Interferon priming is essential for human CD34+ cell-derived plasmacytoid dendritic cell maturation and function

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Plasmacytoid dendritic cells (pDC) are essential for immune competence. Here we show that pDC precursor differentiated from human CD34+ hematopoietic stem and progenitor cells (HSPC) has low surface expression of pDC markers, and has limited induction of type I interferon (IFN) and IL-6 upon TLR7 and TLR9 agonists treatment; by contrast, cGAS or RIG-I agonists-mediated activation is not altered. Importantly, after priming with type I and II IFN, these precursor pDCs attain a phenotype and functional activity similar to that of peripheral blood-derived pDCs. Data from CRISPR/Cas9-mediated genome editing of HSPCs further show that HSPC-pDCs with genetic modifications can be obtained, and that expression of the IFN-α receptor is essential for the optimal function, but dispensable for the differentiation, of HSPC-pDC percursor. Our results thus demonstrate the biological effects of IFNs for regulating pDC function, and provide the means of generating of gene-modified human pDCs.
Plasmacytoid dendritic cells (pDCs) are key effectors in cellular immunity with the ability to not only initiate immune responses but also to induce tolerance to exogenous and endogenous antigens. pDCs are distinct from conventional DCs (cDCs) as they express high levels of interferon regulatory factor 7 (IRF7) and they primarily sense pathogens through Toll-like receptor (TLR) 7 and 9.1-2 Through these pattern-recognition receptors, pathogen-derived nucleic acids can activate pDCs to produce high levels of type I interferon (IFN). Furthermore, activated pDCs link the innate and adaptive immune system together via cytokine production combined with antigen-presenting cell (APC) activity. This dual role makes pDCs essential for maintaining immune homeostasis.3-7 Mice deficient for the type I IFN receptor (IFNAR) and STAT2 show decreased numbers of pDCs within peripheral blood, as well as reduced capacity to mature upon TLR7 stimulation.8,9 Moreover, neonatal mice stimulated with type I IFN were found to have increased numbers of pDCs in the periphery. Accordingly, type I IFNs have been shown to promote the expression of Flt3, the receptor for Flt3-L, which is known to be one of the primary physiological factors mediating DC homeostasis.10-12 On the other hand, type I IFNs that were induced during systemic viral infections were found to negatively regulate pDC numbers via the intrinsic apoptosis pathway.13 Thus, IFNs appear to regulate key aspects of murine pDC biology, including development, apoptosis, migration, homeostasis, and function. However, their role in dictating human pDC development and functions remains incompletely understood. Lack of biological studies of human pDCs can be ascribed to the very low frequencies of pDCs within peripheral blood, as pDCs represent less than 0.1% of peripheral blood mononuclear cells (PBMCs) and have a short lifespan ex vivo of 1-3 days.14 Moreover, genetic manipulation of immune cells, in particular pDCs, remains a major challenge as most attempts of introducing genetic material have thus far not been validated. Importantly, the biological effects of IFNs on human pDC maturation and function have not been investigated.

Here we describe a culture protocol focusing on the yield and biological characteristics of human pDCs. We show that by using an optimized combination of cytokines and growth factors, an average of 81 (±20) pDCs per single HSPC can be generated, which outperforms other available protocols. Upon extensive evaluation of the functional and phenotypic features of the HSPC-derived pDCs in comparison to canonical peripheral blood-derived pDCs, we identify a hitherto unknown pDC phenotype that displays low expression of pDC-specific markers and poor functional activation when stimulated with synthetic TLR7 and TLR9 agonists. Upon priming with type I and II IFNs, we find that this immature phenotype shifts toward a canonical pDC phenotype with full functional activity, highlighting the requirement of positive stimuli in the form of IFNs during in vitro human pDC development. Notably, we also demonstrate that genetically modified pDCs can be derived from CRISPR/Cas9 gene-edited CD34+ HSPCs, and that knockout of the type I IFN receptor abrogates TLR7 and TLR9-mediated innate sensing.

**Results**

**Expansion and differentiation of HSPCs pDC.** Initially, we investigated various culture conditions reported earlier to support HSPC survival, proliferation, and differentiation toward cells with pDC lineage characteristics. In validation of our experimental approach, we first reproduced outcomes from past published protocols and made direct comparisons with our culture system.20-25 (Fig. 1a, b). As baseline condition, we implemented a previously reported pDC differentiation protocol entailing culturing cells 21 days in medium containing the cytokines and growth factors Flt3-L, TPO, and interleukin-3 (IL-3).20 To stimulate expansion of progenitor cells during differentiation we added SCF20 and SRI27, and observed far greater expansion when both factors were included (240 fold ± 42 compared to 35 fold ± 26 in baseline culture; Fig. 1b). Blood-derived pDCs lack the expression of lineage-specific surface markers (i.e. CD3, CD14, CD16, CD19, CD20, and CD56) and the conventional DC marker CD11c.2 Therefore, after the 21-day in vitro differentiation protocol, we enriched for pDCs by negative selection for lin-CD11c-CD123-CD34-CD38 cells by immunomagnetic separation and quantified the cells obtained. For simplicity these cells are hereafter referred to as HSPC-pDCs, despite the fact that no further phenotypic validation had been performed (Fig. 1c, d and Supplementary Fig. 1a-c). Importantly, SCF and SRI proved to be indispensable additives to achieve an abundant and pure population of HSPC-pDC. We obtained an average yield of 81 (±20) HSPC-pDCs per single HSPC, corresponding to a total of 16.2 × 10^6 HSPC-pDCs ± 4.2 × 10^6 per starting 0.2 × 10^6 HSPCs. This protocol is a 57-fold improvement in pDC yield relative to the published protocol of generating HSPC-derived pDCs.20

HSPC-pDCs display an immature precursor phenotype. Next, we examined whether the generated HSPC-pDCs were phenotypically and functionally equivalent to blood pDCs. Blood pDCs are characterized as being lin-CD11c-CD38, while being positive for CD123 (IL3Ra), CD303 (BDCA2), CD304 (BDCA4), CD4, and HLA-DR.1-28 At culture day 21, we observed that the majority of HSPC-pDCs were positive for CD123, CD303, CD4, and HLA-DR, but only about 10% expressed CD304 (Fig. 2a and Supplementary Fig. 2a, b). Compared to blood-collected pDCs, HSPC-pDCs had significantly lower surface expression levels of these receptors as determined by mean fluorescence intensity (MFI) values (Fig. 2b and Supplementary Fig. 2b, c). While the HSPC culture conditions utilized were initially essential for expansion and survival, we hypothesized that continued incubation of cells in this cocktail arrested development of HSPC-pDCs. Such an outcome could result in the observed deficiencies relative
to blood pDCs. To test this, HSPC-pDCs were cultured for an additional 24 h (Day 21 +1) in medium supplemented with only IL-3 to support survival (Fig. 2c, d). Removal of growth factors led to an increase in surface expression of all the pDC-related markers compared to cells on day 21, but the emerging phenotype still did not recapitulate the phenotype of blood pDCs (Fig. 2d and Supplementary Fig. 3a–c). We then examined whether these HSPC-pDCs at day 21 or day 21 +1 were able to respond to classical pDC activators, i.e. synthetic TLR7 and TLR9 agonists. Both cell populations were found to produce significantly less type I interferon and IL-6 compared to blood pDCs (Fig. 2e, f). Nevertheless, upon stimulation with lipofectamin-formulated agonists directed at cytosolic DNA and RNA sensors, HSPC-pDCs produced high levels of type I IFN, indicating that HSPC-pDCs do not lack the capacity to induce innate immune responses (Fig. 2g). However, as was the case for TLR stimulation, the type I IFN responses was found to be lower compared to blood pDCs. Taken together, we hypothesize that day 21 HSPC-pDCs...
pDCs represent a phenotypic and functional precursor to canonical pDCs, defining them as immature HSPC-pDCs.

**Interferon priming leads to a canonical pDC phenotype.** Recent studies have emphasized the role of IFNs in murine pDC development, homeostasis, and function. Thus, we hypothesized that exogenous IFN may be required to drive HSPC-pDCs into a phenotype that more closely resembles blood pDCs and that these cells would then be able to respond efficiently to TLR agonists. To test this, we evaluated the effects of a 24 and
72 h β- and/or γ-interferon exposure on HSPC-pDCs initiated at Day 21 (Day 21 + IFN-β+γ and Day 21 + IFN-β+γ). Consistent with our hypothesis, we found that IFN-primed HSPC-pDCs acquired a surface phenotype profile that strongly resembles blood pDCs with no significant differences in the expression of CD303, CD304, CD4, or HLA-DR (Fig. 3a, b and Supplementary Fig. 4a–d). However, CD123 (IL-3 receptor) expression was still significantly lower, which has been reported to be caused by the supplementation of IL-3 during culture of pDCs31. Next, we challenged IFN-primed HSPC-pDCs with synthetic TLR7 and TLR9 agonists and evaluated the type I IFN and IL-6 responses. Intriguingly, we now observed that type I IFN and IL-6 reached levels comparable to that produced by stimulated blood pDCs (Fig. 3c, d and Supplementary Fig. 5a–d). While the TLR7- and TLR9-mediated type I IFN responses were potent in IFN-primed HSPC-pDCs, the type I IFN response upon stimulation with synthetic agonists directed at cytosolic RNA and DNA sensors was less affected by IFN priming, indicating that IFNs mainly play a role in TLR7- and TLR9-dependent innate immune responses (Supplementary Fig. 6a, b). Notably, IFN-γ priming predominantly promoted the secretion of IL-6, but was less effective at promoting a type I IFN response in comparison to IFN-β priming after synthetic TLR7 and TLR9 agonist stimulation (Supplementary Fig. 5a–d). Expression levels of CD303, CD304, and HLA-DR were increased by IFN-γ priming. Together, these data indicate that the signaling pathways of type I and II IFNs do not merely overlap, but rather lead to synergistic interferon-dependent signaling pathways (Supplementary Fig. 4a–d). By titrating in different amounts of exogenous IFN-β and IFN-γ, we found that HSPC-pDCs were able to acquire the phenotypic and functional traits of blood pDCs using as little as 30 U/mL of recombinant cytokine for priming (Fig. 3c and Supplementary Fig. 7a–c). To reduce potential negative effects of lot-to-lot variations in fetal calf serum, we also evaluated the phenotypic and functional appearance of HSPC-pDCs using commercially available serum-free media (SFM II), which showed similar yield, pDC surface expression, and responses following TLR stimulation (Supplementary Fig. 8a–c).

Previous studies have identified rare pre-DC subsets, which, due to functional and phenotypic similarities to pDCs, may have
inadvertently been considered as mature pDCs in prior studies\textsuperscript{32,33}. We therefore next evaluated if pre-DCs were present within the HSPC-pDC population. Pre-DCs are characterized as Lin\textsuperscript{−}CD123\textsuperscript{−}CD303\textsuperscript{−}CD304\textsuperscript{−}, but in addition express markers unrelated to pDCs, including CD2, CD5, and CD33. Furthermore, they secrete cytokines produced by cDCs such as IL-12 (refs.\textsuperscript{32,33}). After immunomagnetic pDC enrichment on culture day 21, we observed that HSPC-pDCs were negative for the pre-DC marker CD33. Importantly, IFN priming of HSPC-pDCs did not lead to increased CD33 expression, indicating that IFN priming did not lead to generation of pre-DCs (Supplementary Fig. 9a, b). Notably, while it was possible to detect pre-DCs in a PBMC population, the subset was not detectable in the enriched blood pDC population, indicating that the enrichment procedure removes the blood pre-DC subset (Supplementary Fig. 9c, d). We further evaluated cell-surface expression of CD2, CD5, and CX3CR1, which have also been reported to define the pre-DC population. CD2 and CD5 were not expressed by HSPC-pDCs, neither at day 21 nor after IFN priming (Supplementary Fig. 9e, f). An increase in CX3CR1 expression was observed upon IFN priming, which reached an expression level similar to that of blood pDCs (Supplementary Fig. 9e, f). Finally, HSPC-pDCs at day 21 or IFN-primed HSPC-pDCs did not produce IL-12 upon stimulation with cDC-activating stimuli (R848, poly I:C or LPS), further supporting our conclusion that the previously described pre-DCs are not present within the HSPC-pDC population (Supplementary Fig. 10a–c).

Based on our observations that HSPC-pDCs from day 21 of culture displayed a previously undescribed precursor phenotype, we next searched for this population in vivo. We purified Lin\textsuperscript{−}CD11c\textsuperscript{−} cells from CB and analyzed the cells by flow cytometry for the expression of CD123 and CD303. Interestingly, we observed that fresh CB-pDCs contained two distinct populations, one with a mature phenotype (CD123\textsuperscript{high} CD303\textsuperscript{high}) and one with a precursor phenotype (CD123\textsuperscript{dim} CD303\textsuperscript{dim}) (Supplementary Fig. 11). Importantly, as observed for HSPC-pDCs, IFN priming increased the population of CD123\textsuperscript{high} CD303\textsuperscript{high} cells, indicating the presence of a pDC precursor phenotype in CB. However, a maturation was also evident during culture with IL-3 alone, albeit not to the same extent. A recent study argues that human pDCs are capable of self-priming by continuously secreting small amounts of type I IFN, enabling pDCs to retain a response through TLR7 and TLR9. This may partly explain the minor phenotypic shift we observed for unprimed cells\textsuperscript{34}. Finally, we tested whether priming of HSPC-pDCs led to an altered response to TLR7 and TLR9 agonists via increased mRNA expression of genes involved in the TLR signaling pathway. The expression levels of IRF7 and TLR7 were significantly increased after IFN priming, whereas the expression of TLR9 remained unchanged (Supplementary Fig. 12). To test if exogenous priming of HSPC-pDCs initiated self-priming, we removed IFN-β and IFN-γ from HSPC-pDCs after one day in culture, and let the cells rest for an additional day in medium with IL-3 alone (Supplementary Fig. 13a). Priming of HSPC-pDCs led to increased expression of the IFN-α mRNA isotypes tested (α4, α16), and this expression was maintained after the resting phase, suggesting that self-priming occurs in this culture system (Supplementary Fig. 13b).

**HSPC-pDCs transcriptional responses.** To more comprehensively assess the transcriptional response of HSPC-pDCs to priming and TLR7 activation, we next performed whole transcriptome analysis. Cells from blood pDCs (n = 4) and primed HSPC-pDCs (n = 4) were mock-treated or subjected to 24 h of activation through TLR7. RNA was then immediately processed and analyzed by RNA-seq. Pathway analysis on genes differentially expressed between stimulated and non-stimulated cells was used to determine which biological processes were enriched during stimulation of blood pDCs and primed HSPC-pDCs (Fig. 4a). The most highly activated biological processes were shared between the two cell types, although the HSPC-pDCs tended to be more enriched for the shared pathways. When we examined the upstream regulators predicted to be responsible for inducing the transcriptional changes, we found a striking concordance between HSPC-pDCs and blood pDCs (Fig. 4b). Gene expression changes related to TLR7 signaling interactome (visualized in the outer circle) were strongly activated in both blood pDCs and HSPC-pDCs, supporting the fact that both cell types are fully capable of responding to the TLR7 agonist (Fig. 4c). The increased numbers of genes in the interactome of the HSPC-pDCs versus blood pDCs do indicate that a larger genetic shift from precursor HSC-pDCs to fully TLR7-responsive pDCs is needed, supporting the fact that our day 21 HSPC-pDCs are less activated than unstimulated blood pDCs.
Production of genetically modified HSPC-pDCs. To provide evidence that IFN signaling is essential for differentiation and/or maturation of HSPC-pDCs, we set out to genetically remove the IFN receptor. Whereas genetic manipulation of pDCs is currently not possible (discussed in the Introduction), recent advances in the CRISPR/Cas9 technology have resulted in successful and efficient gene editing of CD34+ HSCPs. Thus, we hypothesized that gene-edited pDCs could be generated successfully by initial modification of CD34+ HSCPs and subsequent differentiation into pDCs. We employed a gene editing strategy where synthetic chemically modified sgRNAs and recombinant Cas9 protein, which forms a ribonucleoprotein (RNP) complex, were delivered to CD34+ HSCPs by electroporation35,36 (Fig. 6a). The sgRNAs were designed to target the open reading frame within the first exon of IFNAR1 (subunit of the type I IFN receptor) and MyD88. The latter target was included for its established role in TLR-mediated responses1,10,34. A previously published sgRNA targeting the safe harbor gene CCR5 was used as a negative control38. A range of sgRNAs for each target were then tested for their potency to induce targeted gene disruption by insertions and deletions (Indels) by plasmid electro-poration39. As synthetic sgRNAs with chemically modified nucleotides at both termini by Cas9 RNP delivery by electroporation of CD34+ HSCPs35,36 (Fig. 6a). The three sgRNAs with the highest efficacy for each target were then tested as synthetic sgRNAs with chemically modified nucleotides at both termini by Cas9 RNP delivery by electroporation of CD34+ HSCPs, leading to the selection of a single potent sgRNA for each target gene yielding 73.4 ± 4.5% and 86.7 ± 2.7% Indel frequencies for MyD88 and IFNAR1, respectively (Supplementary Fig. 16b).
The two selected sgRNAs and the CCR5 control sgRNA were then used in CD34+ HSPCs that following a 3-day recovery phase after electroporation were differentiated into pDCs (Fig. 6a, b). Importantly, no change in the proliferation rate and total cell yield was observed during differentiation (comparing Figs. 1b and 6b) and high Indel rates were observed pre- and post differentiation (Fig. 6c and Supplementary Fig. 16c, d). When we evaluated the overall yield of HSPC-pDCs on day 21 we observed a ~4-fold decrease in yield compared to previous experiments. We ascribed this to either the nucleoporation procedure itself, as mock-electroporated cells were equally affected, or to the increased period of cultivation (Fig. 6d). Analysis of Indel frequencies after immunomagnetic pDC enrichment confirmed that HSPC-pDCs had similar levels of Indels as the total non-enriched cell population (Supplementary Fig 16d), and MyD88 protein levels determined by western blotting confirmed potent knock-down (Fig. 6e). IFNAR1-targeted HSPC-pDCs showed reduced capacity to induce downstream signaling through IFNAR, as stimulation with IFN-β failed to induce STAT1 phosphorylation, whereas signaling through the IFN-γ receptor was unaffected (Fig. 6e and...
**Fig. 6** HSPC-pDCs are amenable to genetic modifications. 

**a** Graphical illustration of the protocol for CRISPR/Cas9-mediated gene editing using Cas9 RNP. CD34⁺ HSPCs were initially cultured in CD34⁺ HSPC medium at low density for 3 days before being electroporated with Cas9 RNP complexes. After a 3-day recovery phase, pDC differentiation was initiated. Expansion of cells during differentiation from HSPCs into HSPC-pDCs. 

**b** Indel frequencies after the 3-day recovery phase quantified by TIDE analysis for cells targeted at MyD88, IFNAR1, and CCR5 (control). Numbers of HSPC-pDCs at day 21 for gene-edited HSPC-pDCs. 

**c** Western blot analysis showing induction of pSTAT1 upon 3 h IFN-β stimulation (upper panel) and protein levels of MyD88 (lower panel) in HSPC-pDCs targeted at IFNAR1 and MyD88, respectively (detection Ab for STAT1: CST 9172). Indel frequencies at the respective targets are listed below the western blot. 

**d** Surface expression levels (MFI) of CD123 (f) and CD304 (g) in IFN-primed and unprimed HSPC-pDCs gene-edited at MyD88, IFNAR1, and CCR5. 

**e** Functional levels of type I IFN after stimulation with R837 (TLR7) or CpG-A (TLR9) in unprimed and IFN-primed HSPC-pDCs gene-edited at CCR5, MyD88, or IFNAR1. Levels of IL-6 after stimulation with R837 (TLR7) or CpG-A (TLR9) in unprimed and IFN-primed HSPC-pDCs gene-edited at CCR5, MyD88, or IFNAR1. Data are ±SEM of five (b–d) or three (f–i) donors. Statistical analysis was performed using regular two-way ANOVA followed by Bonferroni post hoc test. Two additional donors are shown in Supplementary Fig. 17.
Supplementary Fig. 16c, f). Next, the HSPC-pDCs were analyzed for two pDC phenotypic markers (CD123 and CD304) and for functionality after IFN priming. CRISPR/Cas9-treated cells showed expression of both surface markers and expression of both markers increased markedly following IFN priming, which recapitulates earlier observations (Fig. 6f, g and Supplementary Fig. 17a, b).

MyD88-targeted HSPC-pDCs were then IFN-primed and stimulated with TLR7/9 agonists. In both cases of TLR stimulation, type I IFN and IL-6 induction were significantly decreased in the KO cells (Fig. 6h, i and Supplementary Fig. 17c). Interestingly, when we evaluated the response to TLR agonists in HSPC-pDCs targeted at the IFNAR1 gene, we found that the cells displayed a decreased capacity to induce type I IFN, supporting our previous findings that type I IFN feedback is a prerequisite for TLR7- and TLR9-dependent type I IFN induction (Fig. 6h).

Moreover, IL-6 production after stimulation was also diminished in IFNAR1 sgRNA-targeted HSPC-pDCs, indicating type I IFN signaling is important for TLR7 and TLR9 activity in pDCs (Fig. 6i). Of note, in some donors, we observed a full abrogation of the type I IFN response, despite ~20% of the cells still possessing the WT allele of IFNAR1 (as estimated from the TIDE analysis), indicating an amplification loop may be required for the TLR7/9-mediated type I IFN response to occur in the remaining cells (Fig. 6h, i and Supplementary Fig. 17d). In summary, our results identify a hitherto unknown role of interferons in promoting the activation of human precursor pDCs into a phenotype that strongly resembles that of pDCs derived from peripheral blood. We also demonstrate that gene-edited HSPC-pDCs can be produced by initially modifying CD34+ HSPCs before differentiation to HSPC-pDCs. Using this system, we confirm that while MyD88 and IFNAR1 do not appear to be important in human pre-pDC development, they are essential for functional pDC immune responses upon stimulation through the TLR pathway.

**Discussion**

Here we present the first evidence that IFN priming is essential for the ability of pDCs to mount a robust and efficient innate immune response. We show this using a novel in vitro approach to expand and differentiate human CD34+ HSPCs, first into precursor pDCs, and then into fully mature and functional pDCs. The power of the system is found within its simplicity, the increased survival of the pDC, high cell yields, and phenotypic and functional resemblance of these HSPC-derived cells to blood-collected pDCs. In addition, the approach is readily amenable for genetic manipulation by CRISPR/Cas9 approaches. This is an important benefit of this culture system, since inhibition of gene expression in by RNAi-based methods in pDCs can be problematic due to non-specific immune activation through, e.g. RIG-I or TRIM37/7/8. Furthermore, pDCs in general have been difficult to genetically modify, and even siRNAs engineered to be specific to certain pDC genes, including Spi-B, IRF7, TLR7, TLR9, ILT7 (CD85g), and CD303 (refs.53–56). Accordingly, we observed up-regulation

While previous culture systems have allowed for the generation of pDCs, our work identified a limitation of those studies, namely that such previously generated HSPC-pDCs display a precursor phenotype, that must be overcome in order to generate pDCs that resemble the majority of pDCs found in the bloodstream. Type I IFN responses in in vitro-generated pDC after stimulation with the synthetic TLR9 agonist CpG-A have previously been reported, while synthetic TLR7 agonists, to our knowledge, have never been tested. Moreover, limited comparisons to blood pDCs have been conducted. We found that unprimed HSPC-pDCs produced type I IFN in response to CpG-A, although at significantly reduced levels compared to blood pDCs. In addition, no type I IFN responses were observed upon stimulation of HSPC-pDCs with synthetic TLR7 agonists. Notably, the observed precursor state of unprimed HSPC-pDCs shares many of the intrinsic features of neonatal pDCs, including a lack of a TLR7-mediated response and decreased surface expression of pDC markers.

Of note, three distinct maturation stages of pDCs have recently been identified by Martin-Martín et al. within adult bone marrow. These stages were separated by their expression levels of pDC phenotypic markers (CD123, CD303, and CD304), and the capacity to produce type I IFN upon synthetic TLR9 agonist stimulation. Martin et al. found that pDCs from peripheral bloodproduced markedly higher levels of type I IFN in comparison to bone marrow-derived pDCs. We identified two populations of lin<sup>−</sup>CD11<sup>C</sup>CD19<sup>−</sup> cells within CB separated by their expression levels of the pDC markers CD123 and CD303. Importantly, IFN priming increased the fraction of cells with high expression levels of these pDC markers, whereas the population with intermediate expression decreased. Of note, an increase in the CD123<sup>high</sup>CD303<sup>high</sup> population was observed upon IL-3 conditioning alone, suggesting that self-priming capabilities exist which can potentially overcome the need of exogenous priming with IFN. Overall, this indicates that pDCs undergo different stages of development before reaching phenotypic and functional maturity.

Our findings indicate that immature pDCs require the withdrawal of precursor-supporting growth factors and the addition of a positive factor in order to attain activity and respond to pathogens. Infection and other physiologic stresses are known to stimulate cytokine output, in particular type I and II IFNs, both regionally and systemically, which would support local priming of pDCs and their trafficking to the site of infection. While IFNs have traditionally been considered to be antiviral, pro-inflammatory, and pro-apoptotic, they have recently also been shown to regulate survival of activated T cells and promote proliferation of dormant HSPCs via a STAT1-dependent pathway. Similarly, Chen et al. reported that IFNAR1<sup>−/−</sup> and STAT2<sup>−/−</sup> mice display significantly decreased numbers of pDCs within the blood, supporting our hypothesis that IFN priming is involved in pDC homeostasis. Moreover, as demonstrated by Vollstedt et al. mouse pDCs are mobilized in neonatal mice upon IFN-α treatment. Although a direct role of type II IFNs in pDC development and activation remains to be elucidated, both type I and II IFNs are known to signal through a JAK–STAT signaling pathway. Accordingly, it is well-established that many ISGs are regulated by both factors. However, other genes are known to be selectively regulated by the two types of IFN, which is likely regulated by differential activation of different STAT proteins. Notably, both type I and II IFNs induce STAT3 phosphorylation, which is known to induce the transcription factor E2-2, which is directly involved in pDC development by inducing a number of pDC-specific genes, including Spi-B, IRF7, TLR7, TLR9, ILT7 (CD85g), and CD303 (refs.53–56). Accordingly, we observed up-regulation
of pDC-related markers, including CD123, CD303, and CD304, upon priming with IFN-β and/or IFN-γ. Furthermore, from the transcriptomic analysis we saw that priming of HSPC-pDCs with IFNs resulted in a shared pattern of differentially expressed genes activating biological pathways as that observed upon stimulation of blood pDC, suggesting the existence of shared functional responses in the blood- vs. HSPC-derived pDCs.

Interestingly, we found IFN-γ to be most potent in upregulating pDC-associated surface markers, which may suggest this cytokine has a higher preference for STAT3-mediated signaling. However, we found that IFNAR knockdown in HSPC-pDCs abrogated type I IFN responses to TLR7 and 9 agonists, suggesting that type I IFN feedback is crucial for the TLR function of pDCs. As type I IFN indirectly signals the presence of a pathogen, this may in turn serve to regulate the function of the receptors, so aberrant immune activity is avoided. This is analogously supported by the concept that many proteins implicated in TLR7 and 9 signaling are recruited to pDCs, according to the manufacturer’s protocol (EasySep™ Human Plasmacytoid DC Enrichment kit, STEMCELL Technologies).

**pDC enrichment from peripheral blood.** PBMCs were isolated by Ficoll-Hypaque density centrifugation of normal healthy blood donor buffy coats obtained from Aarhus University Hospital Blood Bank. Blood pDCs were enriched by negative selection following the instructions by manufacturer (EasySep™ Human Plasmacytoid DC Enrichment kit; STEMCELL Technologies).

**Generation of monocyte-derived dendritic cells (mo-DCs).** CD14⁺ monocytes were initially purified from PBMCs by negative immunomagnetic selection according to the manufacturer’s instructions (EasySep™ Human Monocyte Enrichment kit, STEMCELL Technologies). Purified monocytes were washed twice and cultured in six-well plates at a density of 0.5 × 10⁶ cells/mL in 1.2 mL of RPMI-1640 medium (Lonza), supplemented with 2% normal human serum type AB (NHS AB), 600 µg/mL l-glutamine (Sigma), and 20 µg/mL gentamicin (Gibco). Cultures were established in the presence of 10 ng/mL human GM-CSF (PeproTech) and 20 ng/mL IL-4 (PeproTech). Cells were cultured at 37 °C, 95% humidity, and 5% CO₂ for 6 days. During the cultivation, cultures were supplemented with fresh medium supplemented with GM-CSF and IL-4. At day 6, cells were functionally evaluated.

**Priming of HSPC-pDCs.** HSPC-pDCs were cultured in RPMI-1640 medium with 10% heat-inactivated FCS, 600 µg/mL l-glutamine, 200 µ/mL penicillin, and 100 µg/mL streptomycin, but depleted for growth factors used for the differentiation process described above. As described in individual figure legends, Supplement was supplemented with 250 U/mL IFN-β (PBL Assay Science), 250 U/mL IFN-γ (PeproTech), or both cytokines simultaneously (IFN-β + γ). After 24 (day 21 + 1) or 72 (day 21 + 3) hours, cells were washed in warm medium, and phenotypically or functionally characterized.

**Cell stimulation.** For functional characterization, cells were seeded in 96-well plates. Cells were left untreated or stimulated with stimuli as shown in Supplementary Table 1. During stimulation cells were kept in medium with 20 ng/mL IL-3. Twenty hours post stimulation supernatants were harvested and kept at −20 °C until cytokine quantifications. For phenotypic characterization, IFN-primed or unprimed cells were seeded in 48-well plates and stimulated for 24 h before phenotypic analysis by flow cytometry.

**Measurement of functional type I IFN.** Bioactive functional type I IFN was quantified in supernatants using the reporter cell line HEK-Blue™ IFN-a/β (Invivogen). The cell line was maintained in DMEM+GluMax™-1 (Gibco®, Life Technologies), supplemented with 10% heat-inactivated FCS, 100 µg/mL streptomycin and 200 U/mL penicillin, 100 µg/mL normocin (InvivoGen), and 30 µg/mL blasticidin (InvivoGen), and 100 µg/mL zeocin (InvivoGen). Cells were passaged using 1× tropsyn (Gibco®, Life Technologies). To ensure optimal type I IFN response and stable expression of the plasmids encoding hIRF9, hSTAT2, and hSTAT4 (pHIt9, HiSTAT2, and SEAP, cells were not passaged more than 20 times. For measurement of functional type IFN, cells were seeded at 3 × 10⁴ cells/well in 96-well plates in 150 µL medium. Cells were grown as previously described, but without blasticidin and zeocin. In the following day, 50 µL of supernatant from stimulated cells or a type I IFN standard range was added to the cells. After 24 h of incubation, 20 µL of supernatant from the HEK-blue cells was subsequently added to 180 µL QUANTI-Blue™ SEAP activity was assessed by measuring optical density (OD) at 620 nm on a microplate reader (Titertek, BioTek). The standard range was made with IFN-α (IFNα-22, PBL Assay Science) and ranged from 2 to 500 µU/mL.

**Enzyme-linked immunosorbent assay.** Protein levels of IL-6, IL-12 p70, or IFN-γ in supernatants were evaluated using ELISA kits for IL-6 (Biolegend), IL-12 (Human IL-12 p70 DuoSet ELISA kit, R&D Systems), and IFN-γ (Human IFN-γ DuoSet ELISA kit, R&D Systems), following the manufacturer’s instructions.

**Western blot.** To evaluate protein levels, cells were lysed in Pierce RIPA buffer (Thermo Scientific) supplemented with 10 mM NaF, 0.2% SDS, 1× protease inhibitor (Roche), and 1× XT sample buffer (Bio-Rad). Whole-cell lysates were heated at 95 °C for 5 min before being separated on a 10% SDS Bis-Tris Gel (Criterion TGX gels, Bio-Rad) and blotted onto PVDF membrane (Bio-Rad). Proteins were stained using primary antibodies (1:1000) directed against MyD88 (D68F5; Cell Signaling Technology), STAT1 (C10; Cell Signaling Technology), STAT2 (E14; Cell Signaling Technology), STAT3 (E7; Cell Signaling Technology), STAT4 (D4F9), JAK1 (E9), JAK2 (Tyr701, D4A7; Cell Signaling Technology), and vinculin (hVINV-1; Sigma-Aldrich) and secondary peroxidase-conjugated AffiniPure F(ab′)2 donkey
anti-rabbit (iGg) (1:15000) (Jackson Immuno Research). Proteins were detected using Clarity Western ECL Blotting Substrate (Bio-Rad) followed by visualization with an ImageQuant LAS4000 mini molecular imaging system (GE Healthcare). Unscraped scans of important plots are included in the Supplementary Figure 18.

**Morphology of cells.** To examine the morphology of cells at day 21, cells were smeared out on glass slides and fixed with methanol. May–Grünewald Giemsa Staining (Merck) was performed according to the manufacturer’s protocol. Slides were examined using a Leica DM IL LED microscope.

**Phenotypic analysis with flow cytometry.** Immunophenotypical analysis was performed using flow cytometry. Briefly, 2 × 10^5 cells were spun down and resuspended in staining buffer (PBS with 0.5% BSA and 0.09% NaN₃). Cells were blocked on ice for 30 min before being stained with fluorochrome-conjugated antibodies for 30 min. After three washing steps in staining buffer, cells were fixed in 0.9% formaldehyde. Fluorescence intensities were measured by flow cytometry either on an LSR Fortessa flow cytometer with four lasers (405, 488, 561, and 640 nm) and 13 PMT detectors (ACEA Biosciences, Inc), or on a CytoFlex (Beckman Coulter) with four lasers (405, 488, 561, and 633 nm) and 14 PMT detectors. Antibodies used to determine phenotype of cells, as well as working concentrations, are listed in Supplementary table 2. Data were analyzed using Flowjo software (version 10, Tree Star, Ashland, OR, USA). Individual gating strategies are depicted in relevant figures and outlined in figure legends. FMO controls were utilized to distinguish different populations.

**Viability over prolonged culture.** To assess the viability over prolonged culture of HSPC-pDCs or blood pDCs, cells were collected at each time point and subsequently analyzed using flow cytometry. Cells were stained with Zombie aqua as a viability dye, and viable cell counts were measured and normalized to a fixed number of counting beads (CytoCount beads; Dako).

**Quantitative real-time PCR.** Gene expression levels of IFN-α, IFN-β, IL-12, and TNF were determined by reverse transcription (RT)-qPCR using TaqMan detection systems (Life Technologies). Expression levels were normalized to DDA1, and data are presented as relative expression levels. The following TaqMan assays were used: IFN-α2; Hs00265051_s1, IFN-α4; Hs01681284_s1, IFN-α16, Hs03050575_s1, IL7R; Hs01933259_s1, IL12B; Hs00370913_s1, DDA1; Hs00189308_m1.

**Antigen presentation assays.** Enriched HSPC-pDCs were primed with IFN-γ or IFN-α for 72 h and subsequently stimulated with R837 (InvivoGen, 2.5 µg/mL). After 24 h stimulation, HSPC-pDCs were pulsed with 1 µg per peptide/mL of CMV (Pro-Mix™ CMV Peptide Pool, ProImmune) or left untreated. After 3 h cells were washed and subsequently co-cultured with PBMCs from CMV-seropositive donors. As controls, PBMCs from the same donors were stimulated with 1 µg per peptide/mL of CMV or left untreated. PBMCs and HSPC-pDCs were co-cultured at a cell ratio of 101 (10^5 PBMC and 10^4 HSPC-pDCs) in duplicates in 96-well plates, 200 µL 10% RPMI supplemented with 20 ng/mL IL-2. After 24 h incubation supernatants T cell activation was evaluated by measuring IFN-γ levels in supernatants.

**RNA-seq analysis.** Library preparation was performed using 1 µg of total RNA from each sample, using polyA selection. After fragmentation, first strand cDNA synthesis was performed using random hexamers followed by second strand cDNA synthesis. After double-stranded cDNA purification using AMPure XP beads, the fragments were end-repaired, 3′ adenylated, and NEBNext Adaptor with hairpin loop structure was ligated. cDNA fragments of preferentially 350 bp in length were selected using the AMPure XP system and PCR amplification using primer structure was ligated. cDNA fragments of preferentially 350 bp in length were amplified using the following conditions: 1 cycle at 95°C for 2 minutes, followed by 12 cycles of 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 15 seconds, and finally 1 cycle at 72°C for 1 minute. The resulting cDNA library was sequenced using Illumina 150 bp single-end reads on an Illumina HiSeq, 2500. The resulting reads were imported into Partek Flow and aligned to the human reference genome (UCSC genome browser). The aligned reads were normalized using a parts-per-million (TPM, transcripts per kilobase million). Genes with extremely low expression (<1 TPM) were filtered out. Two annealed oligonucleotides carrying the sgRNA target sequences were cloned into pX330 (pX330-U6-Chimeric-BB-Bb-hSpCas9) was a gift from Feng Zhang, Addgene plasmid #42230) as previously described

**Screening of sgRNAs.** sgRNAs were initially screened for the capacity to induce insertions or deletions (Indels) in the immortalized K562 cell line (ATCC). K565 (ATCC) was maintained in RPMI-1640 (Lonza) supplemented with 10% FCS (HyClone®), 100 µg/mL streptomycin and 200 U/mL penicillin, and 2 mM L-glutamine. Two annealed oligonucleotides carrying the sgRNA target sequences were cloned into pX330 (pX330-U6-Chimeric-BB-Bb-hSpCas9 was a gift from Feng Zhang, Addgene plasmid #42230) as previously described

**Making genetically modified HSPC-pDCs.** sgRNAs directed at MyD88, IFNAR1, and CCR5 were synthesized by Synthego and TriLink Technologies with the three terminal nucleotides in both ends chemically modified with 2′-O-methyl-3′-phosphorothioate. Thawed CD34+ HSPC were initially pre-cultured at low density (10^5 cells/mL) for 3 days in CD34+ HSPC medium (SEFM II medium (STEMCELL Technologies) supplemented with 20 units/mL penicillin, 20 mg/mL streptomycin, 1000 U/mL GM-CSF, 10 µg/mL IL-6, 100 ng/mL TNF, 0.16 µM SR1, 0.16 µM UM171) before being nucleoporated as shown previously. Ribonucleoprotein (RNP) complexes were made by incubating Cas9 protein (Integrated DNA Technologies) with sgRNA at a molar ratio of 1:2.5 at 25 °C for 10 min prior to nucleoporation. Nucleoporation was performed using the Lonza 4D-Nucleofector™ System (program DF100). After a 3-day recovery phase in CD34+ HSPC expansion medium, HSPC medium was changed to the medium promoting pDC differentiation as previously described.

**Statistical analysis.** All data were plotted using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data presented are expressed as means ± standard error of mean (±SEM). Statistical analysis was performed using one-way ANOVA or two-way ANOVA, followed by Bonferroni post hoc test, as indicated in the figure legends. *P ≤ 0.05,* **P ≤ 0.01,** ***P ≤ 0.001,** ****P ≤ 0.0001.

**Data availability** All relevant datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

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Author contributions
A.L., R.O.B., N.R.R., M.P., M.N., P.W.D., and M.R.J. designed the experiments. A.L., C.K., R.O.B., I.K., J.H.E., C.C.P., S.P., H.Q.T., N.U., N.R.R., and M.N. performed the experiments and analysis. A.L., R.O.B., N.R.R., M.N., P.W.D., and M.R.J. wrote the paper and all authors contributed to the manuscript writing.

Additional information
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Competing interests: M.R.J., A.L., and Aarhus University has filed provisional patent application related to the development and usage of HSPC-pDCs. The remaining authors declare no competing interests.

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