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Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived *in vitro* and *in vivo*

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The development of functionally specialized subtypes of dendritic cells (DCs) can be modeled through the culture of bone marrow with the ligand for the cytokine receptor Flt3. Such cultures produce DCs resembling spleen plasmacytoid DCs (pDCs), CD8$^+$ conventional DCs (cDCs) and CD8$^-$ cDCs. Here we isolated two sequential DC-committed precursor cells from such cultures: dividing 'pro-DCs', which gave rise to transitional 'pre-DCs' *en route* to differentiating into the three distinct DC subtypes (pDCs, CD8$^+$ cDCs and CD8$^-$ cDCs). We also isolated an *in vivo* equivalent of the DC-committed pro-DC precursor cell, which also gave rise to the three DC subtypes. Clonal analysis of the progeny of individual pro-DC precursors demonstrated that some pro-DC precursors gave rise to all three DC subtypes, some produced cDCs but not pDCs, and some were fully committed to a single DC subtype. Thus, commitment to particular DC subtypes begins mainly at this pro-DC stage.

Dendritic cells (DCs) are antigen-presenting cells crucial for the innate and adaptive response to infection as well as for maintaining immune tolerance to self tissue. Among the cells classified as DCs there are many subtypes that, despite having many common features, have distinct immune functions[1]. The DC subtypes present in the steady state, before infection, include type 1 interferon–producing plasmacytoid DCs (pDCs), 'migratory' DCs such as Langerhans cells, and 'lymphoid tissue–resident' conventional DCs (cDCs)[1]. The lymphoid tissue–resident cDCs consist of different subtypes, including CD8$^+$ cDCs and CD8$^-$ cDCs[2]. More DC subtypes develop after infection or inflammation, such as the monocyte-derived 'inflammatory' DCs[3–6]. Despite recent insights, the pathways of development of these distinct DC subtypes have not been adequately mapped.

DCs, like other blood cells, derive from hematopoietic stem cells through early progenitor cells. However, there is little evidence of commitment to DC subtype at the 'early' progenitor stage, as either common myeloid or common lymphoid progenitors have the ability to form pDCs, CD8$^+$ cDCs and CD8$^-$ cDCs[7,8] as long as the progenitors express the cytokine receptor Flt3 (refs. 9,10). 'Downstream' of these early progenitors, there is now evidence for restriction at 'intermediate' precursor stages. Precursor cells that can form cDCs but no longer produce pDCs have been found in bone marrow[11,12], including one precursor type that produces both cDCs and macrophages[12]. Further 'downstream', 'immediate' precursors have been identified for several DC types, including Ly6C^{hi} monocytes as precursors of inflammatory DCs[3,4,6] and Langerhans cells[13]. A non-monocyte immediate precursor of cDCs (a 'pre-cDC') that had branched away from pDC development has been isolated from spleen[4]. This population includes CD24^{hi} pre-cDCs producing only CD8$^+$ cDCs, and CD24^{lo} pre-cDCs producing only CD8$^-$ cDCs[4].

A more detailed and sequential map of the pathway of DC development would be possible with a suitable culture model of the process. Culture of precursor cells with granulocyte-macrophage colony-stimulating factor has led to models of the development of migratory DCs and of monocyte-derived inflammatory DCs[1,14]. However Flt3 ligand (Flt3L), rather than granulocyte-macrophage colony-stimulating factor, is the limiting cytokine for the steady-state development of spleen DCs[15,16], so culture of bone marrow precursor cells with Flt3L[1,17–19] is a more appropriate model for studying the origin of pDCs and of lymphoid tissue–resident cDCs. Despite the use of Flt3L in amounts well above steady state amounts[20], the DCs produced by Flt3L culture closely resemble the immature steady-state spleen pDCs, CD8$^+$ cDCs and CD8$^-$ cDCs[19].

Here we evaluate early stages of Flt3L-stimulated bone marrow cultures to demonstrate two successive DC-restricted precursor cells, 'pro-DCs' and pre-DCs, *en route* to the generation of all three DC

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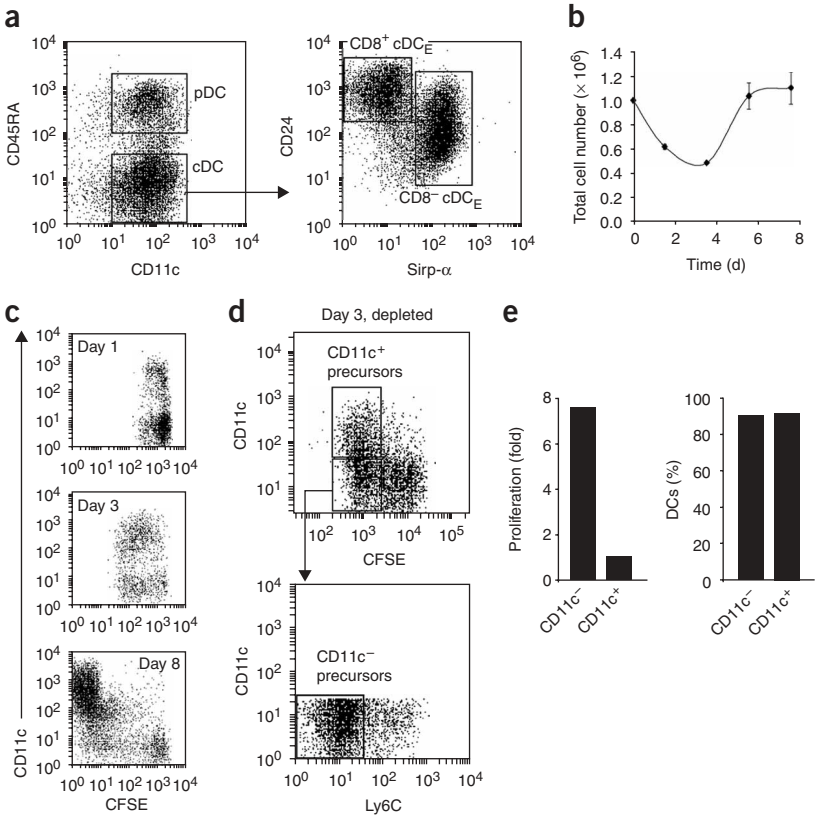

Figure 1 DC precursors in cultures of bone marrow stimulated with Flt3L. (**a**) Flow cytometry of bone marrow cells cultured for 9 d with mouse Flt3L and then analyzed for expression of CD11c (to segregate DCs as CD11c$^+$), CD45RA (to segregate pDCs as CD45RA$^+$) and CD24 and Sirp-α (CD172a; to segregate CD8$^+$ cDC–equivalent cells (CD45RA$^-$CD24^{hi}Sirp-α$^-$) and CD8$^-$ cDC–equivalent cells (CD45RA$^-$CD24^{lo}Sirp-α$^+$)). Similar results were obtained over 50 times. (**b**) Total number of viable cells versus time in the culture conditions in **a**. This result was obtained twice. (**c**) Flow cytometry of bone marrow cells labeled with CFSE and anti-CD11c on days 1, 3 and 8 of the culture conditions in **a**. Three experiments gave similar results. (**d**) Flow cytometry and sorting of CFSE^{lo}CD11c$^+$ and CFSE^{lo}CD11c$^-$ Ly6C$^-$ DC precursors. Similar results were obtained over 30 times. (**e**) Proliferation of the CD11c$^+$ and CD11c$^-$ precursor populations from **d** recultured for a further 5 d in conditioned medium. The left bar graph shows the extent of cell expansion and the right bar graph shows the percentage of DCs among the cells produced. A second experiment gave similar results.

subtypes: pDCs, CD8$^+$ cDCs and CD8$^-$ cDCs. We also identify *in vivo* equivalents of these precursor cells. Clonal analysis of both *in vitro* and *in vivo* pro-DCs demonstrated that in this population, some individual precursor cells produced clones of all three DC subtypes, some produced clones of two DC subtypes, and others produced clones of only one DC subtype. Earlier bone marrow progenitors were more likely to produce clones containing all DC subtypes. Our data show that the process of commitment to DC subtype occurs mainly at the dividing, pro-DC stage.

RESULTS

Dividing DC precursors in Flt3L cultures

When we cultured bone marrow cells with Flt3L for 8–10 d, three populations of DCs (Flt3L-DCs) were generated in good yield, as reported before[19]. We segregated Flt3L-DCs as CD11c$^+$CD45RA^{hi} pDCs and CD11c$^+$CD45RA^{lo} cDCs (**Fig. 1a**). We then separated the cDCs into CD24^{hi}Sirp-α^{lo} and CD24^{lo}Sirp-α^{hi} subpopulations (**Fig. 1a**), shown before to be equivalent to CD8$^+$ and CD8$^-$ spleen cDCs, respectively, despite the lack of expression of CD8α in culture[19]. We therefore called these 'CD8$^+$ cDC–equivalent cells' and 'CD8$^-$ cDC–equivalent cells', respectively. The amount of major histocompatibility complex (MHC) class II and of costimulatory molecules (CD40, CD80 and CD86) on these Flt3L-DCs was similar to that on spleen DCs[1]. Accordingly, we used these cultures to identify precursors of these DC subtypes.

We first evaluated some general properties of the cells in the Flt3L-based cultures. The total number of viable cells dropped over the first 3 d of culture with Flt3L and then increased over the ensuing 5 d; this suggested that the generation of Flt3L-DCs involved extensive cell division (**Fig. 1b**). To directly test this, we labeled bone marrow cells before culture with CFSE, a cytosolic dye that dilutes progressively with cell division. Labeling the cells with CFSE did not affect the development of Flt3L-DCs relative to that of unlabeled control cells (data not shown). We then monitored the dilution of CFSE in the cells and the expression of CD11c during the culture (**Fig. 1c**). After 1 d, only a few cells had divided; by day 3, a higher proportion of cells had undergone several divisions and some of these expressed CD11c. By day 8, most cells were CD11c^{hi} DCs and had low CFSE fluorescence, demonstrating that they were derived from extensive precursor cell division. We therefore reasoned that selection of dividing cells with low CFSE fluorescence during the course of the culture would enrich the cultures for DC precursors. Furthermore, day 3.5 of culture, when most irrelevant cells had died, would be the optimal time to select precursor cells that still had substantial proliferative potential. We used two separate assays to assess the activity of DC precursors: an *in vivo* assay[4] involving transfer into unirradiated recipient mice followed by quantification of donor-derived DCs in the recipient spleens after 5 d; an *in vitro* assay involving the reculture of cells in conditioned medium retained from the Flt3L bone marrow cultures at day 3.5 (called 'conditioned medium' here), with quantification of population expansion and Flt3L-DC production 5 d later.

Isolation of DC precursors

We first labeled bone marrow cells with CFSE, then cultured them with Flt3L for 3.5 d and collected them. In the initial enrichment step, we removed dead cells and most surviving differentiated cells by centrifugation in Nycodenz medium with a density of 1.086 g per cm^3. The pellet of cells of higher density had little precursor activity and was discarded (**Table 1**). Sorting of the fraction of lighter density for cells that had divided (**Fig. 1d**) resulted in substantial selection for DC precursor activity (**Table 1**). However, these cells included some that were Ly6G$^+$, CD19$^+$, MHC class II–positive and positive for interleukin 7 receptor-α (IL-7Rα$^+$). When we sorted the latter cells and evaluated their DC precursor activity, they produced no DC subtypes by either *in vivo* or *in vitro* assay (data not shown). Accordingly, in the

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Table 1 Purification of DC precursors from cultures

Fraction	DCs generated
Uncultured, total bone marrow	1,870 ± 200
Day 3, cultured bone marrow, dense cells	430
Day 3, cultured bone marrow, light-density cells	30,420 ± 9,590
Light-density cells, undivided	7,120 ± 2,070
Light-density cells, divided	57,920 ± 15,400
Final preparation, CD11c$^+$ pre-DCs	121,860 ± 65,470
Final preparation CD11c$^-$ pro-DCs	161,050 ± 60,110

Fractions were recovered during isolation of precursor cells from Flt3L-stimulated CD45.2$^+$ bone marrow cultures at day 3.5, and a fixed number of cells was transferred into unirradiated CD45.1$^+$ recipient mice; recipient spleens were removed 5 d later, samples were enriched for DCs and counted and then the number of CD45.2$^+$CD11c$^+$ DCs was determined by flow cytometry. The DC precursor activity of each fraction was calculated as the number of CD45.2$^+$ DCs generated in the recipient spleens per 1×10^6 cells transferred. Results are the mean ± s.d. of three or more experiments.

final enrichment procedure, we depleted the fraction of lighter density of all cells bearing these cell surface markers using monoclonal antibodies and immunomagnetic particles before sorting for the divided cells.

The divided (CFSE^{dull}) cells, depleted of cells bearing Ly6G, CD19, MHC class II or IL-7Rα, included CD11c$^-$ and CD11c$^+$ cells (**Fig. 1c,d**), both of which had DC precursor activity *in vivo* (**Table 1**) and produced DCs when recultured *in vitro*, although the CD11c$^-$ precursor populations underwent much more expansion *en route* to DC production (**Fig. 1e**). The CFSE^{dull} cell fractions also included some cells that were Ly6C$^+$; in the CD11c$^+$ fraction, both the Ly6C$^+$ and Ly6C$^-$ cells had DC precursor activity, so we retained both in the final selection of the CD11c$^+$ precursor cells (**Fig. 1d**). However, in the CD11c$^-$ fraction, no DC precursor activity was present in the Ly6C$^+$ cells (data not shown), so we sorted only the Ly6C$^-$ cells in the final selection of the CD11c$^-$ precursor cells (**Fig. 1d**). The final CD11c$^-$ and CD11c$^+$ precursor cells (**Fig. 1d**) were substantially enriched for DC-generation capacity *in vivo* (**Table 1**).

Sequential precursor stages in DC development in culture

It was not immediately obvious whether the CD11c$^-$ and CD11c$^+$ DC precursors represented sequential steps in a common pathway or separate branches of DC development. To determine which was the case, we cultured the CD11c$^-$ and CD11c$^+$ DC precursors in conditioned medium and analyzed expression of CD11c and MHC class II at 1, 3 and 5 d later. The CD11c$^-$ precursor cells first became CD11c$^+$ and then expressed MHC class II (**Fig. 2a**); in contrast, the CD11c$^+$ precursor cells did not become CD11c$^-$ cells but instead progressed directly to MHC class II–positive DCs. Both precursor populations generated pDCs and cDCs (**Fig. 2a**). These results suggested that the CD11c$^-$ and CD11c$^+$ precursor cells represented developmental stages of a single pathway, with the CD11c$^-$ precursor cells giving rise to the CD11c$^+$ precursor cells, which then expressed MHC class II and became DCs.

Further support for that sequence of events came from studies of the kinetics of DC generation. When recultured in conditioned medium, the CD11c$^+$ precursor cells demonstrated little proliferation capacity and produced a peak of DC progeny by 3 d, whereas the CD11c$^-$ precursor cells showed extensive proliferation capacity and a peak of DC production at day 5 (**Fig. 2b**). A similar difference in kinetics was apparent *in vivo*; when each population of cells was transferred to unirradiated recipient mice, the CD11c$^+$ precursor cells produced a peak rise of spleen DC production at about day 5 after transfer, whereas the CD11c$^-$ precursor cells produced a larger and later peak at about day 9 after transfer (**Fig. 2c**). Notably, each precursor group produced pDCs, CD8$^+$ cDCs and CD8$^-$ cDCs in the spleens of the unirradiated recipient mice (**Fig. 2d**), and there was also a small amount of DC production, mainly pDCs, in the lymph nodes of recipient mice after transfer of CD11c$^-$ precursor cells. Overall, these findings support the conclusion of a linear developmental sequence: CD11c$^-$ precursor cells give rise to transitional CD11c$^+$ MHC class II–negative precursor cells, which then produce pDCs and both types of cDCs. In accordance with the nomenclature for other hematopoietic lineages, we called the CD11c$^-$ MHC class II–negative precursor cells 'pro-DCs' and the CD11c$^+$ MHC class II–negative precursor cells 'pre-DCs'. We noted additional characteristics of the pro-DC and pre-DC precursors. Both precursor populations had the morphological appearance of monoblast cells, although CD11c$^-$ precursor cells were slightly larger than the CD11c$^+$ precursor cells and lacked the dendritic extensions on the fully formed DCs (**Fig. 2e**). We also assessed the CD11c$^-$ and CD11c$^+$ precursor populations for their expression of over 40 cell surface markers (**Fig. 3** and **Supplementary Table 1** online). Notably, they were negative for surface expression of MHC class II, CD80 and CD86, markers found on fully developed DCs, and they expressed CD43, which has low expression on mature DCs[4]. The CD11c$^-$ precursor cells expressed several markers characteristic of precursor cells (**Fig. 3a**), including CD115 (the receptor for macrophage colony-stimulating factor) and CD135 (Flt3), moderate expression of CD117 (c-Kit), and only low expression of Sca-1 (Ly6A/C). They were negative for IL-7 receptor expression and thus were distinct from the common lymphoid progenitors[21]. The bulk of the CD11c$^-$ precursor cells had relatively low expression of the CD16/32 Fc receptor and of CD34, with only a small portion having the higher expression of both markers characteristic of the common macrophage-cDC precursor[12] or common myeloid progenitors[22] (**Fig. 3b**).

Commitment of pre-DCs and pro-DCs to DC development

We next assessed the capacity of the culture-derived DC precursors to form other hematopoietic lineages (**Fig. 4**) and to form DCs (**Fig. 4a,b**). We cultured samples of pre-DCs and pro-DCs with various cytokines in soft agar and assessed their ability to form various types of hematopoietic cell colonies (**Fig. 4c** and **Supplementary Table 2** online). The pre-DCs did not generate colonies or clusters in response to any of the stimuli tested, which indicated DC commitment; the pro-DCs did not form blast colonies or colonies of eosinophils and less than 2% formed granulocytes or mixed granulocyte-macrophage colonies (**Fig. 4c**). Overall, the pro-DCs were distinctly unresponsive to granulocyte-macrophage colony-stimulating factor. There was some macrophage colony potential in response to macrophage colony-stimulating factor (7%), but this was concentrated in the 20% CD16/32^{hi}CD34^{hi} fraction, leaving only a low macrophage precursor frequency (4%) associated with the bulk of DC precursors (**Fig. 4c**). The pro-DCs did not form any colonies in response to Flt3L, IL-7 or lipopolysaccharide (data not shown), indicating an absence of late-stage B lymphocyte precursor cells. Only 0.3% of pro-DCs generated colonies on OP9 mouse bone marrow stromal cell cultures containing IL-7 and Flt3L, indicating that very few cells in the pro-DC preparations had early B lineage potential.

To test the *in vivo* hematopoietic lineage potential of the culture-derived precursor cells, we adoptively transferred pro-DCs obtained from CD45.2$^+$ bone marrow into lethally irradiated CD45.1$^+$ recipient mice. We assayed recipient spleen and bone marrow 10 or 21 d later for donor-derived myeloid and lymphoid progeny. The adoptively transferred pre-DCs generated only CD11c^{hi} DCs in the spleen, which

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included pDCs, $CD8^+$ cDCs and $CD8^-$ cDCs but no $CD11c^-$ cells or cells with surface markers of other lineages (**Supplementary Fig. 1** online). The adoptively transferred pro-DCs also generated mainly $CD11c^{hi}$ DCs of all three types in the spleen (**Fig. 4b**), $CD11c^{hi}$ DCs in bone marrow (**Supplementary Fig. 1**), no $Ly6G^+$ granulocyte progeny above background (**Supplementary Fig. 1**), in accordance with the colony assays, and no $CD11c^-CD11b^{hi}$ macrophages in the spleen or bone marrow (**Supplementary Fig. 1**). However, some $CD11c^{lo}CD11b^{hi}$ cells were generated in bone marrow (**Supplementary Fig. 1**). These may have represented transitional pre-DCs *en route* to DCs or a small amount of macrophage production; if the latter, the efficiency was less than 0.2% of the macrophage-generating potential of unseparated bone marrow. The adoptively transferred pro-DCs did not generate any $DX5^+$ natural killer cells or $CD3^+$ T cells in the thymus or spleen at day 21 (data not shown). Although we detected no $CD19^+CD45RA^+$ B cells 10 d after transfer, we detected some in the spleen by day 21 (data not shown), presumably derived from the 0.3% early B precursor cells in the OP9 culture assays. The *in vivo* assays thus indicated that both pre-DCs and pro-DCs are DC-committed precursor cells.

Frequency of DC generation from single pro-DCs

Even though pro-DCs produced only DCs when recultured in conditioned medium, it was important to determine by clonal assay what

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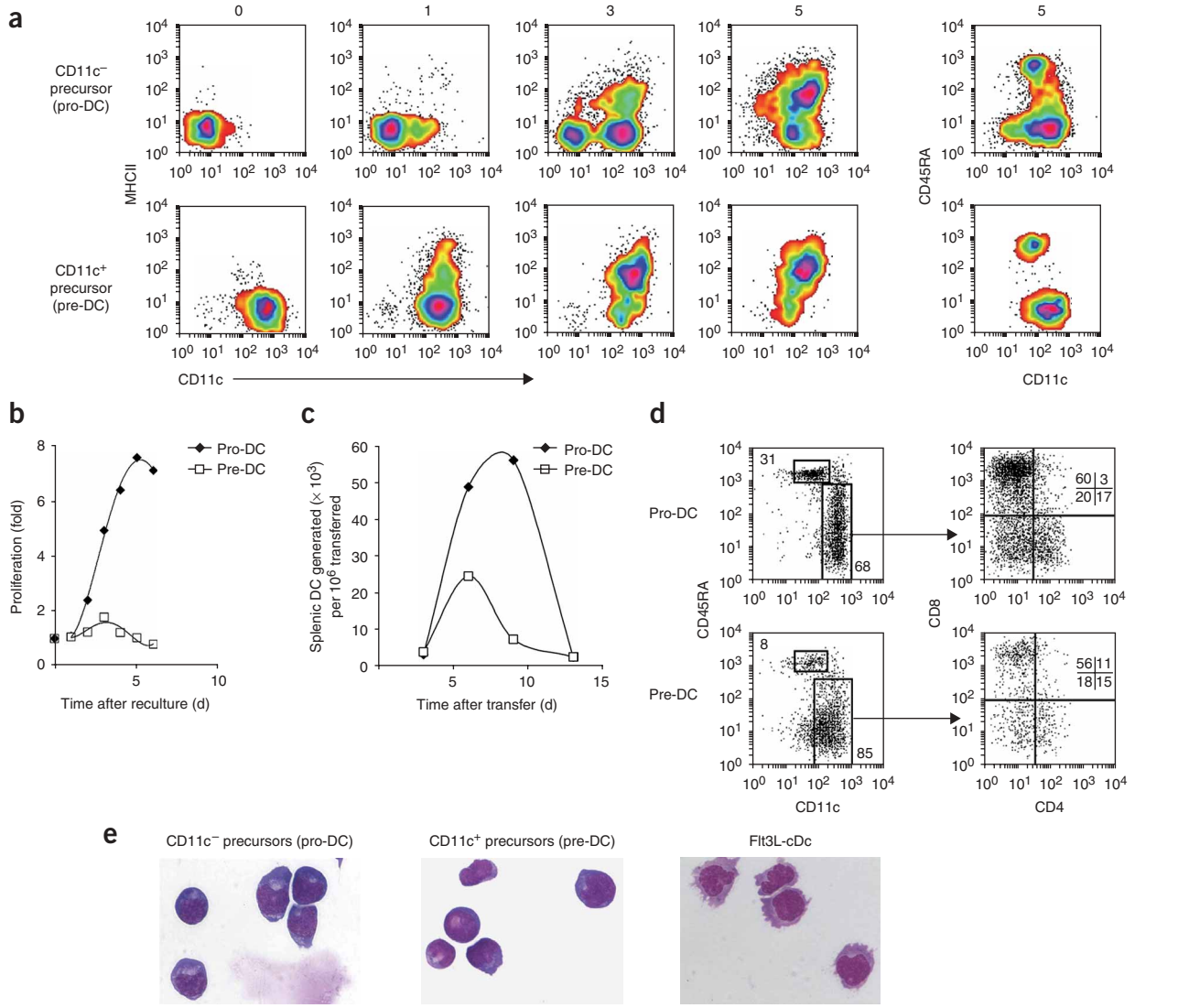

Figure 2 Development potential of $CD11c^-$ and $CD11c^+$ DC precursors *in vitro* after reculture or *in vivo* after transfer to recipient mice. (**a**) Flow cytometry of the acquisition of expression of CD11c and MHC class II by $CD11c^-$ or $CD11c^+$ precursor cells recultured in conditioned medium (reculture time, above plots). (**b**) Population expansion of pro-DCs and pre-DCs over time *in vitro*. (**c**) Splenic DCs generated over time *in vivo* per 1×10^6 pro-DCs and pre-DCs transferred into recipient mice. (**d**) Flow cytometry of spleen DC progeny derived *in vivo* from $CD11c^-$ or $CD11c^+$ precursor cells after transfer into recipient mice. $CD45.2^+$ precursor cells were transferred into $CD45.1^+$ unirradiated mice and recipient spleens were analyzed 5 d later. The DCs were enriched, stained, gated for $CD45.2^+$ and then gated on $CD11c^+CD45RA^+$ pDCs and $CD11c^+CD45RA^-$ cDCs (left); the latter were then analyzed for expression of CD8α and CD4 (right). All spleen DC subtypes were produced by both precursor populations. Numbers in plots indicate percent cells in each outlined area (left) or quadrant (right). (**e**) Purified $CD11c^-$ precursor cells from cultures at day 3.5 (pro-DCs), purified $CD11c^+$ precursor cells from cultures at day 3.5 (pre-DCs) and $CD11c^+CD45RA^-$ cDCs from cultures at day 9 (Flt3L-cDCs), stained with May-Grunwald Giemsa. Original magnification, ×400. All experiments were repeated once with similar results.

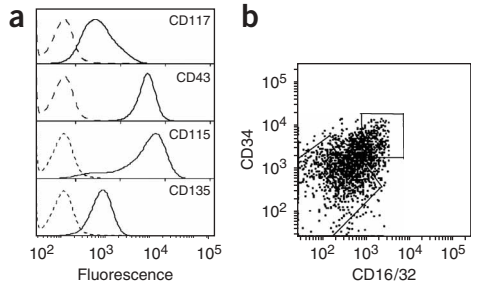

Figure 3 Surface phenotype of pro-DCs derived from cultures. (**a**) Flow cytometry of CD11c$^-$ pro-DCs isolated from Flt3L-stimulated bone marrow cultures at day 3.5 (as in **Fig. 1**), stained for various surface markers (in plots; expression of other cell surface markers, **Supplementary Table 1**). Dashed lines, background. Each analysis was repeated once with similar results. (**b**) Flow cytometry of the surface expression of CD34 and CD16/32 on pro-DCs; outlines indicate gates used to sort the CD34^{hi}CD16/32^{hi} cells. Results are representative of three experiments.

proportion of cells in these preparations had such DC precursor activity. However, single pro-DCs cultured alone in fresh or conditioned medium did not yield detectable progeny, and a minimum of 500 pro-DCs per well was required for optimal expansion to DC development. Therefore, to track the DC progeny of a single pro-DC, we developed an assay consisting of the culture of a single brightly fluorescent pro-DC among nonfluorescent 'filler' pro-DCs. We used bone marrow from mice expressing green fluorescent protein (GFP) under control of the ubiquitin c promoter (UBC-GFP) in Flt3L cultures as a source of fluorescent pro-DCs, except we used PKH26 (a fluorescent cell linker compound incorporated into the cell membrane and dilutes with cell division), rather than CFSE, to select the cells that divided. We then used a fluorescence-activated cell sorter to deposit single PKH26-labeled UBC-GFP (GFP$^+$) pro-DCs into individual wells of a 96-well round-bottomed plate containing 500 nonfluorescent pro-DC filler cells per well in conditioned medium. The average efficiency of deposition of a single GFP$^+$ pro-DC was 96%, as determined by direct microscopy, with no well receiving more than one fluorescent pro-DC. After 5 d of culture, we assigned scores to the wells for GFP$^+$ progeny by fluorescence microscopy. Clones were produced in 43–96% of wells over separate experiments and were generally small, ranging from 2 to over 100 individual cells, with 80% of the single cell–derived clones being less than 20 cells. Subsequent staining and flow cytometry showed that the clones developing from a single GFP$^+$ pro-DC were distinguishable from the GFP$^-$ filler cell products and that almost all GFP$^+$ cells were CD11c$^+$ MHC class II–positive DCs (**Fig. 5a**). The mean DC precursor frequency of the pro-DC preparations in six experiments was 72% ± 18% (**Fig. 4a**). This high DC precursor frequency was maintained in the CD16/32^{lo}CD34^{lo} pro-DC fraction (**Fig. 4a**) with the lowest frequency of macrophage colony–forming cells. As the culture system was probably not 100% efficient, it was apparent that most cells in the pro-DC preparations were DC precursors.

Commitment of pro-DCs to DC subtypes

The ability to analyze individual clones of DCs allowed us to determine whether a single pro-DC was able to generate all three DC subtypes or instead whether the pro-DC population consisted of a mixture of precursor cells, each committed to forming a particular DC subtype. We individually analyzed clones derived from single GFP$^+$ pro-DCs by flow cytometry for surface CD45RA, Sirp-α and CD11c (**Fig. 5**). On average, this procedure captured 70% of the GFP$^+$ cells initially detected by microscopy in each clone. Single pro-DCs produced clones differing in DC composition. Some clones contained all three DC subtypes, including pDCs (CD45RA$^+$Sirp-α$^-$), CD8$^-$ cDC–equivalent cells (CD45RA$^-$Sirp-α$^+$) and CD8$^+$ cDC–equivalent cells (CD45RA$^-$Sirp-α$^-$; **Fig. 5c**). In these cases, the initiating pro-DCs were thus not precommitted to a particular DC type. However, some DC clones contained no CD45RA$^+$ cells, suggesting the initiating pro-DCs were precommitted to cDC development (**Fig. 5e**). Other

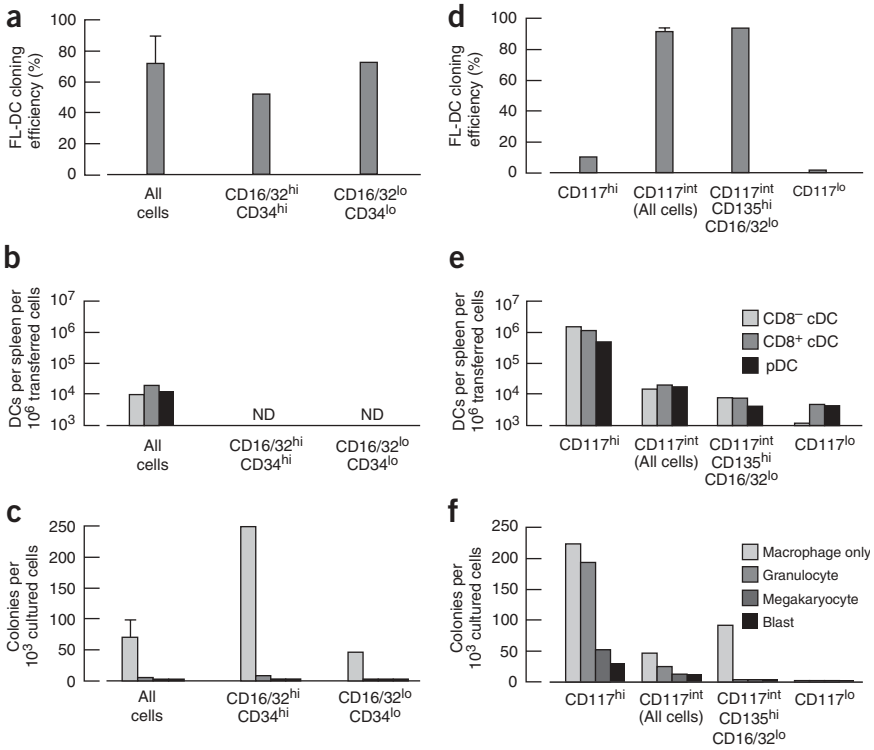

Figure 4 Pro-DCs from *in vitro* culture versus precursor populations isolated from bone marrow. Differentiation potential of pro-DCs isolated from Flt3L-stimulated bone marrow cultures and sorted into fractions (**a**–**c**) and of Lin$^-$ fractions isolated directly from bone marrow (**d**–**f**). (**a**,**d**) Cloning efficiency in cultures of single GFP$^+$ precursor cells sorted into cultures of GFP$^-$ pro-DC filler cells in conditioned medium; the frequency of all GFP$^+$ DC clones was determined 5 d later. (**b**,**e**) DC generation *in vivo* determined by intravenous transfer of precursor cells into unirradiated recipient mice; the number of donor-derived DC progeny of various types in the spleen was evaluated 8 d later. (**c**,**f**) Generation of hematopoietic colonies in soft agar, determined by culture of 1 × 10^2 to 1 × 10^5 precursor cells with various cytokines (detailed colony counts, **Supplementary Table 2**). 'Granulocyte' includes colonies of granulocytes alone and of granulocytes plus macrophages; 'Macrophage only' represents colonies of macrophages alone. ND, not done. Results are the means of two experiments (data without error bars) or the mean + s.d. of three to six experiments (data with error bars).

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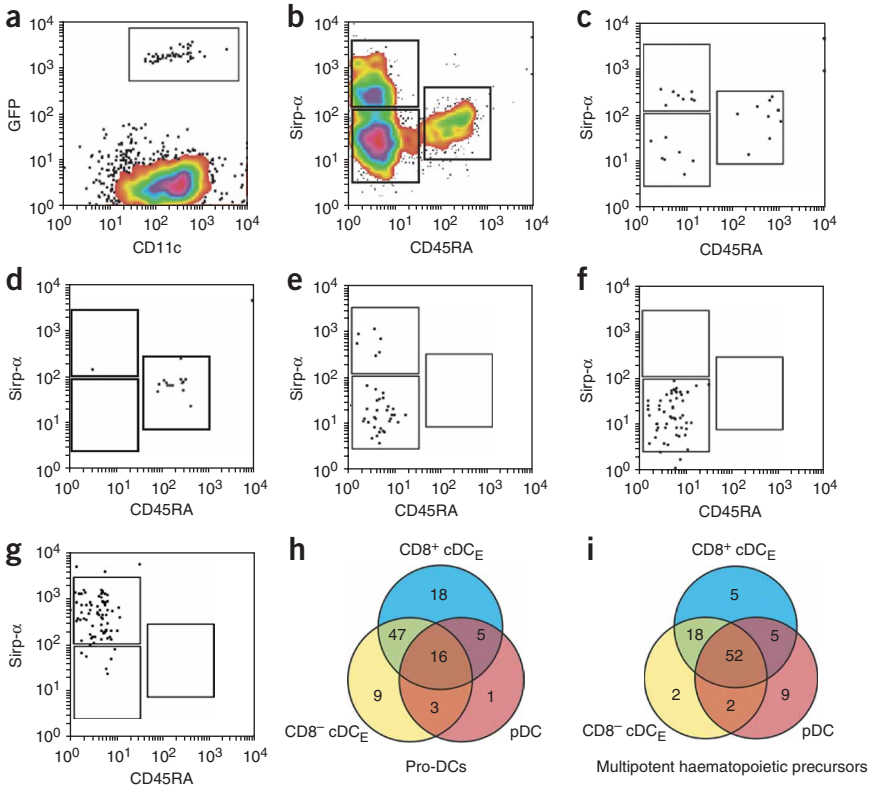

Figure 5 Clones of DCs produced in culture from single pro-DC precursors. Flow cytometry of the *in vitro* differentiation potential of single GFP$^+$ pro-DC precursors isolated from Flt3L cultures at day 3.5, then recultured clonally for 5 d with GFP$^-$ pro-DC filler cells. (**a**) GFP$^+$ clonal DC progeny derived from a single GFP$^+$ pro-DC (gated cells) and GFP$^-$ DC progeny of pro-DC filler cells. This result was obtained over 100 times. (**b**) Segregation of the GFP$^-$ DC progeny of pro-DC filler cells into three populations: CD45RA$^+$ pDCs, CD45RA$^-$Sirp-α$^+$ cells (CD8$^-$ cDC–equivalent cells) and CD45RA$^-$ Sirp-α$^-$ cells (CD8$^+$ cDC–equivalent cells). This result was obtained over 100 times. (**c**–**g**) Representative samples of DC clones derived from single GFP$^+$ pro-DC precursors. These show DC clones composed of all three DC subtypes, derived form uncommitted precursors (**c**), DC clones from mostly pDC-committed precursors (**d**), DC clones from cDC-committed, but cDC subtype–uncommitted precursors (**e**), DC clones from CD8$^+$ cDC equivalent–committed precursors (**f**), and DC clones from mostly CD8$^-$ CDC equivalent–committed precursors (**g**). (**h**,**i**) Distribution of DC subtypes among the 99 pro-DC-derived clones (**h**) and the 93 clones derived from bone marrow multipotent precursor cells (**i**). Results are from three (**h**) or five (**i**) experiments.

DC clones contained mainly pDCs (**Fig. 5d**), mainly CD8$^+$ cDC–equivalent cells (**Fig. 5f**), or mainly CD8$^-$ cDC–equivalent cells (**Fig. 5g**), suggesting the initiating pro-DCs were precommitted to forming a single DC subtype. We determined the incidence of these different types of DC clones among the 99 clones sampled over three experiments (**Fig. 5h**). Three points should be emphasized. First, the nonfluorescent DCs arising from the pro-DC filler cells included all three DC subtypes in all cultures analyzed (as in **Fig. 5b**), which indicated that the conditions in the individual culture wells were not restricting the DC subtypes produced. Second, when we pooled the numbers of product DC subtypes from all the fluorescent clones sampled in **Figure 5h**, the distribution resembled that of the nonfluorescent filler cell–derived DCs, indicating that the clones reflected events in the bulk cultures. Third, when we removed the 20% CD16/32^{hi}CD34^{hi} cells with much of the macrophage potential from the pro-DC preparation, the remaining main pro-DC population had a mixed DC potential similar to that in **Figure 5h** and generated a similar proportion of clones containing all DC subtypes (data not shown).

Because DC clone sizes were small and on average only 70% of each clone was analyzed by flow cytometry, it was important to check statistically that any apparent restriction in the DC subtypes produced was not due to sampling errors. Accordingly, we assessed the distribution of DC subtypes noted with each fluorescent clone for independence from the distribution of the much larger numbers of nonfluorescent DCs derived from the 500 pro-DC filler cells in the same well. The null hypothesis was that each GFP$^+$ pro-DC was multipotent and would produce the same distribution of DC subtypes as the pro-DC filler cells did. Comparison of the data obtained with the data expected if pro-DCs were able to produce all DC subtypes (**Supplementary Fig. 2** online) showed a substantial deviation from this null hypothesis. Over half the clones (57%) showed significant departure from the null hypothesis ($P < 0.05$), yet a substantial proportion (16%) also contained all DC subtypes. We concluded that the pro-DCs included both common DC precursors that generated all DC subtypes as well as DC subtype–committed precursor cells.

DC clones derived from multipotent hematopoietic precursor cells

Because the process of commitment to different DC subtypes seemed to be occurring at the pro-DC precursor stage, it was important to check that individual 'upstream' precursor cells were able to form all DC subtypes. To test this, we isolated from the bone marrow of UBC-GFP mice multipotent GFP$^+$ hematopoietic precursor cells with a surface phenotype of Lin$^-$CD117$^+$Sca-1$^+$CD34$^+$. As described above, we sorted single GFP$^+$ hematopoietic precursor cells into wild-type filler bone marrow cells at the initiation of culture. At day 8, 15% of such cultures contained GFP$^+$ DC clones, larger than those derived from culture-derived pro-DC precursors and ranging from 20 to 1,000 cells. The proportion of DC clones containing all DC subtypes was much higher from these earlier precursor cells than from pro-DCs (**Fig. 5i**). However, some DC clones derived from these multipotent precursor cells (including large clones less subject to sampling errors) were restricted to particular DC types, including some that were restricted to pDC development. Thus, whereas restriction to DC subtypes occurred mainly at the pro-DC stage, some aspects of this process may have begun at an earlier developmental stage. Before concluding that most DC subtype commitment was occurring at the pro-DC stage, however, we ensured that the product Flt3L-DC–derived subtypes were stable and did not interconvert. To test this, we sorted pDCs, CD8$^+$ cDC–equivalent cells and CD8$^-$ cDC–equivalent cells from bulk cultures at day 7 and then recultured the cells individually for 1 or 2 d longer in conditioned medium from day 7. In all cases, the pDCs, CD8$^+$ cDC–equivalent cells and CD8$^-$ cDC–equivalent cells retained their original CD45RA and Sirp-α surface phenotypes (data not shown). Thus, once differentiated, the Flt3L-DC subtypes are stable.

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Equivalents of pro-DCs in normal bone marrow

To determine if the culture-derived pro-DC population had an *in vivo* equivalent, we first selected from bone marrow cells that were, like our pro-DCs, negative for lineage markers (Lin^-), including $CD11c^-Ly6C^-$ and $CD127^-$ ($IL\text{-}7R^-$). We then segregated this population based on expression of CD117 (c-Kit; **Fig. 4d–f** and **Supplementary Fig. 3** online). Cells that were intermediate for CD117 expression were also $CD43^+$ and many were $CD135^+$, $CD115^+$ and $CD16/32^-$, which were the cell surface markers characteristic of our pro-DCs. When separated by CD117 expression and tested for function, the $CD117^{lo}$ cells had little capacity to form DCs or other hematopoietic cells (**Fig. 4f**). The $CD117^{hi}$ cells had a large capacity to form DCs on transfer (**Fig. 4e**) and a moderate cloning efficiency for DC generation in culture (**Fig. 4d**), with a peak at day 9, rather than at day 5, as for pro-DCs. As expected, this $CD117^{hi}$ fraction efficiently formed all types of hematopoietic colonies in agar and thus contained many hematopoietic precursor cells, including multipotent precursor cells (**Fig. 4f** and **Supplementary Table 2**). However, the $CD117^{int}$ bone marrow fraction, with surface markers similar to those of pro-DCs, produced DCs of all types in culture with a very high cloning efficiency (92%; **Fig. 4d**) but relatively small clone size, as did pro-DCs. The peak DC production was at day 5, as it was for pro-DCs. The $CD117^{int}$ fraction produced all DC types after transfer to unirradiated mice (**Fig. 4e**) but with only the same moderate DC yield as culture-derived pro-DCs; the lower transfer efficiency relative to that of the $CD117^{hi}$ fraction (**Fig. 4e**) presumably reflected a lower proliferation potential relative to that of the earlier multipotent cells. Finally, the $CD117^{int}$ fraction showed a low incidence of hematopoietic colony–forming cells relative to that of the $CD117^{hi}$ fraction (**Fig. 4f** and **Supplementary Table 2**).

We further sorted the $CD117^{int}$ bone marrow fraction for cells that were $CD135^+CD16/32^{lo}$; these were all $CD115^+$ (**Supplementary Fig. 3** online). These cells formed DCs of all subtypes after transfer to unirradiated recipients (**Fig. 4e** and **Supplementary Fig. 4a** online) with an efficiency similar to that of culture-derived pro-DCs (**Fig. 4b**). We obtained only MHC class II–positive progeny and detected no B cells, T cells, natural killer cells or granulocytes (**Supplementary Fig. 4b**), which indicated restriction to DC development, similar to culture-derived pro-DCs. This bone marrow fraction formed DCs of all subtypes in our culture system with high cloning efficiency (94%) but small clone size (**Fig. 4d**). When tested in a different culture system of OP9 filler cells supplemented with conditioned medium and Flt3L, this $CD117^{int}CD115^+CD16/32^-$ fraction formed DCs (both pDCs and cDCs) with 25% ± 4% cloning efficiency and large clone size, whereas the $CD117^{int}CD135^-$ fractions showed only a low DC cloning efficiency (3%; **Supplementary Fig. 5** online). The incidence of macrophage colony–forming cells remained 5–9% in the $CD117^{int}CD135^+CD16/32^{lo}$ fraction (**Fig. 4f**), but few other hematopoietic colony-forming cells were present (**Supplementary Table 2**), similar to our culture-derived pro-DCs. Overall, the pro-DCs we isolated from our Flt3L cultures resembled, by surface phenotype and precursor function, a DC-restricted precursor population in steady-state bone marrow, similar to that described before[23].

DISCUSSION

The Flt3L-stimulated bone marrow culture system we used here to model spleen DC development allowed a more detailed examination of the process than is possible with approaches using intact animals. We have demonstrated here that all stages of commitment leading from multipotent hematopoietic precursor cells to three distinct DC subtypes can be studied at a clonal level with this approach. Although the Flt3L-DCs derived from these cultures lacked CD8 and CD4 expression[15,24], it has been shown that other markers allow segregation of DCs that are functionally equivalent to pDCs, $CD8^-$ cDCs and $CD8^+$ cDCs, the main DC subtypes found in the steady-state spleen[17,19,25,26]. In addition, the amount of MHC class II and of costimulatory molecules (such as CD40, CD80 and CD86) on the Flt3L-DCs was very similar to that on spleen DCs[1]. Although Flt3L is known to be the main cytokine required for normal steady-state development, a chief difference in the culture system we used versus the conditions in the steady-state spleen was the greatly enhanced generation of DC progeny from the relatively large amounts of Flt3L in our cultures. However, increased Flt3L is encountered *in vivo* after immune stimulation or in leukopenic conditions[27–30].

We were able to isolate from these cultures two successive DC-committed precursors *en route* to becoming Flt3L-DCs: the earlier $CD11c^-$ pro-DCs, with substantial proliferation potential, and the 'downstream' $CD11c^+$ pre-DCs, closer to the final DC products. Notably, we found that these precursor cells, when transferred into unirradiated recipient mice, generated the three types of DCs normally present in steady-state mouse spleen: pDCs, $CD8^-$ cDCs and $CD8^+$ cDCs. When recultured in conditioned medium, both the pro-DCs and the pre-DCs generated all three Flt3L-DC subtypes: pDCs, $CD8^-$ cDC–equivalent cells and $CD8^+$ cDC–equivalent cells. When these individual Flt3L-DC subtypes were purified and then placed back in conditioned culture medium, they did not interconvert to other subtypes but instead retained their surface phenotype. Thus, commitment to a particular DC subtype occurred at an earlier precursor stage. Such a conclusion is in accordance with *in vivo* evidence that the final DC products instead represent separate developmental lineages originating from dividing precursor cells[31,32].

The enhanced DC precursor proliferation obtained in these Flt3L-stimulated cultures allowed us to do true clonal analysis of the production of the types of DCs present in spleen. We found that the pro-DCs were a mixture of precursor cells at various levels of commitment. Thus, some pro-DCs generated clones containing all three DC types, which indicated that they were 'common' DC precursors not yet committed to DC subtype. However, some pro-DCs generated clones containing both types of cDCs but not pDCs, and others generated clones restricted to a single cDC subtype. Most of the process of commitment to individual DC subtypes seemed to be occurring at this dividing pro-DC stage, a stage at which the ability to form other hematopoietic cells has been lost. However, some of the process of DC subtype commitment, especially to pDCs, seemed to have begun even earlier, at a multipotent hematopoietic precursor stage.

How close are the DC precursors isolated from Flt3L-stimulated cultures to the DC precursors present in steady-state mice? The culture-generated pre-DCs were similar to the immediate precursor cells of spleen cDCs (pre-cDCs) identified before[4,33]. Both are $CD11c^+$, MHC class II negative and $CD43^+$, and both are committed to DC production, after limited population expansion. We were unable to analyze the very small DC clones produced by the culture-derived pre-DCs, but analysis of DC clones derived from the 'upstream' pro-DCs provided crucial information for comparison with the spleen precursor cells. Spleen pre-DCs have been shown to include cells committed to the production of either $CD8^-$ or $CD8^+$ cDCs[4], which fits with our clonal analysis showing some individual 'upstream' pro-DCs were already committed to a single cDC subtype. One characteristic in which the pre-DCs derived from cultures initially

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seemed to differ from the pre-cDCs directly isolated from spleen was that the spleen precursor cells produced only cDCs, whereas the pre-DCs from Flt3L-stimulated bone marrow cultures produced both cDCs and pDCs. Our clonal analysis has now provided an explanation for this discrepancy, as many individual clones derived from the earlier, 'upstream' pro-DCs did not contain pDCs and were cDC restricted. The spleen pre-cDCs therefore closely resemble the many cDC-committed precursor cells in the total population of bone marrow culture–derived DC precursors.

The earlier pro-DCs isolated from our cultures closely resembled the DC-restricted, common DC precursors that have been isolated from bone marrow before[23]. Both pro-DC precursor populations have a $Lin^{-}CD117^{int}CD115^{+}CD135^{+}$ surface phenotype and include precursor cells capable of forming both cDCs and pDCs, as well as precursor cells already committed to one or the other DC type. When we isolated such precursor cells from bone marrow, we found that they were effective precursors of DCs in our culture system (94% cloning efficiency) with kinetics similar to our pro-DCs, and they formed both pDCs and cDCs. Both the culture-derived pro-DCs and the precursor cells from bone marrow were unable to form colonies of most hematopoietic cell types in agar, although in our experiments, both precursor populations retained a 7–9% frequency of macrophage colony–forming cells. However, much of the macrophage precursor activity of the pro-DCs was concentrated in a minor $CD16/32^{hi}CD34^{hi}$ fraction. The final cloning efficiency for forming macrophage colonies (4%) versus DCs (75%) indicated that most of the $CD16/32^{lo}CD34^{lo}$ culture–derived pro-DC population was DC restricted.

Our pro-DC population thus seems distinct from the CX3CR1-expressing macrophage-cDC precursors isolated from bone marrow[12]. Crucially, those precursor cells were unable to form pDCs. A common precursor of macrophages and monocyte-derived 'inflammatory' or 'migratory' DCs would not be unexpected[1]; this possibility may be reflected in the response of macrophage-cDC precursors to the inflammatory cytokine granulocyte-macrophage colony-stimulating factor and the lack of responsiveness of pro-DCs to this cytokine. However, macrophage-cDC precursors have also been found to form $CD8^{+}$ and $CD8^{-}$ spleen DCs after transfer *in vivo*[12]. As that assay[12] was not clonal, the proportion of the precursor cells able to produce spleen cDCs was not clear. However, the boundaries between the common DC-restricted and the macrophage-cDC-restricted precursor cells[12,23] may be blurred, and several routes to spleen cDC production might exist. Thus, our data suggest that some pDC-committed precursor cells do 'branch off' at the multipotent precursor stage before the pre-DC stage. In addition, our pro-DC population did retain some limited capacity for macrophage production that was concentrated in a minor fraction expressing CD16/32 and CD34, both markers of the macrophage-cDC precursors[12]. Nevertheless, in our Flt3L-stimulated system, most generation of cDCs and pDCs flowed through a common DC precursor with little capacity to generate macrophages.

We have shown here that all stages of DC development were present in the Flt3L-stimulated culture system and had *in vivo* equivalents, with differentiation occurring from multipotent hematopoietic precursor cells to DC-committed pro-DCs, then to the development of the final three DC products through the $CD11c^{+}$ pre-DC population. The particular value of our system is the opportunity to analyze the process of commitment to the main subtypes of DC found in spleen, including commitment to the $CD8^{+}$ cDC subtype versus the $CD8^{-}$ cDC subtype. Much of the commitment to a DC subtype occurs at the pro-DC stage. The exogenous (cytokine) controls and endogenous (transcription factor) controls over DC subtype development can now been examined in more detail with this culture model.

METHODS

Mice. C57BL/6J mice (CD45.2) 6–10 weeks of age were the usual source of bone marrow for culture. For tracking of the clonal progeny of single precursor cells, C57BL/6-TG(UBC-GFP)30Scha/J mice, originally obtained from The Jackson Laboratory, were the source of precursor cells. For transfer studies, recipient mice were C57BL/6 Pep^{3b} CD45.1. All mice were bred at The Walter and Eliza Hall Institute Animal Facility in specific pathogen–free conditions.

Flt3L-stimulated bone marrow cultures. Cultures were set up as described[19,34]. Bone marrow cells were extracted, and erythrocytes were removed by brief exposure to 0.168 M NH_4Cl. Cells were cultured at a density of 1.5×10^6 to 3.0×10^6 cells per ml in mouse osmolarity RPMI 1640 medium with 10% (vol/vol) fetal bovine serum containing mouse Flt3L (200 ng/ml), at 37 °C in 10% CO_2. Recombinant mouse Flt3L was produced by a Chinese hamster ovary cell line and was purified in our laboratory.

Isolation of DC precursors from cultures. For labeling of bone marrow cells with CFSE before culture, 1 μl of 0.5 M CSFE (carboxyfluorescein diacetate succinmidyl ester; Molecular Probes) was mixed with 1×10^7 cells in 1 ml medium, followed by incubation for 10 min at 37 °C and washing. UBC-GFP bone marrow cells prelabeled with the membrane dye PKH-26 (Sigma) were cultured as described[4]. After 3.5 d of culture in Flt3L at a density of 3.0×10^6 cells per ml, cells were resuspended and recovered by centrifugation. Medium from cultures at day 3.5 was passed through a 0.22-μm filter and was retained for use as conditioned medium. Cell pellets were resuspended in iso-osmotic Nycodenz medium (1.086 g/cm^3) containing EDTA, then were centrifuged at 1,700*g* for 10 min at 4 °C. Supernatant containing the buoyant DC precursors was diluted and cells were recovered by centrifugation; after initial testing, the pellet of dense cells and dead cells was discarded. The cells of lighter density were then coated with biotinylated antibodies to CD19 (ID3), Ly6G (IA8), IL-7α (A7R34) and MHC class II (M5/114). All mAB were produced, purified and conjugated in our laboratory. For isolation of only the earlier pro-DC precursors, antibody to CD11c (anti-CD11c; N418) and anti-Ly6C (5075-3.6) were also used for depletion. Coated and washed cells were then mixed and were incubated with anti-biotin magnetic beads, followed by depletion on a magnetic column (Miltenyi). The unbound 'flow-through' containing DC precursors was retained; after initial testing, bound cells were discarded. The DC precursor–enriched fraction was then labeled with Alexa Fluor 594–conjugated anti-CD11c (N418) and biotin-conjugated Ly6C (5075-3.6; with streptavidin-phycoerythrin secondary label, Becton-Dickinson Biosciences). Samples were then enriched for DC precursors by fluorescence-activated cell sorting with gating on large viable cells that had undergone division; pro-DCs were selected as $CD11c^{-}Ly6C^{-}$ cells, and pre-DCs were selected as $CD11c^{+}$ cells.

Isolation of DC precursors from fresh bone marrow. Bone marrow cells were suspended in Nycodenz medium (1.086 g/cm^3) and cells of lighter density were isolated by centrifugation as described above. The cells of lighter density were then coated with biotinylated monoclonal antibodies to the following lineage markers: CD3 (KT3-1.1), CD19 (ID3), CD45R (B220, RA36B2), CD11b (M1/70), CD11c (N418), Ly6G (IA8), Ly6C.2 (5075-3.6), NK1.1 (PK136), CD127 (IL-7R; A7R34-2.2) and Ter119. After being washed, cells were incubated with anti-biotin magnetic beads, followed by depletion of positive cells on a magnetic column (Miltenyi) to produce the Lin^{-} fraction. Lin^{-} cells were first stained with phycoerythrin-indotricarbocyanine to ensure any residual Lin^{+} cells could be gated out, then were stained with various combinations of monoclonal antibodies to CD117 (c-Kit; ACK-2; conjugated to fluorescein isothiocyanate or allophycocyanin), CD43 (S7; conjugated to biotin, with streptavidin-phycoerythrin second stage), CD115 (M-CSF receptor; AFS98; conjugated to biotin, with streptavidin-phycoerythrin second stage), CD16/32 (2.4G2; conjugated to allophycocyanin) and Sca-1 (Ly6A/E; D7; conjugated to Alexa Fluor 594). Fractions were then sorted on the basis of expression of CD117 and other markers and were tested for DC precursor activity.

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Isolation of multipotent hematopoietic precursor cells from fresh bone marrow. Lin^- bone marrow cells less dense than 1.086 g per cm^3 were prepared with a lineage-depletion 'cocktail' consisting of monoclonal antibodies produced in our laboratory to the following antigens: CD2 (RM2-1), CD3 (KT3-1.1), CD19 (ID3), CD45R (RA36B2), CD11b (M1/70), Ly6G (IA8), NK1.1 (PK136), Ter119. The Lin^- cells were then stained with monoclonal antibodies to CD117 (c-Kit; ACK-2; conjugated to allophycocyanin), Sca-1 (Ly6A/E, E13161.7; conjugated to Alexa Fluor 594) and CD34 (RAM34; conjugated to biotin, with streptavidin-phycoerythrin second stage). Multipotent hematopoietic precursor cells were then sorted as $CD117^+Sca\text{-}1^+CD34^+$ cells.

Generation of Flt3L-DC from precursor cells in bulk culture. A fixed number of pro-DCs or pre-DCs (over 500 cells) isolated from cultures at day 3.5 were recultured for various times in 0.2 ml conditioned medium. A fixed number of latex beads was then added to each culture to aid in the quantification of DCs. Cells were stained with anti-CD11c (N418), anti-Sirp-α (P84), anti-CD45RA (14.8) and antibody to MHC class II (M5/114), with propidium iodide (1 μg/ml) added to the final wash to stain dead cells. DC progeny were then counted by flow cytometry, with gating on viable $CD11c^+$ MHC class II–positive cells and separate counting of $CD45RA^{hi}Sirp\text{-}\alpha^{lo}$ DCs (pDCs), $CD45RA^-Sirp\text{-}\alpha^{hi}$ DCs ($CD8^-$ cDC–equivalent cells) and $CD45RA^-$ $Sirp\text{-}\alpha^{lo}$ DCs ($CD8^+$ cDC–equivalent cells).

Generation of DCs from precursor cells *in vivo*. The procedure has been described in detail[4]. Between 0.5×10^6 and 2×10^6 pro-DCs or pre-DCs from cultures of $CD45.2^+$ bone marrow at day 3.5 were injected intravenously into unirradiated $CD45.1^+$ recipient mice. At several time points later, recipient spleens were collected and enriched for DCs[4] and then were stained with anti-CD45.2 (S450-15.2), anti-CD4 (GK1.5), anti-CD8 (YTS169.4) and anti-CD45RA (14.8). Donor-derived DCs were counted by flow cytometry and the activity of the transferred precursor cells was determined as the number of donor-type DCs generated in the spleens per 1×10^6 precursor cells transferred.

Clonal development of Flt3L-DCs from single precursor cells. GFP^+ pro-DC precursors were isolated from cultures of UBC-GFP bone marrow. Bone marrow multipotent hematopoietic precursor cells or DC-restricted precursor cells were isolated directly from UBC-GFP bone marrow. Pro-DC filler cells were purified from cultures of wild-type bone marrow at day 3.5. Cultures containing 500 wild-type pro-DC filler cells were set up in 200 μl conditioned medium in the U-bottomed wells of 96-well culture trays. A single UBC-GFP (GFP^+) DC precursor cell was then deposited in each culture well with the single-cell function of a FACSVantage DIVA (Becton-Dickinson). Precursor cells were then cultured for a further 5 d. For earlier hematopoietic precursor cells, a single GFP^+ precursor cell was deposited in a culture of 1.5×10^6 wild type bone marrow cells at the time of culture initiation, followed by culture for 8 d. At the time of collection, fluorescent cells and positive clones could be detected and counted by direct microscopy of the cultures under ultraviolet illumination. Cells were resuspended and were stained with a combination of allophycocyanin-conjugated anti-CD11c (N418), phycoerythrin-conjugated anti-MHC class II (M5/114), phycoerythrin-conjugated anti-CD45RA (14.8) and biotin-conjugated anti-Sirp-α (CD172a; P84; with streptavidin–peridinin chlorophyll protein–cyanin 5.5 second stage), with Fluorogold (2.5 μg/ml) in the final suspension medium to label dead cells. The entire stained culture content was analyzed by flow cytometry. Viable cells were gated and were segregated by GFP fluorescence to distinguish the many DCs derived from wild-type filler bone marrow versus the clone of GFP^+ DC progeny derived from a single UBC-GFP pro-DC. All cells were assessed for expression of CD11c and MHC class II to determine total DC production or were gated as $CD11c^+$ and segregated into the three Flt3L-DC types based on expression of CD45RA and Sirp-α.

Hematopoietic lineage potential by *in vivo* assay. The pro-DCs and pre-DCs from cultures of $CD45.2^+$ bone marrow at day 3.5 were transferred intravenously into lethally irradiated $CD45.1^+$ recipient mice, together with $CD45.1^+$ bone marrow. Donor-derived cells in the spleen and bone marrow were analyzed at 10 d and 21 d after transfer. DCs were identified as $CD11c^{hi}CD11b^-$ cells ($CD8^+$ cDCs and pDCs) and $CD11c^{hi}CD11b^{int}$ cells ($CD8^-$ cDCs), macrophages were identified as $CD11c^-CD11b^{hi}$ cells, granulocytes were identified as $Ly6G^{hi}$ (IA8) cells, natural killer cells were identified as $CD49b^+$ (DX5) cells, T cells were identified as $CD3^+$ (KT3) cells, and B cells were identified as $CD19^+CD45RA^+$ cells.

Analysis of hematopoietic lineage potential by agar colony formation. This procedure has been described[35]. Cells from each population (1×10^2 to 1×10^3) were cultured in 1 ml DMEM containing 0.3% (wt/vol) agar and various cytokines. Recombinant cytokines were used at the following concentrations: granulocyte-macrophage colony-stimulating factor, IL-3, macrophage colony-stimulating factor and leukemia inhibitory factor, 10 ng/ml; stem cell factor, 100 ng/ml; and erythropoietin, 21 U/ml. After 7 d of incubation, differential colony counts were made on fixed whole-mount preparations stained for acetylcholinesterase, Luxol fast blue and hematoxylin.

Assay of B cell precursor frequency. This procedure has been described[36]. OP9 cells (4×10^3 per culture) were plated in the flat-bottomed wells of 96-well culture plates. Then, 1 d later, 'graded' numbers of the DC precursors or of pro–B cells deficient in transcription factor Pax5 (positive control) were plated onto the feeder cells in modified MEM supplemented with 50 mM β-mercaptethanol and 10% (vol/vol) fetal calf serum, containing Flt3L (5 ng/ml) and 2% of the supernatant of an IL-7-producing cell line. After 7 d of culture at 37 °C, the incidence of B cell colonies was assessed and precursor frequency was determined by Poisson statistics.

Statistical analysis of precursor commitment from DC clone data. 'R' software (http://www.r-project.org/index.html) was used for all statistical analyses. The χ^2 test of independence was used to compare DC subtype distribution among fluorescent DCs from the single UBC-GFP pro-DC versus DC subtype distribution among nonfluorescent DCs from the 500 pro-DC filler cells in each well for each of the 96 culture wells sampled. All wells were assumed to act independently. Significance was assessed with a permutation test rather than the usual χ^2 distribution because of the small clone sizes. The observed quantiles of P values from the permutation test results of the χ^2 test statistics of each well were compared with the expected quantiles of a uniform distribution over [0, 1] with a quantile-quantile plot. With the null hypothesis of no difference in the distribution of DC subtypes for fluorescent clones versus nonfluorescent filler cells, a straight line should result. Data were also simulated '*in silico*' with the null hypothesis for each culture well by random sampling of the DCs derived from the nonfluorescent filler cells to produce a model clone the same size as the UBC-GFP clone (**Supplementary Fig. 2**). A two-sample, two-sided Kolmogorov-Smirnov test was used for comparison of the observed P-value distribution with the expected P-value distribution from the null hypothesis. Finally, an 'omnibus test' for all 99 wells was done with a likelihood-ratio test statistic, assuming a multinomial distribution for each well. Its significance was also assessed by permutation tests because of the small clone sizes. All permutation tests used 1,000 permutations.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

S.H.N. designed and did most experiments and wrote the manuscript; K.S. designed experiments and wrote the manuscript; P.S. and H.-Y.P. isolated bone marrow precursor cells and did clonal experiments; J.-Y.K., M.O'K. and L.W. helped to design experiments; D.M. did and analyzed colony assays; A.I.P. and A.D. assisted with precursor characterization; S.C. did the OP9 B precursor assays; M.B. and A.P. designed and did the statistical analysis; and all authors discussed the results and helped to write the manuscript.

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Erratum: Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo

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In the version of this article initially published online, the outlined boxes in the flow cytometry plots in Figure 5d are incorrect. The error has been corrected for all versions of the article.

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In the HTML version of this paper published online, Figure 2 was missing and a duplicate copy of Figure 4 was included in its place. This error has been corrected in the HTML version of the paper.

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