Convergent Differentiation: Myeloid and lymphoid pathways to murine plasmacytoid dendritic cells

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Abstract

The developmental origin of interferon-producing plasmacytoid dendritic cells (pDC) has been uncertain. We tracked the development of pDC in cultures of bone marrow precursors stimulated with Flt3 ligand. Common myeloid precursors (CMP) produced both conventional DC (cDC) and pDC, via the DC-restricted common DC precursor (CDP). Common lymphoid precursors (CLP) produced only a few cDC with variable efficiency, but produced pDC via a transient intermediate precursor with B cell potential. The pDC of both origins produced interferon-α when stimulated with CpG oligonucleotides. The pDC of CLP origin showed evidence of past RAG1 expression and had D-J rearrangements in IgH genes. Most pDC and all cDC of CMP origin lacked these signs of a lymphoid past. However in these cultures a proportion of pDC of CMP origin showed evidence of past RAG1 expression and had D-J IgH gene rearrangements; most of these derived from a subset of CMP already expressing RAG1.
Introduction

Plasmacytoid dendritic cells (pDC) are a subset of dendritic cells (DC) that circulate through the blood and peripheral tissues\(^1,2\). After activation pDC develop dendritic processes, upregulate expression of major histocompatibility (MHC) class II molecules, and become antigen-presenting cells. Further, on activation they secrete type 1 interferon (IFN), hence are also known as interferon producing cells. Despite their classification as DC, pDC have many of the attributes of B cells. pDC and B cells share expression of several surface receptors, use similar antigen presentation machinery and in the unstimulated state have a similar morphology\(^3-5\).

The developmental origin of pDC has long been unclear. Early studies showed that both common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) had the potential, upon transfer into irradiated mice, to produce pDC\(^6,7\), although it was unclear whether these potentials were expressed in steady state. Similar potential has been found in CLP and CMP from human cord blood\(^8\). Later it was shown that some pDC in the bone marrow (BM) and spleen of un-manipulated mice had expressed recombination activating gene 1 (RAG1)\(^9\) and possessed D-J rearranged genes at the immunoglobulin heavy chain (IgH) locus\(^9,10\). Such gene rearrangement events have been considered as indicators of a lymphoid developmental history. A recent paper described a cell intrinsic requirement for IL-7 signaling in the development of a subset of both splenic cDC and pDC, and concluded some DC were normally of lymphoid origin\(^11\). Taken together, these results suggested that the pDC found in normal mice had two different origins, from myeloid and from lymphoid progenitors.

However, in a subsequent study, IgH gene rearrangements were found in pDC derived after transfer from CMP as well as CLP\(^12\). The authors concluded that the IgH gene rearrangements were not necessarily markers of a lymphoid developmental history, but rather an accidental by-product of the similarity in transcriptional programs between B cells and pDC. In addition, a precursor restricted to production of pDC and conventional DC (cDC), termed a common dendritic cell precursor (CDP) or pro-DC, has been isolated from BM\(^13,14\). The CDP is downstream of the CMP\(^13\) and so is considered a myeloid lineage cell. As a result of these findings, only the myeloid origin of pDC tends to be
considered at present.

To investigate in detail the pathway of pDC differentiation, we have used a BM culture system driven by fms-like tyrosine kinase 3 ligand (FL)\textsuperscript{15-17}. We have previously demonstrated that this system models the pathway of steady state spleen DC development, including production of pDC and cDC from a CDP intermediate\textsuperscript{14}. We now combine RAG1 gene expression and IgH gene rearrangement analysis with the isolation of distinct intermediate precursors to demonstrate the existence of separate myeloid and lymphoid pathways, both leading to cells that would be classified as pDC based on their surface phenotype and capacity to produce IFN\(\alpha\). We find that although the CMP fraction includes a proportion of precursors expressing RAG1 and produces some pDC with D-J gene rearrangements, most D-J rearranged pDC appear to derive from lymphoid committed precursors.
Materials and Methods

Mice

Unless otherwise indicated, experiments were performed using C57BL/6J Wehi (CD45.2) mice 6-12 weeks of age. Where indicated, C57BL/6-RAG1<sup>tm1/lmku</sup> (RAG1-GFP) or C57BL/6<sup>TG3OScha/J</sup> (UBC-GFP) mice were used as the source of precursors. C57BL/6 Pep<sup>3b</sup> (CD45.1) mice were used as the source of BM feeder cells for cultures. Mice were bred under specific pathogen free conditions at the Walter and Eliza Hall Institute in accordance with the guidelines of the Animal Ethics Committee.

Monoclonal antibodies

Unless otherwise stated, antibodies were generated, purified and conjugated in house. The mAb hybridoma clones used were: CD2 (RM2-1), CD3 (KT3), CD8 (YTS169.4 or 53-6.7), CD19 (1D3) CD45R (RA36B2), CD45RA (14.8), CD11b (M1/70), TER119 (TER119), CD45.1 (A20.1), CD45.2 (S450-15.2), CD11c (N418), F4/80 (F4/80), sca-1 (E13 161-7), c-kit (ACK2), flt3 (A2F10.1), BST-2 (120G8), CD34 (RAM34), CD127 (A7R34) and CD16/32 (2.4G2). The mAbs were conjugated to one of the following fluorochromes (from Molecular Probes): biotin; phycoerythrin (PE); Alexa Fluor 594 (Alexa594); Alexa Fluor 633 (Alexa633); Alexa Fluor 680 (Alexa680); phycoerythrin-cyanine 7 (PECy7); peridinin chlorophyll protein cyanine 5.5 (PerCP-Cy5.5); fluorescein-5-isothiocyanate or to allophycocyanin (from Prozyne). The commercially produced mAb conjugates used were: A2F10.1 (flt3)-PE and 53-6.7 (CD8α)-PerCP-Cy5.5 (BD biosciences). The mAb were titrated on spleen cells or purified DC to determine the optimal staining concentration.

Isolation of precursor cells from BM

Erythrocytes, dead cells and dense cells were first eliminated from BM cell suspensions by centrifugation in Nycodenz (Nycomed Pharma) medium (1.086g/cm<sup>3</sup>, 4°C, mouse osmolarity). The BM cells were then coated with mAb against the lineage antigens CD2, CD3, CD8, CD19, CD45R, CD11b, TER119 and Ly6G. For isolation of the pDC-B cell progenitor, the cells were coated with mAb against CD3, CD11b, CD19, TER119 and Ly6G. The cells were then incubated with sheep anti-rat IgG magnetic beads (Qiagen) at 8 beads/cell. The beads were removed using a magnet and unbound cells stained for
specific precursor cells. Non-specific binding was blocked with polyclonal rat Ig, and if CD16/32 was not used for sorting, with anti-CD16/32 prior to staining. Progenitors were sorted as follows: LSK cells, lineage-(lin)-c-kit+sca-1+; CMP, lin-c-kit+sca-1-CD16/32lowCD34+; CLP, lin’CD127+c-kitint’sca-1+. The purity of precursors ranged from 95 to 96%. Absence of lineage marker bearing cells was verified by staining with anti-rat Ig. Propidium iodide (PI) was included in the final stains to gate out dead cells.

**Precursor isolation from culture**

CDP were isolated from culture as previously described. BM was labeled with CFSE (Molecular Probes) or PKH-26 (Sigma) and cultured with flt3L (FL) (200ng/mL). After 3.5 days, light density cells were isolated by centrifugation in Nycodenz medium (1.086g/cm³). These cells were coated with biotinylated antibodies against CD19, Ly6G, CD127, MHC class II, CD11c, Ly6C and TER119, incubated with anti-biotin magnetic beads, then removed using a MACS magnetic column (Myltenyi). The depleted fraction was incubated with streptavidin-PE.Cy7 or streptavidin-PerCPCy5.5. CDP were sorted as PECy7’ or PerCPCy5.5’ and CFSElow or PKH-26low. For isolation of the pDC-B cell precursor from cultures, CLP were isolated and cultured with CD45.1 feeder cells and FL (200ng/mL). After 2 days, precursors were sorted as CD45.2’CD45.1’CD11c’BST-2’.

**FL stimulated BM culture systems for pDC development**

Cells extracted from the femur and tibia bones were briefly exposed to red cell removal buffer, re-suspended in 10mL RPMI 1640-FCS, washed by centrifugation, then passed through a sieve. Where indicated, pre-existent pDC were first removed from BM by incubating the cells with anti-BST-2 then eliminating the coated cells with anti-Ig magnetic beads. Cells were suspended in culture medium at 1.5-3 x 10⁶ cells/mL with FL (200 ng/mL).

**Clonal assays**

LSK progenitors or CDP from UBC-GFP mice were sorted from BM or 3.5 day BM cultures. Single cells were deposited by a FACs DiVa into each well of a 96-well U-bottom culture plate containing wild type GFP’ feeder cells (750 culture-derived CDP in 0.2ml 3 day conditioned medium or 0.3 x 10⁶ BM cells in 0.2ml fresh medium with 200ng/mL FL). After 5 days (CDP) or 8 days (LSK cells), wells were screened under
ultraviolet light for the presence of GFP<sup>+</sup> progeny. Wells with detectable GFP<sup>+</sup> cells were harvested and the entire contents of the well stained.

**Re-culture of isolated precursors**

Progenitors were isolated as above and added to erythrocyte-depleted CD45.1 BM to a final concentration of 1.5 x 10<sup>6</sup> cells/mL, then cultured with FL as above. After 5 days, cells were analyzed by flow cytometry for the presence of CD11c<sup>+</sup>CD45RA<sup>−</sup> or BST-2<sup>−</sup> cDC and CD11c<sup>+</sup>CD45RA<sup>+</sup> or BST-2<sup>+</sup> pDC. Progenitor-derived cells were identified as CD45.1<sup>−</sup>CD45.2<sup>+</sup>.

**Adoptive transfers for pDC generation and assessment of developmental potential**

CLP or downstream precursors were isolated from C57BL/6 BM as above and intravenously injected into irradiated CD45.1 recipients. After 6 or 14 days, spleen and BM was harvested and analyzed by flow cytometry for CD3<sup>+</sup> T cells, CD19<sup>+</sup>CD24<sup>+</sup> B cells, CD11c<sup>+</sup>SiglecH<sup>−</sup> cDC, CD11c<sup>int</sup>SiglecH<sup>+</sup> pDC and CD49b<sup>+</sup>CD161c<sup>+</sup> NK cells. Donor derived cells were identified as CD45.2<sup>+</sup>CD45.1<sup>−</sup>. Appropriate gating was determined by reference to the CD45.1<sup>−</sup>CD45.2<sup>+</sup> host cell progeny. For assessment of IFNα production, CLP-derived splenic pDC were sorted at day 14 as CD45.2<sup>+</sup>CD11c<sup>+</sup>BST-2<sup>−</sup>.

**Culture system for B cell development**

OP9 cells (4000) were plated into wells of 96-well flat-bottomed culture plates. Progenitors (<1000) were cultured in modified MEM with 50mM β-mercaptoethanol, 10% fetal calf serum, FL (5ng/mL) and 2% of the supernatant of an IL-7 producing line. Cultures were harvested at 7 days and analyzed for CD19<sup>+</sup> B cells, CD11c<sup>+</sup>BST-2<sup>−</sup> cDC and CD11c<sup>+</sup>BST-2<sup>+</sup> pDC.

**Culture system for NK cell development**

OP9 cells (80000) were plated into wells of 24-well flat-bottomed culture plates and allowed to adhere. CLP (2000) or up to 15000 progenitors were cultured in IMDM with 50mM β-mercaptoethanol, 10% fetal calf serum, 50ng/mL IL-15, 50ng/mL SCF and 5ng/mL FL. Half the medium and the cytokines were replenished after 4 days of culture. Cultures were harvested at 6 and 14 days and analyzed for the presence of CD49b<sup>+</sup>NK1.1<sup>+</sup> NK cells, CD11c<sup>+</sup>BST-2<sup>−</sup> cDC and CD11c<sup>+</sup>BST-2<sup>+</sup> pDC.
**PCR analysis for IgH gene rearrangements**

PCRs for IgH rearrangements were performed as described previously \(^{18}\). Sorted cells were washed with PBS then re-suspended at 10^6 cells/ml in PCR lysis buffer. Amplification of the RAG1 gene served as a template concentration control. DNA was titrated to give an equivalent amount of product in the control reactions, and to ensure that reactions were performed within the linear range. D to J rearrangements were detected as amplified fragments of ~1,033, ~716, or ~333 nucleotides depending on whether JH1, JH2, or JH3 was rearranged. PCR products were quantified using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). PCR products were detected and quantified by Southern blotting and hybridization with appropriate Ig gene probes.

**Assay for IFNα production**

Progenitors were isolated and re-cultured with FL and BM feeders or transferred into irradiated recipients as above. Progenitor-derived pDC were sorted and re-cultured at 10^6 cells/mL with 0.5 μM CpG2216 (Proligo). After 20 hours, culture supernatants were harvested and the concentration of IFNα analyzed by ELISA as previously described \(^{19}\). The capture antibody was RMMA-1 (PBL InterferonSource). Polyclonal rabbit anti-IFNα (PBL InterferonSource) followed by anti-rabbit antibodies conjugated to horseradish peroxidase were used for detection. The reaction was visualized by the addition of ABTS. The optical density was read with a Kinetic microplate reader set to 405-490 nm (Molecular Devices). Cytokine concentrations were interpolated from a standard curve.
Results

Kinetics of pDC development

To investigate the development of pDC in detail, we used FL driven BM cultures that model steady-state DC development. We determined the kinetics of new pDC production from total BM that had been depleted of BST-2+ cells, to remove the population of pre-existing pDC. The peak of pDC production occurred at day 6, earlier than the peak of cDC production at day 8 (Fig. 1a). We had previously demonstrated that in these cultures CDP numbers peak at day 314. Peak production of cDC from CDP occurred at day 5 upon re-culture, consistent with these precursors being the source of the day 8 cDC peak in the standard cultures. Although pDC are produced on re-culture of CDP14, the early peak of pDC development in the total BM cultures made it unlikely this myeloid route via CDP was the only source of pDC. We postulated that the pDC produced in these cultures derived from both myeloid and lymphoid precursors, as previously suggested7,9,10.

To investigate whether this might explain the kinetics of pDC development, we isolated CLP20 and CMP21 from BM according to the surface markers originally described and analyzed the kinetics of DC generation from each precursor. CMP produced both cDC and pDC. The main peak of pDC production from CMP occurred at day 7 (Fig. 1b), consistent with a myeloid pathway in which CMP give rise to CDP, which in turn produce both pDC and cDC. In contrast, CLP produced predominantly pDC and the peak of production occurred earlier, at day 5 (Fig 1c), suggesting such a lymphoid pathway might be responsible for much of the pDC generated in these cultures. cDC production from CLP varied greatly (data not shown), ranging from undetectable to 15% of the total DC progeny. The pDC produced from both precursors displayed the typical pDC surface phenotype, namely expressing high levels of CD11c, BST-2 (CD317), CD45R (B220) and CD45RA and Siglec H (Fig 1d). These markers were subsequently used interchangeably to define pDC depending on particular fluorochrome and mAb staining combinations. Thus, in this model of steady state DC development, pDC developed via both lymphoid and myeloid intermediates.
Production of pDC clones

To assay the developmental potential of different precursors, we had previously devised a clonal assay in which single GFP-expressing precursors were cultured with FL and BM feeder cells. Using this assay we had compared clones derived from CDP (a DC restricted, late myeloid precursor) with those derived from LSK cells (enriched for early multipotent precursors). We found that while 25% of the DC clones derived from CDP contained pDC, almost all of these included cDC (Table 1). However when LSK cells were used as precursors, giving the possibility of both myeloid and lymphoid developmental options, DC clones consisting of only pDC were prevalent. Of the 77% of DC clones containing pDC, almost 10% contained only pDC (Table 1). We reasoned that these pure pDC clones must have been generated from a pathway not involving the CDP intermediate, likely a lymphoid pathway. However, single CLP showed too low a cloning efficiency for analysis by this assay.

An immediate pDC precursor on the lymphoid pathway

As we had found that CLP gave rise to pDC in FL BM cultures, we attempted to map this lymphoid pathway downstream of the CLP. CLP were isolated from BM and cultured with FL and the cultures analyzed at various time points to determine intermediate stages en route to pDC. We noted the emergence after 1-2 days, then disappearance by 3 days, of a population that differed from CLP in expressing BST-2, a pDC marker, but did not yet express the pan-DC marker CD11c (Fig 2a). To determine the fate of this CD11c⁻BST-2⁺ CLP-derived population, it was sorted from cultures at day 2 then re-cultured with FL. After 1-2 days of re-culture, CD11c⁺BST-2⁺ pDC appeared and constituted most of the cells recovered, though a minor population of cDC was also produced (Fig 2b). However it was not clear whether all cells with the CD11c⁻BST-2⁺ phenotype were engaged in pDC production. Since CLP are efficient progenitors of B cells and NK cells, we asked whether the CLP-derived, CD11c⁺BST-2⁺ population retained this potential. The precursors were isolated from cultures, then re-cultured under conditions known to be optimal for the generation of B cells or NK cells. After 7 days of culture these precursors produced B cells (Fig 2c). However, the progeny of the CD11c⁺BST-2⁺ progenitors were uniformly CD49b⁺NK1.1⁻, indicating a lack of NK cell potential (data
not shown). Under the same conditions, CLP efficiently gave rise to CD49b\(^{+}\)NK1.1\(^{+}\)CD161c\(^{+}\) NK cells.

We then asked whether an equivalent of these progenitors existed \textit{in vivo}. We found within BM, the site of pDC production, a population of lin BST-2\(^{-}\)CD11c\(^{-}\) cells, constituting 0.15\% of the BM. Most of these also expressed IL-7R\(\alpha\), suggesting they were lymphoid-derived (Fig 2d). To determine whether these cells were developmentally distinct from CLP, we adoptively transferred them into lethally irradiated congenic recipients. Very few progenitor-derived cells were detectable in recipient BM between day 6 and 14 after transfer but within the spleen we found progenitor-derived pDC, B cells and some cDC (Fig 2e). No significant production of NK cells was seen at any time point, but a marginal production of T cells was seen at day 14. Overall the BM CD11c\(^{-}\) BST-2\(^{-}\)IL-7R\(\alpha\)\(^{+}\) progenitors, like their culture derived counterparts, maintained the B cell and DC potential of CLP, but had lost much of the ability to give rise to other lymphoid lineages. This points to a tight developmental lineage between these pDC and B cells.

\textit{Both CMP and CLP give rise to pDC with a history of RAG1 expression}

The incidence of D-J IgH gene rearrangements and a history of RAG1 expression had suggested that some pDC in steady state were of lymphoid origin\(^9\),\(^10\). However, this was called into question by the finding that some CMP-derived pDC also have IgH gene rearrangements\(^12\). To determine whether the culture generated pDC from both lymphoid and myeloid sources showed the ‘lymphoid’ characteristic of recent RAG1 expression, we isolated CMP and CLP from the BM of RAG1-GFP reporter mice. Cells expressing RAG1 show strong GFP fluorescence when RAG1 is expressed, although this declines once RAG1 expression ceases. The CD45.2 progenitors were cultured with congenic CD45.1 BM feeder cells and FL, and their progeny analyzed after 5 days of culture for GFP expression.

CLP-derived pDC uniformly showed some level of GFP expression (Fig. 3a), indicating that at some point in their developmental history they had expressed RAG1. Interestingly, while most CMP-derived pDC were GFP negative, a subset had detectable GFP expression (Fig. 3b). Thus, in agreement with the analysis of IgH gene rearrangements\(^12\),
some pDC derived from a myeloid precursor showed a marker traditionally associated with development from a lymphoid precursor.

**A history of RAG1 expression in pDC indicates development from RAG1⁻ precursor**

The CMP-derived pDC with a history of RAG1 expression may have “ectopically” activated RAG1 as a consequence of their transcriptional similarity with B cells, as postulated. Alternatively, RAG1 may have already been expressed at the precursor stage. Although CMP were originally described as myeloid restricted, the original report by Akashi et al. indicated a low frequency of B cell progenitors in this fraction. Further, upon adoptive transfer CMP give rise to some B cells. To investigate the possibility of a subset of CMP with some lymphoid characteristics, we examined the CMP from RAG1-GFP mice. We found both RAG1⁺ and RAG1⁻ subsets within the CMP fraction, and these were entirely within the the Flt3 expressing fraction known to contain the DC precursors (Fig. 4a). The RAG1⁺Flt3⁺ CMP subset retained myeloid potential, since it produced macrophages with high efficiency after 7 days of culture with GM-CSF (Fig. 4b), whereas CLP did not produce any macrophages (data not shown). The Flt3⁺, RAG1⁺ and RAG1⁻ subsets of CMP were sorted and cultured with congenic BM cells and FL, then the progeny DC analyzed. Both subsets gave rise to both pDC and cDC. The cDC derived from either subset had no detectable GFP fluorescence, indicating that any initial RAG1 expression had not persisted. However the pDC derived from the RAG1⁺ subset still had GFP expression whereas the pDC derived from the RAG1⁻ subset had no detectable GFP fluorescence (Fig. 4c). Thus, a history of RAG1 expression in CMP-derived pDC largely reflected development from a progenitor already expressing RAG1.

**Most pDC with IgH gene rearrangements derive from RAG1⁻ precursors**

We had found in previous experiments that the DC produced in our cultures were similar in IgH gene rearrangement status to the DC from normal mouse spleen; a proportion of the pDC displayed D-J rearrangements, whereas the cDC were all in germline state. We had assumed D-J IgH gene rearrangements in pDC indicated development from a lymphoid precursor, and such D-J rearrangements in pDC had been shown to correlate
with a history of RAG1 expression. However, in view of the finding of rearrangements in pDC derived from CMP, we re-examined this issue. CLP, RAG1+ CMP and RAG1- CMP were isolated and cultured with congenic BM cells and FL, then 5 days later the pDC progeny sorted and analyzed by PCR for IgH gene rearrangements. The pDC derived from CLP always displayed D-J rearrangements in the IgH genes, but no V-D-J rearrangements (Fig. 5a). The few pDC derived from the RAG1+ CMP also displayed D-J rearrangements. The pDC derived from the RAG1- CMP usually did not display D-J rearrangements (Fig. 5a). However in occasional experiments D-J rearrangements were revealed even in the pDC from the RAG1- CMP fraction (e.g. Fig. 5b). We suggest this reflects a low frequency of rearrangement events not always detected with this non-quantitative PCR assay. We concluded that pDC with D-J rearrangements in IgH genes generally derived from progenitors already expressing RAG1 as had been suggested, but that the downstream process of pDC development does sometimes allow “ectopic” lymphoid-like rearrangement events, as had been proposed.

Type 1 interferon production by myeloid and lymphoid derived pDC

The ability to produce large amounts of type 1 interferon when appropriately stimulated is a key characteristic of pDC. The RAG1+ and RAG1- subsets of splenic pDC had previously been found to differ in the quantity of IFNα they produced following CpG stimulation, suggesting that developmentally distinct pDC may differ in this function. The pDC produced in vivo by adoptive transfer of CMP and CLP had not been tested for IFN production, so their identification as “true” pDC had been questioned. Since in our cultures multiple pathways produced cells with a pDC surface phenotype, we examined whether these pDC differed in this key function. The pDC derived in culture from total BM, from CMP or CDP or CLP were purified, then compared for their ability to produce IFNα in culture in response to CpG stimulation.

When the pDC that developed in the FL stimulated cultures were first tested by stimulation by CpG in fresh culture medium, the pDC from total BM produced IFNα, the pDC derived via the myeloid route from CMP or CDP produced higher levels of IFNα, but the CLP derived pDC appeared inactive (Fig. 6a). However the CLP derived pDC
were found to be dead after overnight culture in the fresh medium of these assays, suggesting cell death due to lack of some survival factor may have been the reason for the failure to produce IFNα. The capacity of CLP-derived pDC to respond to CpG stimulation and produce IFNα was therefore tested under other conditions. Since the pDC were viable in the original cultures, the CpG stimulation experiments were repeated in conditioned medium recovered from day 7 FL stimulated BM cultures; this medium was found to be free of IFNα. The pDC generated from both CLP and CMP survived under these re-culture conditions and both responded to CpG stimulation by upregulation of CD69, indicating that they had a functioning TLR9; importantly, both then produced IFNα, although the CLP-derived pDC produced less (Fig. 6b). Despite showing a similar initial viability (92% for CLP-derived pDC, 96% for CMP-derived pDC, based on PI exclusion) the CLP-derived pDC showed reduced viability by the time of supernatant harvest (28% for CLP-derived pDC, compared to 67% for CMP-derived pDC). This difference in viability may account for the differential IFNα production. To check that pDC developing by the lymphoid route in vivo were interferon-producing cells, CLP were purified and transferred into irradiated congenic recipients, the pDC progeny isolated 7 days after transfer and then tested for IFNα production after CpG stimulation in culture. These pDC derived from CLP in vivo upregulated CD69 and produced IFNα, even when stimulated in fresh culture medium (Fig. 6c). We concluded that pDC derived from both myeloid and lymphoid routes are IFN producing cells and so can be considered as ‘true’ functional plasmacytoid cells.
Discussion

The earlier findings that both CMP and CLP populations from mouse BM have the potential to produce pDC on adoptive transfer to irradiated mice led to the original concept that pDC might in steady state be produced by both myeloid and lymphoid routes. However, since both these precursor populations show heterogeneity and CMP as isolated are able to produce some B cells on transfer to irradiated recipients, doubt may be cast on this interpretation. The presence of D-J rearranged IgH genes in some but not all normal mouse spleen pDC seemed to support the dual origin hypothesis, since these DNA changes serve as an indelible marker of a past rearrangement process normally restricted to lymphoid lineage cells. However, this has also been interpreted as an “ectopic” event that occurs even with a myeloid pDC origin, since in forming pDC transcriptional programs similar to those involved in B cell development must be activated. We now use a culture system that produces DC similar to those found in spleen to demonstrate that indeed separate pathways, with separate intermediate precursors, are involved in pDC development. We also reconcile the two interpretations of the origin of the D-J IgH gene rearrangements in pDC.

One pathway to pDC had already been documented by the detailed studies in this and other laboratories on DC development. It begins with a myeloid precursor (CMP), proceeds via a DC-restricted CDP, which eventually produces both pDC and cDC. Another pathway we now delineate begins with a lymphoid precursor (CLP) and produces pDC but few cDC. This pathway proceeds via a precursor resembling a pro-B cell, and produces pDC and B cells but no NK cells and few T cells. The relative infrequency of these progenitors suggests they are a transient intermediate. Further, it is unclear whether all CLP must transit through this intermediate en route to pDC. Mice deficient in E2-2, a transcription factor essential for pDC development, accumulate within the BM a population of CD11c<sup>+</sup>BST-2<sup>-</sup> cells that were postulated to be an arrested stage in pDC development. This progenitor may represent a normal alternate intermediate between CLP and pDC; however, its appearance may also be an experimental artifact of the inability of these cells to express BST-2, a direct transcriptional target of E2-2. It should be noted that the CDP and the transient B-pDC precursor isolated from these cultures differ in surface phenotype and are the product of distinct developmental
pathways, as CLP could not give rise to CDP in vivo\textsuperscript{13}. Both progenitors have equivalents in normal BM.

Our results suggest that most pDC with D-J IgH gene rearrangements have developed via a lymphoid route, involving a lymphoid-restricted precursor such as the CLP, as previously suggested\textsuperscript{9,10}. However we confirm that some pDC with D-J IgH gene rearrangements do arise from CMP, as reported by Shigematsu et al\textsuperscript{12}. This led to our interesting finding of a small subset of RAG1 expressing Flt3\textsuperscript{+} precursors within the CMP fraction. Despite this lymphoid feature, these RAG1\textsuperscript{+} CMP retained the myeloid potential of forming macrophages, so were functionally distinct from CLP. We suggest they represent the CMP subset able to produce some B cells in vitro and on adoptive transfer\textsuperscript{7,21}. Although we found most pDC that arise via the myeloid, CMP to CDP route do not show a history of RAG1 expression and do not display IgH gene D-J rearrangements, a variant of the myeloid route involves RAG1 expression leading to IgH gene rearrangements in some pDC. RAG 1 expression at the CMP stage may sometimes be transient, since it does not lead to persistent GFP fluorescence or IgH gene rearrangements in cDC. This supports the concept of Shigematsu et al\textsuperscript{12} that the similarity of gene activation profiles in B cells and pDC allows “ectopic” gene rearrangement events during pDC development. Our finding of very occasional D-J gene rearrangements even in pDC derived from RAG1\textsuperscript{−} CMP is also in line with this concept, since it suggests RAG1 expression and other requirements for gene rearrangement may be induced downstream of CMP, along the route to pDC but not along the route to cDC. Overall these findings indicate substantial flexibility in the pathways leading to pDC. It should be noted that bone marrow from E2-2\textsuperscript{−/−} mice fail to produce any pDC in response to FL stimulation\textsuperscript{22}, suggesting that pDC absolutely require E2-2 to develop, regardless of the pathway by which they differentiate.

In our model multiple separate pathways of hematopoietic development converge to produce cells that would be classified as pDC. The important question is whether these pDC products are identical, apart from the DNA changes at the IgH locus. We find they are similar in surface phenotype. Importantly we find that all pDC produced in our cultures are able to produce INF\textalpha in response to CpG stimulation, regardless of lymphoid or myeloid origin. Therefore as well as having the characteristic surface phenotype, they
meet the main functional criterion of pDC.

We found that pDC derived from CLP appear to produce less IFNα in the assay cultures than pDC derived from CMP. Similar differences have been reported between RAG1⁺ and RAG1⁻ pDC from spleen⁹. In addition there are a number of recent reports of cells with a pDC surface phenotype that differ in their capacity to make IFNα²⁴-²⁷. Important in this context is our finding of one set of conditions under which CLP derived pDC failed completely to produce IFNα, due to a high rate of pDC death. This reflected a requirement for a particular survival factors in the assay rather than incapacity to produce IFNα. Overall our study emphasizes that differences in cytokine production between pDC populations may be due to differences in survival in the assay culture, or slightly different maturation or activation states, rather than due to fundamental functional differences. More extensive functional analysis and gene expression profiling would be needed to determine if there are any important functional differences between pDC differing in lineage origin.
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Authorship

Contributions: P.S. designed and performed experiments and wrote the manuscript; K.S. designed experiments and wrote the manuscript; D.V. and L.C. designed and performed experiments; L.W. assisted with experimental design and discussion of data.

The authors declare no conflict of interest.

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Table 1: Clonal analysis of the pDC potential of single progenitors

<table>
<thead>
<tr>
<th>Precursor population</th>
<th>pDC containing clones (% of total)</th>
<th>pDC restricted clones (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSK cells</td>
<td>77.6</td>
<td>11.9</td>
</tr>
<tr>
<td>CDP</td>
<td>25.3</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Lin–c–kit+sca-1+ (LSK) cells and common DC progenitors (CDP) were isolated from BM or day 3 FL-stimulated BM from UBC-GFP mice. Single LSK cells were sorted and cultured with total WT BM feeder cells and FL for 8 days, and single CDP sorted and cultured with WT CDP feeder cells and FL for 5 days. At the end of culture, the single progenitors that had given rise to clonal progeny were identified by observation under UV fluorescence. These clones were analyzed by flow cytometry for the presence of CD11c+CD45RA– cDC and CD11c+CD45RA+ pDC. 15% of LSK cells formed DC clones and 93 were analyzed; 51% of CDP formed DC clones and 99 were analyzed. Clones were classified as ‘pDC containing clones’ if the progeny included any pDC. Clones were classified as ‘pDC restricted clones’ if the progeny were solely pDC.
Figure legends

Figure 1: Kinetics of pDC development in FL BM cultures. (A) BM depleted of erythrocytes and BST-2− cells, (B) lin−ckit+lin−ckit+sca-1+ common lymphoid progenitors (CLP) or (C) lin−ckit+sca-1+CD16/32lowCD34+ common myeloid progenitors (CMP), were cultured with FL. The number of CD11c+CD45RA+ pDC or CD11c+ CD45RA− cDC was assayed each day by flow cytometry. (D) The CD11c+CD45RA+ pDC progeny of CLP and CMP, sorted as in (B) and (C), were analyzed by flow cytometry for the expression of the pDC markers BST-2 (CD317), CD45R (B220) and SiglecH. Results are representative of 3 separate experiments.

Figure 2: Identification of intermediate CLP-derived pDC precursors. (A). The transient pDC precursor. CLP were cultured with FL for 1-2 days. CLP-derived cells were analyzed for expression of the DC markers BST-2 and CD11c. (B) pDC products of this precursor. CD11c−BST-2+ cells were sorted after 2 days of culture, re-cultured with FL and analyzed after 2 days for the presence of CD11c+BST-2+ pDC. (C) B cell products of this precursor. CD11c−BST-2+ precursors were sorted and cultured on OP9 stromal cells with IL-7 and FL. Progeny were analyzed after 7 days for the presence of CD19+ B cells. (D) An equivalent precursor population in BM. BM from C57Bl/6 was depleted for a selection of lineage antigens (CD3, CD11b, CD19, TER119 and Ly6G), then BST-2−CD11c−IL-7Rα+ cells were sorted from the lin− BM. (E). The in vivo products of this BM precursor. The sorted cells were adoptively transferred into lethally irradiated CD45.1 recipients. 6-14 days after transfer, recipient spleens were analyzed for the presence of CD45.2+ donor derived cells, and within the donor-derived cells for the presence of SiglecH−CD11cint pDC; SiglecH+CD11chigh cDC; CD24+CD19+ B cells; CD49b+CD161c+ NK cells; and CD3+ T cells. Results presented represent the peak responses at day 6 for DC, day 14 for lymphoid cells. Results are representative of (A) 5; (B) 3; (C,D,E) 2 independent experiments.

Figure 3: RAG1 expression in pDC from lymphoid and myeloid progenitors. (A) CLP or (B) CMP were isolated from the BM of RAG1-GFP reporter mice and cultured with FL and congenic BM feeder cells. After 5 days, pDC were identified as CD11c+CD45RA+ and their GFP expression assessed by flow cytometry. Results are representative of (A) 2 and (B) 6 independent experiments.
Figure 4: RAG1 expression in CMP and CMP-derived pDC. CMP were isolated from the BM of RAG1-GFP reporter mice and (A) analyzed by for expression of flt3 and RAG1. Flt3⁺ RAG1⁺ (B) or flt3⁺ RAG1⁺ and flt3⁺ RAG1⁻ CMP were sorted from the BM of RAG1-GFP reporter mice and cultured with (B) GM-CSF (10ng/mL) or (C) FL. (B) Progeny were analyzed after 7 days and macrophages identified as CD11b<sup>high</sup>F4/80<sup>+</sup>. (C) After 5 days, pDC were identified as CD11c<sup>c⁺</sup> BST-2<sup>⁺</sup> and cDC identified as CD11c<sup>c⁺</sup> BST-2<sup>⁻</sup>. The GFP expression of RAG1⁺ CMP derived cDC (light gray shaded histogram) and pDC (dark grey shaded histogram) and RAG1⁻ CMP derived cDC (dashed line) and pDC (solid line) was analyzed by flow cytometry. Plots are representative of (A) 5 (B) 2 and (C) 4 independent experiments.

Figure 5: Gene rearrangements in pDC from different sources. RAG1⁺ CMP, RAG1⁻ CMP or CLP were cultured with congenic CD45.1<sup>+</sup> total BM feeder cells and FL. Progenitor derived pDC were identified as CD45.1<sup>⁺</sup>CD45.2<sup>⁻</sup>CD11c<sup>⁺</sup>BST-2<sup>⁺</sup> after 5 days. pDC were analyzed by PCR for the presence of D-J rearrangements at their IgH locus. (A) shows the more frequent result of no detectable rearrangement in pDC from CMP. (B) gives the occasional result of Ig gene D-J rearrangements in pDC from CMP.

Figure 6: Differences in IFNα production by pDC from different lineages. (A) Total BM cells, CMP, CDP or CLP from C57BL/6 BM were cultured with FL and CD45.1 total BM feeder cells. After 5 days, pDC were sorted as CD11c<sup>⁺</sup>BST-2<sup>⁺</sup> and cultured with CpG2216. After 20 hours, supernatant was harvested and analyzed by ELISA for the presence of IFNα. (B) CLP and CMP were cultured, and the resulting pDC at day 5 isolated as in (A). pDC were re-cultured in medium collected from day 7 FL BM cultures with CpG2216. After 20 hours, supernatant was harvested and analyzed by ELISA for the presence of IFNα, and pDC were analyzed by flow cytometry for CD69 expression and viability by PI exclusion. At the time of harvest 67% of CMP-derived pDC but only 28% of CLP-derived pDC remained viable. (C) CLP were isolated from C57BL/6 BM and transferred into irradiated CD45.1 recipients. After 14 days, pDC were sorted as CD11c<sup>int</sup>BST-2<sup>⁺</sup> and cultured with CpG2216. After 20 hours, supernatant was harvested and analyzed by ELISA for the presence of IFNα. Results are pooled from (A) 4 independent experiments and (B) and (C) 3 independent samples from 1 experiment. Error bars show mean ± SEM.
Figure 1

A. pDC depleted BM-derived

Number of cells (x10^6)

Days of culture

B. CLP-derived

Number of pDC (x10^3)

Days of culture

C. CMP-derived

Number of pDC (x10^3)

Days of culture

D. Surface phenotype of CD11c^+ CD45RA^+ pDC

CLP derived

CMP derived

BST-2

CD45R

Siglec H
Figure 6

(A) IFNα (pg/mL) per 10^3 cells

- Total BM-derived pDC
- CDP-derived pDC
- CMP-derived pDC
- CLP-derived pDC

(B) IFNα (pg/mL) per 10^3 cells
- CMP-derived pDC
- CLP-derived pDC

Conditioned assay media

(C) IFNα (pg/mL) per 10^3 cells
- CLP-derived pDC

Fresh assay medium

CD69
Convergent differentiation: myeloid and lymphoid pathways to murine plasmacytoid dendritic cells

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