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Plasmacytoid dendritic cell heterogeneity is defined by CXCL10 expression following TLR7 stimulation

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ABSTRACT

Plasmacytoid dendritic cells (pDCs) play a critical role in bridging the innate and adaptive immune systems. pDCs are specialized type I interferon (IFN) producers, which has implicated them as initiators of autoimmune pathogenesis. However, little is known about the down-stream effectors of type I IFN signaling that amplify autoimmune responses. Here we have used a chemokine reporter mouse to determine the CXCR3 ligand responses in DCs subsets. Following TLR7 stimulation conventional type 1 and type 2 DCs (cDC1 and cDC2 respectively) uniformly upregulate CXCL10. By contrast, the proportion of chemokine positive pDCs was significantly less, and stable CXCL10+ and CXCL10- populations could be distinguished. CXCL9 expression was induced in all cDC1s, in half of the cDC2 but not by pDCs. The requirement for IFNAR signaling for chemokine reporter expression was interrogated by receptor blocking and deficiency and shown to be critical for CXCR3 ligand expression in Flt3-ligand derived DCs. Chemokine producing potential was not concordant with the previously identified markers of pDC heterogeneity. Finally, we show that CXCL10+ and CXCL10- populations are transcriptionally distinct, expressing unique transcriptional regulators, IFN signaling molecules, chemokines, cytokines and cell surface markers. This work highlights CXCL10 as a downstream effector of type I IFN signaling and suggests a division of labor in pDCs subtypes that likely impacts their function as effectors of viral responses and as drivers of inflammation.

INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells that link innate and adaptive immunity. There are multiple DC subsets each with specialized immune-modulatory roles. Conventional (c)DCs are comprised of two broad subsets, cDC1s and cDC2s[1]. cDC1s are recognized for their capacity to cross-present antigens and CD8+ T cell priming, while cDC2 have a high capacity for MHC class II-mediated presentation of exogenous antigen[2]. Plasmacytoid (p)DCs are distinct from cDCs. Although pDCs can present antigen, they are best known for their ability to rapidly produce large amounts of type I IFNs (IFNα/β)[3]. This feature plays an important role in response to viral infection, but also implicates pDCs as key effectors in autoimmune diseases[3-6]. pDCs express Toll-like receptor 7 (TLR7) which recognizes single-strand RNA leading to activation of the transcription factor interferon regulatory factor 7 (IRF7) which initiates the secretion of type I IFNs. This signaling pathway is important in the autoimmune disease Systemic Lupus Erythematosus (SLE), in which gene dosage of TLR7 is linked to susceptibility and active disease is characterized by the expression of a type I IFN signature[5, 7, 8]. IFNs and their downstream signature genes have proven useful biomarkers of disease and allow patients to be stratified for tailored therapeutic intervention[9-11]. The pathogenic role of type I IFNs in autoimmunity has recently be validated in two phase 2 clinical trials[6]. In addition to IFNα/β, pDCs can express other inflammatory cytokines and chemokines[12]. However, the mediators that are down stream of IFNs and amplify autoimmune responses remains in completely understood.

The CXCR3 family chemokine CXCL10 is an IFNα/β signature gene, upregulated in patients with SLE[13, 14]. The CXCR3 chemokines CXCL9 and CXCL10 promote cell recruitment to sites of inflammation[15, 16]. During viral infection, DC-derived CXCL10-CXCR3 interactions can optimise both T and B cell responses[17-19]. Combined, this suggests CXCL10 is a downstream mediator of type I IFNs and may amplify autoimmune immune responses.

In this study, we used chemokine reporter mice to determine the role of type I IFNs in the induction of CXCR3 chemokines following DC TLR7 stimulation. Using IFNAR1 blocking and deficiency, we found that CXCL9 and CXCL10 upregulation exclusively dependent on IFNα/β in all Flt3-ligand-derived DC subsets. Serendipitously, we revealed heterogeneity within the pDC population that is defined by expression of CXCL10. Previous studies have identified heterogeneity within the pDC lineage[3, 20-24], although whether this diversity is functionally relevant to type I IFN signaling and pDC function is incompletely understood. We found CXCL10- and CXCL10+ populations were stably induced and transcriptionally distinct. The functional characterization of pDC subtypes will progresses our understanding of how pDCs act as effectors of viral responses and as drivers of inflammation.

RESULTS

In vitro derived DCs have different CXCR3 ligand expression following TLR7 stimulation

To characterize the expression of CXCR3 chemokines in DC populations, we made use of the REX3 transgenic mouse that reports the expression of CXCL9 and CXCL10 by red fluorescent protein (RFP) and blue fluorescent protein (BFP) respectively[18]. Steady state-DC populations were differentiated *in vitro* from REX3 BM precursors in Flt3-ligand cultures[25]. After 8 days, differentiated cultures including pDC, cDC1 and cDC2 populations were stimulated with the TLR7 agonist, Imiquimod (IMQ, Figure 1a)[1]. Following activation, cells were stained for DC markers and subsets were assessed for the expression of BFP and RFP (Figure 1b). Approximately 30-50% of pDCs upregulated BFP (CXCL10), while RFP-CXCL9 was only expressed in a small number of cells. The remaining pDCs were double negative for chemokine expression, similar to unstimulated cultures. The bimodal distribution of CXCL10 in pDCs, was in contrast to both populations of cDCs, that uniformly upregulated CXCL10. The majority of cDC1 cells upregulated high levels of both BFP and RFP (consistently over 95%), while cDC2s where either BFP+ RFP+ or BFP+RFP-.

We next titrated IMQ to determine if higher concentrations would increase the upregulation of CXCL9 and CXCL10 (Figure 1c). This confirmed that 1ug mL^{-1} IMQ was optimal for CXCL10 expression by pDCs (and cDCs, not shown), as chemokine reporters were not further increased at higher concentrations. Importantly, similar chemokine expression was observed for each DC subset following stimulation with the TLR9 agonist, ODN2216 and a combination of both TLR7 and TLR9 agonists (not shown). Therefore, the bimodal expression of CXCL10 in pDCs was not a result of insufficient stimulation.

To assess the upregulation of CXCL10 on DCs subtypes following TLR7 activation, DCs were isolated directly from REX3 spleens. Unlike unstimulated cells from Flt3-ligand cultures, *ex vivo* unstimulated pDCs showed background expression of BFP. This expression was further upregulated with IMQ (Figure 1d). Isolated REX3 pDCs strongly upregulated BFP, and more cells were double positive for both BFP and RFP than their Flt3-ligand counterparts. Despite this, a population of cells remained chemokine negative following stimulation. Therefore, regardless of the method of purification or differentiation, both Flt3-ligand cultured and *ex vivo* purified pDCs display bimodal expression of CXCL10 following TLR7 stimulation.

To exclude the possibility that CXCL10 negative cells were observed due to contamination of pDC gates, we expanded our staining panel to include the additional pDC marker Bst2 and lineage markers CD11c, CD3 and CD19 in both Flt3-ligand cultures and *ex vivo* purified DC cultures (Supplemental Figure1a and b respectively). Bst2 co-stained SiglecH pDCs, which were CD11c+CD3-CD19- irrespective of CXCL10 expression. Thus, stimulated BFP-expressing and BFP-negative pDCs are both bona fide pDC populations and chemokine-negative cells are not explained by contamination.

IMQ-induced CXCR3 ligand expression requires type I IFN signaling

Despite all CXCR3 ligands being cytokine-inducible, differences exist in the potency of cytokines such as IFNα/β, IFNγ and TNFα that modulate their expression. Additionally, the response to inducing cytokines can

change depending on cell type and experimental setting[15, 26]. We investigated the requirement for type I IFNs in the induction of CXCL9 and CXCL10 following IMQ stimulation of DC populations *in vitro*. Remarkably, following treatment with anti-IFNAR1 antibody[27], chemokine expression was reduced to levels similar to that in unstimulated DCs (Figure 2a,b). REX3 reporters were crossed with IFNAR1-deficient animals (REX3xIFNAR$^{-/-}$) to confirm this major cytokine signaling pathway. Indeed, DCs derived from REX3xIFNAR$^{-/-}$ animals were unable to upregulate either CXCL9 and CXCL10, as marked by the REX3 reporter expression, following IMQ exposure (Figure 2c,d). DCs were isolated directly from REX3 and REX3xIFNAR$^{-/-}$ spleens and cells were stimulated *ex vivo*. Interestingly, unstimulated REX3xIFNAR$^{-/-}$ pDCs showed no chemokine expression, unlike the REX3 mice that exhibited baseline BFP+ cells (Figure 2e). Following stimulation, however, the IFNAR$^{-/-}$ cells did upregulate both CXCL9 and CXCL10. In fact, given the lower baseline, the REX3xIFNAR$^{-/-}$ cells showed higher fold increase of response compared to their REX3 controls. These results indicate that IFNα plays a major role in the regulation of CXCL10 expression by Flt3-ligand-derived DCs, however, there are differences in the chemokine responses of isolated and *in vitro* differentiated pDC populations.

CXCL10- and CXCL10+ pDC subsets are stable

As pDCs are crucial mediators linking the innate and adaptive arms of the immune system, the observation that only a proportion of these cells produce CXCL10 has implications for their role in effector cell recruitment. We therefore examined the division of CXCL10 potential in pDCs. To complement our titration of IMQ stimulation (Figure 1c), we investigated if type-I IFNs were limiting in our culture system, which may indicate that BFP- pDCs represent cells that receive insufficient stimulation, rather than a distinct pDC subpopulation. However, the addition of either IFNα or IFNβ during IMQ stimulation of *in vitro*-derived pDC cultures did not further increase their chemokine expression potential, suggesting that stimulation was saturating (Figure 3a). Further, we reasoned that, if CXCL10 expression defined distinct subtypes of pDCs, this expression potential would remain stable following extended stimulation. To this end, stimulated Flt3-ligand-derived pDC populations were sorted and restimulated in separate cultures. There was no change in expression of either BFP+ or BFP- populations when cells were re-cultured with or without IMQ (Figure 3). Thus, the CXCL10 reporter expression observed during initial stimulation was conserved following subsequent stimulations suggesting these CXCL10-defined populations reflected their overall potential to produce CXCL10. Importantly this further demonstrated that BFP- pDCs were incapable of expressing CXCL10 and not the result of insufficient stimulation and that BFP- and BFP+ cells were distinct and no plasticity was observed between these populations.

Current markers of pDC heterogeneity are insufficient to define CXCL10 expression potential

Several previous studies have defined and characterized heterogeneity within pDC compartment either prior to or following stimulation[3, 21-24, 28, 29]. Heterogeneity within this DC lineage represents an attractive hypothesis to explain the diverse functions of pDCs and the potential division of labor during immune

responses. As CXCL10+ and CXCL10- pDCs may have different roles during infection and inflammation, we wanted to determine if these chemokine-specified subsets could be identified without the use of REX3 mice. Expression of pDC BFP was compared with cell surface markers previously shown to identify pDC subtypes. Flt3-ligand-derived populations of unstimulated, stimulated BFP- and stimulated BFP+ were compared for expression of SiglecH, CD8, Sca1 and CCR9. No single marker alone indicated CXCL10 potential, however there was a correlation between CXCL10 expression with CCR9 and SiglecH downregulation (Figure 4a). We next combined our antibody panel with an unsupervised cluster analysis to determine if the combination of pDC markers could segregate CXCL10 expressing cells. Unsupervised tSNE dimensional reduction was used to analyse expression of SiglecH, CD11b, MHCII, CD8, Sca1, CCR9, CXCR3 with and without the inclusion of BFP expression in the parameter analysis (Figure 4b). When the BFP parameter data was included in this analysis, populations of unstimulated and stimulated BFP+ and BFP- were located in isolated cell clusters. The same analysis was performed with the exclusion of BFP expression data. Here, unstimulated pDCs could still be separated from the stimulated samples. However, BFP+ and BFP- cells were now found in largely overlapping clusters and could not be separated. Therefore, neither direct flow analysis or unbiased tSNE clustering analysis could distinguish a distinct pDC marker (or combination of makers) to identify pDC populations without the inclusion of CXCL10 reporter data. Thus, additional markers are required to further define the CXCL10 potential of pDC populations without REX3 mice.

CXCL10 expression defines transcriptionally distinct pDC subsets

As the current set of surface markers were insufficient to resolve CXCL10- and CXCL10+ pDC subsets, we next identified genes that were differentially expressed between each population using whole genome RNA sequencing analysis. This confirmed that CXCL10- and CXCL10+ pDCs populations were transcriptionally distinct subsets, with 680 gene transcripts differentially regulated between them (Figure 5a-d). The top 20 gene ontology categories defined by Metascape analysis ranked by p-value (log_{10}) for differentially expressed genes highlighted pathways regulated between pDC samples involve cell activation, adhesion, migration and actin dynamics in addition to viral response, cytokine signaling and SLE signature genes (Supplemental Figure 2a). The pDC lineage-defining transcription factor E2-2[30] (also known as TCF4) was unchanged between these groups (Supplemental Figure 2c), however, other transcriptional regulators important for DC fate decisions, such as PU.1, Xbp1 and Batf were differentially regulated between samples (Figure 5a)[2]. Several genes (such as TCF7, CD5, CD19) differentially regulated between our *in vitro*-derived, simulated pDC populations play important roles in T or B cell biology, but are largely uncharacterized in DCs. Although we found no evidence of T or B cell survival following 8 days of Flt3-ligand culture and these cells are absent from our pDC gating strategy (Supplemental Figure 1a), we used reference RNAseq data compiled for haemosphere.org[31] to determine that *in vitro*-derived pDC populations were bona fide pDCs without contamination. Using unbiased multi-dimensional scaling, *in vitro* pDC populations were most similar to *ex vivo* purified, unstimulated splenic pDC and unrelated to sorted spleen and lymph node naïve CD4 T cells,

naïve CD8 T cells and total B cells (Supplemental Figure 2b). Further, by comparing lineage defining gene transcripts, we showed transcripts of *in vitro* pDCs subtypes, were related to *ex vivo* purified, unstimulated pDCs and neither BFP+ or BFP- pDC populations were enriched for T or B cell transcripts (Supplemental Figure 2c). Thus, by cellular and transcriptional analysis, pDC populations are bona fide pDCs without contaminating lymphocytes.

IFNα2, the sole differentially regulated IFNα gene between CXCL10+ and CXCL10-, was found more highly expressed in the CXCL10-BFP- cells (Figure 5c and Table 1). Further, we found no difference in expression of transcription factors IRF7, IRF8 or Runx2 that upregulate IFNα production or differences in IFNAR receptor or TLR7 expression (Supplemental Figure 2c, Table 1 and data not shown), both subsets upregulated unique TLR and IFN signaling pathways (Figure 5b). Consistent with the observation that CXCR3 chemokines are induced secondary to type I IFN expression, IFN-regulated genes were found differentially expressed. Indeed, 11 type I IFN signature genes that are found overexpressed in autoimmune patients were expressed (Figure 5b-d)[9-11]. The majority of these were found in the BFP+ population, although Oasl1 was more highly expressed in the BFP- cells. Together with the observed increase in transcripts for chemokines and cytokines in this population (Figure 5c), this confirms that CXCL10- pDCs are an activated and signaling population.

RNAseq data provided multiple differentially regulated genes that transcribe cell surface proteins (Figure 5d). We next attempted to use this list to find markers to distinguish pDC subpopulations. Consistent with our previous work, CCR9 was found to be down regulated on BFP+ expressing pDCs. CCR9 expression was co-analysed with antibodies for Ly6C.2, CCR2, CD9, CD5 and CD19 (Figure 5e and data not shown). This confirmed our RNAseq approach as Ly6C.2 and CCR2 expression correlated with the CXCL10+ pDC population. However, these markers were not able to completely segregate the pDC populations. We next used these additional markers (SiglecH, CD11b, MHCII, CCR9, CCR2, Ly6C.2, CD19, CD8 and CD9) in unsupervised clustering analysis with and without inclusion of BFP expression data (Figure 5f). Again, when the BFP parameter data was included in this analysis, populations of unstimulated and stimulated BFP+ and BFP- were located in isolated cell clusters. The use of additional surface markers allowed BFP+ and BFP- expressing cells to be more segregated than our previous analysis (Figure 5f and Figure 4b). However, this combination of markers could still not fully distinguish pDCs based on CXCL10 expression without the inclusion of the BFP parameter. This work suggests that pDCs are more heterogeneous following activation than currently appreciated, and the CXCL10+ and CXCL10- compartments may comprise of further subpopulations.

DISCUSSION

This study sought to characterize the expression of CXCR3 chemokines in Flt3-ligand derived DC subsets. Following TLR7 activation, we found pDCs, cDC1s and cDC2s express reproducible and distinct profiles of

CXCL9 and CXCL10 that are primarily induced by IFNα/β. We further identified heterogeneity within the pDC population that is characterized by CXCL10 expression. The BFP+CXCL10+ and BFP-CXCL10- activation-induced subpopulations of pDCs were transcriptionally distinct. BFP+ pDCs expressed increased PU.1. Conversely, BFP-CXCL10- pDCs had higher expression of Batf and Xbp1. These transcription factors regulate DC lineage fate, however, little is known about how they may act to specify cell function in mature DC subsets[2, 3].

pDCs have been implicated in the pathogenesis of autoimmune diseases that are characterized by a type I IFN signature, such as SLE[4, 32]. Using cellular and molecular approaches, we show that IFNAR signaling plays a major role in the regulation of CXCL9/10 expression by all Flt3-ligand DCs. As unsorted DC cultures conditions were used for stimulation, we suspect that pDCs are the cellular source of IFNα/β leading to cDC chemokine expression. In contrast to Flt3-ligand derived pDCs, IFNAR1-deficient *ex vivo* isolated pDCs are able to upregulate both CXCL9 and CXCL10. Potentially, isolated DCs can produce multiple inflammatory cytokines, such as TNFα, in addition to IFNα/β which may override this pathway. Alternatively, *ex vivo* pDCs contain an additional subtype with CXCL9 expression potential that is not found in Flt3-ligand cultures and this population is increased in IFNAR1-deficient mice. While IFNAR-deficiency does not alter the potential for pDCs to produce IFNβ, some DC populations are altered in IFNAR$^{-/-}$ animals[23, 33]. Together, this suggests that IFNAR$^{-/-}$ mice are more complicated than a simple receptor knockout and that compensatory mechanisms may exist in this model. Consistent with IFNα/β signaling, we found multiple type I IFN signature genes were differentially regulated in our pDC populations[9-11]. These were genes predominantly upregulated in the BFP+CXCL10+ population. The observation that IFNα2 is expressed by CXCL10- pDCs further supports the notion that chemokine negative cells are not the result of insufficient stimulation, and indeed are a likely cellular source of IFNs for BFP+CXCL10+ pDCs. Combined, this suggests that pDC-derived IFNα/β can act on all DC subsets leading to chemokine expression, further emphasizing CXCL10 as a critical downstream mediator and biomarker of type I IFNs[13, 14].

CXCL10- and CXCL10+ pDC populations express transcriptionally regulated cell surface markers, cytokines and chemokines that highlight the diverse range of pDC functions[3]. This emphasizes CXCL10 expression an essential consideration when determining pDC function *in vivo* and suggests that different populations may influence adaptive immune responses via distinct mechanisms. Chemokine receptor expression by pDCs may indicate preferential homing between populations. BFP+ cells showed increased expression of CCR2 which promotes guidance to the skin, while BFP- cells expressed the gut homing chemokine CCR9[34, 35]. The CXCL10+BFP+ pDC population may be superior at T cell recruitment and priming[17, 18]. Not only due to the expression of CXCL10, but also expression of activating immune-modulatory molecules such as Ly6c, Clec7a, CD74, Slamf7, Ly86 and CD53. Interestingly, this population also expressed higher levels of inhibitory receptors, such as CD300A, Clec4a, BTLA/CD272, CD200/CD200r1[3]. Recently it was shown following viral infection that pDCs promote cDC1 DC maturation[36]. CD8+ T cells were shown to promote

interactions between themselves and cDC1s through XCL1 expression. Our data suggests specific pDC populations may be involved in these cell clusters, either through pDC CXCL10+ expression, or through XCL1 expression by CXCL10- pDCs. Our data suggests a correlation between pDC subtypes defined by IFNβ expression that express chemokines such as CCL3 and have different potential for attracting T cells, however CXCL10 was production was not assessed in this study[23].

Unfortunately, surface markers that define pDC populations based on CXCL10 potential remain elusive. The strongest correlation with chemokine expression was the expression of CCR9, which was highest on the BFP- pDC population. Although, CCR9 expression in BM pDCs is a marker of heterogeneity[37, 38], others have proposed that alternatively, these cells are precursors of cDCs[39]. In our hands, the BFP+ pDCs are largely CCR9- and have higher expression of PU.1, similar to cDC precursors. However, CCR9-BFP+ cell chemokine expression was distinct from the cDC compartment and expresses Bst2 and increased levels of SiglecH, which specifies them within pDC subset. Interestingly, DC populations defined by CXCL10 do not segregate with previously described populations of mouse pDCs described as Sca1+ and Sca1- populations or CD9+Ly6C-CCR9- that produce IFNα and CD9-Ly6C+CCR9+ that do not[22, 24]. These pDC subpopulations are distinguishable prior to stimulation and likely represent different maturation states, whereas CXCL10 expression only defines pDC heterogeneity following TLR-ligand stimulation.

Recently, a study of human pDCs has also revealed activation-induced pDC subpopulations[21]. This study demonstrated that influenza virus treatment of pDCs resulted in three dominant populations with specific molecular features. These populations were distinguished based on the PD-L1 and CD80 surface expression. Parallels can be drawn between these newly described human pDC subpopulations and those identified here. Some common markers, CD80 and CD83 were both more highly expressed in the BPF+ cells in our study and the PD-L1-CD80+ population (termed P3). Similar to our hypothesis for BFP+ pDCs, this study demonstrated the P3 strongly induce T cell activation. The BFP- population shares similarities to the described PD-L1+CD80+ (P1) population due to their common expression of TACI, TNF and IFNα2. In human pDCs, this population was shown to exclusively produce IFNα and TNFα[21]. Finally, human and mouse pDC populations exhibit distinct morphology[21, 22]. Our Metascape gene ontology analysis (Supplemental Figure 2a) highlighted differentially expressed pathways relating to adhesion and actin dynamics suggesting this may also the case for pDC populations defined by CXCL10 expression. One intriguing hypothesis is that CXCL10 expression potential may align with the lineage precursor cell that gave rise to each pDC subtype. In Flt3-ligand cultures, both common myeloid precursors (CMP) and common lymphoid precursors (CLP) give rise to functional, IFNα expressing pDC populations[29, 40, 41]. Differences between CMP and CLP-derived pDC populations remain incompletely characterized.

This study highlights the complexity that exists within the pDC population and a division of labor between pDC populations following stimulation. This work begins to unravel this complexity and relate this to how pDCs may function *in vivo* to attract as effectors of viral responses and as drivers of type I IFN inflammation.

METHODS

Mice

Mice were maintained on a C57BL/6 background in specific-pathogen-free conditions. Animal experiments were performed an accordance with the Walter and Eliza Hall Institute animal ethics committee. REX3 (Reporting the Expression of CXCR3 ligands) transgenic mice[18] were crossed with IFNAR1$^{-/-}$ [42] for analysis.

Generation and activation of Flt3-ligand dendritic cells

Flt3-ligand DCs were generated as previously described[25] with some modifications. Bone marrow was flushed and washed in RPMI1640 3% FCS, through an FCS underlay. Red cells were removed and cells were washed two more times through an FCS underlay. Cells were cultured at 1.5x10^6 cells mL^{-1} in specific DC-media (KDS RPMI, 2-ME, 10% FBS) containing 200 ng mL^{-1} recombinant Flt3-ligand (BioXcell), for 8 days at 37C in 10% CO_2. Flt3-ligand DCs were resuspended in cultured DC-media at a concentration of 5x10^5 cells mL^{-1} and cultured alone or with 1 μg mL^{-1} IMQ (Invivogen) with or without 10 μg mL^{-1} anti-IFNAR1 (MAR1, PJ. Hertzog[27]) 24 h at 37C in 10% CO_2. IFNα and IFNβ (PBL Assay Science) was added to some stimulating IMQ cultures.

Splenic DC preparation

Small spleen fragments were digested successively for 20 min and two times for 10 min at 37C with 0.2 mg mL^{-1} of collagenase P (Roche), 0.8mg mL^{-1} of Dispase II (Roche) and 0,1 mg mL^{-1}of DNase I (Roche)[43]. After each incubation, the supernatant was collected and remaining tissue pieces were incubated with fresh digestion medium. Light-density cells were isolated from digested cell suspensions using Nycodenz density medium (Nycomed Pharma, Oslo, Norway) at 1.076 g cm^{-3} and centrifuged 15 min at 1700g at 4C[44]. Cells were collected, washed and depleted of non-DC lineage using an mAb cocktail (CD3 (KT3-1.1), Thy-1 (T24/31.7), Ly6G (1A8), CD19 (ID3), erythrocyte (TER119)) and BioMag anti-Ig beads (Qiagen).

Flow Cytometry

DCs were stained with a combination of Siglec-H (eBio44c), MHCII (M5/114.15.2), CD11c (N418), and CCR9 (eBioCW-1.2); CD24 (M1/69), CD11b (M1/70), Bst2 (120G8) and Fixable Viability Stain (A700), CD8 (53.6.7), CD19 (1D3), Sca-1 (E13-161.7), Ly6C (HK14), CCR2 (MC-21, M Mack[45]). Cell analysis and sorting was performed on LSR Fortessa x-20, FACS-Aria Fusion (BD Biosciences). Lineage reference cells were *ex vivo* purified, unstimulated splenic pDC (CD11c+, Bst2+, F4/80- (BM8)) and unrelated to sorted spleen and lymph node naïve CD4 T cells (CD4+ (GK1.5), CD25- (PC61.5), CD62L+ (MEL14), CD44-

(IM7)), spleen and lymph node naïve CD8 T cells (CD8+, CD62L+, CD44-) and spleen and lymph node total B cells (B220+ (RA6-8C3), CD19+, Ter119-, F4/80-, CD11b-)

RNA sequencing

RNA was isolated independently from two biological replicates (RNeasy plus micro kit, Qiagen). mRNA reverse transcription and cDNA libraries were prepared using the SMART-Seq v4 Ultra low Input RNA kit (Clontech Laboratories). Samples were sequenced with Illumina NextSeq 500 or Illumina Genome Analyzer sequencers. Sequence reads were aligned to the GRCm38/mm10 build of the Mus musculus genome using the Subread aligner[46] and genewise counts were obtained using featureCounts[47]. Genes were filtered from downstream analysis if they failed to achieve a CPM (counts per million mapped reads) value of 0.1 or greater in at least two libraries. Counts were converted to log2 counts per million, quantile normalized and precision weighted with the 'voom' function of the limma package[48, 49]. Empirical Bayes moderated t-statistics were used to assess differences in expression[50]. Empirical Bayes moderated-*t* P values were computed relative to a fold-change cutoff of 1.2-fold using treat[51]. Genes were called differentially expressed if they achieved a false discovery rate of ≤0.01. (RNAseq data uploaded to GEO). Metascape analysis was used to find the top 20 gene ontology categories defined by ranked by p-value ($\log_{10}$).

Statistical analysis

Flow cytometry was analysed using Flowjo (Treestar) and Prism 6 (Graphpad). Flowjo downsample and tSNE plugin was used for unsupervised cluster analysis. Significance of CXCL10 blocking experiments was determined by two-way *ANOVA* with *Tukey's* multiple comparison post-test.

Accession codes

Raw sequence reads, read counts and normalized expression values have been deposited into the Gene Expression Omnibus (GEO) database under accession numbers GSE110975 and GSE113770.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1 REX3 chemokine reporter expression in IMQ stimulated DCs. (**a-c**) 8 day *in vitro*-derived Flt3-ligand cultured REX3 DCs stimulated with IMQ at indicated concentrations for 24h and analyzed by flow cytometry. (**d**) *Ex vivo* purified DCs were isolated from REX3 spleens, stimulated with IMQ for 24h and pDCs were analyzed by flow. Results are representative of at least 3 individual experiments.

Figure 2 IMQ-induced CXCR3 ligand expression requires type I IFN signaling. (**a**) *In vitro*-derived Flt3-ligand cultured REX3 DCs were stimulated with IMQ with anti-IFNAR1 blocking antibody as indicated. (**b**) Normalized CXCL10 expression of a. IMQ alone CXCL10 is set to 100% expression for each DC population. (**c**) *In vitro*-derived Flt3-ligand cultured REX3 and REX3xIFNAR1$^{-/-}$ DCs were stimulated with IMQ with anti-IFNAR1 blocking antibody as indicated. (**d**) Frequency of CXCL10+ cells of c. (**e**) *Ex vivo* purified pDCs isolated from REX3 and REX3xIFNAR1$^{-/-}$ spleens were stimulated with IMQ for 24h. (**a, c, e**) Results are representative of 3 individual experiments. (**b, d**) Data is compiled from 3 individual experiments. Results are expressed as mean +/- SD of individual experiments. ***$P < 0.0001$, ** $P < 0.001$, * $P < 0.05$, two-way *ANOVA* with *Tukey's* multiple comparison post-test.

Figure 3 CXCL10 expression is stable in pDCs following IMQ stimulation. (**a**) *In vitro*-derived Flt3-ligand cultured REX3 DCs were stimulated with IMQ for 24 hours with additional IFNα or IFNβ as indicated. (**b**) *In vitro*-derived Flt3-ligand cultured REX3 pDCs were sorted on CXCL10-BFP expression after 24h stimulation with IMQ. Sorted BFP+ and BFP- populations were returned to culture either left unstimulated, or restimulated for an additional 24h prior to flow analysis. Results are representative of 3 individual experiments.

Figure 4 Current pDC markers are insufficient to define heterogeneity in CXCL10 expression. (**a**) *In vitro*-derived Flt3-ligand cultured REX3 DCs were left unstimulated (grey) or IMQ (1ug mL^{-1}) stimulated REX3 for 24h (BFP+ blue; BFP- red) pDCs were analyzed for expression of SiglecH, CD8, Sca1 and CCR9. Populations gated based on CXCL10 expression, downsampled to achieve equivalent population sizes and overlaid to compare marker expression. (**b**) tSNE analysis using concatenated pDC populations from unstimulated and stimulated REX3 cultures of a. Analysis used SiglecH, CD11b, MHCII, CD8, Sca1, CCR9, CXCR3 flow parameters +/- CXCL10 expression data. Results are representative of 3 individual experiments.

Figure 5 CXCL10+ and CXCL10- pDCs are transcriptionally distinct, but cannot be identified by additional surface markers. (**a-d**) Differentially expressed genes from *in vitro*-derived Flt3-ligand cultured, IMQ (1ug mL^{-1}) stimulated REX3 pDCs BFP+CXCL10+ and BFP-CXCL10- populations analyzed by RNAseq. Genes are categorized by function. Type 1 IFN signature genes (red) and surface molecules analyzed (green) are highlighted. (**e**) *In vitro*-derived, IMQ (1ug mL^{-1}) stimulated REX3 (BFP+ blue; BFP- red) pDCs were

analyzed for expression of Ly6C.2 and CCR2. Populations gated based on CXCL10 expression, downsampled to achieve equivalent population sizes and overlaid to compare marker expression. (**f**) tSNE analysis using concatenated pDC populations from unstimulated and stimulated REX3 cultures. Analysis used SiglecH, CD11b, MHCII, CCR9, CCR2, Ly6C.2, CD19, CD8 and CD9 flow parameters +/- CXCL10 expression data. RNAseq samples were collected in 2 independent experiments. Libraries were made and analyzed together. Flow results are representative of 3 individual experiments.

Supplemental Figure 1 Gating strategy for pDCs. (**a**) 8 day *in vitro*-derived Flt3-ligand cultured REX3 DCs were unstimulated or stimulated with IMQ for 24h and analyzed by flow cytometry. Panels show co-expression of SiglecH with Bst2 and lineage specifying markers CD11c, CD3 and CD19 compared to BFP CXCL10 induction. (**b**) *Ex vivo* purified DCs were isolated from REX3 spleens were stimulated and analyzed as in a.

Supplemental Figure 2 Transcriptional analysis of pDC subpopulations with lineage reference data. (**a**) Metascape analysis of the top 20 gene ontology categories defined ranked by p-value ($\log_{10}$) for differentially expressed genes between stimulated, *in vitro*-derived Flt3-ligand cultured REX3 pDC populations. (**b**) Multi-dimensional scaling of transcripts from *in vitro*-derived pDC populations compared to *ex vivo* purified, unstimulated splenic pDC and sorted spleen and lymph node naïve CD4 T cells, naïve CD8 T cells and total B cells. (**c**) Comparison of specific lineage defining gene transcripts between samples analyzed in a. Note that the pDC genes, Tcf4, Tlr7 and Tlr9 are also expressed in B cells.

Table 1 Raw data counts of IFNα genes transcripts in pDC populations. Log2 Reads Per Kilobase of transcript per million mapped reads from pDC populations analyzed in Figure 5.