c\(^{+}\)MHC II\(^{+}\) bone marrow cells
The methodology used to isolate DCp in the present study was modeled after the strategy described by del Hoyo et al. (9) for the isolation of CD11c\(^{+}\)MHC II\(^{+}\) DCp from murine blood. However, to ensure high purity, we used a combination of magnetic bead enrichment for CD11c\(^{+}\) cells and FACS rather than immunomagnetic depletion to isolate CD11c\(^{+}\)MHC II\(^{+}\) cells from murine bone marrow (Fig. 1). This technique enabled specific elimination of autofluorescent cells and CD11c\(^{+}\) NK cells (17, 18). The purified cell population comprises ~0.5% of total nucleated bone marrow cells. Flow cytometric analysis detected the presence of CD44 and CD24 on all cells; CD4 and CD8\(\alpha\) were absent; 50–60% of the cells expressed CD62L and CD11b. Ly-6c was expressed by most cells, whereas F4/80 was absent. The costimulatory molecules CD40, CD80, and CD86 were absent or expressed at very low levels. Lineage markers for T cells (CD3), B cells (CD19), and NK cells (DX5) were undetectable.

To investigate the developmental potential of these cells in vivo, \( 3–5 \times 10^5 \) CD11c\(^{+}\)MHC II\(^{+}\) bone marrow cells from CD45.2\(^{+}\) mice, along with \( 5 \times 10^4 \) bone marrow cells from CD45.1\(^{+}\) congenic mice were transplanted into lethally irradiated CD45.1\(^{+}\) recipients (Fig. 1d). At 3 days after transplantation, <2000 CD45.2\(^{+}\) cells were recovered from recipient spleen (\( n = 3 \)). By 7 days, the mean number of CD45.2\(^{+}\) cells recovered from the spleen had increased to \( 21 \pm 6 \times 10^3 \) in six independent experiments. Immunophenotypic analysis revealed that virtually all CD45.2\(^{+}\) cells were CD11c\(^{+}\); lineage markers for T cells (CD3), B cells (CD19), NK cells (DX5), or granulocytes (Gr-1) were not detected (data not shown). Most CD11c\(^{+}\) cells expressed MHC class II; CD11b was expressed on 55%, B220 on 42%, and CD8\(\alpha\) on 26% of cells. These results indicate that CD11c\(^{+}\)MHC II\(^{+}\) bone marrow cells are immediate DCp with the capacity to generate the commonly recognized mature DC subsets in spleen (i.e., CD8\(\alpha^{+}\) DC, CD8\(\alpha^{-}\) DC, and B220\(^{+}\) DC).

CD11c\(^{+}\)MHC II\(^{+}\) bone marrow cells can be separated into distinct B220\(^{+}\) and B220\(^{-}\) subpopulations
Although these data were consistent with the view that CD11c\(^{+}\) MHC II\(^{+}\) cells are immediate common DCp (9), the bimodal expression pattern of B220 and Gr-1 suggested that more than one precursor population might be present. We therefore sorted CD11c\(^{+}\)MHC II\(^{+}\) bone marrow cells into B220\(^{+}\) DCp and B220\(^{-}\) DCp subpopulations to >99% purity (Fig. 2). Other surface Ags were found to cosegregate with this cell separation strategy. Thus, B220\(^{+}\) cells were Gr-1\(^{-}\) and >50% are CD11b\(^{+}\), whereas B220\(^{-}\) cells are CD11b\(^{-}\) and >50% are Gr-1\(^{+}\). The expression of hematopoietic growth factor receptors was analyzed in detail. Both populations expressed CD123 (IL-3R) at low levels, and neither expressed CD117 (c-kit receptor). Flt3 was expressed by a small proportion of B220\(^{+}\) cells and by none of the B220\(^{-}\) cells, whereas M-CSFR was detected on some B220\(^{+}\) cells and was absent on B220\(^{-}\) cells. IL-7R was not detected on either population (data not shown).

![FIGURE 2. Immunophenotype and morphology of B220\(^{+}\) DCp and B220\(^{-}\) DCp.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)

CD11c\(^{+}\) bone marrow cells were enriched by anti-CD11c magnetic beads, then labeled with anti-I-A\(^{\beta}\) biotin allophycocyanin, antilineage markers (anti-CD4, anti-CD8\(\alpha\), anti-CD19, and anti-DX5 biotin allophycocyanin), anti-CD11c PE, and anti-B220 FITC mAbs. CD11c\(^{+}\)MHC class II\(^{+}\) lineage\(^{+}\) cells were separated into B220\(^{+}\) and B220\(^{-}\) subpopulations and labeled with the indicated fluorescence-labeled mAb (filled histograms) or isotype control (open histogram). Transmission electron micrographs of each population (original magnification ×2,500 on left, and ×20,000 on right). Data are representative of at least three experiments.
Electron microscopy revealed striking differences in the morphologic characteristics of these two cell populations. B220+ DCp were 4–5 μm in diameter and had a villous plasma membrane; the cytoplasm contained lysosomes and some large vacuoles; and the nucleus was indented or clefted. B220+ DCp were larger (5–6 μm) and less electron dense than B220− DCp and had the appearance of typical lymphocytes; the cytoplasm was agranular and contained ribosomes, mitochondria, small Golgi, and scant endoplasmic reticulum.

These immunophenotypic and morphologic characteristics of B220+ and B220− DCp resemble, respectively, murine plasma-cytoid pre-DC, which have been identified in blood, bone marrow, and spleen (19–22), and murine CD11c+ conventional DCp that were isolated recently from blood (18).

**Functional response of B220+ DCp and B220− DCp to CpG and LPS**

We next compared the response of these precursor populations to stimulation with CpG and LPS, which are known agonists for TLR 9 and 4, respectively (23, 24). IL-3 was included in the culture medium for these experiments because most of the cells died within 24 h in its absence, as reported by others (14, 21). In the presence of IL-3 alone, 70% of B220+ DCp were recovered alive; addition of CpG, but not LPS, induced the formation of many cell aggregates and reduced the number of viable cells (Fig. 3a). B220− DCp tended to form aggregates in the presence of IL-3 alone, and ~50% of the recovered cells were viable; neither CpG nor LPS affected their morphology or viability (Fig. 3b).

Both populations of cells expressed MHC II and CD86 (data not shown) after culture in IL-3 alone (Fig. 3c). CD8α was expressed de novo by 25–30% of B220+ DCp and 10% of B220− DCp, and increased further in both populations after treatment with CpG, but not with LPS. CD8α expression was detected predominately on cells that coexpressed MHC II. Interestingly, a small proportion (10–15%) of the cells retrieved from B220+ DCp cultured in IL-3 alone did not express B220, and this proportion increased (25–30%) after CpG treatment. CD11b was persistently absent on the B220− subset and detected on 49% of B220+ cells. LPS did not have any apparent effect on the expression level of the cell surface Ags analyzed on either subset as compared with IL-3 alone.

**FIGURE 3.** Functional properties of B220− DCp and B220+ DCp. B220− DCp and B220+ DCp were cultured in IL-3 with and without CpG (0.2 μM) or LPS (1 μg/ml). At 36 h, the cells were analyzed to determine: a, viability; b, morphology; c, immunophenotype; d, stimulatory capacity in allogeneic MLR; and e, capacity to produce IFN-α and IL-12 p70, as described in Materials and Methods. Results are expressed as mean ± SD (*, p < 0.01).
Regardless of the culture conditions, the recovered cells derived from both precursor populations could stimulate proliferation of allogeneic lymphocytes, although cells derived from B220⁺ DCp were consistently less potent than those from B220⁻ DCp. Treatment with CpG increased the potency of B220⁺ cells, but not B220⁻ cells, whereas LPS had the converse effect (Fig. 3d). IFN-α and IL-12 p70 were detected in the supernatants from CpG-stimulated B220⁺ cells, but not from CpG-stimulated B220⁻ or from either cell population cultured in IL-3 alone or IL-3 and LPS (Fig. 3e). Taken together, these results show that B220⁺ DCp and B220⁻ DCp are functionally distinct populations.

Adoptive transfer of B220⁺ DCp and B220⁻ DCp

Having established that CD11c⁺ MHC II⁺ bone marrow cells are comprised of two subpopulations, it was next important to clarify the developmental potential of each subpopulation in vivo. For these studies, 2–5 × 10⁵ B220⁻ or B220⁺ DCp from CD45.2 mice, along with unfractionated bone marrow cells (5 × 10⁶) from CD45.1 mice, were injected i.v. into sublethally (6 Gy) irradiated CD45.1 congenic recipients. Seven days after transplantation, the spleen was recovered and the mononuclear fraction was analyzed by flow cytometry. Almost all CD45.2⁺ donor cells from both subsets were CD11c⁺ (Fig. 4), and none expressed markers for T cells, B cells, NK cells, or granulocytes (data not shown), which confirmed that their developmental fate is restricted to the DC lineage. The mean number of cells recovered per 5 × 10⁵ cells injected was 15 ± 9 × 10³ (range 5–26 × 10³; n = 5 experiments) for B220⁺ DCp and 36 ± 23 × 10³ (range 21–71 × 10³; n = 6) for B220⁻ DCp, respectively. In paired experiments, the number of DC recovered from B220⁺ DCp was 3–4-fold higher than from B220⁻ DCp (p = 0.03).

B220⁺ DCp gave rise to CD11c⁻ low and CD11c⁻ high DC populations at a ratio of ~1.5:1, whereas B220⁻ DCp gave rise only to CD11c⁻ high DC. The CD11c⁻ low DC were B220⁺, CD11b⁻, a mean of 19 ± 3% expressed CD8α, and most were MHC II⁻ low. The CD11c⁻ high cells from either precursor population were CD11b⁺, MHC II⁺, and B220⁻, and 11 ± 6% expressed CD8α. These studies therefore indicate that there is a clear difference in both the number and the phenotype of DC generated by these two precursor populations. B220⁻ DCp are immediate precursors of conventional DC, whereas B220⁺ DCp are precursors of plasmacytoid DC and have the potential to give rise to conventional DC.

Proliferation capacity of B220⁺ DCp and B220⁻ DCp

To investigate the capacity of B220⁺ DCp to generate conventional DC, we used a stromal cell coculture system established in our laboratory that supports survival and expansion of conventional DC from primitive hemopoietic progenitors and purified CD11c⁺ MHC II⁺ bone marrow cells. B220⁺ and B220⁻ DCp were cocultured with S17 monolayers in the presence of GM-CSF for 12 days. Compared with the input number of cells, there was a 25-fold increase in the number of nonadherent cells per culture in the B220⁺ DCp cultures, but no increase in cell number in the B220⁻ DCp cultures (Fig. 5a). The progeny of both B220⁺ and B220⁻ precursors exhibited immunophenotypic features of immature conventional DC (CD11c⁺CD11b⁻B220⁻CD8α⁻; Fig. 5b). When stimulated with TNF-α or LPS (data not shown), they developed characteristics of mature DC with up-regulation of MHC II, CD40, and CD86 molecules, and increased potency to stimulate allogeneic lymphocyte proliferation (Fig. 5c).

These findings indicate that B220⁻ DCp have a significantly higher capacity to expand than B220⁺ DCp, consistent with the adoptive transfer studies, and that both generate conventional DC under these culture conditions. An alternative explanation for the apparent generation of conventional DC from B220⁺ DCp is that they were derived from a small number of contaminating B220⁻ DCp. Based on the high purity of the cell populations used in these studies, we estimate that <1% of the cells in the B220⁺ population could be non-B220⁺ DCp (i.e., <500 cells per culture). When we cultured 500 B220⁺ DCp under the same conditions, however, few cells were recovered (data not shown).

B220⁺ DCp have a limited capacity to generate B220⁻ CD11b⁻ DC

We next modified the in vitro culture system to allow us to analyze cell surface Ag expression dynamically with each cell division. B220⁺ and B220⁻ DCp from CD45.2 mice were labeled with CFSE and cocultured with S17 stromal cells in the presence of...
GM-CSF. All cells were harvested at 2 and 3 days; stained with propidium iodide and fluorochrome-labeled mAbs to CD45.2, CD11c, and B220; and analyzed by multicolor flow cytometry (Fig. 6). Cells derived from B220–DCp showed increased expression of CD11b and MHC II on the second and third day of culture, and all were consistently B220–. Based on CFSE expression, ~30% of the cells had divided at day 2; at day 3, 80% of the cells had divided, with ~15% completing five division cycles. Of the cells derived from B220+ DCp, ~20% were B220– at day 2, increasing to 60% by day 3; this was accompanied by an increase in the number of cells that expressed CD11b (10% at day 2, and 30% at day 3). CFSE labeling showed no evidence of cell division of B220– DCp at day 2, which indicates that the B220– cells identified at this time point arose from B220+ DCp through a transformation process; by day 3, a small population of CD11c+ B220– cells showed evidence of cell division.

Discussion

In this study, we show that CD11c+MHC II+ DCp in bone marrow can be subdivided into B220– and B220+ subpopulations, which are morphologically, immunophenotypically, and functionally distinct. We are the first to compare the developmental fate of these defined precursor populations in vivo. B220– DCp generate exclusively CD11b+ DC, whereas B220+ DCp are precursors of B220+ DC with some capacity to generate CD11b+ DC. Our study also highlights the proliferative capacity of B220– DCp, which challenges the prevalent view that immediate DCp are non-dividing cells.

The expression of B220 and CD11b on CD11c–MHC II– bone marrow cells in our study was mutually exclusive, which allowed us to separate these cells into two distinct precursor populations. This finding contrasts with the phenotype of CD11c+MHC II– blood cells described by del Hoyo et al. (9), of which >70% co-expressed both markers. Whether this discrepancy reflects different stages of differentiation of bone marrow and blood CD11c–MHC II– cells or is indicative of entirely different precursor populations is unclear. A CD11c+MHC II–Ly-6c+CD11b+ DCp population has been isolated from murine bone marrow and blood (25). However, these cells differ from those that we and del Hoyo et al. describe because they do not proliferate and they generate macrophages as well as DC. A recent study by O’Keeffe et al. (18) has shown that murine blood DCp can be separated into plasmacytoid and conventional DCp based on the expression of CD45RA, an isofrom of B220. CD45RA+ conventional and CD45RA– plasmacytoid precursors share many immunophenotypic, morphologic, and functional similarities with B220– DCp and B220+ DCp, respectively, and it seems likely that they may be equivalent populations. This hypothesis is consistent with the view that blood precursor cells are brief transients of those in bone marrow (8).

The generation of CD11b+B220– DC from B220+ DCp raised the possibility that this population may be the precursor of B220– DCp. The rate of proliferation of B220– DCp in vitro was less than B220+ DCp, however, which argues against this idea. Although it is conceivable that the culture conditions were not optimal for B220+ DCp expansion, the in vivo adoptive transfer experiments also indicate that this cell population has a reduced capacity to expand and generate CD11b+B220– DC as compared with B220+ DCp. Moreover, O’Keeffe et al. (14) has shown that plasmacytoid DCp have a much slower rate of turnover than other DC subsets, and as such could not be the predominate source of the more rapidly turning-over CD11b+B220– DC. Whether conventional CD11c–CD11b–B220+ DC generated from B220+ DCp are equivalent to those generated from B220– DCp is not entirely resolved. The morphologic, immunophenotypic, and functional similarity of these cells is unclear.

FIGURE 5. Coculture of B220– DCp and B220+ DCp on S17 monolayers. A total of 5 × 10⁴ B220– DCp or B220+ DCp was cocultured on S17 monolayers in the presence of GM-CSF (1000 U/ml) for 12 days, then stimulated with TNF-α (100 U/ml) for 48 h. a. Cell recovery after 12-day culture period. Nonadherent cells were removed and counted to determine mean ± SD fold increase over the initial number of cells placed in culture (*, p < 0.001). b. Flow cytometric analysis showing expression of the indicated cell surface markers before and after TNF-α stimulation; c. stimulation of 10⁵ allogeneic BALB/c splenic lymphocytes by graded numbers of TNF-α-treated stimulator cells. Proliferation was assessed by [³H]thymidine incorporation; results are expressed as mean cpm × 10⁵ ± SD. Results are representative of at least three independent experiments.
analyses performed in this study did not reveal any differences between these cell populations. However, we cannot rule out the possibility that other differences might exist. Analysis of gene expression profiles, which are currently underway in our laboratory, may provide more insight into this issue.

De novo expression of CD8α was detected on B220+ DCp after culture in IL-3 and further augmented by CpG, which is consistent with other studies of plasmacytoid DCp. Surprisingly, however, these culture conditions also induced CD8α expression on a small proportion of B220- DCp. Despite this phenotypic similarity, there were clear differences in stimulatory capacity in MLR and cytokine production between these cell populations. These findings support the current opinion that CD8α expression cannot be used in isolation to define either lineage origin or functional characteristics of DC (16, 26, 27).

The relationship between B220+ DCp and B220- DCp with earlier progenitor populations remains to be defined. It has been suggested that CD11c+MHC II+ DCp are located downstream from common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) based on absence of expression of the transcription factors Pax-5 and SCL, and the receptor IL-7Rα (9, 26). Independent developmental pathways for conventional DC and plasmacytoid DC are supported by experimental evidence from IFN consensus-binding protein knockout mice and Id2/Id3-induced hematopoietic stem cells (28, 29). Other studies, however, indicate that both populations can arise in vivo from CLP and CMP in bone marrow (8, 7). Further analysis of gene expression profiles of these DCp and their upstream parents may allow delineation of the factor(s) that converts progenitors with olig lineage potential into DC-restricted precursors and to define these developmental pathways more precisely. In Fig. 7, we propose a

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**FIGURE 6.** Analysis of proliferating B220+ DCp and B220- DCp. CD45.2 B220+ DCp and B220- DCp were labeled with CFSE and cocultured with stromal cells in the presence or absence of GM-CSF and recovered at days 2 and 3. CD45.2+-gated cells were analyzed for expression of CD11c, CD11b, B220, and CFSE. Dot-plot quadrants were determined by isotype-matched controls. Cell proliferation is measured by loss of 50% fluorescence intensity with each division, as indicated. Data are representative of three independent experiments.

**FIGURE 7.** Model of DC development. HSC, hematopoietic stem cell. The relationship between CLP and CMP with B220+ DCp and B220- DCp has not been defined precisely.
model of the developmental pathways of DC from B220+ DCp and B220− DCp and earlier progenitors.

To our knowledge, we are the first to report expansion and differentiation of DC from a defined population of highly purified DCp in vitro. Previous attempts to expand purified DCp in vitro with various cytokines have been unsuccessful and are consistent with our results when B220+ DCp and B220− DCp were cultured with cytokines alone (18, 25). It is unlikely that contamination of DCp with another population of highly purified DC from a different developmental pathway is involved.

We are currently using our stromal coculture system to investigate regulatory processes involved in expansion and differentiation of DCp. In conclusion, we have identified two distinct populations of immediate DCp in murine bone marrow. B220+ DCp appear to be equivalent to the recently described IFN-producing plasmacytoid DCp alone (18, 25). It is unlikely that contamination of DCp with MHC II−/H11001 cells surface markers for primitive progenitors, were absent or detected at very low levels. Second, CD11c+ MHC II− cells did not proliferate when cultured in a cytokine mixture designed for stem cell expansion (our unpublished observations). Third, the progeny of CD11c+ DCp in vitro with DCp in vitro. Previous attempts to expand purified DCp under steady-state conditions.

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References