Interferon-producing killer dendritic cells (IKDCs) arise via a unique differentiation pathway from primitive c-kit^{Hi}CD62L^{+} lymphoid progenitors

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Interferon-producing killer dendritic cells (IKDCs) have only recently been described and they share some properties with plasmacytoid dendritic cells (pDCs). We now show that they can arise from some of the same progenitors. However, IKDCs expressed little or no RAG-1, Spi-B, or TLR9, but responded to the TLR9 agonist CpG ODN by production of IFNγ. The RAG-1^{-}pDC2 subset was more similar to IKDCs than RAG-1^{+} pDC1s with respect to IFNγ production. The Id-2 transcriptional inhibitor was essential for production of IKDCs and natural killer (NK) cells, but not pDCs. IKDCs developed from lymphoid progenitors in culture but, unlike pDCs, were not affected by Notch receptor ligation. While IKDCs could be made from estrogen-sensitive progenitors, they may have a slow turnover because their numbers did not rapidly decline in hormone-treated mice. Four categories of progenitors were compared for IKDC-producing ability in transplantation assays. Of these, Lin^{-}Sca-1^{+}c-Kit^{Hi}Thy1.1^{-}L-selectin^{+} lymphoid progenitors (LSPs) were the best source. While NK cells resemble IKDCs in several respects, they develop from different progenitors. These observations suggest that IKDCs may arise from a unique differentiation pathway, and one that diverges early from those responsible for NK cells, pDCs, and T and B cells. (Blood. 2007;109:4825-4831)

Introduction

Functionally specialized cells in the innate and adaptive immune systems are still being discovered,[1-3] and each needs to be fully understood in terms of developmental history. Replacement of immune effector cells from hematopoietic stem cells (HSCs) is an ordered process where multiple lineage potentials are gradually lost coincident with gain of specialized functions, and within the context of patterns of transcriptional activity and surface marker expression.[4-9] The focus of the present study was on recently identified interferon-producing killer dendritic cells (IKDCs). IKDCs exhibit "hybrid" phenotypic and functional characteristics of dendritic and natural killer (NK) cells.[2,3] Shared properties include expression of B220, CD11c, CD122, and NK1.1, as well as production of interferons, capability of antigen presentation, and strong cytotoxic or antitumor activities. Thus, IKDCs are unique, multifunctional cells that merit study with respect to development.

HSCs and several categories of primitive progenitors reside in the lineage marker–negative Sca1^{+}c-kit^{hi} (LSK) fraction of BM. Up-regulation of Flk-2 and corresponding loss of erythroid/megakaryocytic potential is an early event that sets the stage for lymphopoiesis.[10,11] LSKs include 2 overlapping subsets of lymphopoietic cells identified as Flk-2$^{+/-}$Thy1.1^{-}L-selectin^{+} progenitors (LSPs) and RAG-1^{+}Flk-2^{+}CD27^{+} early lymphoid progenitors (ELPs).[12-14] Although clearly B- and T-lymphoid specified, LSPs and ELPs retain some potential for myeloid, NK, and dendritic cell (DC) lineages. Firm commitment in these pathways and repression of alternative fates involve expression of key transcription factors, such as Pax5, Notch-1, and Spi-B, as well as environmental cues.[15]

All lymphoid progenitors in BM are rapidly and preferentially depleted in estrogen-treated mice.[16,17] Progenitors that can more quickly give rise to lymphocytes reside in a Lin^{-}Flk-2^{+} c-Kit^{Lo} prolymphocyte (Pro-L) fraction that includes Lin^{-}c-Kit^{Lo}Sca-1^{+}IL-7Rα^{+} common lymphoid progenitors (CLPs).[16,18] While CLPs have some T-lineage potential under experimental circumstances, most of them appear to be biased toward B and NK lineages.[14,17,19-21] Although a bipotential T/NK progenitor is recognizable in fetal tissues,[22,23] the earliest steps in adult NK cell development appears to be shared with B lymphocytes. Cells in the Pro-L fraction may represent an important intermediate in the production of NK cells, as single cell analysis and 2-step cultures revealed that NK cells are made from segregating Pro-L whose fate is established with expression of CD122.[17] CD122 is a cytokine receptor that identifies committed NK cell precursors (NKPs).[23] NK cell development is uniquely dependent on the Id-2 transcriptional repressor.[24]

Recent studies reveal a surprising degree of diversity in DCs, and they can be resolved into functionally specialized subsets.[25] B220^{-}CD11c^{+}CD11b$^{+/-}$Ly6C$^{lo/-}$ conventional DCs (cDCs) are antigen-presenting cells for optimal T-cell activation, whereas B220^{+}CD11c^{lo}CD11b^{-}Ly6C^{+} plasmacytoid DCs (pDCs) represent a principal source of IFN-α and are believed to function in defense against viral infection and T-cell tolerance. Two stable categories of pDCs were distinguished according to RAG1 expression, and these cells may have unique roles in immune responses.[1] They represented conspicuous subsets of BM fraction A, along with a companion population of B220^{+}CD11c^{lo}CD19^{-}Ly6C^{-} cells

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(referred to by us as pDC cohort). We previously showed that the pDC cohorts did not convert to pDCs in culture, but they expressed the NK-lineage markers NK1.1 and DX-5 and expanded in response to IL-15[1] (R.P., unpublished data, February 2005).

Developmental relationships between cDCs, pDCs, NK cells, and IKDCs have not been explored. Flk-2$^+$ common myeloid progenitors (CMPs) and CLPs represent good precursors for cDCs.[26,27] While small numbers of pDCs can also be generated from these precursors,[27-30] our experiments indicated that most pDCs derive from the more primitive LSK fraction.[1]

We now report that the BM Ly6C$^-$ pDC cohort population corresponds to the recently described IKDCs and, unlike one pDC subset, does not express RAG-1. Furthermore, IKDCs have a pattern of cytokine and gene expression distinct from pDCs. Similar to pDCs, IKDCs could be generated in culture from RAG-1$^+$ ELPs. However, formation of IKDCs was unaffected by Notch receptor ligation. IKDCs were not quickly depleted in the BM of estrogen-treated mice but may be slowly replaced from hormone-sensitive progenitors. Furthermore, hormone exposure diminished their ability to make IFNγ. IKDCs were developmentally dependent on Id-2 and most efficiently arose in transplant recipients from T-lineage–biased LSP BM. These and other findings suggest a differentiation sequence for production of IKDCs from very primitive lymphoid progenitors in adult BM.

Materials and methods

Mice

RAG-1/GFP knock-in mice have been described.[13] Heterozygous F1 RAG-1/GFP mice were generated at the OMRF Laboratory Animal Resource Center (LARC). C57BL6 (B6; CD45.2 alloantigen), B6-Thy1.1, BALB/c, and B6-SJL (CD45.1 alloantigen) mice (Jackson, Bar Harbor, ME) were bred and maintained in the LARC. B6-Thy1.1 mice were crossed with B6-RAG-1/GFP knock-in[13] mice to produce animals expressing Thy1.1, RAG-1/GFP, and the CD45.2 alloantigen. Id-2$^{-/-}$ have been described.[24]

Isolation of cell populations and flow cytometry

Dendritic cells were sorted as follows. BM cells from RAG-1/GFP mice were treated with Fc-receptor block (2.4G2). After staining with APC-Cy5-anti-CD45R/B220 (RA3/6B2), APC-Cy7-anti-CD19 (1D3), biotin-anti-Ly6C (AL-21), APC-anti-CD11c (HL3), and PE-anti-NK1.1, pDC1s were double sorted as GFP$^+$B220$^+$CD19$^-$CD11c^{Lo}Ly6C$^+$NK1.1$^-$; pDC2s, as GFP$^-$B220$^+$CD19$^-$CD11c^{Lo}Ly6C$^+$NK1.1$^-$; and IKDCs, as B220$^+$CD19$^-$CD11c^{Lo}Ly6C$^-$NK1.1$^+$, on a FACSAria (BD, San Jose, CA). Biotinylated antibodies were revealed using R613-conjugated streptavidin. All antibodies came from BD Pharmingen (San Diego, CA) unless otherwise stated. Purification of each population was confirmed by post-sorting analyses. To isolate progenitor populations for culture, BM cells from *RAG1/GFP* knock-in mice were enriched by negative selection using mAbs anti-Gr1 (RB6-8C5), anti–CD11b/Mac-1, anti-CD19, anti-CD45R/B220, and anti-Ter119, followed by the BioMag goat anti–rat IgG system (Qiagen, Valencia, CA). After staining with biotin-antilineage markers (Gr-1, Mac-1, CD19, CD45R/B220, Ter119, CD3ϵ, CD8α, and pan-NK), lineage$^-$GFP$^{+/-}$ populations were sorted and stained with APC-anti–c-kit (2B8) and PE-anti-Sca1 (D7). The LSK fraction was obtained as Lin$^-$ckit^{Hi}Sca1$^+$GFP$^-$, while ELPs were sorted as Lin$^-$ckit^{Hi}Sca-1$^+$GFP$^+$. For progenitor transfers, lineage-negative cells were stained with PE-anti–IL-7Rα (SB/14), biotin-anti-Thy1.1 (Caltag, Burlingame, CA), PE-Cy5-anti-Sca-1 (eBioscience, San Diego, CA), APC-anti–c-Kit, and L-selectin APC-Cy7 (MEL-14, eBioscience), then isolated using a FACSAria Flow Cytometer (Figure S1, available on the *Blood* website; see the Supplemental Figures link at the top of the online article). Dead cells were excluded by propidium iodide staining (Molecular Probes, Eugene, OR). Cells harvested were stained with antibodies, and flow cytometry analyses were performed on a FACSCalibur (BD Biosciences, San Jose, CA), using the Cell Quest software (BD).

Reverse-transcription–polymerase chain reaction (RT-PCR) analysis of gene expression

mRNAs were isolated from sorted cells using MicroPoly (A) pure (Ambion, Austin, TX) and converted to cDNA with murine Moloney leukemia virus (MMLV) reverse transcriptase (Invitrogen, Carlsbad, CA). The PCR was conducted using ampli-Taq DNA polymerase (TaKaRa, Shiga, Japan) and TaqStart antibody (Clontech, Palo Alto, CA). Anti-Taq Ab was inactivated by heating at 95°C for 7 minutes prior to amplification 30 seconds at 94°C, 30 seconds at 56°C, and 45 seconds at 72°C. Gene-specific primers were described in Pelayo et al.[1]

Cell stimulation and cytokine production

Freshly isolated IKDCs and pDCs were cultured for 18 hours in RPMI-1640 with phosphorothiolated CpG A–containing oligonucleotide (CpG-ODN) 1826 0.6 μg/mL (InvivoGen, San Diego, CA). Supernatants were assayed using enzyme-linked immunosorbent assay (ELISA) kits for IFNα (PBL, Piscataway, NJ), IFNγ, and TNFα (R & D Systems, Minneapolis, MN).

Stromal cell cocultures

Sorted cells were cocultured for 8 days with Delta-like-1 and GFP retrovirally transduced OP9 stromal cells (OP9-DL1 and OP9-vector, respectively) kindly provided by Dr J. C. Zúñiga-Pflücker (University of Toronto, ON). The α-MEM 10% FCS contained 100 ng/mL Flt3-L, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. For production of NK1.1$^+$ cells, 20 ng/mL SCF and 50 ng/mL IL-15 were included in the media.

Hormone treatment in vivo

Time-release pellets of 17β-estradiol, 0.1 mg/pellet, 21-day release (Innovative Research of America, Sarasota, FL) were implanted subcutaneously with a 10-gauge precision trochar. After 1 or 2 weeks, mice were killed and BM cells were analyzed by fluorescent-activated cell sorter (FACS) or sorted for culture.

Intravenous progenitor transfers

Recipient mice were given 6.5 Gy radiation from a ^{137}Cs source (Mark I gamma irradiator; J. L. Shepard and Associates, Glendale, CA). Mice were anesthetized with isofluorane (Isosol; Vedco, St Joseph, MO), and sorted populations were infused intravenously. Host mice were 6- to 12-week-old B6-CD45.1; donor cells were from 4- to 10-week-old B6-RAG-1/GFP-Thy1.1 mice expressing the CD45.2 alloantigen. Spleen and marrow from one femur were collected separately from recipient mice, and donor-derived cells were assayed by flow cytometry. Cells were treated with blocking Fc receptors (Fc gamma RIII/II) with 2.4G2 antibody to minimize nonspecific staining. Staining for IKDCs and other populations is described above with the addition of CD45.2 (104), and flow cytometry analyses were performed on a BD LSRII (BD Biosciences).

Statistics

The Prism V3.02 software (GraphPad, San Diego, CA) was used for statistical analysis. Intergroup comparisons were performed with the unpaired *t* test. *P* values were 2-tailed and considered significant if less than .05.

Results

Ly6C$^-$ cohort cells correspond to IKDCs and are distinct from pDCs

We previously showed that a CD11c$^+$CD45R/B220$^+$CD19$^-$Ly6C$^-$ cohort population was often found together with pDCs but displayed the NK1.1 and DX-5 NK lineage markers.[1] These properties, together with a high density of CD62L, are identical to the more recently described IKDCs[2] (and data not shown). While a subset of pDCs (pDC1s) was stably marked by the RAG-1/GFP reporter, no IKDCs were found among BM RAG-1$^+$ cells (Figure 1A). Reciprocally, marrow cells resolved for IKDC markers lacked GFP and detectable RAG-1 transcripts (Figure 1B). Only a minority (11.2%) of IKDCs in spleen were GFP$^+$ (not shown). Additional analyses were performed with B220$^+$CD19$^-$CD11c$^+$ DC populations rigorously double sorted from BM as Ly6C$^-$NK1.1$^+$ IKDCs and Ly6C$^+$NK1.1$^-$ pDCs. The Spi-B and ICSBP transcription factors are essential for pDC development,[31,32] and we report that their levels distinguished IKDCs from pDCs (Figure 1C). TLR9 is conspicuous in pDCs,[1] while we found a relatively low amount in IKDCs. Levels of Notch-1 in IKDCs were much lower than in pDCs. On the other hand, IKDCs contained more transcripts for TLR4 and the GATA-3 transcription factor. Both express at least some RelB, a transcription factor known to be critical for cDC development.[33] The Id-2 transcriptional repressor is essential for NK cell formation, and we found that the same was true for IKDCs (Figure 1D). In contrast, pDC numbers were normal in Id-2 knock-out mice.

Cytokine profiles are characteristic for particular DC subsets. Therefore, we compared IKDCs with 2 pDC subsets following stimulation with CpG ODN (Figure 1E). As previously reported, RAG-1$^-$ pDCs (pDC2s) produce impressive amounts of IFNα and TNFα.[1] While that was not a property of IKDCs, significant amounts of IFNγ were made following CpG stimulation. This is despite the fact that the receptor (TLR9) is not highly expressed (Figure 1C). We conclude that the Ly6C$^-$ pDC cohort population we observed previously is identical to IKDCs. IKDCs are distinct from pDCs with respect to gene expression, Id-2 dependence, and cytokine production following CpG ODN exposure.

IKDCs arise independently of Notch receptor ligation in culture

Our previous experiments revealed that ELPs and perhaps other LSKs are highly effective progenitors for pDCs.[1] Therefore, we isolated 2 of these subsets and placed them in OP9 stromal cell cocultures along with Flk-2/Flt-3 ligand (Figure 2A). Cytokines associated with lymphopoiesis (IL-7, IL-15) or cDC production (GM-CSF) were not added, but cDCs, pDCs, and IKDCs were obtained within 8 days. CMPs held in the same culture conditions produced no pDCs or IKDCs (data not shown). Transduction of OP9 stromal cells with Delta-like ligand 1 provides a stimulus for T-lineage commitment while suppressing entry to B and pDC lineages.[1,34,35] We now show that yields of cDCs were reduced by this Notch receptor ligand (on OP9-DL1), but formation of IKDCs was unaffected (Figure 2B). Therefore, IKDC formation is Notch independent and, thus, unlike T, B, pDC, or cDC lineages. The culture studies also suggest that primitive lymphoid progenitors are a good source, a point further investigated by transplantation (below).

IKDCs are relatively resistant to estrogen but can be generated from hormone-sensitive progenitors

Lymphopoiesis is arrested by high estrogen concentrations,[16,17] while a population of estrogen-resistant common myeloid progenitors (CMPs) generate pDCs on transplantation,[30] and GM-CSF–mediated differentiation of Langerhans cells is actually dependent on this hormone.[36] Therefore, estrogen treatment experiments were

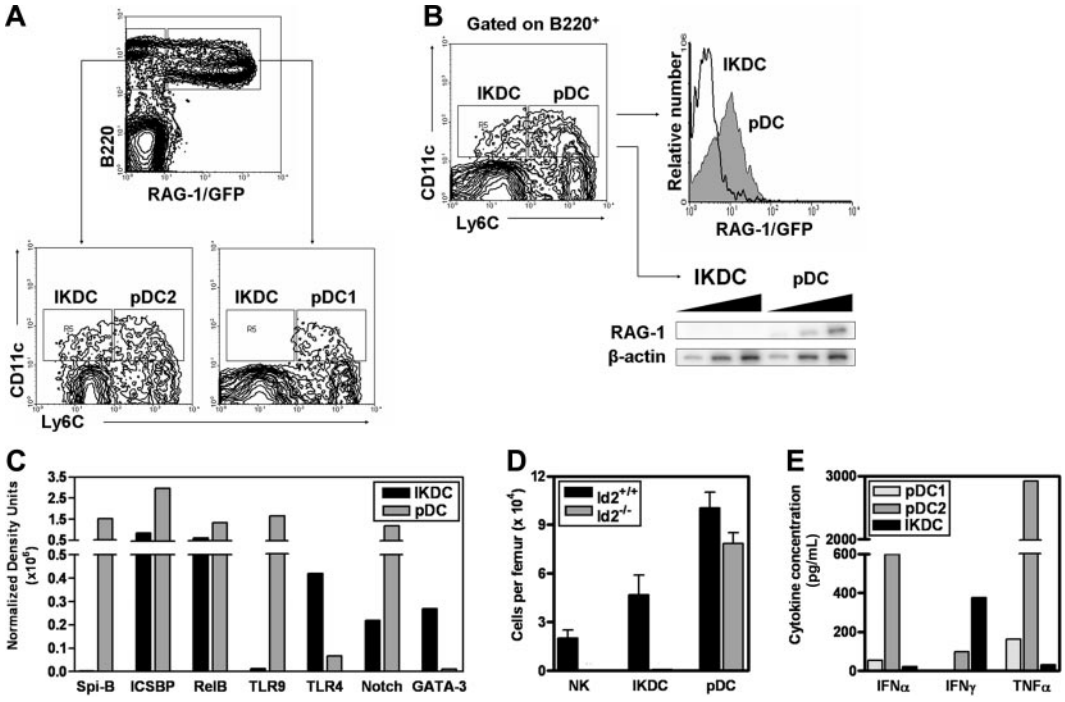

Figure 1. IKDCs differ from otherwise similar Ly6C$^+$ pDCs with respect to RAG-1 and patterns of gene expression. (A) The B220$^+$RAG-1/GFP$^+$ population freshly isolated from BM did not include IKDCs. (B) Conversely, BM IKDCs do not express RAG-1 detectable in RAG-1/GFP reporter mice or by RT-PCR. (C) Transcripts for a series of genes were measured in highly purified cells by RT-PCR. (D) Wild-type mice were compared with Id2 gene–targeted animals (Id2$^{-/-}$) by flow cytometry. Mean ± SEM values are shown for NK1.1$^+$ CD11c$^-$ NK cells, B220$^+$CD11c$^+$CD19$^-$Ly-6C$^-$ NK1.1$^+$ IKDCs, and B220$^+$CD11c$^+$CD19$^-$Ly-6C$^+$NK1.1$^-$ pDCs in BM. (E) Rigorously sorted DC subsets were placed in overnight cultures with CpG ODN, and supernatants were tested for the 3 indicated cytokines. The results are representative of 3 independent experiments.

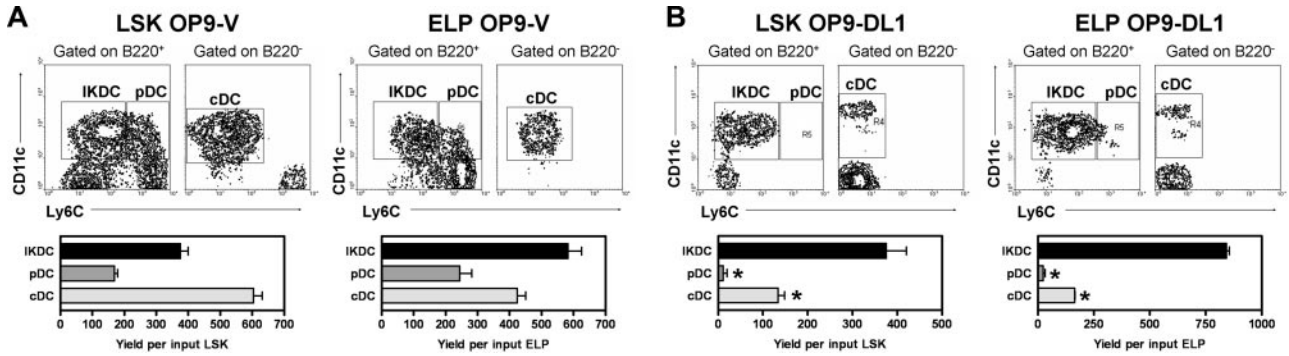

Figure 2. Lymphoid progenitors are a source of IKDCs, and their formation is insensitive to Notch receptor ligation. LSKs and RAG-1$^+$ ELPs were sorted from BM and placed in coculture with either vector (A) or Delta-like-1–transduced (B) OP9 stromal cells for 8 days in the presence of Flk-2/Flt-3 ligand. The indicated flow cytometry gates were used to discriminate B220$^+$CD19$^-$CD11c$^+$Ly6C$^-$ IKDCs, B220$^+$CD19$^-$CD11c$^+$Ly6C$^+$ pDCs, and B220$^-$CD19$^-$CD11c$^+$ cDCs. Total numbers of recovered cells of each type were calculated and expressed in the graphs as yields per input progenitor (mean ± SEM). Asterisks denote significant differences ($P < .05$) by *t* test.

used as another way to establish lineage relationships. We recorded no significant changes in absolute numbers of pDC1s, pDC2s, or IKDCs one week after time-release estrogen pellets were implanted into the mice (Figure 3A). IKDCs recovered from placebo- and estrogen-treated mice were indistinguishable in terms of morphology (not shown). NK cells declined approximately 40% in this interval. An additional week of hormone treatment caused significant reductions in numbers of all 3 DC subsets and NK cells (Figure 3A right panel), raising the possibility that they are slowly renewed from estrogen-sensitive progenitors. However, frequencies of all 4 of these effector cells were actually increased 2 weeks after treatment, reflecting some decrease in marrow cellularity (Figure 3B). In contrast, the treatment reduced frequencies and absolute numbers of LSPs by more than 60% and of ELPs by more than 97%.[13,16,37] This point was further investigated by isolation of LSKs from control and hormone-treated mice before stromal cell coculture (Figure 3C). Numbers of IKDCs that emerged in one-week cultures initiated from hormone-treated LSKs were substantially reduced.

Given the importance of DCs to autoimmune disease and the sex bias of such conditions, it was informative to test the competence of DCs recovered from hormone-treated mice. IFNγ was of particular interest because its production is a distinctive property of IKDCs.[2,3] In each of 3 experiments, IFNγ production was reduced by half in IKDCs isolated after one week of hormone treatment (Figure 3D). Low level synthesis of TNF and IFNα was not changed. To summarize, short-term elevations in estrogen did not rapidly change numbers of IKDCs; however, production may eventually decline due to exhaustion of progenitors, and IFNγ-producing capability was diminished.

Primitive CD62L$^+$ lymphoid progenitors are an efficient source of IKDCs

Our culture results (Figures 2-3) suggested that IKDCs may arise from lympho-hematopoietic cells, and our attention focused on 4

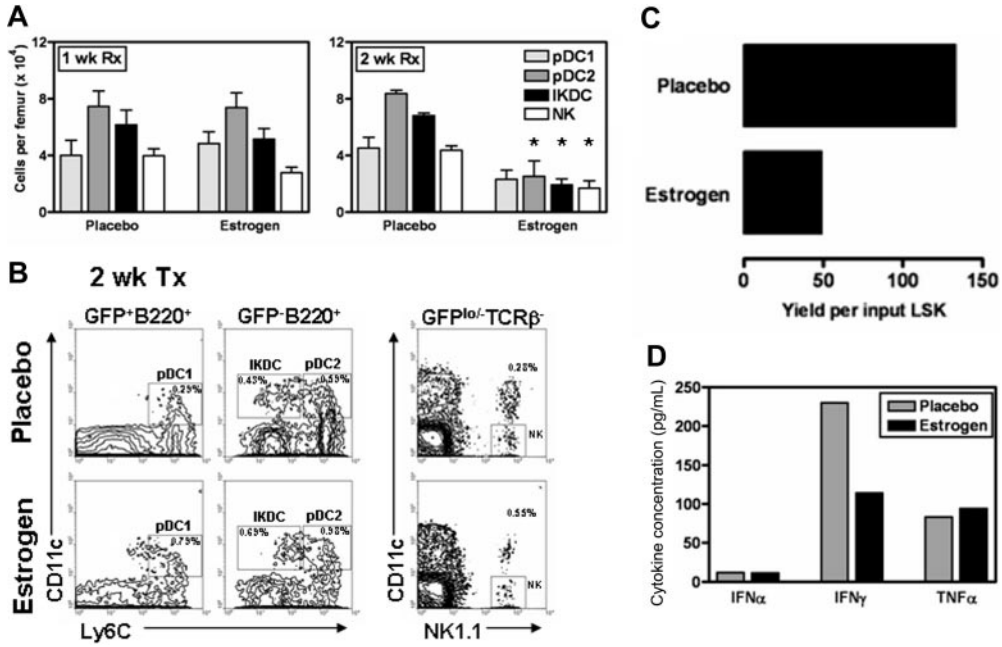

Figure 3. IKDCs are not rapidly depleted in estrogen-treated mice, but their functions may be hormone modulated. Mice were given subcutaneous time-release pellets containing placebo or 17β-estradiol. (A) IKDCs, pDC1s, pDC2s, and NK cells in BM were enumerated by flow cytometry and mean ± SEM values are shown (*$P < .05$). (B) Typical flow cytometry analyses at 2 weeks after treatment are shown. Average frequencies for each of the gated populations are given in the figure. (C) LSKs remaining in BM of mice treated for one week were also sorted and placed in stromal cell cocultures (OP9 + Flk-2/Flt-3 ligand) for an additional week. The results are given as yields of IKDCs per input progenitor. (D) IKDCs were recovered and double sorted from BM of mice treated for one week before being evaluated in overnight CpG-containing cultures for cytokine production. The data are representative of 3 similar experiments.

well-studied categories of progenitors. We purified LSPs, ELPs, CMPs, and CLPs from Thy1.1 congenic RAG-1/GFP reporter mice and transferred 5×10^3 cells to sublethally irradiated (6.5 Gy) recipients. Serial analyses established that peak numbers of BM and splenic pDCs and IKDCs were detected around day 16 after transplantation (not shown). Therefore, absolute numbers of each recovered cell type on day 16 are given in Figure 4A and Figure S2. While LSPs and ELPs more efficiently restored pDCs in recipient femurs, LSPs were superior in generating IKDCs in the bone marrow and spleen (Figure 4A and Figure S2). Some groups have shown that myeloid-restricted CMPs can produce small numbers of pDCs,[27,28,30,38,39] but this was not a significant source of IKDCs (Figure 4A). Although NK cells resemble IKDCs in multiple ways, the transplantation results indicate that they arise from distinct differentiation pathways. Of particular importance is the fact that NK1.1$^+$ CD11c$^-$ NK cells were much more efficiently made from CLPs.

LSPs were then isolated and placed in OP9 stromal cell cocultures with IL-15 to establish a precursor-product relationship to IKDCs (Figure 4B). B220$^+$ CD11c$^+$ Ly6C$^-$ cells were present by one week of culture, whereas only small numbers expressed CD122 and even fewer were positive for NK1.1 (Figure 4 and data not shown). There was complete acquisition of CD122 and NK1.1 after an additional week of culture. We conclude that IKDCs are efficiently replenished in vivo from LSPs. The timing and sequence of maturation in culture are consistent with the very primitive nature of the progenitor cells. B220 and CD11c are probably acquired first, followed by CD122, NK1.1, and DX-5.

Discussion

IKDCs may arise via a unique differentiation pathway. Like lymphocytes and NK cells, they can be efficiently produced from estrogen-sensitive lymphopoietic cells. However, IKDCs are not rapidly depleted in hormone-treated mice, and their formation is not influenced by Notch receptor ligation. Different from T and B cells, but similar to NK cells, IKDCs depend on the Id-2 transcriptional repressor. Furthermore, transplantation experiments suggest that IKDCs and NK cells are not made with equal efficiency from the same types of progenitors.

Our lab originally referred to IKDCs as cohort of pDCs because both are part of Hardy B220$^+$CD24^{Lo}CD19$^-$CD43$^+$ BM fraction A, express CD11c, and were produced together in cultures initiated with LSKs or ELPs.[1] However, IKDCs lack Ly6C and display the DX5 and NK1.1 markers associated with NK cells. We now show that these cells can be distinguished in other ways. There was little evidence for expression of RAG-1 in IKDCs, although they could be generated in transplant recipients and in culture from RAG-1$^+$ ELPs. Similarly, some NK progenitors transiently express RAG proteins and undergo immunoglobulin D_H-J_H rearrangements.[13,40] In contrast, pDC1 continue to be RAG-1 positive even outside of the BM.[1] As another distinction, pDCs (and especially pDC2s) express large amounts of IFNα and TNFα when stimulated with CpG DNA. IKDCs were more like NK cells in being specialized to produce IFNγ. Although recognition of CpG is thought to be mediated by TLR9,[41-43] and IKDCs responded to this stimulus, relatively low levels of TLR9 transcripts were detected by RT-PCR. Low expression of TLR9 is a characteristic of NK cells and another distinction from pDCs.[1] IKDCs had negligible Spi-B and reduced levels of ICSBP, transcription factors essential for pDC development. Finally, IKDCs had higher levels of the GATA-3 needed for T-cell development and NK functions.[23,44]

Notch-1 transcript levels were lower in IKDCs than in pDCs. Notch signals control many steps in hematopoiesis, ranging from stem cell self-renewal to development of mature B lymphocytes.[45] Of particular relevance, Notch receptor ligation can block formation of B- and pDC-lineage cells in culture.[1,46] While Notch sustains several aspects of T lymphopoiesis, it causes partial suppression of myelopoiesis, and NK-lineage progenitors are largely unaffected.[34,47,48] We now report that IKDC production in culture was Notch independent, and formation of cDCs was slightly suppressed. Notch-1 signaling directs the pDC versus T-cell lineage decision in human progenitors in part by downregulating Spi-B, an essential transcription factor for pDC formation.[49] IKDCs express little Spi-B and are unaffected by a Notch ligand. In contrast to these reports[49] and our new results, one study found that production of BDCA-2$^+$ pDCs from human CD34$^+$ cells was stimulated by Notch signaling.[50]

Notch is thought to control the B/T branchpoint, but lower levels of Notch signaling are needed to suppress B lymphopoiesis than are required to support T-lineage differentiation.[35] Lineage restriction is probably a gradual process, and several distinct types of BM progenitors can generate at least some IKDCs. Evidence is accumulating that the same is true for T and DC differentiation.[4,6,20] Thus, early specification is followed by progressive, rather than stepwise, commitment to a unique lineage. Recent studies suggest that fate decisions may be determined by microbial/viral products in addition to endogenous cues.[15]

Lymphoid progenitors are estrogen sensitive,[13,16,37,51] but IKDCs persisted during one week of exposure to this hormone. This may reflect a slow rate for their replacement, because IKDCs did decline following prolonged treatment, and the IKDC/pDC-containing fraction of BM has a low rate of BrdU incorporation.[1] LSKs from

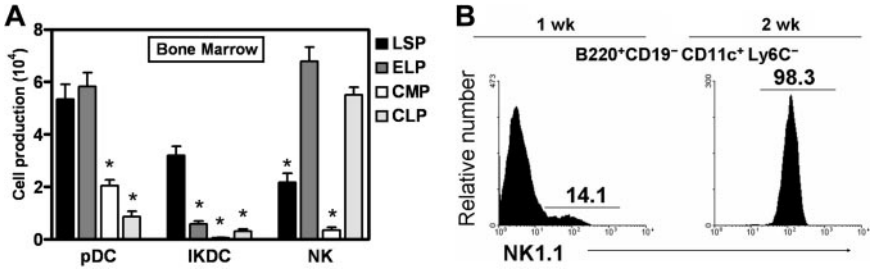

Figure 4. IKDCs develop efficiently from CD62L$^+$ lymphocyte progenitors. (A) LSPs, ELPs, CMPs, and CLPs were sorted from Thy1.1 congenic RAG-1/GFP reporter mice, and 5×10^3 cells of each were transferred to sublethally irradiated recipients. Flow cytometry analyses were then performed 16 days after transplantation. Absolute numbers of donor-derived pDC (B220$^+$ CD19$^-$CD11c$^+$ Ly6C$^+$), IKDC (B220$^+$ CD19$^-$CD11c$^+$ Ly6C$^-$ NK1.1$^+$), and NK (NK1.1$^+$ CD11c$^-$) BM cells per femur are given (* denotes significant differences [$P < .05$]). (B) LSPs (10^3/well) were placed in OP9 cocultures with Flk-2/Flt-3 ligand and IL-15. They were subcultured to fresh stromal cell monolayers after one week, and gated B220$^+$CD19$^-$CD11c$^+$ Ly6C$^-$ cells were assessed for NK1.1 expression by flow cytometry at the indicated intervals.

estrogen-treated mice produced only one third as many IKDCs as untreated progenitors. Additionally, the estrogen-sensitive LSP and ELP subsets of lymphopoietic cells were effective in producing IKDCs in culture and following transplantation. However, some IKDCs could derive from estrogen-independent progenitors, as has recently been proposed for pDCs.[30] It is interesting that GM-CSF–mediated differentiation of Langerhans cells actually requires estrogen,[36] and some studies suggest that dendritic cell functions are hormone regulated.[52] The IFNγ–producing potential of IKDCs from estrogen-treated mice was subnormal, a finding with possible relevance for the sex bias associated with autoimmune diseases.

IKDCs share many properties with NK cells,[2,3] and some findings suggest they have a developmental relationship. That is, both are unaffected by Notch receptor ligation and dependent on the Id-2 transcriptional repressor. Also, IL-7Rα$^{-/-}$ mice are not deficient in pDCs, NK cells, or IKDC cells[5] (R.P., unpublished observations, February 2006). IL-15 signals in part via the IL-2Rβ chain (CD122), and mice lacking either IL-15 or CD122 have severe NK cell deficiencies.[53,54] Furthermore, CD122 has been used to identify committed NKPs.[17,55,56] Consequently, it is interesting that cells with IKDC features arose in one-week stromal cell cocultures even though the cytokine was not deliberately added. Full expression of the NK1.1 and DX-5 markers on IKDCs required an additional week of culture, and IL-15 was present in that circumstance. Nevertheless, the transplantation results suggest that pathways devoted to NK and IKDC differentiation are distinct. For example, the CD62L^{+} subset of LSKs (LSPs) was a good progenitor for IKDCs, but not NK cells (Figure 4A).[14] In contrast, CLPs were an excellent source of NK cells, but not IKDCs.

The LSP fraction was superior to 2 other types of lymphoid progenitors in generating IKDCs in marrow of transplant recipients. As noted above, it was not a good source of NK cells under the same conditions.[14] Therefore, competence for IKDC lineage differentiation must be established in very primitive c-Kit^{Hi} cells as suggested in the model shown in Figure 5. However, it remains to be determined if committed progenitors for IKDCs with no other options can be identified. Furthermore, it remains possible that 1 of these 2 cell types is an activated form of the other or that there is conversion during disease circumstances.[15,57]

There was no CD122 on LSPs, the best progenitors of IKDCs. Our findings suggest that CD122 is acquired just before NK1.1 and DX5, but well behind B220 and CD11c. B220^{+}CD11c^{+}Ly6C^{-}NK1.1^{-} may represent an intermediate stage because they were present in one-week OP9 cocultures initiated with LSPs. All of them were NK1.1^{+} IKDCs one week later. This sequence resembles that found in 2-step culture studies of NK cell differentiation.[17,21]

IKDCs represent a distinct, but multifunctional, component of the innate immune system and our observations provide insight into their formation within BM. They appear to arise via a dedicated differentiation pathway that diverges from other lineages at an early stage. Indeed, very primitive, lymphoid-specified LSPs were the most efficient source.

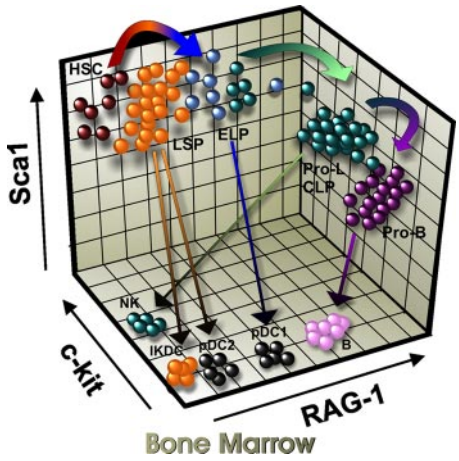

Figure 5. Possible relationship between IKDCs and other lineages in bone marrow. Lymphoid progenitors can be conveniently resolved on the basis of c-Kit, Sca-1, and RAG-1 densities[6] as depicted here. Our new findings suggest that CD62L/L-selectin^{+} progenitors (LSPs) are the most effective source of IKDCs. In contrast, NK cells are normally produced from c-Kit^{Lo} RAG-1^{+} prolymphocytes (including IL-7Rα^{+} common lymphoid progenitors [CLPs]). Although IKDCs do not express RAG-1, they could be made under experimental conditions from progenitors that express RAG-1 (not illustrated; see Figure 2A). Among other distinctions from pDCs, IKDC development was insensitive to Notch and dependent on the Id-2 transcriptional repressor (not illustrated).

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Authorship

Contribution: R.S.W. designed research, performed research, analyzed data, and wrote the paper; R.P. designed research, performed research, analyzed data, and wrote the paper; K.P.G. designed research, performed research, analyzed data, and wrote the paper; X.C. designed research, performed research, analyzed data, and wrote the paper; S.S.P. designed research, performed research, and wrote the paper; X.-H.S. designed research and wrote the paper; B.L.K. contributed vital reagents or analytical tools, and wrote the paper; P.W.K. designed research, analyzed data, and wrote the paper. R.S.W. and R.P. contributed equally to this work.

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