**Cell Reports**

**Notch Signaling Facilitates In Vitro Generation of Cross-Presenting Classical Dendritic Cells**

**Graphical Abstract**

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**Highlights**

- DL1-Notch2 signaling induces differentiation of murine CD8α⁺ CD103⁺ cDC1s *in vitro*
- Notch-induced cDC1s show improved expression profile and CCR7-dependent migration
- Notch-induced cDC1s mediate superior T cell cross-priming and antitumor vaccination
- DL1 signaling facilitates *in vitro* generation of human IRF8-dependent CD141⁺ cDC1s

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**In Brief**
Dendritic cells (DCs) are critical inducers of immune responses, but current methods to generate them *in vitro* are suboptimal. Kirkling et al. report that Notch signaling facilitates the generation of DCs that closely resemble their *in vivo* counterparts and show superior capacity to vaccinate against tumors *in vivo*.

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Notch Signaling Facilitates In Vitro Generation of Cross-Presenting Classical Dendritic Cells

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SUMMARY

The IRF8-dependent subset of classical dendritic cells (cDCs), termed cDC1, is important for cross-priming cytotoxic T cell responses against pathogens and tumors. Culture of hematopoietic progenitors with DC growth factor FLT3 ligand (FLT3L) yields very few cDC1s (in humans) or only immature “cDC1-like” cells (in the mouse). We report that OP9 stromal cells expressing the Notch ligand Delta-like 1 (OP9-DL1) optimize FLT3L-driven development of cDC1s from murine immortalized progenitors and primary bone marrow cells. Co-culture with OP9-DL1 induced IRF8-dependent cDC1s with a phenotype (CD103+ Dec205+ CD8α+) and expression profile resembling primary splenic cDC1s. OP9-DL1-induced cDC1s showed preferential migration toward CCR7 ligands in vitro and superior T cell cross-priming and antitumor vaccination in vivo. Co-culture with OP9-DL1 also greatly increased the yield of IRF8-dependent CD141+ cDC1s from human bone marrow progenitors cultured with FLT3L. Thus, Notch signaling optimizes cDC generation in vitro and yields authentic cDC1s for functional studies and translational applications.

INTRODUCTION

Dendritic cells (DCs) link innate and adaptive immunity by recognizing pathogens through pattern recognition receptors such as Toll-like receptors (TLRs) and recruiting diverse immune cells to orchestrate antigen (Ag)-specific adaptive responses (Pulendran, 2015; Steinman, 2012). Classical or conventional DCs (cDCs) are specialized Ag-presenting cells with a characteristic dendritic morphology, high major histocompatibility complex (MHC) class II expression, and a unique capacity for priming naïve T cells. Upon Ag capture, cDCs upregulate chemotactic receptors such as CCR7, migrate from tissues into the T cell areas of regional lymphoid organs, secrete cytokines and chemokines, and present Ag to Ag-specific T cells. As such, cDCs hold great promise as cellular vaccines for eliciting Ag-specific immune responses, in particular to tumor antigens (Palucka and Banchereau, 2013).

In the mouse, cDCs are comprised of two main subsets: CD8α+CD103+ cDCs capable of Ag cross-presentation to CD8+ T cells and CD11b+ cDCs specialized in the presentation of exogenous Ag to CD4+ T cells (Merad et al., 2013; Mildner and Jung, 2014; Schraml and Reis e Sousa, 2015). Both subsets are conserved in humans (Hariffa et al., 2015) and have recently been designated as cDC1 and cDC2, respectively (Guilliams et al., 2014). All DCs, including cDCs and the related lineage of interferon-producing plasmacytoid DCs (pDCs), develop in the bone marrow (BM) in a process driven mainly by the cytokine FLT3 ligand (FLT3L). Progenitors committed to cDC subsets (pre-DCs) exit the BM and undergo terminal differentiation in peripheral lymphoid organs and tissues. The development of DC subsets is driven by several transcription factors, such as IRF8, which is absolutely required for cDC1 differentiation in mice (Aliberti et al., 2003; Sichien et al., 2016) and in humans (Bigley et al., 2017; Hambleton et al., 2011). Additional factors, such as BATF3 and other BATF family members, cooperate with IRF8 to facilitate optimal development of cDC1s (Hildner et al., 2008; Murphy et al., 2016). In addition to these cell-intrinsic factors, terminal cDC differentiation in the periphery is guided by tissue-specific signals, such as lymphotixin-β and Notch.

Notch is an evolutionarily conserved pathway of cell-cell communication that informs cells of their surroundings and, thereby, guides their differentiation. Vertebrate Notch receptors (NOTCH1–4) transmit signals from membrane-bound ligands of the Delta-like (DL) and Jagged (Jag) families through the
common transcription factor CSL (also called RBPJ). Notch signaling plays an essential role in the development of immune cell types that differentiate in distinct anatomical niches. For instance, DL4-NOTCH1 and DL1-NOTCH2 signaling is required for the specification of T cells in the thymus and of marginal zone (MZ) B cells in the spleen, respectively (Radtke et al., 2013). Indeed, co-culture of stem/progenitor cells with a murine stromal cell line OP9 expressing DL1 (OP9-DL1) has become a standard approach to generate T cells in vitro (Schmitt et al., 2004; Mohtashami et al., 2016). Using DC-specific gene targeting, we have established the role of NOTCH2 receptor signaling in the differentiation of a cDC2 subset in the spleen and intestine (Caton et al., 2007; Lewis et al., 2011). In particular, splenic cDC2 contains a lymphotixin-β- and NOTCH2-RBPJ-dependent Esamhi subset that is required for optimal CD4+ T cell priming. These studies also revealed the reduction of Notch2-deficient splenic CD8α+ cDC1s (Lewis et al., 2011), which was subsequently ascribed to their impaired differentiation and aberrant phenotype (Satpathy et al., 2013). Finally, DL1 expression on fibroblasts has been identified as the relevant ligand of NOTCH2 on splenic cDCs (Fasnacht et al., 2014). Thus, NOTCH2 signaling mediated by DL ligands on stromal cells controls the phenotypic and functional differentiation of both cDC subsets.

Because primary DCs (particularly cDC1s) are rare in vivo, their study and translational applications require methods to generate functional DC subsets in vitro. Commonly used cultures of primary BM with the cytokine granulocyte-monocyte colony-stimulating factor (GM-CSF) produce a mixture of cDC2-like cells and macrophages (Helft et al., 2015) but no cDC1s. Cultures of murine BM supplemented with the physiological cytokine FLT3L produce a mixture of pDCs, cDC2s and cDC1-like cells (Naik et al., 2005). The latter express appropriate transcription factors, including IRF8, but have an abnormal phenotype, including a lack of key cDC1 markers (e.g., CD8α, CD103, and Dec205) and aberrant expression of cDC2 markers (e.g., CD11b). Human hematopoietic stem and progenitor cells cultured with FLT3L and other cytokines and/or stromal cells can produce CD141+ cDC1s with the expected expression profile and in vitro functional properties (Balan et al., 2014; Lee et al., 2015; Poulin et al., 2010; Proietto et al., 2012). However, the yield of cDC1s has been very low in all reported protocols. Thus, new approaches are necessary to produce the full spectrum and high numbers of fully differentiated DCs, particularly of functional cDC1s.

Given the important role of Notch signaling in cDC differentiation in vitro, we hypothesized that it would facilitate cDC differentiation in vitro. We now report that combination of FLT3L-driven differentiation of murine hematopoietic progenitors with the well-established OP9-DL1 system produces optimally differentiated cDC subsets, including bona fide CD8α+ Dec205+ cDC1. The resulting cDC1s showed improved migration properties and superior T cell cross-priming capacity in vivo. Furthermore, co-culture of human hematopoietic progenitors with OP9-DL1 enhanced the generation of functional human cDC1s. These results emphasize the key role of Notch signaling in terminal cDC differentiation and facilitate the generation of functional cDC1s for translational applications.

RESULTS

Notch Signaling Enables cDC1 Differentiation of Immortalized DC Progenitors

To optimize DC production in vitro, we initially studied the differentiation of DC progenitors that were conditionally immortalized with the estrogen-dependent HoxB8 oncogene (Redecke et al., 2013). In this system, progenitors can be grown indifferently in the presence of FLT3L and estrogen, whereas estrogen withdrawal induces spontaneous FLT3L-driven DC differentiation within ~7 days. Although HoxB8-FL (FLT3L) cells were originally reported to produce all DC subsets, we never observed the production of CD24+ or CD8α+ cDC1s from the original HoxB8-FL line or from any newly derived lines (Figure 1A). To mimic Notch signals received by committed cDC progenitors in vivo, we differentiated HoxB8-FL cells for 3 days to initiate DC development and then plated them on a monolayer of OP9-DL1 cells or control GFP-transduced OP9 cells for the last 4 days of culture. Control OP9 cells inhibited the development of B220+ pDCs and yielded only CD11b+ CD24− CD8α+ cDC2s in lower numbers (Figure 1A and data not shown). Co-culture with OP9-DL1 largely abolished pDC development and reduced the yield of cDC2s; however, it induced the generation of a distinct cDC subset with the CD11b− CD24+ CD8α+ phenotype of cDC1s (Figures 1A and 1B).

To confirm that the observed DL1-driven cDC1 generation is NOTCH2-dependent, we used an antibody (Ab) that blocks the activation of NOTCH2 (anti-N2) (Wu et al., 2010). The administration of this anti-N2 Ab in vivo recapitulated the effects of DC-specific NOTCH2 blockade, including ablation of splenic Esamhi cDC2s and of intestinal CD11b+ CD103+ cDCs and loss of CD8α+ splenic cDC1s (Figure S1 and data not shown). The addition of anti-N2 to HoxB8-FL co-cultures with OP9-DL1 did not rescue pDC loss (Figure 1C), further suggesting that this loss is caused by OP9 cells independent of DL1 expression. On the other hand, anti-N2 reduced the development of cDC2s and largely abolished the development of cDC1s (Figure 1C). To test the function of NOTCH2 ligands, we used OP9 cells transduced with DL4 (OP9-DL4), which are as efficient as OP9-DL1 in driving T cell development (Mohtashami et al., 2016). We found that OP9-DL4 were able to induce cDC1 differentiation of HoxB8-FL cells, but less efficiently than OP9-DL1 (Figure 1D). Collectively, these data show that DL1-NOTCH2 signaling can elicit de novo generation of cDC1s from immortalized DC progenitors.

Notch Signaling Induces Optimal cDC1 Differentiation of BM Progenitors

Having established the OP9-DL1 co-culture system of DC differentiation, we applied it to the cultures of primary BM. Total BM cells were either cultured for 7 days using a standard DC differentiation protocol in FLT3L-containing medium or transferred on day 3 to monolayers of OP9-DL1 in the continued presence of FLT3L (hereafter referred to as FL or FL-Notch cultures, respectively). As described previously (Naik et al., 2005), DCs generated in FL cultures (DCFL) comprised B220+ pDCs, CD11b+ CD24− cDC2s, and CD11b+/low CD24+ cDC1-like cells (Figure 2A). Co-culture with control OP9 cells impaired the
Figure 1. DL1-NOTCH2 Signaling Induces Differentiation of cDC1s from a DC Progenitor Cell Line

The HoxB8-FL cell line was induced to differentiate in vitro by estrogen withdrawal in the presence of FLT3L alone (FL), FLT3L with control OP9 cells (FL+OP9), or FLT3L with OP9 cells expressing the Notch ligand DL1 (FL+Notch). OP9 cells were added on day 3, and HoxB8-FL cells were analyzed on day 7 of differentiation.

(A) Representative staining plots of differentiated HoxB8-FL cells. The top row shows total live cells with B220⁺ MHC class II⁺ pDCs and B220⁻ MHC class II⁺ cDCs highlighted; the other rows show gated cDCs with CD11b⁺ cDC2s and CD11b⁻/⁻ cDC1s highlighted.

(B) The subset composition of differentiated HoxB8-FL cells. Shown are fractions of pDCs (of total live cells) and cDC subsets (of gated cDCs) and the absolute number of these subsets per 10⁶ initial undifferentiated cells. Data represent mean ± SD of 6 parallel cultures, representative of 3 experiments.

(C) The effect of NOTCH2 blockade on HoxB8-FL cell differentiation. HoxB8-FL cells were differentiated in FL+Notch cultures in the presence of anti-NOTCH2 blocking Ab (anti-N2) at 50 ng/ml (gray open bars) or 500 ng/ml (gray textured bars); the fractions of DC subsets are shown as above. Data represent mean ± SD of 5 parallel cultures.

(D) The effect of Notch ligands on HoxB8-FL cell differentiation. HoxB8-FL cells were differentiated in co-cultures with OP9 cells expressing the Notch ligand DL1 or DL4; the fractions of DC subsets are shown as above. Data represent mean ± SD of 6 parallel cultures.

Statistical significance: ****p < 0.0001; ***p < 0.001; **p < 0.01; ns, not significant.
generation of pDCs and cDC1-like cells, yielding primarily cDC2s (Figures 2A–2C). In contrast, co-culture with OP9-DL1 inhibited pDC but not cDC development; moreover, the resulting DC population (DCFL-Notch) contained two fully resolved subsets, including a distinct CD11b<sup>+</sup> CD24<sup>-</sup> cDC1 population that expressed Dec205 and CD8<sup>-</sup> (Figures 2A–2C). In contrast to cDC1-like cells from FL cultures (cDC1<sup>FL</sup>), CD24<sup>+</sup> cDC1s from FL-Notch cultures (cDC1<sup>FL-Notch</sup>) downregulated CD11b, acquired expression of Dec205 and CD8<sup>-</sup>, and upregulated CD103 (Figure 2D). Staining for additional subset markers revealed a similarly improved resolution of the two cDC subsets in FL-Notch cultures (Figure S2A). In particular, cDC2<sup>FL-Notch</sup> expressed low levels of the cDC1 markers Xcr1 and Clec9a, which were reduced in cDC2<sup>FL-Notch</sup>, conversely, cDC1<sup>FL-Notch</sup> expressed Xcr1 and Clec9a but lacked the cDC2 markers CD11b and CD172a/Sirpx (Figure S2A). All DCFL-Notch had higher levels of CD11c and reduced levels of surface MHC class II; the latter was particularly evident in cDC1s (Figure 2E). This is consistent with the phenotype of resident cDCs in lymphoid organs, which have higher CD11c and lower MHC class II surface levels than migratory tissue-derived cDCs. Furthermore, cDC2s from FL-Notch cultures showed induction of Esam, a marker of NOTCH2-dependent splenic cDC2s (Figure 2F). Thus, DL1-NOTCH2 signaling refines the phenotypes of BM-derived cDC subsets and brings them closer to those of the primary resident cDCs in lymphoid organs.

The induction of CD8<sup>-</sup> on cDC1s and of Esam on cDC2s was blocked by anti-N2 Ab (Figure 2G), confirming NOTCH2 as the relevant receptor. Both DL1- and DL4-expressing OP9 cells were able to induce differentiation of CD8<sup>+</sup> cDC1s; however, DL1 induced higher levels of CD8<sup>-</sup> expression than DL4 (Figures S2B and S2C). Addition of OP9-DL1 at an earlier (day 1) or later (day 5) time point was less efficient (Figure S2D), suggesting that a durable Notch signal delivered after the initial DC lineage commitment provides optimal DC subset resolution.

As expected, global or DC-specific deletion of Irf8 spared cDC2s, impaired pDCs, and fully abolished the development of cDC1-like cells in FL cultures (Figure 2H). On the other hand, deletion of Batf3 only mildly affected cDC1<sup>FL</sup> development. cDC1s in FL-Notch cultures showed the same strict dependence on Irf8 but also a stronger dependence on Batf3 (Figure 2H). Furthermore, Batf3 deletion reduced the expression of CD103 on cDC1s, as described previously (Jackson et al., 2011); this effect was particularly prominent in CD103<sup>-</sup> cDC1<sup>FL-Notch</sup> (Figure S2E). Altogether, these results suggest that FL-Notch cultures of primary BM yield cDC1 cells with the appropriate phenotype and genetic requirements.

**Notch Signaling Optimizes the Global Expression Profile of In Vitro-Derived DCS**

To further explore the effect of DL1-Notch signaling on in vitro-derived DCS, we sorted cDC1s and cDC2s from FL and FL-Notch cultures and interrogated their expression profiles by global mRNA sequencing (RNA-seq). The resulting expression profiles were merged with those of primary splenic cDCs (Lau et al., 2016; Table S1) and compared using multidimensionality scaling (MDS) analysis. As expected, the first dimension separated culture-derived DCS from primary DCS (Figure S3A), whereas the second and third dimensions separated DC subsets (Figure 3A). By both the second and third dimension, cDC2<sup>FL-Notch</sup> clustered closer than cDC2<sup>FL</sup> to primary cDC2 samples, particularly to the NOTCH2-dependent Esam<sup>hi</sup> subset (Figure 3A). Moreover, cDC1<sup>FL-Notch</sup> clustered much closer to primary cDC1 compared with cDC1-like cells from FL cultures (Figure 3A).

Pairwise comparison between cDC1 and cDC2 for each culture condition showed a greater divergence of the two subsets in FL-Notch cultures (Figure 3B). In particular, characteristic markers of cDC1 (Xcr1, Clec9a, CD36, and Tlr11) and cDC2 (Itgam and Lyz2) showed greater differential expression. Pairwise comparison between FL and FL-Notch cultures for each subset (Figure 3C; Table S2) showed that cDC2<sup>FL-Notch</sup> induced the expression of NOTCH2-dependent genes overexpressed in Esam<sup>hi</sup> cDC2 (Lewis et al., 2011; Satpathy et al.,...
Figure 3. Notch Signaling Optimizes the Expression Profile of BM-Derived DCs

Duplicate samples of sorted DC subsets from FL and FL-Notch cultures of primary BM were analyzed by RNA-seq.
(A) Multidimensionality scaling (MDS) analysis of RNA-seq profiles of DC subsets derived from FL cultures (DC1FL, DC2FL), FL+Notch cultures (DC1Notch, DC2Notch), and primary splenic DC subsets from WT mice (DC1WT, EsamhighWT, and EsamlowWT DC2s). All samples are plotted on the second and third dimension of MDS.
(B) Pairwise comparison of RNA-seq profiles of cDC1 versus cDC2 subsets from the indicated culture conditions. Shown are volcano plots of individual genes, with select subset-specific marker genes highlighted.
Notch Signaling Optimizes Migratory Properties of In Vitro-Derived DCs

We examined the distinct functional properties of cDC1s from FL and FL-Notch cultures in vitro. Despite the increased baseline expression of Itifb (Figure 3C), cDC1FL-Notch did not show an enhanced interleukin-12 (IL-12) response to the TLR11 ligand profilin (data not shown). Cross-presentation of exogenous protein Ag to CD8+ T cells is a hallmark property of cDC1 (den Haan et al., 2000; Hildner et al., 2008). To measure Ag cross-presentation in vitro, we pulsed DCs from either culture method with ovalbumin (OVA), washed and incubated them with H-2Kb-OVA peptide-specific T cell receptor (TCR) transgenic OT-I CD8+ T cells, and measured OT-I proliferation by the dilution of the cell tracer dye carboxyfluorescein succinimidyl ester (CFSE). Total DCs and enriched cDC1s from both culture types induced comparably strong OT-I proliferation at a 1:1 DC:T cell ratio (data not shown). At lower DC:T cell ratios (1:5–1:10), total DCs and enriched cDC1s from FL-Notch cultures induced more extensive CFSE dilution in T cells (Figures 4A and 4B). Thus, Notch signaling is not strictly required for the cross-presentation capacity of cDC1 but facilitates T cell cross-priming under limiting conditions in vitro.

Comparison of DCFL and DCFL-Notch expression profiles identified regulators of cell migration among the most differentially expressed genes (Figures 3C and 3D). We therefore analyzed the expression of chemokines and their receptors in culture-derived and primary DCs (Table S4). Unsupervised clustering by chemokine expression did not group DCFL-Notch with primary DCs (Figure S3C). Nevertheless, several chemokines were induced in cDC1FL-Notch compared with cDC1FL, including Cxcl9 (Figure S3D). Notably, CXCL9 is preferentially expressed in primary cDC1s (Figure S3D) and plays a major role in cDC1-mediated priming of tumor-specific T cell responses (de Mingo Pulido et al., 2018; Spranger et al., 2017). The expression of chemokine receptors separated DCFL from other samples but clustered DCFL-Notch with their respective splenic DC counterparts (Figure 4C). Furthermore, cDC1FL-Notch showed a profound downregulation of Ccr1, Ccr2, and Ccr5 and induction of Ccr7, aligning the expression of these receptors with primary cDC1 (Figure 4D). The induction of Ccr7 in cDC1FL-Notch was particularly notable given its very low levels in cDC1FL. Cell surface staining confirmed the induction of CCR7 expression on DCFL-Notch (Figure 4E). Accordingly, transwWell migration assays showed that all DCFL preferentially migrated toward the CCR2 ligand CCL2, with lower migration toward the CCR1 and CCR5 ligand CCL5 and CCR7 ligand CCL19 (Figure 4F). In contrast, DCFL-Notch failed to migrate toward CCL2 or CCL5, but showed increased migration toward CCL19, with the difference being particularly notable in the cDC1 subset (Figure 4F). These data suggest that Notch signaling induces a more physiological pattern of chemokine receptor expression and migration in cultured DCs, specifically favoring CCR7-dependent over CCR2- and CCR5-dependent migration.
into wild-type syngeneic H-2Kb recipients. One week later, OVA-specific endogenous CD8+ T cells were detected with an H-2Kb-OVA peptide (SIINFEKL) tetramer. We found that up to 10^6 cells/animal of DCFL failed to elicit OVA-specific T cells in the spleen (Figure S4B) and in the peripheral blood (Figures 5A and 5B). In contrast, DCFL-Notch induced robust T cell responses even at lower doses (0.25–0.5 × 10^6 cells/animal) (Figure S4B; Figures 5A and 5B). Furthermore, a major dose, whereas total DCFL-Notch and DCc1FL-Notch were comparably efficient.

To test the consequences of differential T cell priming, recipient mice were challenged with the OVA-expressing syngeneic melanoma cell line B16 (B16-OVA). After intravenous (i.v.) retroorbital administration of 2.5 × 10^6 B16-OVA cells, all untreated mice became moribund with respiratory distress within ~2 weeks and had to be sacrificed (Figure 5D). Histological

### Figure 4. Notch Facilitates CCR7-Dependent Migration of DCs

DCs were generated by culturing murine BM cells in the presence of FLT3L alone (FL) or FLT3L with OP9 cells expressing the Notch ligand DL1 (FL+Notch).

(A and B) Cross-priming of CD8+ T cells in vitro. FL or FL-Notch cultures on day 7 were incubated with OVA, and either total cultures or enriched cDC1s were incubated with CFSE-labeled OT-I cells at a 1:10 ratio for 3 days.

(A) The levels of CFSE versus the activation marker CD44 in gated CD8+ T cells from cultures with the indicated DCs, with the individual peaks of CFSE dilution highlighted. No CFSE dilution was observed in the absence of OVA (data not shown).

(B) The fraction of T cells in each peak (mean of 4 parallel cultures ± S.D., representative of 2 experiments).

(C) Unsupervised clustering of cultured and primary splenic DCs by the expression of chemokine receptors (Table S4). Shown is the clustering dendrogram with individual replicates of the indicated samples.

(D) Heatmap of select chemokine receptor expression in cultured and primary splenic DCs as determined by RNA-seq. The color scale represents the row Z score.

(E) The expression of CCR7 on the surface of culture-derived DCs. Shown is a representative staining profile and averaged mean fluorescence intensity (MFI) of CCR7 on gated CD11b+ cDC2s or CD24+ cDC1s. Data represent mean ± range of 2 cultures.

(F) DC migration in vitro in a transwell assay. Total DCs from FL or FL+Notch cultures were seeded in top chambers with the indicated recombinant chemokine in the bottom chamber and allowed to migrate for 3 hr. Shown is the fraction of each DC subset that migrated into the bottom chamber. Data represent mean ± SD of 4 parallel transwell cultures, representative of 3 experiments. Statistical significance: *p < 0.05.

Improvement in T cell priming was also observed when the same experiments were done with enriched cDC1FL-Notch (Figures 5A and 5C). The comparison of total DCs to cDC1s in these experiments was confounded by the cDC1 isolation procedure and by potential saturation of the T cell response. Nevertheless, it is notable that neither total DCFL nor enriched cDC1FL could cross-prime at any
analysis of the lungs showed disseminated infiltration of the alveolar space by B16 cells, likely causing respiratory failure (Figure S4C). A similar mortality was observed in all mice vaccinated with OVA-pulsed total DCFL (Figure 5D). In contrast, vaccination with OVA-pulsed total DCFL-Notch conferred near-complete protection against B16-OVA challenge (Figure 5D). The single DCFL-Notch recipient that succumbed to the tumor harbored a low fraction (<0.2%) of OVA-specific T cells, confirming the dependence of protection on T cell priming. We also performed these experiments injecting B16-OVA cells i.v. through the tail vein; this route of administration yielded the characteristic focal growth of melanoma cells in the lungs. Again, vaccination with OVA-pulsed DCFL had no effect, whereas vaccination with DCFL-Notch strongly reduced the growth of melanoma foci (Figure S4D). We conclude that FL-Notch culture generates cDC1s with a superior capacity for T cell cross-priming and antitumor vaccination.

**Notch Signaling Facilitates Development of cDC1s from Human Hematopoietic Progenitors**

The potential therapeutic properties of cDC1s, but their rarity in vivo, led us to explore in vitro differentiation approaches in humans. Culture of BM CD34+ progenitors in liquid medium supplemented with the cytokines FLT3L, stem cell factor (SCF), and GM-CSF (FSGM) generated only CD14+ and CD1c+ monocyte-like or cDC2-like cells (Figure 6A). Addition of an OP9 feeder layer supported the simultaneous generation of all three DC subsets with a low yield of 0.4 cDC1s per input CD34+ progenitor (Figure 6B). Addition of a Notch signal by co-culture with OP9-DL1 cells resulted in selective expansion of cDC1s, increasing the cDC1 output 11-fold (4.4 cDC1s per input progenitor) on day 14 of culture. A lower expansion of cDC1s (2.8 cDC1s/progenitor) was seen on exposure of progenitors to OP9-DL4 under similar culture conditions (Figures 6A and 6B). cDC1 expansion was dependent on a continuous Notch signal because withholding or withdrawing Notch ligand for the first or last 7 days, respectively, decreased the effect (Figure S5A).

Culture-generated cDC1s expressed the human cDC1-specific markers CD141 and CLEC9A at levels comparable with or higher than ex vivo blood cDC1s (Figure S5B). However, they expressed low levels of CD11c and high levels of CD1c, differing from peripheral blood (PB) cDC1s but resembling the phenotype of peripheral tissue cDC1s (Figure S5B). Next we performed a gene expression analysis on the NanoString nCounter platform

**Figure 5. Notch Facilitates cDC1-Mediated T Cell Cross-Priming In Vivo**

DCs from FL or FL-Notch cultures of primary murine BM were incubated with OVA, and total DCs or enriched cDC1s were injected i.v. into naive WT syngeneic recipient mice. The priming of endogenous OVA peptide-specific CD8+ T cells was determined 7 days later by staining of PB leukocytes with H-2Kb-OVA peptide tetramer.

(A) Representative staining profiles of gated CD44+ TCRβ+ T cells in the PB, with the CD8+ tetramer+ cells highlighted.

(B) The fraction of OVA-specific T cells among total CD44+ TCRβ+ CD8+ cells after vaccination with the indicated numbers of total DCs. Mice that received no injection (control) or a mock PBS injection are shown as well. Data points represent values from individual mice pooled from 2 experiments; bars represent mean.

(C) The fraction of OVA-specific T cells after vaccination with 3 x 10^5 enriched cDC1s. Data points represent values from individual mice pooled from 3 experiments; bars represent mean.

(D) Kaplan-Meier survival plot of animals that were vaccinated with OVA-pulsed total DCs and subsequently challenged with the OVA-expressing melanoma cell line.

Statistical significance: ****p < 0.0001, ***p < 0.001.
Figure 6. Notch Facilitates cDC1 Development from Human BM Progenitors

(A and B) Sorted CD34+ stem and progenitor cells purified from human BM were cultured for 2 weeks in the presence of a FLT3L-containing cytokine mix (FSGM) or on monolayers of control OP9, OP9-DL1, or OP9-DL4 cells.

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running the Human Immunology v2 panel (594 genes) with the addition of 30 DC-specific genes (Table S5). Expression profiles were compared after removal of the genes with low expression levels and of the genes that were differentially expressed between all culture and all ex vivo subsets (the cell culture signature). By subsequent principal-component analysis based on 339 genes, the first component separated pDCs from cDCs and the second cDC1s from cDC2s (Figure 6C). Notably, OP9-DL1-differentiated cDC1s grouped closely with OP9-derived cDC1s and with primary cDC1s from the PB. Accordingly, genes encoding subset-specific transcription factors, surface markers, and TLRs were expressed faithfully, including CLEC9A, XCR1, TLR3, IRF8, and BATF3 in cDC1s (Figure S5C). The expression of chemokine receptors was similarly segregated by subset (Figure S5D), although a higher expression of CCR7 in OP9-derived cDC1s and cDC2s was noted. In contrast, the expression of chemokines segregated OP9-derived cDC1s and cDC2s, which showed higher levels of CXCL10, CXCL12, and CCL22 than either DL1-derived DCs or primary DCs (Figure S5E). Thus, unlike in the mouse system, OP9-DL1 does not appear to affect the overall expression profile or the expression of migration regulators in cDC1s. Nevertheless, we conclude that DL1-induced Notch signaling greatly increases the number of differentiated cDC1s with the appropriate expression profile.

**Biallelic IRF8 mutation in human abrogates pDC, cDC1, and cDC2 development in vivo (Bigley et al., 2017; Hambleton et al., 2011). To interrogate the IRF8 requirement for in vitro Notch-induced cDC1 expansion, IRF8108E/108E and IRF883C/291Q CD34+ progenitors were co-cultured with either OP9 or OP9-DL1. cDC1s and pDCs failed to emerge under either condition, whereas some cDC2 generation was observed (Figures 6D and 6E). Thus, Notch signaling from DL1 specifically facilitates the development of human cDC1s with the appropriate phenotype, expression profile, and genetic requirements.**

**Notch-Driven Differentiation Results in Functional Human cDC1s**

To interrogate the functional capacity of in vitro-generated DCs, we examined cytokine production in response to TLR agonists. PB mononuclear cells or in vitro-derived DCs were exposed to a cocktail of TLR agonists (CL075, CpG, lipopolysaccharide [LPS], and poly-I:C), and subset-specific cytokine production was assessed by intracellular flow cytometry (Figures 7A and 7B). Tumor necrosis factor (TNF) production by cDC1s was significantly increased in OP9-DL1-derived compared with OP9-derived cells and comparable with blood cDC1s. No significant increase in IL-12 production was observed in cDC1s or cDC2s generated in vitro, but a decrease in both TNF and interferon α (IFN-α) was observed in DL1-derived pDCs (Figure 7B).

Culture-derived cDC1s showed appropriate migration in a transwell assay toward the XCR1 ligand XCL1 as well as weaker migration toward CCR2, CCR5, and CCR7 ligands; no major differences between OP9- and OP9-DL1-derived DCs were noted (data not shown). To assess their T cell-stimulatory capacity, cDC1s and cDC2s from the PB or from in vitro cultures were sorted and co-cultured with allogeneic T cells. The proportion of CD4+ or CD8+ T cells that underwent division was determined by CFSE dilution (Figure 7C). It should be noted that no differences were expected between CD4+ and CD8+ T cells in this assay, which measures direct T cell activation rather than cross-priming. cDC1s generated on OP9-DL1 stimulated proliferation in a significantly greater proportion of both CD4+ and CD8+ T cells compared with those generated on OP9; this effect was not observed in cDC2s (Figure 7D). We conclude that Notch signaling induces the development of functionally intact cDC1s, which show a significant improvement in their in vitro T cell priming capacity. Together with the drastic increase of cDC1 yield, these data underscore the improvement of cDC1 generation in Notch-driven cultures.

**DISCUSSION**

We describe an in vitro method of DC generation based on the combination of two cell-extrinsic signals, FLT3L and Notch. FLT3L is the key cytokine for the DC lineage and alone is sufficient to drive DC development and subset specification from murine hematopoietic progenitors. Murine FLT3L-derived cDCs harbor a population of IRF8-dependent cDC1-like cells (cDC1FL) that manifest functional hallmarks of cDC1s, such as IL-12 production and T cell cross-priming in vitro (Naik et al., 2005). However, these cells have an abnormal CD11b+ CD8α− Dec205− CD103+ phenotype, fail to migrate toward CCR7, and are shown here to have a poor capacity for T cell cross-priming in vivo. A combination of FLT3L with the cytokine GM-CSF in a two-step culture selectively expands cDC1-like cells and confers CD103 expression, yielding so-called iCD103-DCs (Mayer et al., 2014). However, iCD103-DCs have the same aberrant CD11b+ CD8α− Dec205− phenotype and lack CCR7 expression unless stimulated with TLR ligands. Moreover, their expression profile and functional properties were never directly compared with those of cDC1FL, and their T cell priming capacity in vivo was tested only after TLR-induced activation (Mayer et al., 2014). Thus, apart from the expected induction of CD103 (Zhan et al., 2011), GM-CSF does not appear to improve the quality of FLT3L-derived DCs. Conversely, stromal cells expressing the
Notch ligand DL1 were reported to improve DC differentiation in GM-CSF cultures, but these do not generate any cDC1 output (Cheng et al., 2007).

Here, we show that the introduction of the Notch ligand DL1 into FLT3L-driven culture using the OP9-DL1 stromal cell line (Schmitt et al., 2004) yields the two main cDC subsets, including...
**bona fide** CD11b− CD8α+ Dec205+ CD103+ cDC1s. Indeed, cDC1s and cDC2s became more distinct by key surface markers and by global expression profile, at the same time better resembling their primary counterparts in the spleen. Notably, cDC2s showed higher similarity to the Esamhi subset of splenic cDC2s, which manifest a superior ability to prime T cells (Lewis et al., 2011). Most importantly, the cDC1 subset manifested the appropriate surface phenotype and expression profile and an improved ability to cross-prime CD8+ T cell responses. The latter underscores a dramatic improvement in the quality of resulting cDC1s, even as the fraction and numbers of cDC1s were not increased. Indeed, Notch-derived cDC1s dramatically outperformed conventional cDC1-like cells in T cell priming in vivo, even at much lower numbers. Moreover, the potential limitation of cell numbers can be overcome by our system’s application to immortalized DC progenitors, which can be scaled up indefinitely.

The improved functionality of Notch-derived cDC1s does not appear to reflect an effect on Ag cross-presentation capacity per se because cDC1-like cells from FL cultures could cross-present in vitro, as described previously (Naik et al., 2005). Instead, it is likely due to an enhanced capacity to recruit T cells (e.g., through the elevated expression of CXCL9) and/or to migrate toward appropriate locations in lymphoid organs. These functions are less important when DCs and T cells interact in the context of in vivo cultures (except at lower DC:T cell ratios) but are critical for T cell priming in vivo. FL-Notch co-cultures switched the chemokine receptor expression pattern of the resulting DCs from CCR1, CCR2, and CCR5 toward CCR7, recapitulating that of DCs in lymphoid organs. This switch facilitated the migration toward CCR7 ligands, the process that guides DCs into T cell zones and is essential for T cell priming in vivo (Worbs et al., 2017). Collectively, Notch signaling increases the similarity of in vitro-generated DCs to their primary counterparts in the lymphoid organs, optimizing the functional properties of cDC1s.

Notch signaling is important for the differentiation of cDC2s in the spleen and intestine; indeed, these cells express Notch target genes such as Hes1 and Dlx1, are located in DL1-expressing splenic niches, and depend on NOTCH2 and the canonical RBPJ-mediated signaling downstream of it (Caton et al., 2007; Lewis et al., 2011). In contrast, cDC1s express few or no Notch target genes and are unaffected by DC-specific loss of Rbpj (Caton et al., 2007); however, Notch2 deletion impairs their phenotype and expression profile (Lewis et al., 2011; Satpathy et al., 2013). Consistent with the latter observation, our results identify the DL1-NOTCH2 axis as a signal that is missing in conventional FLT3L cultures and whose induction can drive differentiation into authentic functional cDC1s. To reconcile these data, we propose that DL1-NOTCH2 signaling is an important extrinsic signal for cDCs in the spleen (and possibly other lymphoid organs) that acts on committed DC progenitors to facilitate cDC1 and cDC2 subset specification and terminal differentiation. This signal then stays “on” in Esamhi cDC2, which remain in contact with DL1-expressing stroma and are fully dependent on DL1 signaling through NOTCH2-RBPJ for their survival. In contrast, the DL1-NOTCH2 signal may be turned “off” as cDC1s migrate throughout the spleen and lose Notch target gene expression and NOTCH2-RBPJ dependence. Overall, our studies emphasize the critical role of Notch signaling in the functional differentiation of both cDC subsets and demonstrate its utility to facilitate cDC generation in vitro.

FLT3L alone appears to be insufficient to drive DC development from human hematopoietic progenitors and has to be supplemented with other cytokines (Balan et al., 2014; Poulin et al., 2010; Prietto et al., 2012) or stromal cell lines, including MS5 alone (Lee et al., 2015) or a mixture of MS5 and OP9 (Lee et al., 2017). These conditions can generate mature CD141+ cDC1s with the appropriate phenotype, expression profile, and in vitro functionality. However, the fraction and absolute numbers of the resulting cDC1s are low in all cases, hampering their detailed functional characterization and practical use. This situation appears to be different from that in the mouse, where FLT3L alone can drive the development of abundant but not fully mature cDC1-like cells. It was recently reported that co-culture of human thymic progenitors with OP9-DL1 blocked the emergence of DC progenitors but increased the yield of cDCs from the latter (Martín-Gayo et al., 2017); neither the net effect on DC development nor the resulting DC phenotypes were investigated. We report that, although co-culture of human BM progenitors with OP9 generates all DC subsets, addition of the Notch ligand DL1 resulted in a more than 10-fold increase in cDC1 output per progenitor cell. The resulting cDC1s aligned closely with their ex vivo counterparts by gene expression analysis, were strictly IRF8-dependent, and showed increased T cell-stimulatory capacity. In contrast to mouse BM cultures, DL1 was required continuously from the beginning of the culture; furthermore, unlike in the mouse system, no major effects of DL1 on the phenotype, expression profile, or migration of the resulting cDC1s were noted. This may reflect different comparisons for OP9-DL1 cultures (FLT3L only” in the mouse and “FLT3L with other cytokines and OP9” in the human); differences in the effect of murine OP9 cells and their products (cytokines, adhesion molecules) on murine versus human cells; and/or biological differences between the two species. In each case, however, Notch signaling appeared to solve a major hurdle to cDC1 differentiation; i.e., it improves the suboptimal expression profile and functionality in the mouse and increase low numbers in humans. Collectively, these results emphasize the conserved positive effect of Notch signaling in in vitro cDC1 differentiation.

The long-standing idea of DC-based vaccination against tumors (Palucka and Banchereau, 2013) is now being actively pursued in human patient studies, especially in combination with other immunotherapies (Garg et al., 2017). cDC1s appear to play a particularly important role in antitumor responses through their efficient cross-priming of tumor-specific cytotoxic T cells (Bottcher et al., 2018; Roberts et al., 2016; Salmon et al., 2016; Spranger et al., 2017). The utility of cDC1s for antitumor vaccination is further illustrated by our results in the mouse, in which Notch-derived cDC1s protected against tumor challenge whereas larger numbers of total FL-derived DCs did not. The utility of human cDC1s for antitumor vaccination so far has been severely limited by their rarity in vivo and low yield in vitro. Our system overcomes this hurdle by increasing the output of human cDC1s by an order of magnitude, facilitating their potential use for vaccination. Notably, Notch-derived cDC1s are functional in vivo without prior TLR-induced activation, an important
advantage for translational applications. Furthermore, the activation-independent functionality of Notch-derived DCs supports their potential application for tolerogenic DC vaccination; e.g., against autoimmune diseases. Collectively, the methods of in vitro DC generation described here should facilitate mechanistic and translational studies focused on the therapeutic potential of DCs.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and five tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.05.068.

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**AUTHOR CONTRIBUTIONS**

M.E.K., U.C., C.M.L., and K.L.L. performed and interpreted experiments. C.W.S., H.S., and M.M. provided reagents and expertise. A.K.-J., under A.T.’s supervision, and A.R. performed the bioinformatic analysis. M.E.K., U.C., M.C., V.B., and B.R. wrote the manuscript with input from all coauthors.

**DECLARATION OF INTERESTS**

C.W.S. is an employee of Genentech, Inc.

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**REFERENCES**


## STAR METHODS

### KEY RESOURCES TABLE

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**Chemicals, Peptides, and Recombinant Proteins**

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<td>Invivogen</td>
<td>Cat# vac-ova</td>
</tr>
<tr>
<td>Streptavidin microbeads</td>
<td>Miltenyi Biotec</td>
<td>Cat# 130-048-101</td>
</tr>
<tr>
<td>DNase I</td>
<td>QIAGEN</td>
<td>Cat# 70254</td>
</tr>
<tr>
<td>Recombinant murine SLC (CCL21)</td>
<td>Peprotech</td>
<td>Cat# 250-13</td>
</tr>
<tr>
<td>Recombinant murine RANTES (CCL5)</td>
<td>Peprotech</td>
<td>Cat# 250-07</td>
</tr>
</tbody>
</table>

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Boris Reizis (Boris.Reizis@nyumc.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human studies
The study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from participants prior to recruitment. The study was approved by NRES Committee North East-Newcastle and North Tyneside (08/H0906/72, 14/NE/1212 and 14/NE/1136). Peripheral blood or bone marrow was obtained from healthy volunteers or from the previously described patients with biallelic *IRF8* mutations (Bigley et al., 2017; Hambleton et al., 2011).

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#### Critical Commercial Assays

- **ARCTURUS PicoPure RNA Isolation Kit**
  - **SOURCE:** Applied Biosystems
  - **IDENTIFIER:** Cat# KIT0204

- **FCS, charcoal stripped**
  - **SOURCE:** GIBCO
  - **IDENTIFIER:** Cat# 10296028

- **TRIZOL Reagent**
  - **SOURCE:** Invitrogen
  - **IDENTIFIER:** Cat# 12676-029

#### DEPOSITED DATA

- **RNA-seq analysis of cultured dendritic cells**
  - **SOURCE:** This paper
  - **IDENTIFIER:** GEO: GSE110577

- **RNA-seq analysis of primary splenic dendritic cells**
  - **SOURCE:** Lau et al., 2016
  - **IDENTIFIER:** GEO: GSE76132

- **Microarray analysis of Notch2-deficient dendritic cells**
  - **SOURCE:** Salpathy et al., 2013
  - **IDENTIFIER:** GEO: GSE45681

#### EXPERIMENTAL MODELS: CELL LINES

- **Mouse: OP9-GFP**
  - **SOURCE:** Mohtashami et al., 2016
  - **IDENTIFIER:** N/A

- **Mouse: OP9-DL1**
  - **SOURCE:** Mohtashami et al., 2016
  - **IDENTIFIER:** N/A

- **Mouse: OP9-DL4**
  - **SOURCE:** Mohtashami et al., 2016
  - **IDENTIFIER:** N/A

- **Mouse: B16-OVA**
  - **SOURCE:** Falo et al., 1995
  - **IDENTIFIER:** N/A

- **Mouse: B16-FLT3L**
  - **SOURCE:** Mach et al., 2000
  - **IDENTIFIER:** N/A

- **Mouse: HoxB8-FL**
  - **SOURCE:** Redecke et al., 2013
  - **IDENTIFIER:** N/A

#### EXPERIMENTAL MODELS: ORGANISMS/STRAINS

- **Mouse: C57BL/6**
  - **SOURCE:** The Jackson Laboratory
  - **IDENTIFIER:** Stock #000664

- **Mouse: Rag2/OT-I**
  - **SOURCE:** Taconic
  - **IDENTIFIER:** 2334

- **Mouse: *irf8*<sup>−/−</sup>:B6(Cg)-Irf8<sup>tm1.2Hm</sup>/J**
  - **SOURCE:** Ouyang et al., 2011
  - **IDENTIFIER:** N/A

- **Mouse: *irf8*<sup>fl/fl</sup>:B6(Cg)-Irf8<sup>tm1.1Hm</sup>/J**
  - **SOURCE:** Feng et al., 2011
  - **IDENTIFIER:** N/A

- **Mouse: *itgax-Cre: B6.Cg-Tg*(Itgax-cre)<sup>1-1Reiz</sup>/J**
  - **SOURCE:** Caton et al., 2007
  - **IDENTIFIER:** N/A

- **Mouse: *batf3*<sup>−/−</sup>: B6.129S(C)-Batf3<sup>tm1Kmm</sup>/J**
  - **SOURCE:** Hildner et al., 2008
  - **IDENTIFIER:** N/A

### SOFTWARE AND ALGORITHMS

- **FlowJo**
  - **SOURCE:** FlowJo, LLC
  - **IDENTIFIER:** https://www.flowjo.com/

- **GraphPad Prism**
  - **SOURCE:** GraphPad
  - **IDENTIFIER:** https://www.graphpad.com/

- **DESeq2 (v3.0)**
  - **SOURCE:** Love et al., 2014
  - **IDENTIFIER:** http://bioconductor.org/packages/release/bioc/html/DESeq2.html

- **R (v.3.3.2)**
  - **SOURCE:** N/A
  - **IDENTIFIER:** https://www.r-project.org/

- **STAR aligner (v2.5.0c)**
  - **SOURCE:** Dobin et al., 2013
  - **IDENTIFIER:** https://github.com/alexdobin/STAR

- **Picard tools (v.1.126)**
  - **SOURCE:** http://broadinstitute.github.io/picard/

- **HTSeq (v0.6.0)**
  - **SOURCE:** Anders et al., 2015
  - **IDENTIFIER:** https://github.com/simon-anders/htseq

- **BEDTools (v2.17.0)**
  - **SOURCE:** Quinlan and Hall, 2010
  - **IDENTIFIER:** https://github.com/arq5x/bedtools2

- **nSolver (advanced analysis module v.1.1.4)**
  - **SOURCE:** Nanostring
  - **IDENTIFIER:** https://www.nanostring.com/products/analysis-software/nsolver
Animals
All animal studies were performed according to the investigator’s protocol approved by the Institutional Animal Care and Use Committees of New York University School of Medicine and of Columbia University Medical Center. Wild-type C57BL/6 mice (Jackson Laboratories) and Rag2-deficient OT-I TCR transgenic mice (Rag2/OT-I, Taconic) were maintained by intercrossing in the animal facility at New York University School of Medicine. Mice deficient for Batf3 (Hildner et al., 2008) or Irf8 (Ouyang et al., 2011), mice with a conditional LoxP-flanked allele of Itgax (ItgaxFlox/Flox) (Feng et al., 2011) and the Itgax-Cre deleter strain (Caton et al., 2007) have been described previously and were on pure C57BL/6 background. Irf8Flox/Irf8Flox and Itgax-Cre mice were intercrossed to obtain Irf8Flox/Irf8Flox Itgax-Cre+ mice with a specific deletion of Irf8 in CD11c+ cells. Mice were group-housed in individually ventilated cages and maintained under specific pathogen-free conditions. Male and female mice were used between 8 and 16 weeks of age. No obvious difference between sexes was observed within the parameters analyzed for our experiments.

Cell lines
FLT3L-secreting (Mach et al., 2000) and OVA-expressing (Falo et al., 1995) clones of the C57BL/6-derived B16 melanoma cell line (B16-FLT3L and B16-OVA, respectively) were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% sodium pyruvate, 1% MEM-NEAA and 1% penicillin/streptomycin (full DMEM) at 37°C in a humidified atmosphere at 5% CO2. The murine progenitor Hoxb8-FL cell line (Redecke et al., 2013) was cultured in RPMI medium supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, 10% supernatant from cultured B16-FLT3L cell line and 1 μm β-estradiol at 37°C in a humidified atmosphere at 5% CO2. For differentiation, Hoxb8-FL cells were cultured in the same medium without estradiol and with charcoal-stripped FCS to ensure the absence of estradiol. OP9 cell lines transduced with retroviruses encoding green fluorescent protein (GFP) or Notch ligands DL1 or DL4 (Mohtashami et al., 2016; Schmitt et al., 2004) were cultured in MEM-supplemented with 20% FCS and 1% penicillin/streptomycin (OP9 medium) at 37°C in a humidified atmosphere at 5% CO2. Prior to use in co-cultures, OP9 cells were treated with mitomycin C at 10 μg/mL for 2 hr, harvested, washed three times in PBS and resuspended in OP9 medium. For co-culture with human cells, OP9 were maintained in MEM-α supplemented with 10% FCS and 1% penicillin/streptomycin.

METHOD DETAILS

Methods: mouse
Cell preparations
Spleens were minced and digested with collagenase D (1 mg/mL) and DNase I (20 μg/mL) in full DMEM for 30 min at 37°C. Tissues were pressed through a nylon 70 μm cell strainer to yield single-cell suspensions and then subjected to red blood cell (RBC) lysis (155 mM NH4Cl, 10 mM NaHCO3, 0.1 mM EDTA) for 5 min at room temperature before being filtered. Bone marrow (BM) was prepared by flushing femurs and tibias with phosphate buffer saline (PBS) using a 27-gauge needle followed by RBC lysis and filtering through a sterile 70 μm cell strainer. Peripheral blood (PB) was obtained by submandibular bleed and subjected to RBC lysis for 5 minutes, followed by 3 min, at room temperature (RT).

Flow cytometry
Single-cell suspensions of cultured DCs or primary cells were stained for multicolor analysis with the indicated fluorochrome- or biotin-conjugated antibodies. Antibodies were diluted in FACS buffer (PBS, 1% FCS, 0.02% NaN3). With the exception of Hoxb8-derived DCs, staining of surface molecules with fluorescently labeled antibodies was performed for 20 min at 4°C in the dark. Hoxb8-derived DCs were stained at room temperature. For in vivo cross-presentation experiments, the OVA peptide/H-2Kb tetramer was used and staining was performed for 30 min at room temperature. Samples were acquired on LSR II (BD) flow cytometer using FACSDiva software (BD Biosciences) or Attune NxT (Invitrogen) using Attune NxT software and further analyzed with FlowJo software (Tree Star).

FLT3L-driven DC differentiation of Hoxb8-FL cultures
Hoxb8-FL progenitor cells were expanded in culture and differentiated as previously described (Grajkowska et al., 2017; Redecke et al., 2013). Briefly, progenitor cells were removed from β-estradiol-supplemented medium and washed three times in PBS with 10% charcoal-stripped FBS at room temperature. The cells were then plated in fresh medium without estradiol at 2x10^5 cells per well in 6-well plates and cultured for 7 days without replating.

Notch-driven DC differentiation of Hoxb8-FL cultures
FLT3L-driven Hoxb8-FL DC differentiation was initiated as described above. On day 3 of differentiation, cells were harvested and resuspended in fresh medium. The cells were then plated at 2.5x10^5 cells per well in 24-well plates containing a monolayer of mitomycin-treated OP9-DL1 cells. Where indicated, control OP9-GFP or OP9-DL4 cells were used in a similar fashion. For NOTCH2 blocking experiments, cells were treated with anti-NOTCH2 antibody or control IgG (50 ng/mL or 500 ng/mL) on day 3 at the time of co-culture initiation. Cell cultures were analyzed by flow cytometry on day 7.

FLT3L-driven DC differentiation of primary BM cultures
Single cell suspensions of primary murine BM cells were obtained as described above. The cells were suspended in DMEM medium supplemented with 10% FCS, 1% L-glutamine, 1% sodium pyruvate, 1% MEM-NEAA and 1% penicillin/streptomycin, 55 μM 2-mercaptoethanol and, 10% supernatant from cultured B16-FLT3L cell line (DC medium). Cells were plated at 2x10^5 cells
per well in 2 mL of DC medium in 24-well plates and cultured at 37°C in a humidified atmosphere at 5% CO2 for 7 days without replating.

**Notch-driven DC differentiation of primary BM cultures**

Primary BM cultures were initiated as described above. On day 3 of differentiation, half of the volume of cells in DC medium from each well was transferred to a single well containing a monolayer of mitomycin-treated OP9 cells in 24-well plates. Where indicated, control OP9-GFP or OP9-DL4 cells were used in a similar fashion. For NOTCH2 blocking experiments, cells were treated with anti-NOTCH2 antibody or control IgG at various concentrations on day 3 at the time of coculture initiation. Cell cultures were analyzed on day 7.

**In vitro DC migration assay**

DC migratory capacity was evaluated using a transwell assay using 24-well plates of 6.5 mm transwells with 5.0 μm pore polycarbonate membrane (Corning). Cultured DCS were harvested on day 7 and resuspended in DMEM medium supplemented with 2% FCS, 1% L-glutamine, 1% sodium pyruvate, 1% MEM-NEAA and 1% penicillin/streptomycin (migration medium) at 5 x 10⁵ cells/mL. Migration medium containing chemokines (100 ng/mL in 0.6 mL total volume) was placed at the bottom of each well. DC suspension (100 μL) was added to the top chamber and incubated at 37°C in a humidified atmosphere at 5% CO2 for 3 hr. Migrated cells at the bottom of the wells were recovered in 500 μL of cold PBS supplemented with 2% FBS and 0.5 mM EDTA and analyzed by flow cytometry. For each DC type, frequency of migrated cells was calculated as # cells migrated out of total input per DC type as determined flow cytometry.

**In vitro T cell stimulation**

Endotoxin-free ovalbumin (OVA) protein (Sigma Aldrich) was added to DC cultures on day 7 to a final concentration of 100 μg/mL. Cells were then harvested, washed three times with PBS and filtered using sterile 70 μm cell strainers. Single cell suspensions were obtained from the spleens and lymph nodes of Rag2/OT-I mice as described above. CD8+ T cells were isolated by magnetic-activated cell separation (MACS) by negative selection using biotinylated antibodies (B220, Ter119, Gr1, CD11c, NK1.1, F4/80, CD4, DX5) with streptavidin microbeads, and MACS columns (Miltenyi Biotec). CD8+ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE, 5 μM), washed and 4x10⁴ T cells were cultured with DCs at 1:1-10:1 ratio for 3 days. T cell proliferation was assessed by CFSE dilution using flow cytometry.

**In vivo cross presentation assay**

DC cultures were pulsed with endotoxin-free OVA protein for 2 hr as above. Cells were pooled, harvested, washed three times with PBS and filtered twice using sterile 70 μm cell strainers. Single cell suspensions were obtained from the spleens and lymph nodes of Rag2/OT-I mice as described above. cDC1, cells were stained with biotinylated antibodies against B220 and CD172a (FL cultures) or B220 and CD11b (FL+Notch cultures), and purified by negative selection on MACS columns. OVA-pulsed total DCs (2.5x10⁵ - 1x10⁶) or OVA-pulsed enriched cDC1 (3x10⁵) were resuspended in 0.1 mL PBS and injected i.v. into the retroorbital sinus. Spleens were harvested or PB was collected and analyzed by flow cytometry using OVA peptide/H-2Kb tetramer staining 7 days after DC vaccination.

**Tumor challenge**

B16-OVA melanoma cells were harvested, washed twice with PBS, and filtered. A total of 2.5x10⁵ B16-OVA cells in 0.1 mL PBS were injected i.v. into the retroorbital sinus 7-10 days following vaccination with OVA-pulsed DCs. Mice were monitored daily and sacrificed when moribund or on day 25 of observation if healthy. Lungs from perfused mice were harvested, fixed in 4% paraformaldehyde for 24 hr, sectioned and stained with hematoxylin/eosin for histological examination using light microscopy. Where indicated, 4x10⁵ B16-OVA cells in 0.2 mL PBS were injected i.v. into the tail vein.

**Cell sorting and sample preparation for RNA-seq**

DC populations were stained and sorted on BD FACSaria II as follows: cDC1 (CD11c<sup>hi</sup> MHCl<sup>+</sup> B220<sup>−</sup> CD24<sup>+</sup>) and cDC2 (CD11c<sup>hi</sup> MHCl<sup>−</sup> B220<sup>−</sup> CD11b<sup>+</sup>). Sorted cells (1–3x10⁵) were resuspended in 750 μL Trizol LS (Invitrogen), and RNA was extracted using the Arcturus PicoPure kit (Thermo Fisher Scientific). Equal volume of 70% ethanol was added to the aqueous phase of TRizol samples and applied to columns from the PicoPure kit. Up to 250 μL of ethanol/aqueous phase mix was loaded onto the column and spun at 100 g for 2 min for each load. Bound RNA was washed, treated with DNase I (Qiagen), and eluted as per manufacturer’s instructions. To remove phenol contamination, eluate was resuspended in 100 μL of Wash Buffer 1 and reloaded onto a fresh column followed by elution. RNASeq libraries were prepared using the Clontech Ultra low RNA kit, starting with 3 ng, with 10 cycles of PCR for cDNA amplification, and the Clontech Low Input Kit for library prep, with 7 cycles of PCR amplification, following the manufacturer’s protocol. The amplified library was purified using Ampure beads, quantified by Qubit and QPCR, and visualized in an Agilent Bioanalyzer. The libraries were pooled equimolarly, and run on a HiSeq 2500 as paired, 50 nucleotide in length.

**RNA-Seq data processing**

Sequencing reads were mapped to the mouse reference genome (GRCm38.85/mm10) using the STAR aligner (v2.5.0c) (Dobin et al., 2013). Alignments were guided by a Gene Transfer Format (Ensembl GTF GRCm38.85). The mean read insert sizes and their standard deviations were calculated using Picard tools (v.1.126) (http://broadinstitute.github.io/picard/). The read count tables were generated using HTSeq (v0.6.0) (Anders et al., 2015), normalized based on their library size factors using DESeq2 (v3.0) (Love et al., 2014), and differential expression analysis was performed. The Read Per Million (RPM) normalized BigWig files were generated using BEDTools (v2.17.0) (Quinlan and Hall, 2010) and bedGraphToBigWig tool (v4), and downstream statistical analyses and generating plots were performed in R environment (v3.1.1) (http://www.r-project.org).
**Methods: human**

**Cell isolation, flow cytometry and cell sorting**

Peripheral blood or bone marrow mononuclear cells (PBMC) were isolated by density centrifugation. For flow cytometry or fluorescence-activated cell sorting (FACS) (purity > 98%), cells were stained in aliquots of 1-3 x 10⁶ cells/50 μl of Dulbecco’s phosphate-buffered saline with 0.1%-2% fetal calf serum and 0.4% EDTA. Dead cells, usually < 5%, were excluded by DAPI (Partec) or Zombie (Biolegend) staining. Analysis was performed with an LSRFortessa X-20 and sorting with a FACSaria III (BD Biosciences) running BD FACSDIVA 8.0.1 or 8.0 software, respectively. Data were processed with FlowJo 10.4.1 (Treestar, Inc). Intracellular staining was performed after surface staining, lysis, and fixation (eBioscience) according to manufacturer’s instructions.

**In vitro generation of dendritic cells**

FACS-purified CD34⁺ bone marrow progenitors were cultured (typically 3000/well) in 96 well U-bottomed plates with or without pre-seeded OP9, OP9-DL1 or OP9-DL4 stromal cells (5000/well). Culture media consisted of 200 μL α-MEM (GIBCO) supplemented with 1% penicillin/streptomycin (Sigma), 10% fetal calf serum (GIBCO), 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D systems), 100 ng/ml FLT3-ligand (FL, Immunotools), 20 ng/ml stem cell factor (SCF, Immunotools), and 1% penicillin/streptomycin (Sigma). The half volume of media, with cytokines, was replaced weekly. At day 14 or 21, cells were harvested on ice, passed through a 50 μm filter, washed and stained for flow cytometric analysis or cell sorting. Cell output was normalized to 3000 input progenitors per well.

**Dendritic cell functional analysis**

For cytokine production, PBMC from healthy controls or in vitro-generated cells were cultured in the presence of polysinosinic: polycytidyllic acid (poly(I:C), 10 μg/ml, Invivogen), Lipopolysaccharide (LPS, 5ng/ml, Sigma), CL075 (1 μg/ml, Invivogen) and CpG (ODN 2216, 7.5 μM, Invivogen) for 14h at 37 °C, 5% CO₂ with addition of Brefeldin A (10 μg/ml, eBioscience) after 3 hr. Dead cells (usually < 30%) were excluded with Zombie amine dye (Biolegend). Intracellular cytokine staining was performed after surface staining, fixation, and permeabilization (eBioscience) according to manufacturer’s instructions.

For T cell proliferation, FACS-purified ex vivo or in vitro generated DCs (2,500-8,000 DC/well) were cultured with FACS purified allogeneic CD3⁺ T cells at a ratio of 1:10 DC:T cell (n = 2-9 DC/T cell pairs). Positive controls were generated by T cell co-culture with CD3⁺CD28⁺ beads (Dynabeads®, Thermo Fisher Scientific) at T cell:bead ratio 1:1. T cell proliferation was assessed by CFSE dilution on day 5 of culture.

**NanoString nCounter Gene expression analysis**

Ex vivo or culture-generated DCs were FACS purified (> 98% purity) and lysed in RLT buffer containing 1% β-mercaptoethanol, at a concentration of 2000 cells/μl. Samples were analyzed on the NanoString nCounter® FLEX platform according to manufacturer’s instructions. Briefly, 5 μl of lysate (10,000 cells) was mixed with reporter probes, hybridization buffer, and capture probes and hybridized at 65 °C for 12-30 hr. Samples were then processed on the NanoString Prep station and cartridges were read on the NanoString Digital Analyzer to yield a reporter code count (RCC) dataset. The human Immunology V2 panel was used, supplemented with the following 30 genes: ASIP, DAXX, MERTK, C19orf59, DBN1, Ki67, CCL17, F13A1, NDRG2, CD1c, FGD6, PACSIN1, CD207, FLT3, PPM1N, CLEC10A, GCSAM, PRAM1, CLEC9A, GGT5, S100A12, CLNK, LPAR2, TMEM14A, COBLL1, LYVE1, UPK3A, CXCL5, MAFF, ZBTB46.

Counts were normalized within the nSolver software (advanced analysis module version 1.1.4). The log2 transformed output data were analyzed using R (version 3.3.3). For principal component analysis (PCA), genes expressed below 24.8 in all samples were excluded from further analysis. The remaining 339 genes were used to construct the PCA plot. Heatmaps were generated in R and display log2 transformed expression.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

For experimental results, all statistical calculations were performed using Prism (GraphPad, La Jolla, CA). In animal experiments, statistical significance of differences between experimental groups was determined by non-parametric Mann-Whitney test. Differences in the Kaplan-Meier survival plots (Figure 5) were analyzed using log-rank test. In human cell culture experiments, statistical significance of differences between experimental groups was determined by unpaired, two-tailed Student’s t test.

For RNA-seq data, Wald test p values and adjusted p values provided by DESeq2 package were used for differential expression analysis (adjusted p < 0.1, FC > 2) which is based on estimating dispersions and uses a negative binomial generalized linear model. For sample clustering, we performed a classical multidimensional scaling (MDS) and a Euclidean distance based clustering. For pathway and enrichment analysis, we used hypergeometric distribution tests performed by clusterProfiler package (adjusted p < 0.1). The datasets were individually and comprehensively analyzed and visualized all in the R statistical environment (v3.2.5).

**DATA AND SOFTWARE AVAILABILITY**

The complete processed expression data from RNA-seq and Nanostring experiments are attached as Tables S1 and S5, respectively. The accession number for the raw RNA-seq sequencing data reported in this paper is GEO: GSE110577.