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Plasmacytoid Dendritic Cell Dichotomy: Identification of IFN-α Producing Cells as a Phenotypically and Functionally Distinct Subset

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Plasmacytoid dendritic cells (pDC) produce large amounts of type I IFN in response to invading pathogens, but can also suppress immune responses and promote tolerance. In this study, we show that in mice, these functions are attributable to two distinct pDC subsets, one of which gives rise to the other. CD9<sup>pos</sup>Siglec-H<sup>high</sup> pDC secrete IFN-α when stimulated with TLR agonists, induce CTLs, and promote protective antitumor immunity. By contrast, CD9<sup>neg</sup>Siglec-H<sup>high</sup> pDC secrete negligible amounts of IFN-α, induce Foxp3<sup>+</sup> CD4<sup>+</sup> T cells, and fail to promote antitumor immunity. Although newly formed pDC in the bone marrow are CD9<sup>pos</sup> and are capable of producing IFN-α, after these cells traffic to peripheral tissues, they lose CD9 expression and the ability to produce IFN-α. We propose that newly generated pDC mobilized from the bone marrow, rather than tissue-resident pDC, are the major source of IFN-α in infected hosts. The Journal of Immunology, 2011, 186: 1477–1485.

In this study, we show that a phenotypically and morphologically distinct subset of pDC is responsible for all, or nearly all, IFN-α production by pDC. These cells, which are found mainly in bone marrow (BM) and spleen, ultimately differentiate into pDC that produce little or no IFN-α and instead promote immune tolerance. The latter cells, which also comprise a phenotypically distinct subset, are the main pDC in peripheral tissues. Thus, our data support a model in which immature pDC are proinflammatory, whereas mature, tissue-resident pDC are tolerogenic. Control of the frequency and tissue distribution of these functionally distinct pDC subsets provides an elegant mechanism by which IFN-α can be tightly regulated.

Materials and Methods

Mice and cell lines

Female or male C57BL/6 and Sv129 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used at 8–12 wk of age. OT-I and OT-II OVA-TcR transgenic mice crossed onto Rag-1<sup>−/−</sup> background were purchased from Taconic Farms (Germantown, NY) and bred in-house. Congenic Ly5.1 (Ptp11c) mice, Flt3 ligand (Flt3-L)<sup>−/−</sup> and STAT-1<sup>−/−</sup> mice were obtained from Taconic Farms. All experiments were performed according to approved protocols. The B16 melanoma cell line stably transfected with mouse Flt3-L was a kind gift of Dr. G. Dranoff, Harvard University (13). Three to four million cells were injected s.c., and animals were sacrificed after 10–14 d. EG7 cells (EL4 thymoma transfected with OVA) were purchased from American Type Culture Collection (Manassas, VA).

Cell preparation and flow cytometry

BM-derived DC were prepared by flushing the femur and tibia using a needle. DC derived from lymph node (LN), thymus, or liver were dispersed into single-cell suspensions. Mouse blood was obtained by cardiac puncture, layered onto Ficoll-Hypaque, and centrifuged for 20 min at 800 × g. After washing, total leukocytes were stained with directly FITC-conjugated Abs to CD3, CD19, DX5, and Ly6G (to exclude T cells, B cells, NK cells, and granulocytes), CD11b-Pacific Blue, CD11c-APC-Cy7 (all from Biolegend, San Diego, CA), and CD9-Alexa Fluor 647 (Biolegend) and sorted using an FACSARia (BD Biosciences, Mountain View, CA). Propidium iodide was included to gate out dead cells. Gates were set on forward and side scatter, and lineage-positive cells and myeloid DC were excluded, whereas CD11c<sup>+</sup>B220<sup>+</sup> cells were sorted into CD9<sup>pos</sup> and CD9<sup>neg</sup> subsets. Purity of sorted cells typically exceeded 95% (data not shown). Splenic
pDC and total CD11c DC were isolated using CD11c-conjugated magnetic beads (Miltenyi Biotec) and then stained as above. OVA-specific CD4+ or CD8+ T cells were isolated from the spleens of OT-II-Rag2−/− or OT-I-Rag2−/− mice. Spleens were dispersed into a single-cell suspension, anti-CD4– or anti-CD8–conjugated magnetic beads (Miltenyi Biotec) were added, and cells were incubated for 20 min at 4˚C, after which they were separated using an MACS magnet. Purity was typically >90%. T cells were labeled with CFSE (1 μM) for 10 min at 37˚C, washed, and resuspended in HEPES-buffered RPMI 1640 supplemented with 10% FCS, antibiotics, and 2 mM t-glutamine. A total of 1 × 10^5 DC and 2 × 10^5 T cells were cultured in 96-well round-bottom plates at a final volume of 200 μl. Cultures were supplemented with OVA peptide. Fluorochrome-labeled Abs were obtained from eBioscience (CD4, CD8, CD40, CD80, CD86, CD135, CCR7), BD Biosciences (ICOS-L, OX40-L, Ly-6C, IAb/d, H2-Kb, CD62L, CD74, CD103), R&D Systems (CCR6, CCR9), Invitrogen (B220 Pacific Orange), Serotec (CD200R), or Biolegend (CD3, CD4, CD8, CD40, CD80, CD86, ICS-L, OX40L).

FIGURE 1. A phenotypically distinct subset of pDC produces IFN-α. A. Only a fraction of BM-derived pDC produce IFN-α after stimulation with CpG 2336. Cells were gated on size and expression of CD11c and B220. B. Sorted, CpG-stimulated CD9pos pDC produce more IFN-α by comparison with identically stimulated CD9neg pDC (Student’s paired t test, CpG 2336, p = 0.0472; CpG 1585, p = 0.0210; CpG 1826, p = 0.2074). CD9neg pDC do not produce IFN-α above background levels (CpG 2336, p = 0.059; CpG 1585, p = 0.132; CpG 1826, p = 0.344) (SD). C. Both CD9pos and CD9neg pDC express the pDC-specific transcription factor E2-2, whether freshly isolated or after stimulation with CpG 2336. Both pDC subsets also express similar levels of IRF-7. D. Both pDC subsets express similar levels of the CpG receptor TLR9 as determined by intracellular staining. The results shown are representative of three independent experiments.

FIGURE 2. Many cell-surface Ags are differentially expressed by the two pDC subsets. A. FACS analysis of freshly sorted CD9pos and CD9neg subsets reveal differences in expression of the pDC selective Ags Siglec-H, PDCA-1, and G120.8. Additional surface Ags were also examined, as seen in Supplemental Fig. 3. The results shown are representative of five independent experiments. B. CD9pos pDC contain abundant RER. CD9neg pDC contain many vacuoles and lysosomes. C. Size of CD9pos and CD9neg pDC based on forward and side scatter.
CD11b, CD11c, CD19, DX-5, Siglec-H, CD38). G120.8 was obtained from Scherping-Plough Biopharma and conjugated with Alexa Fluor 488 (Invitrogen) according to the manufacturer’s instruction. pDC Ag-1 (PDCA-1) was purchased from Miltenyi Biotec.

**Electron microscopy**
Transmission electron microscopy (EM) was performed using standard procedures (2). Briefly, freshly FACS-sorted DC were washed and fixed in 2.5% glutaraldehyde in cacodylate buffer and postfixed in 1% OsO$_4$ solution. Cells were dehydrated in a series of alcohol solutions and embedded in epoxy. Sections were examined using a JEOL 1230 microscope at the Stanford Core Facility for biologic imaging.

**Cytokine assays**
Supernatants from DC were harvested after 24–48 h of culture and stored at −20°C until measured by ELISA. TLR agonists (CpG 2336, 1826, and 1585 [TLR9]), gardiquimod, Pam3CSK4 [synthetic triacylated lipopeptide, TLR1/2], FSL-1 [TLR2/6], and R848 [TLR7/8]) were obtained from InvivoGen (San Diego, CA). Polyinosityidine (TLR3 agonist) and LPS (TLR4 agonist) were from Sigma-Aldrich. DC–T cell cocultures were restimulated after 7 d using PMA and ionomycin (Sigma-Aldrich). Supernatants were analyzed by ELISA. Detection of intracellular IFN-α was performed as described (14). Briefly, total BM-derived cells were cultured for 9 h with or without CpG 2336 motifs. Brefeldin A was added after 3 h. Cells were fixed and permeabilized as described by the manufacturer (eBioscience), and a mixture of rat anti-mouse IFN-α Abs RMMA (R&D Systems) and F18 (Abcam) was added. Cells were further incubated with rabbit anti-rat IgG Alexa Fluor 488 (Invitrogen), and total rat IgG (Sigma-Aldrich). Supernatants were analyzed by ELISA. Detection of intracellular IFN-α was performed as described (14). Briefly, total BM-derived cells were cultured for 9 h with or without CpG 2336 motifs. Brefeldin A was added after 3 h. Cells were fixed and permeabilized as described by the manufacturer (eBioscience), and a mixture of rat anti-mouse IFN-α Abs RMMA (R&D Systems) and F18 (Abcam) was added. Cells were further incubated with rabbit anti-rat IgG Alexa Fluor 488 (Invitrogen), and total rat IgG (Sigma-Aldrich) was then used for blocking. Directly fluorochrome-labeled Abs to CD11c and B220 were added, and cells were analyzed by flow cytometry.

**PCR**
PCR was performed using as above and frozen in 10% DMSO, 50% FCS in PBS in liquid nitrogen. Cells were further washed in PBS, and RNA was isolated using an RNeasy microkit (Qiagen, Valencia, CA), according to the manufacturer’s instruction. cDNA was synthesized using Applied Biosystems’s High Capacity Reverse Transcription kit (Foster City, CA) according to the manufacturer’s instruction. PCR was performed using Taq DNA polymerase and buffer mix (Qiagen) in the presence of oligonucleotide primers for E2-2, IFN regulatory factor 7 (IRF-7), and β-actin (control) genes. E2-2 primers: forward 5′-AGACCAAGCTCTCTTCTC-3′; reverse 5′-AGGCTCTAGGACACCTTC-3′ (134 bp product). IRF-7 primers: forward 5′-CTCTGTGTAAGCAGGAATGT-3′; reverse 5′-GTA-CAGGACACGATCTG-3′ (821 bp product). β-actin primers: forward 5′-TGGATCTCTGTGGCAGTCAtCAATGAAAC-3′; reverse 5′-TAAAACGCG-AAGCTCTAGAAGAAGTCG-3′ (349 bp product). The cycling conditions were as follows: initial denaturation at 94°C for 3 min 45 s, followed by 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 1 min 30 s, and a final extension step at 72°C for 10 min. Amplified cDNA was run on 2% agarose gels, stained with propidium iodide, and visualized using a UV-trans-illuminator.

**CTL assay and tumor model**
For detection of CTL activity, EG7 (OVA-transfected) tumor cells were labeled with a high concentration of CFSE, and control EL4 cells were labeled with a low CFSE concentration (15), mixed in equal parts, and used as target cells. Graded numbers of effector OT-I T cells, either unstimulated or cultured with the respective peptide-pulsed (SINFEKL, 100 ng/ml) pDC subsets for 3 d, were added to the labeled target cells. Specific lysis was determined by FACS according to the following formula: ratio = (percentage of CFSE$^{low}$/CFSE$^{high}$) and percentage of specific lysis = 1 – [ratio unprimed/ratio primed] × 100.

Groups of mice were given peptide-pulsed (OVA257-264, 100 ng/ml) pDC subsets by s.c. injection in the footpad (0.5 × 10$^6$/mouse) at day −14 and day −7. On day 0, mice were challenged by injection of 0.1 × 10$^6$ EG7 cells into the flank. Tumor growth was measured every other day. At the termination of the experiment, mice were sacrificed, and tumor-draining LN and contralateral LN were harvested and analyzed for the presence of Foxp3$^+$ CD4 T cells by flow cytometry.

**In vivo pDC differentiation**
Sorted pDC or pDC subsets were labeled with CFSE (Invitrogen), washed, and injected i.v. into congenic CD45.1 recipient mice. Two to 5 d after injection, various organs were harvested and assessed for the presence of CFSE$^+$ pDC using multicolor flow cytometry. On day 4 after transfer, spleen and LN were harvested, pooled, and CFSE$^+$ pDC were isolated by sorting...
with an FACSaria II (BD Biosciences). The sorted cells were cultured in the presence of CpG motifs for 48 hours, after which supernatants were harvested and stored at −20°C until assayed for IFN-α production by ELISA. pDC from S+129 mice were sorted into subsets and injected i.v. into STAT-1−/− mice, which are unable to secrete type I IFN (16). Mice were given a simultaneous injection of CpG 2336 (100 µg/mouse). These recipients were chosen due to their inability to secrete type I IFN. Hence, only adoptively transferred cells are able to respond to TLR9 ligation. Six hours after injection, serum was collected and frozen until analyzed for the presence of IFN-α.

**Statistical analysis**

Comparison of means was performed using paired or unpaired two-tailed Student’s t test (GraphPad Prism, GraphPad). Tumor growth was evaluated by one-way ANOVA.

**Results**

**CD9pos pDC, but not CD9neg pDC, produce IFN-α**

Activation of pDC with oligonucleotides containing CpG motifs leads to IFN-α production. However, as shown in Fig. 1A, only a fraction of pDC produce this cytokine, suggesting that the IFN-α-secreting cells may comprise a discrete pDC subset. In an effort to identify markers that might distinguish such a subset, we surveyed the expression of selected surface markers on BM-derived pDC. Among these, CD9, a tetra-membrane spanning protein (17), appeared to define a clearly discernable subpopulation. To confirm this observation and study the functions of CD9pos and CD9neg pDC, we injected mice with an Flt3-L–secreting tumor cell line that increases the yield of pDC (13). Ten to 14 d later, we stained BM cells with Abs to CD11c, B220, and CD9. Myeloid DC and lineage-positive cells were excluded (Supplemental Fig. 1). pDC were defined as coexpressing CD11c and B220 (2, 3) and were further subdivided into CD9-negative (CD9neg) and CD9-positive (CD9pos) fractions.

To assess the function of these cells, sorted CD9pos and CD9neg pDC were cultured with various TLR agonists for 48 h, before measuring IFN-α and other cytokines in the supernatants. As shown in Fig. 1A, the CD9pos pDC are the major IFN-α–producing cells, and only small amounts of IFN-α could be detected in the cultures of CpG-stimulated CD9neg pDC. Because different CpG motifs may induce different levels of IFN-α, we cultured our pDC...
subsets in the presence of CpG 2336 and 1585 (both CpG-A type) and 1826 (CpG-B) for 48 h and analyzed the supernatants for IFN-α. Only the CD9pos pDC produced IFN-α in response to CpG-A, whereas neither subset produced IFN-α in response to CpG-B (Fig. 1B). In fact, CD9neg pDC did not produce IFN-α above background levels, regardless of the source of CpG (Fig. 1B). It is possible that CD9neg pDC lack the synthetic machinery proximal signaling components or TLR receptors needed to respond to exogenous stimuli and produce IFN-α. It is also possible that these cells are not pDC at all, but simply share a similar surface phenotype with pDC. To help address this question, we determined the expression of E2-2, a pDC-specific transcription factor (18), in CD9pos and CD9neg pDC. Both subsets expressed equal amounts of E2-2 irrespective of IFN-α secretion as detected by RT-PCR (Fig. 1C). Likewise, IRF-7, the main transcription factor responsible for IFN-α expression in pDC (19), was present in both subsets (Fig. 1C). The level of expression of these two transcription factors remained unchanged after stimulation with CpG. As CpG motifs signal through TLR9, we examined the expression of this receptor by intracellular staining. Fig. 1D shows that the two subsets express similar levels of TLR9.

Mouse pDC can produce other cytokines, mainly IL-6, TNF-α, and IL-12, in contrast to human pDC, which do not produce IL-12 (20). Furthermore, and unlike human pDC, mouse pDC express all TLRs (21). On this basis, we examined the cytokine response to the different TLRs (Supplemental Fig. 2A). IL-6, TNF-α, and IL-12 were all produced by the CD9pos cells, whereas the CD9neg cells produced TNF-α but little IL-6 or IL-12. To validate these in vitro data, we examined the capacity of CD9pos and CD9neg pDC to produce IFN-α in vivo. Sorted pDC derived from Sv129 mice, in combination with CpG 2336, were injected into Stat1−/− deficient recipients (Sv129 background), which are unable to produce IFN-α in response to CpG motifs. Sera were collected and analyzed for IFN-α by ELISA. Only mice that received CD9pos pDC showed detectable serum levels of IFN-α (Supplemental Fig. 2B).

**CD9 identifies a subset of pDC with a distinct surface phenotype**

DCs isolated from mice injected with an Flt3-L–secreting tumor cell line were harvested from BM. As shown in Fig. 2A, CD9neg and CD9pos pDC differ in their expression of many surface Ags. The CD9neg pDC express high levels of the inflammatory chemokine receptor CXCR3, the pDC Ags Siglec-H and PDCA-1, CD4, and CD8αα. CD9pos pDC express low levels of Siglec-H and PDCA-1, and little CD4 and CD8αα, but intermediate levels of MHC class II, CD86, ICOS-L, and the gut homing receptor α4β7 and high levels of CD62L and CXCR4 (Fig. 2A). A more complete phenotype of each subset is shown in Supplemental Fig. 3. CCR9 was recently reported to be expressed on tolerogenic pDC (22). Not surprisingly, this molecule is highly expressed on CD9pos pDC and absent from the surface of CD9neg pDC (Fig. 2A). Interestingly, however, when the CD9pos subset was cultured overnight in medium alone, CCR9 appeared rapidly on the cell surface, suggesting that this molecule may have been preformed in these cells (Supplemental Fig. 4, upper panel). Intracellular staining of freshly isolated CD9pos cells for CCR9 confirmed this notion (Supplemental Fig. 5).

**Morphological differences between pDC subsets**

The designation plasmacytoid refers to the similar appearance of pDC and plasma cells when examined using EM, with both cell types showing well-developed rough endoplasmic reticulum (RER) (1). Given the dramatic differences in the capacity of CD9pos and CD9neg subsets to produce IFN-α, we wanted to determine whether these cells also differed when examined by EM. Fig. 2B shows that whereas the BM-derived CD9pos pDC contain abundant RER and have a very electron-dense cytoplasm, the CD9neg DC have little RER, larger nuclei, and many vacuoles. Thus, the ultrastructure of the CD9neg pDC does not correspond to that of a classical IFN-α–secreting pDC.

We further analyzed the relative frequencies of the CD9pos and CD9neg pDC subsets in different tissues of Flt3-L–treated mice. Although pDC could be obtained in greater numbers from BM and spleen, they were also present in peripheral LN and liver, as reported previously (2, 3, 23). Interestingly, in the thymus and mediastinal LN, the CD9pos subset predominated (Fig. 3A). Mediastinal pDC have been reported to induce tolerance to airborne Ags (24), and FACS analysis showed that these cells were almost exclusively of the CD9neg subtype while expressing high levels of the Siglec-H, PDCA-1, and the chemokine receptor CCR9 [an early response gene for pDC (18)] (Fig. 3B). We therefore stimulated pDC from thymus and mediastinal LN and assessed their capacity to secrete IFN-α. The results show that pDC from these tissues produce very small amounts of IFN-α compared with BM-derived pDC (Fig. 3C).

Moreover, when isolating total pDC (CD11c+B220+) versus myeloid DC (CD11c+CD11b+) from BM, spleen, or LNs of Flt3-L–treated mice, pDC from BM secreted at
least twice as much IFN-α compared with pDC from spleen or LNs (Supplemental Fig. 6).

**CD9pos and CD9neg pDC induce the production of different cytokines from CD4+ T cells in vitro**

We further examined the T cell stimulatory capacity of the two pDC subsets. To this end, CFSE-labeled CD4+ OT-II T cells were cultured together with an immunodominant OVA peptide (OVA257-264) and CD9pos or CD9neg pDC for 6 to 7 d. Cells were restimulated using PMA and ionomycin, and the T cell culture supernatant was harvested and analyzed by ELISA for cytokine secretion. Whereas the Ag-pulsed CD9pos pDC induced vigorous CD4+ T cell proliferation, T cells cultured with CD9neg pDC underwent few divisions (Fig. 4A). Moreover, CD4+ T cells cultured with CD9pos pDC produced IFN-γ but little or no IL-4 and IL-10, whereas the T cells cultured with the CD9neg subset produced mainly IL-4 and IL-10 (Fig. 4B). In addition, CD9neg pDC could induce the production of TGF-β, a cytokine typically associated with Tregs (Fig. 4C).

**CD9pos pDC promote CD8+ T cell activation and CTL formation**

We proceeded to examine the response of CD8+ T cells to CD9pos and CD9neg pDC, as such T cells are an important component of antiviral and antitumor immunity and are known to be activated by IFN-α–producing cells (1, 4). We pulsed each pDC subset with the immunodominant OVA peptide SINFEKL (OVA257–264) and cultured these pDC with CD8+ OT-I T cells. CD9pos pDC induced a strong CD8+ T cell response, whereas the CD9neg subset induced little proliferation (Fig. 4D). To assess whether CD9pos pDC could induce functional CTL as determined by lysis of EG7 tumor cells, we cultured each pDC subset together with OT-I T cells for 4 d. After washing, the T cells were incubated with CFSE-labeled EL-4 and EG7 tumor cells to analyze their cytotoxic capacity. As shown in Fig. 4E, CD9pos pDC alone induced CTL capable of causing EG7 cell lysis in a dose-dependent manner.

To test the activity of CD9pos and CD9neg pDC in a tumor model, we injected OVA257–264-pulsed CD9pos or CD9neg pDC (0.5 × 10^6 cells/animal) into the footpads of groups of tumor-naive, syngeneic mice. Control animals were given PBS. One week after the first injection, the mice were given a second injection of similarly prepared pDC. One week after the second injection, mice were challenged with EG7 tumor cells (0.1 × 10^6/animal) s.c. in the flank. Tumor growth was monitored every 2 d. As shown in Fig. 5A, injection of CD9pos pDC completely prevented tumor growth, whereas CD9neg pDC had little or no antitumor effect such that tumors in these mice grew to the same extent seen in the saline control group. When examining the LN of treated mice, we found that mice that received CD9neg pDC had increased numbers of Foxp3+CD4+ T cells in their tumor-draining LN compared with the contralateral LN (Fig. 5B). Vα2TcR+Foxp3+CD4+ T cells were also present (Fig. 5B, lower panel). A similar increase could not be found in the draining LN of the control group or in mice given CD9pos pDC.

**CD9 expression is gradually lost on pDC in vivo**

To assess the stability of CD9 expression on pDC in vivo, total BM-derived pDC or purified CD9pos pDC (2–5 × 10^6 cells) from Flt-3L–treated mice were labeled with CFSE and injected i.v. into congenic CD45.1 mice. After 2–4 d, various organs were harvested and examined for CD9 expression using flow cytometry (Fig. 6A). As shown in Fig. 6B, most CD9pos cells had become CD9neg in vivo and upregulated Siglec-H. To determine whether pDC that matured in vivo lose their ability to secrete IFN-α, we...
transferred CFSE-labeled CD9pos pDC into congenic recipients. Four days after transfer, spleen and LN were harvested and pooled into a single-cell suspension. Cells were sorted based on their expression of CFSE and CD45.2, excluding lineage-positive and CD45.1 cells. As shown in Fig. 6C, the transferred pDC secreted less IFN-α compared with pDC freshly isolated from BM.

**Flt3-L promotes pDC viability**

As Flt3-L is a crucial growth factor for pDC development and differentiation in vitro and in vivo (25–27), we asked whether addition of Flt3-L to the respective subsets would affect their differentiation in vitro. To this end, sorted CD9pos and CD9neg pDC were cultured in the presence or absence of Flt3-L (100 ng/ml) and/or CpG 2336 motifs (10 μg/ml) for 4 d, and viability and phenotypic changes were assessed daily. As shown in Fig. 7A, pDC cultured in medium alone rapidly lost their viability, and after 3 d, all of the cells were dead. By contrast, pDC culture in Flt3-L survived longer, and half of the cells were still viable after 3 d of culture. The CD9pos cells remained viable longer than their CD9neg counterparts. Activation of CD9pos pDC with CpG motifs in the presence of FL resulted in reduced survival. Moreover, whereas the phenotype of the CD9neg pDC appeared stable in medium or Flt3-L, the CD9pos cells gradually lost expression of CD9 and upregulated that of Siglec-H, PDCA-1, and CCR9, indicative of differentiation into CD9neg pDC (Fig. 7B). In the presence of CpG 2336, CD9 expression was rapidly downregulated on CD9pos pDC, and Siglec-H was not induced (Supplemental Fig. 4). However, stimulation with CpG 2336 had little effect on the CD9neg pDC.

To assess the effect of Flt3-L on pDC subset differentiation in vivo, we transferred sorted pDC subsets (CD45.1) into Flt3-L knockout recipients (CD45.2). Two to 4 d later, we harvested the spleens of the Flt3-L knockout mice and examined the expression of CD9 and Siglec-H on the transferred pDCs. Fig. 7C shows that CD9pos pDC differentiate into CD9neg cells even in the absence of Flt3-L, although not to the same extent as in wild-type C57BL/6 recipients (Fig. 7B).

**Discussion**

We initiated this study with the objective of understanding how a single cell type can mediate both proinflammatory (1–4) and suppressive (5–8) immune responses and how these activities relate to the most critical function of pDC, namely the production of IFN-α (1–4, 28). Our data show that the opposing functions of pDC are not mediated by a single phenotypically stable cell type, but by distinct subsets that differ in surface phenotype, morphology, and function. Furthermore, the results indicate that these subsets represent pDC in different stages of maturation: an early stage cell with the capacity to produce IFN-α and other proinflammatory mediators and a later stage cell that can no longer produce these factors but is instead tolerogenic. Thus, the two pDC subsets do not originate from different precursor populations, but rather from a single precursor undergoing a natural differentiation process. Flt3-L plays an important part in promoting...
viability of the pDC during this differentiation process (Fig. 7A) (26, 27). However, the transition into CD9<sup>neg</sup>Siglec-H<sup>high</sup>pDC can proceed in the absence of exogenous Flt3-L, suggesting that other factors may promote pDC differentiation. The data also support a model in which immature pDC are proinflammatory, whereas mature pDC are regulatory or suppressive.

We considered the possibility that one or both of our DC populations studied in this paper might contain myeloid DC, in light of a recent publication suggesting that some CD11c<sup>B220</sup> cells are precursors of conventional myeloid DC (29). However, the CD11c<sup>B220</sup> conventional DC precursors are PDCA-1 (CD317) and Siglec-H negative and produce little IFN-α, whereas the CD9<sup>CD11c<sup>B220</sup></sup> subset identified in our study is PDCA-1<sup>-</sup> and produces large amounts of IFN-α. Moreover, our Siglec-H<sup>high</sup>CD9<sup>neg</sup>pDC cells produce little IFN-α but express more PDCA-1 than CD9<sup>pos</sup>pDC. These data argue strongly that few, if any, of the CD9<sup>pos</sup> cells studied in this paper are conventional DC.

Because IFN-α is a potent cytokine with direct effects on many other cell types, such as NK cells and B cells, it is important that the production of IFN-α be tightly regulated. Our data show that under steady-state conditions, only immature CD9<sup>pos</sup>pDC, which are found mainly in the BM and spleen, can produce IFN-α and other inflammatory cytokines. In contrast, pDC found in tissues such as thymus, liver, and the LN draining the lung and gut are mostly CD9<sup>neg</sup>pDC and no longer produce inflammatory cytokines. Indeed, these cells appear to be tolerogenic. A schematic representation of these findings is shown in Supplemental Fig. 7.

The mechanism responsible for the inability of CD9<sup>neg</sup>pDC to produce substantial quantities of IFN-α is unknown. These cells, like CD9<sup>pos</sup>pDC, express at least some of the synthetic machinery required for IFN-α production, including IRF-7, the main IFN-α transcription factor (19) (Fig. 1C). They also express TLR9 at levels similar to that seen in CD9<sup>pos</sup>pDC. Further, there is no question of their identity as pDCs because they express the pDC-defining transcription factor E2-2 (18) as well as the pDC-specific molecule Siglec-H (30). Indeed, Siglec-H, which mediates signals via DAP-12 that inhibit IFN-α production, is expressed at higher levels on CD9<sup>neg</sup>pDC than CD9<sup>pos</sup>pDC, and exposure of CD9<sup>pos</sup>pDC to CpG motifs results in upregulation of MHC class II and CD86, but not of Siglec-H (Supplemental Fig. 4), thus providing a feedback mechanism by which IFN-α production can be maintained (31).

Regardless of the molecular basis for the differences in IFN-α production between CD9<sup>pos</sup>pDC and CD9<sup>neg</sup>pDC, the sequestering of the most potent IFN-α--producing cells away from peripheral tissues during the steady state may provide a mechanism that helps avoid the development of autoimmune disease. In contrast, activation of CD9<sup>pos</sup>pDC during infection may not only induce their secretion of IFN-α, but may also induce their migration to LN and/or sites of infection.

In a recent report (22), mouse pDC were divided into functionally distinct subsets based on their expression of CCR9, which correlated with the capacity to home to the small intestine (32). Our results show that the CCR9<sup>-</sup>pDC are contained in, and correspond to, the CD9<sup>neg</sup>pDC. Interestingly, we find abundant CCR9 in the cytoplasm of CD9<sup>pos</sup>pDC, which would explain the rapid appearance of this molecule on the surface of cultured or injected CD9<sup>pos</sup>CCR9<sup>-</sup>pDC. Although experiments described in the earlier report suggested that CCR9<sup>-</sup> and CCR9<sup>-</sup>pDC were equally capable of producing IFN-α, the CpG oligonucleotide (1826) used in that study to activate pDC is not a strong inducer of IFN-α production (Fig. 1C) and, therefore, probably would not have revealed the differences we observed.

Several other surface Ags that were differentially expressed on the pDC subsets may contribute to their functional differences.

CD9, a coreceptor for MHC class II on DC (17), likely plays a role in the enhanced T cell stimulation by the CD9<sup>pos</sup>pDC. PDCA-1 and G120.8, two pDC-related molecules recognizing the BM stromal cell Ag-2 (33) are also upregulated upon pDC maturation. The function of BM stromal cell Ag-2 is at the present time unknown, although it has been postulated to participate in the downregulation of IFN-α secretion (33). ICOS-L and OX40-L, both T cell costimulatory molecules, are higher on the CD9<sup>pos</sup>pDC population and are likely to contribute to the superior ability of this subset to activate T effector cells. In contrast, expression of CD4 and CD8α is highest on CD9<sup>neg</sup>pDC and does not appear to correlate with T cell stimulatory capacity, as reported for myeloid DC (34). e<sup>4β7</sup> and CD62L, molecules involved in homing to the small intestine and to peripheral LNs, respectively, are highly expressed on the CD9<sup>pos</sup>pDC.

Our data show that CD9<sup>pos</sup>pDC can induce potent antitumor immunity. Conversely, the CCR9<sup>-</sup>pDC (22), which correspond to the CD9<sup>neg</sup>pDC subset, can protect against graft versus host disease, which is consistent with our finding that CD9<sup>neg</sup>pDC induce Foxp3<sup>+</sup> Tregs in our tumor model. As recently shown (35), ablation of all DC in vivo in the steady state leads to uncontrolled autoimmunity. Although we do not have any evidence at present that the pDC subsets described in this paper contribute to disease pathogenesis, it is tempting to speculate that the CD9<sup>pos</sup>pDC may participate in the development of autoimmune diseases [e.g., systemic lupus erythematosus, psoriasis, and diabetes (10–12)] in which IFN-α has been implicated. Conversely, accumulation of mature CD9<sup>neg</sup>pDC in tumors may have negative clinical consequences, as our data suggest that these cells induce the formation of Foxp3<sup>+</sup>Tregs in tumor-draining LN. A number of published studies indicate that tumor prognosis is inversely related to the number of tumor-infiltrating pDC and Tregs (36–38). Thus, despite the fact that CD9<sup>pos</sup>pDC can induce strong antitumor immunity, using these cells, or pDC TLR agonists, for the treatment of cancer presents a risk that their maturation into tolerogenic cells may contribute to tumor growth.

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Disclosures
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