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9-*O*-acetyl sialic acid levels identify committed progenitors of plasmacytoid dendritic cells

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Abstract

The origins of plasmacytoid dendritic cells (pDCs) have long been controversial and progenitors exclusively committed to this lineage have not been described. We show here that the fate of hematopoietic progenitors is determined in part by their surface levels of 9-*O*-acetyl sialic acid. Pro-pDCs were identified as lineage negative 9-*O*-acetyl sialic acid low progenitors that lack myeloid and lymphoid potential but differentiate into pre-pDCs. The latter cells are also lineage negative, 9-*O*-acetyl sialic acid low cells but are exclusively committed to the pDC lineage. Levels of 9-*O*-acetyl sialic acid provide a distinct way to define progenitors and thus facilitate the study of hematopoietic differentiation.

Key words: 9-*O*-acetyl sialic acid, hematopoietic progenitors, plasmacytoid dendritic cells

Introduction

A prevailing model of hematopoiesis proposes that a multipotent and self-renewing hematopoietic stem cell (HSC) gives rise to either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs), making an irrevocable choice between myelo-erythroid and lymphoid fates (Kondo et al. 1997; Akashi et al. 2000). Several studies have recognized, however, that early progenitor pools are comprised of heterogeneous cells with often multipotent lineage capacity that cross the myelo-erythroid/lymphoid boundary; these have challenged such an obligatory myeloid vs. lymphoid split (Mebius et al. 2001; Chi et al. 2011; Luc et al. 2012; Bell et al. 2008; Wada et al. 2008).

Single cell cloning studies have helped dissect the heterogeneity of progenitor populations and underscore the difficulty in identifying specific progenitors for many hematopoietic lineages, including plasmacytoid dendritic cells (pDCs) (Naik et al. 2013; Yamamoto et al. 2013). These latter cells specialize in type I interferon (IFN)

production that is crucial to the antiviral response, but may also contribute to the pathogenesis of autoimmune diseases like psoriasis and lupus. In addition, pDCs can induce central and peripheral tolerance (Liu 2005; Swiecki and Colonna, 2010; Reizis et al. 2011). In spite of their critical roles in the immune system, the molecular and cellular bases for pDC development remain poorly understood.

Common dendritic cell progenitors (CDPs) that only give rise to conventional dendritic cells (cDCs) and pDCs have been defined among the descendants of CMPs (Fig 1A) (Onai et al. 2007; Naik et al. 2007; Liu et al. 2009). In cell transfer studies, CDPs were seven times more effective at differentiating into cDCs than pDCs (Onai et al. 2007). Single cell cloning of CDPs revealed their skewed heterogeneity as the largest number of clones gave rise to only cDCs, a smaller fraction to cDCs and pDCs and the fewest to pDCs only (Naik et al. 2007). While CDPs differentiate into committed precursors of cDCs (pre-cDCs) that lack pDC potential, attempts to identify committed progenitors of pDCs downstream of these cells have been

unsuccessful (Liu et al. 2009; Naik et al. 2006; Schlitzer et al. 2011; Schlitzer et al. 2012). “CDP-like” cells are very similar to CDPs and have greater pDC potential than CDPs; however, approximately 50% of the progeny of CDP-like cells are cDCs (Onai et al. 2013), underscoring the absence so far of any defined progenitor that exclusively gives rise to pDCs (Figure 1A). That CDPs, which are myeloid in origin, may not be the major source of pDCs is also supported by a lineage-tracing study demonstrating CDPs to be largely cDC-restricted precursors, lacking pDC potential (Schraml et al. 2013).

A lymphoid origin for pDCs has also been proposed. In side-by-side adoptive transfer experiments, CLPs gave rise to more pDCs than CDPs (Onai et al. 2007). Estrogen treatment that effectively depleted lymphoid progenitors, however, did not affect pDC development (Harman et al. 2006). More recent work from Rodrigues et al. (2018) suggest that the majority of pDCs develop from IL-7R⁺ lymphoid progenitors.

We have used a separate unbiased approach to the study of the origins of pDCs that involves the analysis of a post-synthetic modification of sialic acid (Sia) on hematopoietic cells, namely acetylation at the 9-OH position (9-O-AcSia) (Figure S1A) (Pillai et al. 2012).

The *in vivo* biological function of 9-O-AcSia has been explored in lymphocytes, where it has been linked to maturation of thymocytes (Krishna et al. 1997); low levels of 9-O-AcSia have also been described on B cells (Cariappa et al. 2009). One tool utilized to detect 9-O-acetyl Sia is a reagent, termed “CHE-FcD”, which is a fusion protein of the Fc portion of human IgG with the Influenza C hemagglutinin, catalytically inactivated by diisopropylfluorophosphate and that binds specifically to 9-O-acetyl Sia (Klein et al. 1994).

We show that levels of 9-O-AcSia segregate early Lin[−] bone marrow (BM) progenitors into two large pools that, as we demonstrate, represent a new approach to dissecting the heterogeneity of hematopoietic progenitors and have the potential to provide new insights regarding hematopoiesis. *In vivo* studies of the 9-O-AcSia^{Lo} pool of BM progenitors delineated unique sequential progenitors of pDCs that are not linked to either CLPs or CMPs and include a precursor exclusively committed to pDC development.

Results

9-O-AcSia levels define hematopoietic cell progenitor pools

Flow cytometric analysis (FCA) of wild-type murine splenocytes stained with CHE-FcD showed that CD11c^{+/Lo}CD11b[−]B220⁺PDCA-1⁺Siglec-H⁺ pDCs, like B lymphocytes, exhibit low levels of 9-O-AcSia, while CD11c^{Hi} cDCs and other myeloid cells express high levels of this carbohydrate modification (Figure 1B and C). Further inspection revealed that distinct levels of 9-O-AcSia correlate with functionally distinct cDC subsets: cross-presenting CD8α⁺ cDCs stained with lower intensity for CHE-FcD compared to the high levels observed on CD4⁺ and CD4[−]CD8α[−] cDCs (Figure S1B).

We considered that low levels of 9-O-AcSia on pDCs and lymphoid cells vs. high levels observed in myeloid cells including cDCs might reflect their respective origins. BM progenitor populations were surveyed for levels of 9-O-AcSia. CHE-FcD staining of the “LSK” HSC compartment (Lin[−]Sca-1^{Hi}c-Kit^{Hi}), comprised of long-term HSCs and short-term HSCs with multi-lineage potential (Wilson et al. 2006; Hardy et al. 1991; Taylor et al. 2005), revealed intermediate surface levels of 9-O-AcSia (Figure 2A). In contrast, the vast majority of CLPs expressed low levels of 9-O-AcSia, similar to those observed on their B-cell progeny and pDCs (Figure 2B). Most CMPs, on the other hand, resembled their myeloid progeny including cDCs

and exhibited robust levels of 9-O-AcSia (Figure 2C). Furthermore, the majority of macrophage-dendritic cell progenitors (MDPs) that are immediate descendants of CMPs (Fogg et al. 2006) as well as most CDPs, which derive directly from MDPs (Liu et al. 2009), maintained these high levels (Figure 2D and E). The similar CHE-FcD staining of MDPs and CDPs is consistent with their highly overlapping phenotypes that differ only in the degree of c-Kit expression (Auffray et al. 2009). A sizable fraction of CDP-like cells defined by Onai et al. (2013) had high levels of 9-O-AcSia consistent with the considerable cDC potential that remains in this pool (Figure 2F). Although these progenitor pools are heterogeneous with respect to 9-O-AcSia levels and developmental potential, there appears to be a correspondence between the relative proportion of cells with low 9-O-AcSia levels and pDC potential.

We wished to determine if BM progenitors with low levels of 9-O-AcSia might be predisposed to pDC development. CHE-FcD staining of the Lin[−]Sca-1^{Lo} pool revealed clearly distinguishable 9-O-AcSia^{Lo} and 9-O-AcSia^{Hi} cells (Figure 3A). To test if the Lin[−]Sca-1^{Lo}9-O-AcSia^{Lo} cells contained progenitors that preferentially gave rise to pDCs, we used fluorescent activated cell sorting to isolate these cells from the BM and examined their differentiation potential. Of note, this pool includes CLPs and the minor group of CMPs with low 9-O-AcSia. The cells were cultured *in vitro* with Flt3L, a cytokine critical for DC development, and with which supplementation of BM cultures generates only pDCs and cDCs.¹³ As shown in Figure 3B, the Lin[−]Sca-1^{Lo}9-O-AcSia^{Lo} BM progenitors differentiated substantially into pDCs. CD11c⁺ cells from parallel cultures of Lin[−]Sca-1^{Lo}9-O-acetyl^{Hi} cells, however, were almost exclusively cDCs, and CDPs generated four times fewer pDCs than the Lin[−]Sca-1^{Lo}9-O-acetyl^{Lo} cells (Figure 3C and D). Taken together, the data suggest that exclusive precursors of pDCs might exist in the Lin[−]Sca-1^{Lo}9-O-acetyl^{Lo} BM progenitor pool.

Identifying exclusive precursors of pDCs

We considered that the Lin[−]Sca-1^{Lo}9-O-acetyl^{Lo} pool might be enriched for a spectrum of pDC progenitors, since all the cells in this population did not express lineage markers, including B220 and CD11c, at levels that are found on mature pDCs. We reasoned that fully committed precursors of pDCs in this pool might express low levels of the pDC-specific markers, Siglec-H (Blasius et al. 2004; Blasius et al. 2006; Zhang et al. 2006) and PDCA-1 (Blasius et al. 2006). Siglec-H is found only on the surface of murine pDCs; PDCA-1, however, has been described on certain B-cell subsets (Bao et al. 2011; Vinay et al. 2012) limiting its use to discriminating pDCs from cDCs.

Our strategy to identify committed pDC progenitors was to interrogate expression of Siglec-H and PDCA-1 in conjunction with 9-O-AcSia levels in the Lin[−]Sca-1^{Lo} BM progenitor pool. FCA revealed a small group of cells that co-expressed low levels of both Siglec-H and PDCA-1 among progenitors with the lowest 9-O-AcSia levels (Figure 3E). Comparison of Siglec-H and PDCA-1 levels between these cells and mature BM pDCs confirmed that they were distinct populations (Figure 3F). We next investigated whether these Siglec-H^{Lo}PDCA-1^{Lo} cells represented a fully committed pDC precursor population.

We injected freshly sorted Lin[−]Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H^{Lo} BM cells (Figure 4) from CD45.2 donor mice into sub-lethally irradiated CD45.1 recipients and analyzed all transferred cells 9–10 days later. We found that the transferred Siglec-H^{Lo} cells gave rise exclusively to pDCs (Figure 4, upper panel; Figure S7C), but not to B220[−]CD11c^{Hi} cDCs (Figure 4, upper panel). Markers used to

Fig. 1. Low surface levels of 9-O-AcSia distinguish pDCs from cDCs and other myeloid cells. **(A)** A current model for pDC development. **(B)** CHE-FcD profiles of pDCs, cDCs and B cells. **(C)** CHE-FcD staining of pDCs and other myeloid cells [RPM, neutrophil (PMN), monocyte (Mo)]. Splenocytes from 10-week-old WT mice were stained with CHE-FcD and surface phenotype markers and then examined by FCA. Data are representative of three independent experiments. See also Figure S1.

identify pDCs included B220, which is not found on cDCs, and Siglec-H, which is thought to be expressed exclusively on pDCs. Further analyses failed to reveal other myeloid or lymphoid cells among any of the donor-derived cells in the spleen (Figure 4, lower panel). Markers not found on pDCs were used to identify other lineages. Ly6G was used to identify granulocytes, as antibodies to the commonly used Gr.1 marker also cross-react with Ly6C, a known marker of pDCs (Asselin-Paturel et al. 2001; Dalod et al. 2002; Wrangmert et al. 2002). These studies demonstrate that Lin[−]Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H^{Lo} BM cells represent a novel precursor-pDC (pre-pDC) population that is fully committed to pDC development.

pDCs have been identified among the progeny of CDPs and MDPs, although these progenitors are far more effective at generating cDCs (Onai et al. 2007; Naik et al. 2007; Liu et al. 2009; Auffray et al. 2009). We searched for CDPs and MDPs within the pre-pDC population to confirm that the latter is a distinct cell type unrelated

to these cDC progenitors. MDPs and CDPs are rigorously defined to co-express Flt3 and M-CSFR; only a small minority of pre-pDCs expressed both of these, of which an even smaller fraction expressed c-Kit at levels found on these progenitors (Figure S2A). This result is consistent with the robust 9-O-AcSia levels found on most myeloid progenitors (Figure 2C–E), which are minimally represented in the 9-O-AcSia^{Lo} pool that houses pre-pDCs. Siglec-H^{Lo} pre-pDCs are also distinct from CDP-like cells that lack expression of Siglec-H (Onai et al. 2013). In addition, pre-pDCs were defined in the Lin[−]BM fraction that lacks CD11c expression and are therefore distinct from CD11c^{int} pre-cDCs that are committed cDC precursors (Liu et al. 2009; Naik et al. 2006; Diao et al. 2006). Moreover, while pre-cDCs, which are derived from CDPs in the BM, circulate through the blood to secondary lymphoid organs prior to completing their differentiation into mature cDCs, cells with the pre-pDC phenotype could not be found in the spleen (Figure S2B). Taken together, the

Fig. 2. Low surface levels of 9-O-AcSia separate Lin⁺ hematopoietic progenitors into distinct pools. (A–F) CHE-FcD staining of BM progenitors [(A) LSK HSC, (B) CLP, (C) CMP, (D) MDP, (E) CDP, (F) CDP-like cell]. BM cells from 10-week-old WT mice were stained with CHE-FcD and surface phenotype markers and then examined by FACS. Data are representative of three independent experiments

data suggest that commitment to the pDC lineage is distinct from the myeloid process of cDC development.

Pre-pDCs are distinct from mature pDCs

Pre-pDCs are phenotypically distinct from mature pDCs based in part on levels of staining for the pDC markers Siglec-H and PDCA-1 (Figure 3F), but we sought to determine if they exhibited other defining features of pDCs. A functional hallmark of pDCs is their rapid and massive production of type I IFN when triggered through nucleic-acid sensing TLRs such as TLR7 and TLR9 (ten Boekel et al. 1995; Barchet et al. 2002; Cao et al. 2007). We compared the ability of BM pre-pDCs and mature pDCs to make type I IFN in vivo in response to intravenous injection with CpG, a TLR9 agonist. Intracellular staining for IFN- α was observed in a significantly greater fraction of pDCs than in pre-pDCs underscoring that the latter have not yet acquired the full functional capacity of mature pDCs (Figure S3A and B). Electron microscopy revealed that similar to mature pDCs, pre-pDCs have multiple mitochondria and contain nuclei with marginal heterochromatin (Figure S3C, left panel); mature pDCs, however, also have an abundance of lysosomes and vacuoles as well as more extensive endoplasmic reticulum (Figure S3C, right panel) that is a characteristic morphological adaptation of mature pDCs (Liu 2005; Bjorck et al. 2011). Taken together, these studies suggest that pre-pDCs and mature pDCs are discrete cells with distinct phenotypic, functional and structural attributes.

PDC progenitors lack myeloid potential

We identified pre-pDCs as cells exclusively committed to pDC development that expressed low levels of Siglec-H in the pool of early

BM progenitors with low levels of 9-O-AcSia. We reasoned that the larger Siglec-H⁺ fraction of this pool (Figure 5A) might also contain a progenitor pDC population from which fully committed pre-pDCs arise. To investigate the pDC potential of the Siglec-H⁺ subset, we performed in vivo transfers with these cells. Freshly sorted Lin⁺Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁺ from CD45.2 mice was injected into CD45.1 recipients, and donor cells were analyzed. Siglec-H^{Lo} pre-pDCs were identified among donor-derived progeny in the BM at d 4 post-transfer (Figure 5B). Further examination of the BM and the spleen revealed that transferred cells differentiated preferentially into mature pDCs (Figure 5C and D) that had low 9-O-AcSia levels similar to their endogenous counterparts (Figure S4A). The Siglec-H⁺ cells did not give rise to pre-cDCs (Figure 5C) or cDCs (Figure 5D). These data indicate that the Siglec-H⁺ fraction of the Lin⁺Sca-1^{Lo}9-O-AcSia^{Lo} BM pool contains progenitors of Siglec-H^{Lo} pre-pDCs that further differentiate into mature pDCs.

Evaluation at later time points (d 8) revealed that donor cells still differentiated preferentially not only into pDCs (Figures S5A and S7C), but also into other myeloid and lymphoid cells, including cDCs (Figure S5A), granulocytes, NK cells and T cells (Figure S5B). Lin⁺Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁺ cells are thus a heterogeneous pool that is biased toward pDC development.

We devised an approach to exclude the residual cDC potential from the Lin⁺Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁺ progenitor pool (Figure S5A). Our strategy was based on the distinct cytokine requirements for pDC vs. cDC development, and our phenotypic analysis of pre-pDCs. Flt3L and its receptor Flt3 are essential for cDC and pDC development; DC potential is restricted to the Flt3⁺ fractions of CLPs and CMPs in transfer experiments, and Flt3L supplementation of BM cultures drives differentiation of pDCs and cDCs only

Fig. 3. Identification of putative Siglec-H^{Lo}PDCA-1^{Lo} pDC progenitors in Lin⁻Sca-1^{Lo}9-O-AcSia^{Lo} BM pool. (A) CHE-FcD staining of the total pool of non-HSC early BM progenitors (Lin⁻Sca-1^{Lo}). (B) In vitro DC differentiation from sorted 9-O-AcSia^{Lo} Lin⁻Sca-1^{Lo} and 9-O-AcSia^{Hi} Lin⁻Sca-1^{Lo} cells. Data is shown at time of peak pDC differentiation (d 6). A total of 100,000 9-O-AcSia^{Lo} cells in culture gave rise to mean of 22,028 pDCs and a mean of 55,028 cDCs. In contrast, 100,000 9-O-AcSia^{Hi} cells gave rise to 738 pDCs and 45,739 cDCs. (C) In vitro DC differentiation from sorted CDPs. Cells (1 × 10⁵) were cultured with murine Flt3L (100 ng/mL) and absolute numbers of DCs were determined on d 2, 4, 6, 8 and 10. Data is shown at time of peak pDC differentiation (d 6). A total of 100,000 CDPs in culture gave rise to a mean of 6,283 pDCs and 50,508 cDCs. (D) Summary of data in (B) and (C) showing mean and standard error (n = 3) of pDC vs. cDC generation from cultured progenitors. The pDC/cDC ratios depicted are derived from absolute numbers of pDCs (i.e., "p") and cDCs (i.e., "c") as shown. *P < 0.05, **P < 0.01, ***P < 0.001. (E) FCA for co-expression of Siglec-H and PDCA-1 among Lin⁻Sca-1^{Lo} BM cells stratified by 9-O-AcSia levels. (F) Siglec-H and PDCA-1 staining of total Lin⁻ cells and of mature pDCs. Data in (A–C) and (E–F) are representative of three independent experiments.

Fig. 4. Lin[−]Sca-1^{Lo}9-*O*-AcSia^{Lo}Siglec-H^{Lo} BM cells represent pre-pDCs— exclusive precursors of pDCs. 1×10^3 sorted Lin[−]Sca-1^{Lo}9-*O*-AcSia^{Lo}Siglec-H^{Lo} cells from the BM of CD45.2⁺ mice were intravenously (i.v.) injected into sub-lethally irradiated CD45.1⁺ mice and donor-derived cells were examined at time points between 4 and 14 d. FCA of (upper panel) pDCs and cDCs, and (lower panel) non-DC cell types among donor-derived cells. Data is shown in the spleen for d 9 when the number of progeny cells peaked. Percentages are of donor-derived (CD45.2⁺CD45.1[−]) cells. Data are representative of three independent experiments. See also [Figures S2, S3](#) and [S7C](#).

(Schmid et al. 2010). In addition to the role of Flt3, there is a presumed dependence of cDC and myeloid cell development on the M-CSFR (CD115) (Onai et al. 2007; Naik et al. 2007). Injected BM Lin[−]CD115⁺ cells give rise to spleen cDCs and marginal zone macrophages, but not to pDCs (Waskow et al. 2008).

Our analysis revealed that most Siglec-H^{Lo} pre-pDCs express Flt3, but not CD115 (Figure S2A), the majority of Lin[−]Sca-1^{Lo}9-*O*-AcSia^{Lo}Siglec-H[−] cells express only Flt3 or CD115 (Figure S4B), and cDCs express much higher levels of CD115 than pDCs (Figure S4C). Based on these findings, we reasoned that the Flt3⁺CD115[−] fraction of the Lin[−]Sca-1^{Lo}9-*O*-AcSia^{Lo}Siglec-H[−] BM progenitor pool might

contain pDC progenitors that are precursors of pre-pDCs and devoid of cDC potential. We transferred freshly sorted Flt3⁺CD115[−] or Flt3[−]CD115⁺ cells from the Lin[−]Sca-1^{Lo}9-*O*-AcSia^{Lo}Siglec-H[−] pool of CD45.2 mice into CD45.1 recipients. Donor cells were analyzed to compare the ability of Flt3⁺CD115[−] and Flt3[−]CD115⁺ cells to generate pre-pDCs and pDCs. At early time points in the BM (d 4), we found that Flt3⁺CD115[−] cells were more effective at giving rise to pre-pDCs than their Flt3[−]CD115⁺ counterparts (Figure 6A). In line with this data, Flt3⁺CD115[−] cells gave rise exclusively to pDCs, but not to cDCs (Figure 6B and Figure S7C) at later time points (d 8) in the spleen. In contrast, Flt3[−]CD115⁺ cells lacked pDC potential (Figure 6B). Analysis of the Flt3⁺CD115[−] cells for non-DC

Fig. 5. Lin⁻Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁻ cells give rise to pre-pDCs and pDCs. (A) 1×10^4 sorted Lin⁻Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁻ cells from the BM of B6 mice (CD45.2⁺) were intravenously injected into sub-lethally irradiated B6.SJL mice (CD45.1⁺), and donor derived cells were examined at time points between 4 and 14 d. Percentages are of donor-derived (CD45.2⁺CD45.1⁻) cells. Data are representative of three independent experiments. (B) Pre-pDC potential of Lin⁻Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁻ donor cells in the BM. (C) Examination of Lin⁻Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁻ donor progeny at early time points for pDCs and exclusive precursors of cDCs (pre-cDCs). (D) pDC and cDC potential of Lin⁻Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁻ donor cells at early time points in the spleen. See also [Figures S4 and S5](#).

lineage potential demonstrated that over time (d 10–d 14), these cells maintained the ability of the broader Lin⁻Sca-1^{Lo}9-O-acetyl^{Lo}Siglec-H⁻ pool to give rise to NK, B and T cells, but lacked any myeloid potential ([Figure 6C](#)).

These findings indicate that the Flt3⁺CD115⁻ members of the Lin⁻Sca-1^{Lo}9-O-acetyl^{Lo}Siglec-H⁻ BM pool are progenitors of pre-pDCs and mature pDCs that lack potential for cDCs or any other

myeloid cells. Previous attempts to separate pDC from cDC development have exploited disparate expression patterns of growth factor receptors but have been unsuccessful at identifying committed pDC progenitors ([Onai et al. 2013](#)). Our data reveal, however, that identification of commitment to the pDC lineage and the concomitant absence of cDC potential requires stratifying BM progenitors by levels of 9-O-AcSia.

Fig. 6. Lin⁻Sca-1^{Lo}9-*O*-AcSia^{Lo}Siglec-H⁺ Flt3⁺CD115⁻ BM cells give rise to pre-pDCs and to pDCs, but not to cDCs or any other myeloid cells. 1×10^4 sorted Flt3⁺CD115⁻ or Flt3⁺CD115⁺ cells from the Lin⁻Sca-1^{Lo}9-*O*-AcSia^{Lo}Siglec-H⁺ BM pool of CD45.2⁺ mice were i.v. injected into CD45.1⁺ recipients and donor-derived cells were examined at time points between 2 and 14 d. Percentages are of donor-derived cells. Data are representative of three independent experiments. (A and B) Donor-derived cells examined for (A) pre-pDCs in the BM (d 4) and (B) pDCs and cDCs in the spleen (d 8) of recipients. (C) Donor-derived cells assessed for non-DC lineage potential in animals receiving Flt3⁺CD115⁻ cells. Data on d 14 is shown for animals that received 4×10^4 cells. See also Figure S7C.

PDC and lymphoid progenitors are distinct

We identified Siglec-H⁺Flt3⁺CD115⁻ cells in the 9-*O*-AcSia^{Lo} BM progenitor pool devoid of cDC and myeloid potential, but these cells were still a heterogeneous group that gave rise to lymphoid cells (Figure 6C). This result was not unexpected since most CLPs are

9-*O*-AcSia^{Lo} (Figure 2B) and would be expected to overlap with this pool. IL-7R-mediated signals play a non-redundant role in murine T- and B-cell development, and IL-7R expression was a key marker in the original studies that identified CLPs (Kondo et al. 1997). IL-7 signaling is not required for pDC development or maintenance as

demonstrated in IL-7R $\alpha^{-/-}$ and IL-7 $^{-/-}$ mice (Yang et al. 2005; Vogt et al. 2009), and IL-7R $\alpha^{-/-}$ and wild type (WT) donor cells reconstitute the pDC compartment equally well in sub-lethally irradiated mice (Takeuchi et al. 2006). On the other hand, recent studies by Rodrigues et al. (2018) showed that pDC potential was significantly greater from IL-7R $^{+}$ than from IL-7R $^{-}$ progenitors, and in vitro experiments identified a precursor committed to pDC development in the former pool. In their investigations, however, pDCs were identified as CD45RA $^{+}$ PDCA-1 $^{+}$ cells. While PDCA-1 is enriched on pDCs, it is also found on several subpopulations of B lymphocytes (Bao et al. 2011; Vinay et al. 2012), the latter of which would be expected to be found among the progeny of IL-7R $^{+}$ cells.

In our own studies, we noted that most Lin $^{-}$ Sca-1 Lo 9-O-AcSia Lo Siglec-H $^{-}$ Flt3 $^{+}$ CD115 $^{-}$ cells, as well as pre-pDCs and mature pDCs, do not express IL-7R α (Figure 7A and Figure S6A), and we wished to determine whether pDC developmental potential could be ascribed to a more circumscribed pool of progenitors distinct from IL-7R α^{+} CLPs. We used adoptive transfers to compare the lineage potential of IL-7R α^{-} and IL-7R α^{+} Lin $^{-}$ Sca-1 Lo 9-O-AcSia Lo Siglec-H $^{-}$ Flt3 $^{+}$ CD115 $^{-}$ cells, in which CLPs would be restricted to the latter group of cells. Inspection of donor-derived progeny revealed that IL-7R α^{-} cells maintained the characteristics of the broader Lin $^{-}$ Sca-1 Lo 9-O-AcSia Lo Siglec-H $^{-}$ Flt3 $^{+}$ CD115 $^{-}$ pool and gave rise to pre-pDCs at early time points (Figure 7A, upper panel) and to pDCs at later time points (Figure 7A, lower panel; Figures S6B and S7C), without giving rise to cDCs (Figure S6D). In contrast, their IL-7R α^{+} counterparts lacked the ability to give rise to pDCs (Figure 7A, lower panel). Additional analyses revealed that IL-7R α^{-} cells continued to show robust pDC potential at later time points (Figure S6C). This data suggests that pDC potential can be attributed to the IL-7R α^{-} group of Lin $^{-}$ Sca-1 Lo 9-O-AcSia Lo Siglec-H $^{-}$ Flt3 $^{+}$ CD115 $^{-}$ cells that is completely distinct from conventionally defined IL-7R α^{+} CLPs.

Assessment for other lineage potential, however, revealed that at the time of maximum pDC potential (d 8), IL-7R α^{-} cells also gave rise to a small population of NK cells, while IL-7R α^{+} cells had differentiated into B and T lymphocytes (Figure S7A). The latter is consistent with the CLP potential of the IL-7R α^{+} transferred pool. Several days later, IL-7R α^{-} cells gave rise to B and T lymphocytes (Figure S7B), potentially by traversing an intermediate IL-7R α^{+} CLP stage. Based on these results, we term this heterogeneous pool of Lin $^{-}$ Sca-1 Lo 9-O-AcSia Lo Siglec-H $^{-}$ Flt3 $^{+}$ CD115 $^{-}$ IL-7R α^{-} cells that is broadly committed to pDC development and separate from conventionally defined IL-7R α^{+} CLPs, as progenitor-pDCs (pro-pDCs).

These adoptive transfer studies demonstrate separate cellular origins for pDCs and lymphoid cells. We aimed to determine, however, if these developmental pathways might be differentiated at a molecular level. B lymphocyte differentiation absolutely requires the introduction of indelible genetic rearrangements through the process of VDJ recombination at the *IgH* locus. Examination of pDCs revealed the occurrence of D-J rearrangements at the *IgH* locus in a minority of these cells (Corcoran et al. 2003; Shigematsu et al. 2004; Harman et al. 2006; Onai et al. 2013), lending support to a lymphoid affiliation. Complete VDJ events necessary to create a complete antigen receptor heavy chain are never observed, however, bringing into question the potential function of this type of recombination in pDCs, which are innate immune cells. Moreover, D-J rearrangements cannot always be demonstrated in mature pDCs (Pelayo et al. 2005; Sathe et al. 2013; Schlitzer et al. 2011). These inconsistencies, as well as those in studies of pDC origin from lymphoid progenitors, are likely due to the difficulty in isolating pure pDCs. While B220, PDCA-

1 (as noted above) and CD11c are all markers used routinely in pDC purification schemes, each is also found on B cells (Bao et al. 2011; Vinay et al. 2012; Coffman et al. 1981; Rubtsov et al. 2011).

We examined pro-pDCs, pre-pDCs and mature pDCs (purified using low 9-O-AcSia as an additional marker for stringency) and found that these cells lacked D-J rearrangements that were clearly detectable in both pro-B and mature B cells and absent from neutrophils (Figure 7B). We also studied Rag2-GFP mice and confirmed that Rag-2 is expressed in pro-B cells but not in pre-pDCs or pDCs (Figure 7C). These results also suggest that the pDC and lymphoid pathways of development are distinct. They also emphasize that the tight definition of pDCs using low levels of sialic acid 9-O-acetylation in combination with Siglec-H expression unequivocally establishes that pDCs are not of lymphoid origin.

Pro-pDCs, pre-pDCs and mature pDCs differentially express key pDC genes

To further characterize pro- and pre-pDCs and their relationship to mature pDCs, we interrogated the expression of key genes implicated in pDC development, migration and function (reviewed in Swiecki and Colonna 2010) in these populations (Figure 8; Table S1). Hierarchical clustering analysis of the three populations revealed two main branches, one with mature pDCs and the other with two distinct subgroups, comprised of pro- and pre-pDCs, respectively.

Consistent with their unique commitment to pDC development, pre-pDCs expressed the highest levels of genes required for pDC differentiation. These include *Tcf4*, which encodes the basic helix-loop-helix transcription factor, E2-2, essential for pDC differentiation in mice and humans (Cisse et al. 2008; Ghosh et al. 2010) as well as the E2-2 transcriptional target *Irf8* (Schiavoni et al. 2002) and *Ikaros* (Allman et al. 2006), whose deficiencies result in the absence of pDCs.

We also found that the expression of critical cDC-lineage genes decreased over the course of pDC development. These included *Batf3* (Hildner et al. 2008; Edelson et al. 2010) and *Id2*, (Cisse et al. 2008; Hacker et al. 2003) which support CD8 α^{+} and CD103 $^{+}$ cDC development, the latter of which also represses E2-2 to inhibit pDC development. *Batf3* expression decreased in the transitions from pro-pDCs to pre-pDCs and pre-pDCs to mature pDCs, while *Id2* levels declined in the latter.

In line with the importance of the Flt3 pathway in promoting pDC development from progenitors (Schmid et al. 2010), *Flt3* and transcription factors, *Sfp1*, which controls Flt3 expression in a dose-dependent manner (Carotta et al. 2010) and *Stat3*, a downstream mediator of Flt3 signaling (Laouar et al. 2003) that stimulates E2-2 expression (Li et al. 2012), were expressed at similar, higher levels in pro-pDCs and pre-pDCs compared to mature pDCs.

Expression of genes that regulate pDC trafficking reflected the fact that while mature pDCs migrate from the BM to the periphery, pro- and pre-pDCs reside primarily in the BM and pre-pDCs cannot be isolated from the periphery (Figure S2B). *SpiB*, which supports differentiation (Schotte et al. 2004) and BM retention of pDCs (Sasaki et al. 2012) was expressed at higher levels in pro-pDCs and pre-pDCs vs. mature pDCs, while *Runx2*, which drives expression of chemokine receptors CCR2 and CCR5 to enable pDC migration from the BM to the periphery (Sawai et al. 2013) and *Cxcr3*, which modulates pDC migration to inflamed tissues (Krug et al. 2002; Diacovo et al. 2005) were expressed at higher levels in mature vs. pro- and pre-pDCs.

Expression of key pDC functional genes corroborates our results demonstrating the greater functional capacity of mature vs. pre-pDCs (Figure S3). Levels of genes involved in type I IFN production

Fig. 7. Separate progenitors develop into pDCs and lymphocytes. (A) 2×10^3 IL-7Rα⁺ or IL-7Rα⁺ cells from the Lin⁺Sca-1^{Lo}9-O-AcSia^{Lo}Siglec-H⁺Flt3⁺CD115⁺ BM pool of CD45.2⁺ mice were injected into CD45.1⁺ recipients and donor progeny were examined. Percentages are of donor-derived (CD45.2⁺CD45.1⁺) cells (Upper panel) recipients of IL-7Rα⁺ cells were examined for pre-pDCs. (Lower panel) IL-7Rα⁺ and IL-7Rα⁺ donor progeny examined for pDCs. (B) D-J rearrangement of the *IgH* gene assessed by genomic PCR in pDCs and their progenitors. PMNs served as a negative control and pro-B and B cells served as positive controls. (C) Rag2 expression in pDCs and their progenitors in Rag2-GFP mice. Pro-B cells served as a positive control for Rag2 expression. GFP levels in pro-B cells from wild-type animals are representative of background staining in all populations shown. Data in (A–C) are representative of three independent experiments. See also Figures S6 and S7.

including *Tlr9* and *Prkca* (Esashi et al. 2012) increased in transitions from pro-pDCs to pre-pDCs and pre-pDCs to mature pDCs, while *Blnk* (Cao et al. 2007) levels increased in the latter. Expression of *Ciita*, an E2-2 target that promotes MHC class II expression (LeibundGut-Landmann et al. 2004) also increased from pre-pDCs to mature pDCs.

Gene expression of characteristic pDC markers including *Siglech*, *Bst2* (PDCA-1) and *Ccr9* is greater on mature vs. pre-pDCs, reflecting our own phenotypic analyses (Figure 2F). This was also the case for

Klra17 (Bjorck et al. 2011) and *Ly6a* (Sca-1) (Miller et al. 2012), which are expressed heterogeneously on the surface of BM pDCs. Conversely, levels of CD11b, a characteristic marker of certain cDCs and monocytes that is absent from pre-pDCs and mature pDCs (Figure 2E) declined in the transition from pre-pDCs to mature pDCs.

Expression of genes reported to be upregulated in pDCs vs. cDCs including *Cdc14b*, *Cdh1*, *Klk1*, *Lynx*, *Pdcd4* and *Slc40a1* (Miller et al. 2012) increased in the trajectory from pro-pDCs to mature

Fig. 8. Pro- and pre-pDCs are molecularly distinct progenitors of pDCs. Gene expression patterns of pro-pDCs, pre-pDCs and mature pDCs. Heat map of transcripts with differential expression among pro-pDCs, pre-pDCs and mature pDCs (>2 -fold, $P < 0.05$). Relative mRNA expression of key pDC-lineage genes was quantified in pro-pDCs, pre-pDCs and mature pDCs sorted from primary BM cells by quantitative RT-PCR. Results were log transformed and populations and genes were clustered by pairwise centroid linkage with the Pearson correlation. Data are representative of three independent experiments with three replicates each. See also Table S1.

pDCs. Among these, *Cmah* (Varki and Gagneux 2012), an enzyme that catalyzes an irreversible conversion from the Neu5Ac to Neu5Gc type of sialic acid was expressed at higher levels in pre-pDCs and mature pDCs compared to pro-pDCs.

Discussion

Our studies underscore the value of separating BM progenitors into two broad pools: one in which cell surface 9-O-AcSia levels are low and the other in which they are high. This separation facilitates the delineation of progenitors with distinct developmental potentials.

An initial stratification based on 9-O-AcSia levels complements and enhances prevalent cell segregation strategies that rely largely on selective expression of distinct growth factor receptors on the cell surface.

Segregating progenitors using levels of 9-O-acetyl sialic acid was a critical step in identifying progenitors of pDCs. While the use of antibodies to growth factor receptors does contribute to the process of defining specific progenitors, their use alone has previously failed to eliminate cDC potential (Onai et al. 2007; Naik et al. 2007; Liu et al. 2009; Onai et al. 2013). Focusing on cells with low levels of 9-O-acetyl sialic acid not only facilitated the categorization of progenitor populations, but also permitted the stringent definition of pDCs, eliminating some of the long-standing ambiguities about the origins and functions of these cells. Recent studies on human dendritic cell precursors integrating single cell RNA-seq and CyTOF have also revealed the existence of multiple pre-DC populations, reinforcing the notion that our views of pDC and cDC development are still evolving (See et al. 2017).

We have established the existence of two sequential progenitor populations in the 9-O-AcSia^{Lo} pool of Lin[−] hematopoietic cells that comprise the BM stages in the pathway of pDC development. These studies identified unique pro-pDCs that are distinct from myeloid or lymphoid progenitors, and which differentiate into precursors that we call pre-pDCs. These pre-pDCs are wholly committed to the pDC lineage and have no potential for any other cell types, including cDCs. This cellular pathway is schematically described in Figure 9. Our findings, taken together, support a distinct pathway for pDC development whose inception occurs in the pool of BM progenitors with the lowest levels of 9-O-AcSia and in which the earliest progenitors, broadly committed pro-pDCs, are distinct from CLPs and CMPs.

By revealing that pro-pDCs are indeed distinct from CLPs and any progenitors with myeloid potential these data reinforce the notion developed in recent studies that early hematopoietic progenitors may commit to distinct lineages (Naik et al. 2013; Yamamoto et al. 2013) suggesting that “common” progenitors may not be obligate cellular intermediates during the ontogeny of hematopoietic cells.

CasD1 is a 9-O-acetyl transferase that O-acetylates CMP-NANA in the Golgi (Arming et al. 2011; Baumann et al. 2015). We have completely abolished the expression of 9-O-acetyl sialic acid on all cells by knocking out the CasD1 gene in mice (Mahajan et al. 2019). Although higher 9-O-acetyl sialic acid levels broadly separate myeloid progenitors from lymphoid progenitors, knocking out CasD1 results in a total loss of 9-O-acetyl sialic acid expression but reveals no defects in lymphoid or myeloid development, suggesting that the acetylation of sialic acid is a useful marker for progenitors but is not functionally required for hematopoietic cell development.

Following the original submission of this report, a study from Ginhoux and colleagues using a different approach has established the unique nature of pDC precursors (Dresser et al. 2019). These data are fully consistent with the conclusions that we have made.

Materials and methods

Animals

Eight- to twelve-week-old C57Bl/6 (CD45.2) and 10-week-old B6.SJL-Ptprc^hPepc^b/BoyJ (CD45.1) mice were used. *Rag2-Gfp* knockin mice were kindly provided by Duane R. Wesemann (Brigham and Women’s Hospital).

Fig. 9. The pathway of pDC development. In this model, distinct levels of 9-O-AcSia segregate early BM progenitors into two large pools. Progenitors with low levels of this modification include the majority of CLPs, while the pool with high levels includes most CMPs. A three-step cellular sequence for pDC differentiation, distinct from the lymphoid and myeloid streams, can be delineated within the 9-O-AcSia^{Lo} pool. This pathway originates from post-HSC pro-pDCs that are broadly committed to the pDC lineage, but are distinct from CLPs and CMPs. Pro-pDCs give rise to pre-pDCs that are committed to pDC development. Pre-pDCs differentiate exclusively into mature pDCs that maintain the low 9-O-AcSia levels of their progenitors.

Antibodies, staining and flow cytometry

Single cell suspensions were made from spleen and BM (two femurs and two tibias) using standard methodology, with procedural modifications to isolate and enrich mouse DCs (Naik et al. 2007; Vremec 2010).

Multiparameter FCA was performed as previously described (Cariappa et al., 2009).

Populations were gated on as described (see Supplemental Methods for details): BM: LSK HSCs (Wilson and Trumpp, 2006; Yamamoto et al., 2013); CLPs (Kondo et al., 1997); CMPs (Akashi et al., 2000); monocyte and dendritic cell progenitors (MDPs) (Liu et al., 2009); cDC and pDC CDPs (Onai et al., 2007; Naik et al., 2007); CDP-like (Onai et al., 2013); pro-pDCs: Lin⁻ Sca-1^{Lo} 9-O-acetyl^{Lo} Siglec-H⁻ PDCA-1⁻ Flt3⁺ CD115⁻ IL-7Rα⁻; pre-pDCs: Lin⁻ Sca-1^{Lo} 9-O-acetyl^{Lo} Siglec-H^{Lo} PDCA-1^{Lo}; pDCs: Lin⁻ CD11c^{+/Lo} CD11b⁻ B220⁺ PDCA-1⁺ Siglec-H⁺; neutrophil (PMN): CD11b⁺ Ly6G⁺ in the granulocyte gate; FrB/C pro-B (Hardy et al., 1991).

Spleen: B cells: CD3ε⁻ NK1.1⁻ Ly6G⁻ TER119⁻ Siglec-H⁻ CD19⁺ B220⁺; red pulp macrophages (RPMs): Lin⁻ CD11c^{Dim} CD11b^{Dim} F4/80^{Hi} (Taylor et al., 2005); polymorphonuclear leukocytes (PMNs): Lin⁻ CD11c^{Dim} CD11b^{Hi} Ly6G⁺; Monocytes (Mo): Lin⁻ CD11c^{Dim} CD11b^{Hi} Ly6G⁻ CD115⁺ (Idoyaga et al., 2009); cDCs: Lin⁻ CD11c^{Hi} B220⁻ PDCA-1⁻ Siglec-H⁻; pDCs: Lin⁻ CD11c^{+/Lo} CD11b⁻ B220⁺ PDCA-1⁺ Siglec-H⁺.

Ex vivo detection of 9-O-acetylated sialic acid on murine cells

The CHE-FcD probe was generated as previously described (Krishna and Varki, 1997; Martin et al., 2003). The chimeric CHE-FcD protein was pre-complexed with PE-goat anti-human IgG (Fcγ-specific) (Ebioscience; 1.875 μL of a 1:10 dilution of CHE-FcD in PBA with 6 μL of a 1:4 dilution of the secondary antibody in a total volume of 50 μL PBA) for 2 h at 4°C in the dark. This pre-complexing step

increases the overall avidity of the probe toward 9-O-acetylated sialic acids. 3–5 × 10⁶ cells in 100 μL PBA were pre-incubated for 45 min at 37 °C, added to the pre-complex, and incubated on ice for an additional 1.5 h. The cells were washed once with cold PBA, reacted with 2.4G2, an FcγR III/II receptor-blocking antibody and surface stained above.

In vivo adoptive transfer assay to assess lineage potential

To assess differentiation potential for BM progenitor populations, freshly sorted cells from the BM of 40 10-week-old CD45.2 donor mice were injected intravenously into 8-week-old CD45.1 congenic recipient mice that were sub-lethally irradiated with one dose of 4.5 Gy from a Cesium 137 source. Lethal irradiation was not used as it causes Flt3L up-regulation that may favor DC-biased differentiation of transferred cells, and therefore distort the steady-state situation (Chklovskaya et al., 2004). Spleen and BM were harvested at time points ranging from day 4 through 14 post-transfer and donor-derived (CD45.2⁺ CD45.1⁻) and host cells (CD45.2⁻ CD45.1⁻) were assessed using isolation, staining and flow cytometry strategies described above.

Induction of type I IFN production by in vivo treatment with TLR ligands

Ten-week-old mice were intravenously injected with 200 μL of PBS or resiquimod (R-848, 5 μg in 200 μL of PBS; Invivogen) using an approach adapted from that previously described (Asselin-Paturel et al., 2005).

PCR assay for IgH gene rearrangements in pro-pDCs, pre-pDCs and mature pDCs

Genomic DNA was isolated from freshly sorted cells using the DNAeasy Blood and Tissue Kit (QIAGEN). Primers specific for the D_HQ52 element were used amplify D_H to J_H (DJ) rearranged and

non-rearranged germline (GL) *IgH* loci in a nested PCR approach adapted from that previously described (ten Boekel et al., 1995).

Supplementary data

Supplementary data are available at *Glycobiology* online.

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Conflict of interest statement

None declared.

Authors' roles

I.A.N., A.C. and S.P. designed the studies. I.A.N. and A.C. performed and analyzed experimental studies. W.N.H. and K.Y. were consulted on gene expression studies. I.R. contributed to gene expression analyses. I.A.N. and S.P. wrote the manuscript with input from A.C.

References

- Akashi K, Traver D, Miyamoto T, Weissman IL. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 404:193–197.
- Allman D, Dalod M, Asselin-Paturel C, Delale T, Robbins SH, Trinchieri G, Biron CA, Kastner P, Chan S. 2006. Ikaros is required for plasmacytoid dendritic cell differentiation. *Blood*. 108:4025–4034.
- Arming S, Wipfler D, Mayr J, Merling A, Vilas U, Schauer R, Schwartz-Albiez R, Vlasak R. 2011. The human Cas1 protein: A sialic acid-specific O-acetyltransferase? *Glycobiology*. 21:553–564.
- Asselin-Paturel C, Boonstra A, Dalod M, Durand I, Yessaad N, Dezutter-Dambuyant C, Vicari A, O'Garra A, Biron C, Briere F et al. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol*. 2:1144–1150.
- Auffray C, Fogg DK, Narni-Mancinelli E, Senechal B, Trouillet C, Saederup N, Leemput J, Bigot K, Campisi L, Abitbol M et al. 2009. CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation. *J Exp Med*. 206:595–606.
- Bao Y, Han Y, Chen Z, Xu S, Cao X. 2011. IFN- α -producing PDCA-1+ Siglec-H- B cells mediate innate immune defense by activating NK cells. *Eur J Immunol*. 41:657–668.
- Barchet W, Cella M, Odermatt B, Asselin-Paturel C, Colonna M, Kalinke U. 2002. Virus-induced interferon α production by a dendritic cell subset in the absence of feedback signaling in vivo. *J Exp Med*. 195:507–516.
- Baumann AM, Bakkers MJ, Buettner FF, Hartmann M, Grove M, Langereis MA, de RJ, Muhlenhoff M. 2015. 9-O-acetylation of sialic acids is catalysed by CASD1 via a covalent acetyl-enzyme intermediate. *Nat Commun*. 6:7673.
- Bell JJ, Bhandoola A. 2008. The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature*. 452:764–767.
- Bjorck P, Leong HX, Engleman EG. 2011. Plasmacytoid dendritic cell dichotomy: Identification of IFN- α producing cells as a phenotypically and functionally distinct subset. *J Immunol*. 186:1477–1485.
- Blasius A, Vermi W, Krug A, Facchetti F, Cella M, Colonna M. 2004. A cell-surface molecule selectively expressed on murine natural interferon-producing cells that blocks secretion of interferon- α . *Blood*. 103:4201–4206.
- Blasius AL, Cella M, Maldonado J, Takai T, Colonna M. 2006. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood*. 107:2474–2476.
- Cao W, Zhang L, Rosen DB, Bover L, Watanabe G, Bao M, Lanier LL, Liu YJ. 2007. BDCA2/fc epsilon RI gamma complex signals through a novel BCR-like pathway in human plasmacytoid dendritic cells. *PLoS Biol*. 5:e248.
- Cariappa A, Takematsu H, Liu H, Diaz S, Haider K, Boboila C, Kalloo G, Connole M, Shi HN, Varki N et al. 2009. B cell antigen receptor signal strength and peripheral B cell development are regulated by a 9-O-acetyl sialic acid esterase. *J Exp Med*. 206:125–138.
- Carotta S, Dakic A, D'Amico A, Pang SH, Greig KT, Nutt SL, Wu L. 2010. The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity*. 32:628–641.
- Chi AW, Chavez A, Xu L, Weber BN, Shestova O, Schaffer A, Wertheim G, Pear WS, Izon D, Bhandoola A. 2011. Identification of Flt3(+)/CD150(–) myeloid progenitors in adult mouse bone marrow that harbor T lymphoid developmental potential. *Blood*. 118:2723–2732.
- Chklovskaya E, Nowbakht P, Nissen C, Gratwohl A, Bargetzi M, Wodnar-Filipowicz A. 2004. Reconstitution of dendritic and natural killer-cell subsets after allogeneic stem cell transplantation: Effects of endogenous flt3 ligand. *Blood*. 103:3860–3868.
- Cisse B, Caton ML, Lehner M, Maeda T, Scheu S, Locksley R, Holmberg D, Zweier C, den NS, Kant SG et al. 2008. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell*. 135:37–48.
- Coffman RL, Weissman IL. 1981. B220: A B cell-specific member of the T200 glycoprotein family. *Nature*. 289:681–683.
- Corcoran L, Ferrero I, Vremec D, Lucas K, Waithman J, O'Keeffe M, Wu L, Wilson A, Shortman K. 2003. The lymphoid past of mouse plasmacytoid cells and thymic dendritic cells. *J Immunol*. 170:4926–4932.
- Dalod M, Salazar-Mather TP, Malmgaard L, Lewis C, Asselin-Paturel C, Briere F, Trinchieri G, Biron CA. 2002. Interferon α/β and interleukin 12 responses to viral infections: Pathways regulating dendritic cell cytokine expression in vivo. *J Exp Med*. 195:517–528.
- Diacovo TG, Blasius AL, Mak TW, Cella M, Colonna M. 2005. Adhesive mechanisms governing interferon-producing cell recruitment into lymph nodes. *J Exp Med*. 202:687–696.
- Diao J, Winter E, Cantin C, Chen W, Xu L, Kelvin D, Phillips J, Catral MS. 2006. In situ replication of immediate dendritic cell (DC) precursors contributes to conventional DC homeostasis in lymphoid tissue. *J Immunol*. 176:7196–7206.
- Dress RJ, Dutertre C-A, Giladi A, Schlitzer A, Low I, Shadan NB, Tay A, Lum J, Kairi MFBM, Hwang YY et al. 2019. Plasmacytoid dendritic cells develop from Ly6D+ lymphoid progenitors distinct from the myeloid lineage. *Nat Immunol*. 20:852–864.
- Edelson BT, Ko W, Juang R, Kohyama M, Benoit LA, Klekotka PA, Moon C, Albring JC, Ise W, Michael DG et al. 2010. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8 α + conventional dendritic cells. *J Exp Med*. 207:823–836.
- Esashi E, Bao M, Wang YH, Cao W, Liu YJ. 2012. PACSIN1 regulates the TLR7/9-mediated type I interferon response in plasmacytoid dendritic cells. *Eur J Immunol*. 42:573–579.
- Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR, Cumano A, Geissmann F. 2006. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science*. 311:83–87.
- Ghosh HS, Cisse B, Bunin A, Lewis KL, Reizis B. 2010. Continuous expression of the transcription factor e2-2 maintains the cell fate of mature plasmacytoid dendritic cells. *Immunity*. 33:905–916.

- Hacker C, Kirsch RD, Ju XS, Hieronymus T, Gust TC, Kuhl C, Jorgas T, Kurz SM, Rose-John S, Yokota Y *et al.* 2003. Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat Immunol.* 4:380–386.
- Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med.* 173:1213–1225.
- Harman BC, Miller JP, Nikbakht N, Gerstein R, Allman D. 2006. Mouse plasmacytoid dendritic cells derive exclusively from estrogen-resistant myeloid progenitors. *Blood.* 108:878–885.
- Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, Calderon B, Schraml BU, Unanue ER, Diamond MS *et al.* 2008. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science.* 322:1097–1100.
- Idoyaga J, Suda N, Suda K, Park CG, Steinman RM. 2009. Antibody to Langerin/CD207 localizes large numbers of CD8alpha+ dendritic cells to the marginal zone of mouse spleen. *Proc Natl Acad Sci U S A.* 106:1524–1529.
- Klein A, Krishna M, Varki NM, Varki A. 1994. 9-O-acetylated sialic acids have widespread but selective expression: Analysis using a chimeric dual-function probe derived from influenza C hemagglutinin-esterase. *Proc Natl Acad Sci U S A.* 91:7782–7786.
- Kondo M, Weissman IL, Akashi K. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell.* 91:661–672.
- Krishna M, Varki A. 1997. 9-O-acetylation of sialomucins: A novel marker of murine CD4 T cells that is regulated during maturation and activation. *J Exp Med.* 185:1997–2013.
- Krug A, Uppaluri R, Facchetti F, Dorner BG, Sheehan KC, Schreiber RD, Cella M, Colonna M. 2002. IFN-producing cells respond to CXCR3 ligands in the presence of CXCL12 and secrete inflammatory chemokines upon activation. *J Immunol.* 169:6079–6083.
- Laouar Y, Welte T, Fu XY, Flavell RA. 2003. STAT3 is required for Flt3L-dependent dendritic cell differentiation. *Immunity.* 19:903–912.
- LeibundGut-Landmann S, Waldburger JM, Reis e Sousa C, Acha-Orbea H, Reith W. 2004. MHC class II expression is differentially regulated in plasmacytoid and conventional dendritic cells. *Nat Immunol.* 5:899–908.
- Li HS, Yang CY, Nallaparaju KC, Zhang H, Liu YJ, Goldrath AW, Watowich SS. 2012. The signal transducers STAT5 and STAT3 control expression of Id2 and E2-2 during dendritic cell development. *Blood.* 120:4363–4373.
- Liu K, Victora GD, Schwickert TA, Guernonprez P, Meredith MM, Yao K, Chu FF, Randolph GJ, Rudensky AY, Nussenzweig M. 2009. In vivo analysis of dendritic cell development and homeostasis. *Science.* 324:392–397.
- Liu YJ. 2005. IPC: Professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol.* 23:275–306.
- Luc S, Luis TC, Boukarabila H, Macaulay IC, Buza-Vidas N, Bouriez-Jones T, Lutteropp M, Woll PS, Loughran SJ, Mead AJ *et al.* 2012. The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential. *Nat Immunol.* 13:412–419.
- Mahajan VS, Alsufyani F, Mattoo H, Rosenberg I, Pillai S. 2019. Alterations in sialic-acid O-acetylation glycoforms during murine erythrocyte development. *Glycobiology.* 29:222–228.
- Martin LT, Verhagen A, Varki A. 2003. Recombinant influenza C hemagglutinin-esterase as a probe for sialic acid 9-O-acetylation. *Methods Enzymol.* 363:489–498.
- Mebius RE, Miyamoto T, Christensen J, Domen J, Cupedo T, Weissman IL, Akashi K. 2001. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. *J Immunol.* 166:6593–6601.
- Miller JC, Brown BD, Shay T, Gautier EL, Jojic V, Cohain A, Pandey G, Leboeuf M, Elpek KG, Helft J *et al.* 2012. Deciphering the transcriptional network of the dendritic cell lineage. *Nat Immunol.* 13:888–899.
- Naik SH, Metcalf D, van Nieuwenhuijze A, Wicks I, Wu L, O’Keeffe M, Shortman K. 2006. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat Immunol.* 7:663–671.
- Naik SH, Perie L, Swart E, Gerlach C, van Rooij N, de Boer RJ, Schumacher TN. 2013. Diverse and heritable lineage imprinting of early haematopoietic progenitors. *Nature.* 496:229–232.
- Naik SH, Sathe P, Park HY, Metcalf D, Proietto AI, Dakic A, Carotta S, O’Keeffe M, Bahlo M, Papenfuss A *et al.* 2007. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol.* 8:1217–1226.
- Onai N, Kurabayashi K, Hosoi-Amaike M, Toyama-Sorimachi N, Matsushima K, Inaba K, Ohteki T. 2013. A clonogenic progenitor with prominent plasmacytoid dendritic cell developmental potential. *Immunity.* 38:943–957.
- Onai N, Obata-Onai A, Schmid MA, Ohteki T, Jarrossay D, Manz MG. 2007. Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol.* 8:1207–1216.
- Pelayo R, Hirose J, Huang J, Garrett KP, Delogu A, Busslinger M, Kincade PW. 2005. Derivation of 2 categories of plasmacytoid dendritic cells in murine bone marrow. *Blood.* 105:4407–4415.
- Pillai S, Netravali IA, Cariappa A, Mattoo H. 2012. Siglecs and immune regulation. *Annu Rev Immunol.* 30:357–392.
- Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. 2011. Plasmacytoid dendritic cells: Recent progress and open questions. *Annu Rev Immunol.* 29:163–183.
- Rodrigues PF, Alberti-Servera L, Eremin A, Grajales-Reyes GE, Ivanek R, Tussiwand R. 2018. Distinct progenitor lineages contribute to the heterogeneity of plasmacytoid dendritic cells. *Nat Immunol.* 19:711–722.
- Rubtsov AV, Rubtsova K, Fischer A, Meehan RT, Gillis JZ, Kappler JW, Marrack P. 2011. Toll-like receptor 7 (TLR7)-driven accumulation of a novel CD11c(+) B-cell population is important for the development of autoimmunity. *Blood.* 118:1305–1315.
- Sasaki I, Hoshino K, Sugiyama T, Yamazaki C, Yano T, Iizuka A, Hemmi H, Tanaka T, Saito M, Sugiyama M *et al.* 2012. Spi-B is critical for plasmacytoid dendritic cell function and development. *Blood.* 120:4733–4743.
- Sathe P, Vremec D, Wu L, Corcoran L, Shortman K. 2013. Convergent differentiation: Myeloid and lymphoid pathways to murine plasmacytoid dendritic cells. *Blood.* 121:11–19.
- Sawai CM, Sisirak V, Ghosh HS, Hou EZ, Ceribelli M, Staudt LM, Reizis B. 2013. Transcription factor Runx2 controls the development and migration of plasmacytoid dendritic cells. *J Exp Med.* 210:2151–2159.
- Schiavoni G, Mattei F, Sestili P, Borghi P, Venditti M, Morse HC 3rd, Belardelli F, Gabriele L. 2002. ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. *J Exp Med.* 196:1415–1425.
- Schlitzer A, Heiseke AF, Einwachter H, Reindl W, Schiemann M, Manta CP, See P, Niess JH, Suter T, Ginhoux F *et al.* 2012. Tissue-specific differentiation of a circulating CCR9+ pDC-like common dendritic cell precursor. *Blood.* 119:6063–6071.
- Schlitzer A, Loschko J, Mair K, Vogelmann R, Henkel L, Einwachter H, Schiemann M, Niess JH, Reindl W, Krug A. 2011. Identification of CCR9+ murine plasmacytoid DC precursors with plasticity to differentiate into conventional DCs. *Blood.* 117:6562–6570.
- Schmid MA, Kingston D, Boddupalli S, Manz MG. 2010. Instructive cytokine signals in dendritic cell lineage commitment. *Immunol Rev.* 234:32–44.
- Schotte R, Nagasawa M, Weijer K, Spits H, Blom B. 2004. The ETS transcription factor Spi-B is required for human plasmacytoid dendritic cell development. *J Exp Med.* 200:1503–1509.
- Schraml BU, van Blijswijk J, Zelenay S, Whitney PG, Filby A, Acton SE, Rogers NC, Moncaut N, Carvajal JJ, Reis e Sousa C. 2013. Genetic tracing via DNCR-1 expression history defines dendritic cells as a hematopoietic lineage. *Cell.* 154:843–858.
- See P, Dutertre CA, Chen J, Gunther P, McGovern N, Irac SE, Gunawan M, Beyer M, Handler K, Duan K *et al.* 2017. Mapping the human DC lineage through the integration of high-dimensional techniques. *Science.* 356.
- Shigematsu H, Reizis B, Iwasaki H, Mizuno S, Hu D, Traver D, Leder P, Sakaguchi N, Akashi K. 2004. Plasmacytoid dendritic cells activate lymphoid-specific genetic programs irrespective of their cellular origin. *Immunity.* 21:43–53.
- Swiecki M, Colonna M. 2010. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. *Immunol Rev.* 234:142–162.

- Takeuchi S, Katz SI. 2006. Use of interleukin 7 receptor- α knockout donor cells demonstrates the lymphoid independence of dendritic cells. *Blood*. 107:184–186.
- Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S. 2005. Macrophage receptors and immune recognition. *Annu Rev Immunol*. 23:901–944.
- ten Boekel E, Melchers F, Rolink A. 1995. The status of Ig loci rearrangements in single cells from different stages of B cell development. *Int Immunol*. 7:1013–1019.
- Varki A, Gagneux P. 2012. Multifarious roles of sialic acids in immunity. *Ann N Y Acad Sci*. 1253:16–36.
- Vinay DS, Lee SJ, Kim CH, Oh HS, Kwon BS. 2012. Exposure of a distinct PDCA-1⁺ (CD317) B cell population to agonistic anti-4-1BB (CD137) inhibits T and B cell responses both in vitro and in vivo. *PLoS One*. 7: e50272.
- Vogt TK, Link A, Perrin J, Finke D, Luther SA. 2009. Novel function for interleukin-7 in dendritic cell development. *Blood*. 113:3961–3968.
- Vremec D. 2010. The isolation of mouse dendritic cells from lymphoid tissues and the identification of dendritic cell subtypes by multiparameter flow cytometry. *Methods Mol Biol*. 595:205–229.
- Wada H, Masuda K, Satoh R, Kakugawa K, Ikawa T, Katsura Y, Kawamoto H. 2008. Adult T-cell progenitors retain myeloid potential. *Nature*. 452:768–772.
- Waskow C, Liu K, Darrasse-Jeze G, Guernonprez P, Ginhoux F, Merad M, Shengelia T, Yao K, Nussenzweig M. 2008. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat Immunol*. 9:676–683.
- Wilson A, Trumpp A. 2006. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol*. 6:93–106.
- Wrammert J, Kallberg E, Agace WW, Leanderson T. 2002. Ly6C expression differentiates plasma cells from other B cell subsets in mice. *Eur J Immunol*. 32:97–103.
- Yamamoto R, Morita Y, Ouehara J, Hamanaka S, Onodera M, Rudolph KL, Ema H, Nakauchi H. 2013. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell*. 154:1112–1126.
- Yang GX, Lian ZX, Kikuchi K, Moritoki Y, Ansari AA, Liu YJ, Ikehara S, Gershwin ME. 2005. Plasmacytoid dendritic cells of different origins have distinct characteristics and function: Studies of lymphoid progenitors versus myeloid progenitors. *J Immunol*. 175: 7281–7287.
- Zhang J, Raper A, Sugita N, Hingorani R, Salio M, Palmowski MJ, Cerundolo V, Crocker PR. 2006. Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors. *Blood*. 107:3600–3608.