Regulation of the multifaceted functions of human plasmacytoid dendritic cells: a polyphonic policy

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NOTCH4 IS A NOVEL REGULATOR IN THE DEVELOPMENT AND FUNCTION OF HUMAN PLASMACYTOID DENDRITIC CELLS

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ABSTRACT

Plasmacytoid dendritic cells (pDCs) are a subset of DCs that are important for antiviral immune responses. They sense viral RNA and DNA via Toll-like-receptors (TLR)-7 and TLR9, respectively, inducing production of pro-inflammatory cytokines, and differentiation into mature pDCs able to prime T cells. To better understand the molecular mechanisms underlying the regulation of pDC biology we focused on Notch receptors, which play a role in differentiation, survival and cell-fate decisions in different cell types. We report that pDC prominently express Notch4, which is further increased upon TLR engagement. Overexpression of the active Notch4 intracellular domain (NICD4) in human hematopoietic progenitor cells impaired pDC development. Notch ligation on primary pDCs, but also ectopic expression of NICD4 in the pDC cell line Gen2.2 induced apoptosis over time possibly due to upregulation of BID expression. Additionally, Notch signaling increased the levels of co-stimulatory molecules, i.e. CD80, CD86, CD40, and of HLA-DR, reminiscent of a mature pDC phenotype, as compared to control-transduced cells. As a consequence, NICD4-transduced Gen2.2 had an increased ability to induce T cell proliferation that was independent of TLR activation. Collectively, our data indicate that Notch4 is a novel player in the regulation of pDC development that may affect pDC-induced immune responses.
INTRODUCTION

Plasmacytoid dendritic cells (pDCs), which develop from bone marrow-derived hematopoietic progenitor cells (HPCs), play a role in the immune system by activating immune responses. pDC development depends on FMS-like-tyrosine-kinase-3-ligand (Flt3L) and the transcription factors E2-2, Spi-B and signal transducer and activator of transcription (STAT)-3. PDCs express Toll-like receptor (TLR)-7 and TLR9, which sense viral RNA and DNA, respectively, leading to production of type I Interferons (IFNs) that impair viral replication. In addition, IFNs regulate innate- and adaptive immune responses. Following TLR activation, pDCs also differentiate into mature DC that display increased expression of major-histocompatibility-complex (MHC) and co-stimulatory molecules, and secrete IL-6 and TNF-α.

TLR signaling is known to induce pDC maturation via MyD88-induced activation of NF-κB. Much about this process remains incompletely understood. Regulation of pDC development and function is important given the potency of type I IFNs and pro-inflammatory cytokines to activate a wide range of immune responses. Uncontrolled TLR signaling can lead to chronic inflammation, tissue damage and septic shock. Excessive production of IFN-α by pDCs is involved in autoimmune diseases, such as psoriasis, systemic lupus erythematosus (SLE) and Sjögren’s syndrome (SS).

Human pDC development from HPCs is adversely affected by Notch1 signaling. Four Notch receptors have been identified in mammals (Notch1-4), which can be engaged by five different transmembrane ligands: Jagged (Jag)-1 and Jag2, Delta-like (DLL)-1, DLL3 and DLL4. Notch receptors are transmembrane glycoproteins consisting of an N-terminal extracellular and a C-terminal transmembrane-intracellular subunit. Activation of Notch receptors by ligand engagement allows translocation of the intracellular Notch domain (NICD) after a two-step proteolytic cleavage involving a metalloprotease followed by γ-secretase. NICD releases co-repressors from the transcription factor CSL/CBF-1 (called RBP-Jκ in the mouse) and recruits co-activators to form a complex inducing transcription of Notch target genes. Well-known direct Notch/CSL targets are members of the Hairy/Enhancer of Split (Hes) family of bHLH proteins, including HES1 and HEY, which are transcriptional repressors that function as feedback inhibitors of Notch signaling. Other target genes of Notch are C-MYC, CD25, NF-κB, cyclin D3 and p21.

In the hematopoietic system, Notch affects development, proliferation, differentiation, and survival of T cells. On pDCs, expression of Notch1 and -2, Jag1, Jag2 and DLL1 and DLL4 was detected and TLR7 triggering increased expression of Notch1. A role for Notch in pDCs function was suggested, since expression of CD86 and CD83, as well as IFN-α production were reduced after TLR ligation in the presence of the γ-secretase inhibitor DAPT. This suggested that Notch may regulate pDC function, but it remains elusive which of the Notch receptors are implicated and importantly whether Notch may have a role independent of TLR signaling.

Here we investigated the role of Notch4 in regulating pDC development and function. PDCs expressed all Notch receptors, but Notch4 expression was most prominent and increased upon TLR
ligation. Overexpression of NICD4 in HPCs impaired pDC development likely due to induction of apoptosis. Notch ligation on primary human pDCs, but also ectopic NICD4 expression in the pDC model cell line Gen2.2 induced apoptosis over time, likely attributed to increased expression of BID. NICD4 also induced TNF-α secretion in a TLR-independent manner, which in part increased expression of co-stimulatory molecules on the viable NICD4-transduced Gen2.2 cells. In accordance, NICD4-transduced Gen2.2 cells, but not control-transduced cells, induced allogeneic T cell proliferation. Notably, NICD4 acted in synergy with TLR activation in pDC maturation. Together, our results suggest that active Notch4 has a decisive effect not only on the development, but also on the function of pDCs. This increases our understanding on the functioning of pDCs particularly in conditions where Notch ligands are available either in the presence or absence of TLR ligands.

METHODS
Cell lines and reagents
The pDC cell line Gen2.2 was cultured as described before. HPC were co-cultured with OP9 cells in MEM-α (Invitrogen) with 20% fetal calf serum (FCS) (HyClone). Primary pDCs or Gen2.2 cells were activated with CpG-A (ODN2216), CpG-B (ODN2006) or R848 (InvivoGen). Human Fc-tagged DLL1 and DLL4 protein was produced in HEK293T cells transfected with expression plasmids for DLL1-Fc or DLL4-Fc. Isotype-matched control-Ig (palivizumab; Synagis no.54874TF) was a gift from AIMM Therapeutics (Amsterdam, The Netherlands). The TNF-α antibody Adalimumab (Humira) was a kind gift of Prof. D. Baeten. Supernatants were collected and analyzed for the secreted human cytokine TNF-α by ELISA (eBioscience, CA, USA).

Flow cytometry and cell sorting
For flow cytometry we used anti-human monoclonal antibodies coupled to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cyanine (PE-Cy7), allophycocyanin (APC) or APC-Cy7 directed against CD1a, CD3, CD19, CD34, CD38, CD40, NOTCH4, HLA-DR (Biolegend), CD123 (eBioscience), BDC2A, BDC2A4 (Miltenyi), CD20, CD56, CD86, CD16, CD45RA, CD80 (BD) and matching isotype controls (Dako, BD). Apoptotic cells were stained using AnnexinV-PE or -APC and 7-amino-actinomycin (7AAD) (BD) viability staining solution or DAPI. Samples were measured using a LSRII or sorted using a FACSria (BD) and analyzed with FlowJo software (Treestar).

Isolation of primary human cells
Human fetal livers were obtained from elective abortions (Bloemenhoven clinic, Heemstede, The Netherlands). Gestational age was determined by ultrasonic measurements and ranged from 14 to 20 weeks. Postnatal thymus tissue was obtained from surgical specimens removed from children up to three years of age undergoing open-heart surgery (Leids Universitair Medisch Centrum, Leiden, The Netherlands). Tonsils were obtained from routine tonsillectomies (department of Otolaryngology, AMC, Amsterdam, The Netherlands). Peripheral blood of healthy volunteers was used upon donor consent (Sanquin Bloodbank, Amsterdam, The
Netherlands). The use of all human tissues was approved by the Medical Ethical committee of the Academic Medical Center and was contingent on obtaining informed consent, in accordance with the Declaration of Helsinki. Fetal liver, postnatal thymus and tonsil were disrupted by mechanical means and pressed through a stainless steel mesh to obtain a single-cell suspension. Leukocytes were isolated via a Ficoll-Hypaque density gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). CD34+ cells were enriched using an immunomagnetic cell sorting CD34-kit (VarioMACS, Miltenyi Biotec). Further enrichment of HPCs was done by sorting lineage CD34+CD38- fetal liver cells or CD56 CD3 CD34+CD1a+ postnatal thymocytes. For thymic pDC isolation, BDCA4+ cells were enriched using a BDCA4 cell separation kit (varioMACS, Miltenyi Biotec) followed by sorting of CD123+CD45RA+ cells. BDCA4+ pDCs from tonsil and blood were sorted after negative selection using immunomagnetic anti-FITC beads to CD3/CD14/CD16/CD19/CD20 (Miltenyi Biotec). Purity of all sorted populations was >99%.

Retroviral transductions

The human NICD4 cDNA (provided by Dr. A. Karsan) was subcloned into retroviral vector LZRS-IRES-green fluorescent protein (GFP) or was fused to the estrogen receptor (ER) sequence after replacing the stop codon of NICD4 and subcloned into LZRS-IRES-yellow fluorescent protein (YFP).\textsuperscript{24,42} The empty LZRS-IRES-YFP or LZRS-IRES-GFP vectors were used for control transductions. LZRS-HES1-IRES-GFP was a gift from prof. G. Leclercq, Gent, Belgium. Supernatants from Phoenix-GALV\textsuperscript{43} packaging cells were obtained after transfection following manufacturer’s protocol (X-tremeGENE 9 DNA transfection reagent, Roche). For transduction experiments HPCs were cultured overnight in Yssel’s medium\textsuperscript{44} with 2% normal human serum (NHS), 20ng/ml stem cell factor (SCF) and 10ng/ml IL-7 (R&D systems, Abingdon, United Kingdom) followed by incubation with virus supernatant in Retronectin-coated plates (30µg/ml; Takara Biomedicals) for six hours. Development of pDCs was assessed by co-culturing transduced HPCs on OP9 cells in MEM-α medium (Gibco) with 20% FCS (Hyclone), 5ng/ml IL-7 (R&D systems) and 5ng/ml Flt3L (Tebu-bio). Gen2.2 cells were transduced by addition of virus supernatants on Retronectin-coated plates for six hours. To induce nuclear translocation of ER-tagged NICD4, cells were treated with 0.5µM 4-hydroxytamoxifen (4HT; Sigma-Aldrich) or DMSO as solvent control.

PCR

Total RNA was extracted using Trizol reagent (Invitrogen). RNA concentration and quality were determined via Nanodrop spectrophotometer (Thermo Fisher Scientific). Equal amounts of RNA were reverse transcribed into cDNA using the High Fidelity cDNA Synthesis Kit (Roche). cDNA was synthesized using the PCR machine (PTC-200, MJ Research) and analysis was performed on the iCycler (Biorad) for quantitative PCR (qPCR) using SYBR Green supermix (Biorad) and specific primer sets (Supplementary table 1). Samples were analyzed in triplicates and normalized to housekeeping genes GAPDH, HPRT and β-actin.
**Notch ligands binding assay**

Round-bottom 96-well plates were coated for three hours at 37°C with human Fc-tagged DLL1 or Fc-DLL4 protein or the isotype-matched control-Ig. After washing with PBS, primary pDCs were cultured for the time indicated and analyzed by flow cytometry.

**Allogeneic T cell stimulation**

Peripheral blood CD4+ T cells were enriched using CD4 T cells negative isolation kit (Miltenyi Biotec). CD4+ T cell enrichment was >95%. Gen2.2 cells transduced with NICD4-ER or EV were pre-cultured for two days with 4HT or DMSO. T cells were labeled with CellTrace Violet dye (CellTrace Violet Proliferation kit; Invitrogen) according to manufacturer’s instructions and co-cultured with irradiated (60Gy) Gen2.2 cells at a 1:1 ratio for six days in RPMI medium supplemented with 8% FCS. Human T cell expander CD3/CD28 beads (Dynabeads, Dynal, Invitrogen) were used as a positive control. T cell proliferation was measured by detecting loss of the cell membrane dye CellTrace Violet by flow cytometry.

**Statistical analyses**

Data were subjected to two-tailed paired Student’s t-test analysis using Graphpad Prism 5 for Windows (Graphpad software, San Diego, USA) unless stated otherwise. Data are considered significant when at least \( P < .05 \).

**RESULTS**

**Human pDCs express Notch4**

To identify immune regulatory receptors expressed on pDCs, we examined Notch receptors and observed that all Notch members (1-4) were expressed (data not shown). As expression of Notch4 was most prominent on pDCs, we focused on the role of Notch4 in pDC biology. Notch4 mRNA was expressed significantly higher in pDCs from thymus, peripheral blood and tonsil (650- to 1249-, and 444-fold increase, respectively) than in PBLs (Figure 1A) or PBMCs. Consistent with the mRNA data, flow cytometric analysis revealed that Notch4 protein was expressed on peripheral blood pDCs, but not on B and T cells (Figure 1B). Thymic pDCs expressed Notch4 at lower levels than blood pDCs, while unexpectedly Notch4 surface expression on tonsil pDCs was virtually absent (Figure 1C).

To investigate whether TLR activation may affect Notch4 expression we cultured peripheral blood pDCs with agonist for TLR9 (CpG-A, CpG-B) or TLR7 (R848) overnight. Expression of Notch4 significantly increased in all conditions as compared to medium alone (mean fluorescence intensity (MFI) 347±39) (Relative MFI difference (\( \Delta \text{MFI} \)) compared to medium alone \( \pm \) standard deviation (SD) for CpG-A: \( \Delta \text{MFI} 409\pm66 \); CpG-B: \( \Delta \text{MFI} 592\pm46 \); R848: \( \Delta \text{MFI} 787\pm145 \)) (Figure 1D-E).

Notch4 expression on the model pDC cell line Gen2.2 39 was relatively low (MFI 111±5) compared to primary pDCs (MFI 347±39) (Figure E and G), also at the mRNA level (data not shown). Notch4 expression on Gen2.2 cells also increased after TLR9 and TLR7 ligation compared to medium (CpG-A: \( \Delta \text{MFI} 18\pm10 \); CpG-B: \( \Delta \text{MFI} 58\pm13 \); R848: \( \Delta \text{MFI} 116\pm19 \)) (Figure 1F-G).
NICD4 overexpression in hematopoietic progenitor cells impairs in vitro pDC development

To assess the role of Notch4 in pDC development we employed an in vitro model system in which functional pDCs develop from precursor cells in co-culture with OP9 mouse stromal cells, in the presence of Flt3L and IL-7.24 We retrovirally transduced NICD4 or the control empty vector (EV) into CD34+CD38− HPCs from human fetal liver. GFP co-expressed from the same retroviral promoter distinguished transduced and non-transduced cells. After one week of culture on OP9 cells, we observed that significantly lower percentages (Figure 2A-B) and cell numbers (Figure 2C) of GFP+BDCa2+CD123+ pDCs had developed from the NICD4-transduced than from the EV-transduced HPCs. These results indicate that NICD4 overexpression inhibits pDC development.

NICD4 affects development and growth of differentiated pDCs

To determine whether NICD4 impacted also at a later stage in pDC development we generated a 4-hydroxy-tamoxifen (4HT)-regulatable NICD4-estrogen receptor (NICD4~ER) fusion that allows 4HT-induced nuclear transport of active NICD4. YFP was used to identify transduced cells. NICD4~ER-YFP was transduced in thymic CD34+CD1a− multipotent precursors and cocultured on OP9 cells in the absence of 4HT. The percentage of YFP+ pDCs was analyzed after one week, followed by the addition of 4HT or DMSO for three days. Cultures were analyzed by flow cytometry. Within these three days, both the relative percentage of pDCs (Figure 3A) as well as the absolute pDC number (Figure 3B) in the NICD4~ER transduced cultures were significantly reduced as compared to the EV-control culture. This suggested that NICD4 affected development of HPCs into pDCs, but possibly also affected pDCs once developed. To investigate this we overexpressed NICD4~ER in Gen2.2 cells.39 Following addition of 4HT, we observed significantly fewer NICD4~ER-transduced YFP+ Gen2.2 cells, both in percentages and absolute cell numbers, as compared to either NICD4~ER Gen2.2 cultured with the 4HT solvent control DMSO or EV-transduced Gen2.2 (Figure 3C-D). NICD4~ER expression in Gen2.2 cells cultured in DMSO reduced growth to some degree suggesting 4HT-independent leakage of NICD4~ER into the nucleus as was observed previously with similar ER-fusion constructs for Notch1.41 Together, we conclude that NICD4 impacts on pDCs both during and after their development.

NICD4 induces growth inhibition of Gen2.2 cells

To understand the cause of reduced Gen2.2 cell growth by NICD4, we first studied the proliferative capacity of Gen2.2 cells by measuring loss of the cell membrane dye CellTrace Violet over time. We observed impaired proliferation of NICD4~ER-transduced YFP+ cells cultured with 4HT as compared to EV-control cells (Figure 4A). Non-transduced YFP+ cells in the NICD4~ER culture proliferated equally well compared to EV-transduced cells, suggesting a cell intrinsic effect of NICD4 (Figure 4A). As C-MYC is a Notch target gene involved in proliferation,46-47 we measured C-MYC mRNA levels by qPCR in Gen2.2 cells over time. In line with our observation of reduced Gen2.2 cell proliferation, C-MYC mRNA levels were rapidly down-regulated after addition of 4HT to NICD4~ER-transduced Gen2.2 cells, but not control cells (Figure 4B). As expected, expression...
Figure 1: Notch4 is expressed on pDCs and upregulated upon TLR activation. (A) Freshly isolated human PBL (n=4), PBMC (n=4), thymic pDCs (n=5), peripheral blood pDCs (n=5) and tonsil pDCs (n=4) were compared for expression levels of Notch4 mRNA by qPCR. Samples were normalized to the average of GAPDH, β-actin and HPRT expression. Expression levels in PBL were set to 1. Shown are the mean values ± SD of at least four independent donors measured in triplicate. Data were analyzed by Mann-Whitney U test (*P<.05). (B) Flow cytometric analysis of Notch4 expression (black line) on peripheral blood B-cells, T-cells and pDCs. Isotype control staining is depicted in filled grey histograms. RCN, relative cell number. (C) Notch4 protein expression was measured on freshly isolated human PBLs (n=3), PBMCs (n=3) and compared to pDCs isolated from thymus (n=3), tonsil (n=4) and peripheral blood (n=3) by flow cytometry. Shown is the mean fluorescent intensity ± SD of at least three donors (*P<.05). (D) Sorted peripheral blood pDCs were cultured overnight in medium only or in the presence of CpG-A, CpG-B or R848 (10µg/ml). Notch4 expression was analyzed by flow cytometry after overnight TLR ligation (black line) or medium (filled dark grey histograms) and compared to isotype control stained cells (filled light grey histogram). RCN, relative cell number. (E) Cells were analyzed as in panel D. Statistical analysis of the relative MFIs ± SD of three independent donors (*P<.05, **P<.01). (F) Gen2.2 cells were cultured overnight in medium only or in the presence of the TLR ligands and depicted as in panel D. (G) Gen2.2 cells were cultured and analyzed as in panel F. Statistical analysis of the relative MFIs ± SD of five independent experiments (*P<.05, ***P<.001).

Figure 2: Notch4 blocks pDC development in vitro. (A) Gating strategy showing the percentage of in vitro generated pDCs from CD34+CD38- FTL progenitors either transduced with EV or NICD4 and measured after one week of culture. Samples were analyzed by flow cytometry, gated on GFP+BDCA2+CD123+ cells. (B) Percentage of in vitro generated pDCs from CD34+CD38- FTL progenitors either transduced with EV or NICD4 gated as in panel A. Indicated are mean percentages ± SD of three independent experiments (*P<.05). (C) Absolute cell numbers of in vitro generated pDCs from CD34+CD38- FTL progenitors after one week of culture. Indicated are mean cell numbers ± SD of three independent experiments (*P<.05).
of another Notch target, HES1, was up-regulated in the NICD4–ER-transduced cells cultured in the presence of 4HT (Supplementary Figure 1). In conclusion, NICD4 inhibits proliferation of Gen2.2 cells possibly as a result of down-regulating C-MYC expression.
NICD4 overexpression increases apoptosis of pDCs

In contrast to the Gen2.2 cell line, primary pDCs do not proliferate in vitro and lack significant proliferative capability in vivo. Therefore, we also studied whether NICD4 may induce apoptosis. We measured the percentages of apoptotic cells by flow cytometry after staining with Annexin V and DAPI on sorted YFP+ Gen2.2 cells transduced with either NICD4-ER or EV and cultured for 72 hours with 4HT or DMSO. We observed an increase in Annexin V+ cells among NICD4-ER-transduced Gen2.2 cells (average 2.2-fold) as compared to EV-transduced Gen2.2 and DMSO control cells (Figure 4C). Notably, higher numbers (3-fold) of Annexin V+ pDCs were also detected when they were generated in vitro from thymic CD34+CD1a- progenitor cells that were retrovirally transduced with NICD4 as compared to in vitro generated EV-transduced pDCs (Figure 4D).

To test the role of Notch in a more physiological context, pDCs from peripheral blood were cultured for three days on recombinant Notch ligands DLL1 or DLL4 or control-Ig. We observed increased apoptosis of primary pDCs after culture with Notch ligands as compared to control-Ig, in line with the results obtained using NICD4-transduced Gen2.2 cells and in vitro generated NICD4-pDCs (Figure 4E).

The increase in apoptosis of NICD4-ER-transduced pDCs may result from increased expression of pro-apoptotic factors. Hence, we analyzed expression of several factors, including BID, BAX, and BAD by qPCR. Increased expression of BID mRNA was observed in the NICD4-ER-transduced Gen2.2 cells as compared to EV-control transduced cells (Figure 4F). While a slight increase in expression of BAX was also detected, BAD expression did not change (data not shown).

Given that the Notch target HES1 induced apoptosis in B-cell lines, we also determined the consequences of ectopic expression of HES1 in Gen2.2 cells. This recapitulated the NICD4-ER effect on apoptosis suggesting that HES1 contributed to the Notch4-induced apoptosis (Supplementary Figure 2). Taken together, our results show that NICD4 induces apoptosis both in in vitro generated pDCs and Gen2.2 cells, which may be the result of increased expression of BID and HES1. In line with this, the Notch ligands DLL1 and DLL4 induced apoptosis in primary pDCs.

NICD4 overexpression in Gen2.2 induces maturation

Next, we studied the impact of NICD4 on pDC maturation and observed that overexpression of NICD4-ER increased CD80 expression on viable Gen2.2 cells after addition of 4HT over time (Figure 5A-B-C). In addition, increased expression of other co-stimulatory molecules including CD40, CD86, and of HLA-DR was observed (Figure 5D). HES1 was not sufficient for the maturation of pDCs as ectopic HES1 expression in Gen2.2 cells did not up-regulate co-stimulatory molecules (Supplementary Figure 3).

Given the relatively late up-regulation in mRNA and protein expression of maturation markers after addition of 4HT (>3 days) to NICD4-transduced Gen2.2 cells (Supplementary Figure 4), we hypothesized that NICD4 may indirectly regulate expression of these molecules. We considered TNF-α, which induces DC maturation, as a likely candidate. Culture supernatants were analyzed by ELISA and revealed that 4HT-treated NICD4-transduced Gen2.2 cells produced significantly higher amounts of TNF-α, but not IL-6 (data not shown), as compared to control
C-MYC

**B**

Relative mRNA expression over time (hours)

**C**

Percentage of AnnexinV+ cells

**D**

Percentage of AnnexinV+ in vitro generated pDCs

**E**

Percentage of AnnexinV+ pDCs

**F**

BID

Relative mRNA expression over time (hours)
Notch4 activation regulates pDC functions

Cells (Figure 5E). TNF-α mRNA levels were consistently higher in NICD4–ER-transduced Gen2.2 cells compared to control cells as well (Supplementary Figure 5). Addition of Adalimumab (i.e. blocking antibody to TNF-α) to NICD4-transduced Gen2.2 cells partially impaired the upregulation of CD80 (ΔMFI:55.5), CD40 (ΔMFI:201) and HLA-DR (ΔMFI:2876) reinforcing the notion that TNF-α at least in part contributed to their maturation (Figure 5F).

Flow cytometric analysis of peripheral blood pDCs showed that both recombinant DLL1 and DLL4 significantly up-regulated expression of CD40 on pDCs as compared to control-Ig (Figure 5G-H). In conclusion, our results suggest that Notch4 activation may contribute to maturation of pDCs at least in part by inducing production of TNF-α.

Synergistic impact of NICD4 and TLR co-activation on maturation of Gen2.2

Given that TLR ligation induces pDC maturation,[14] we also investigated the role of NICD4 in combination with TLR activation. When NICD4–ER transduced Gen2.2 cells were treated overnight with 4HT together with a low concentration of the TLR7 ligand R848 we noticed that NICD4–ER had a significant synergistic effect on CD80 protein levels as compared to control cultured cells with R848 alone (Figure 6A-B). TNF-α and IL-6 mRNA expression levels further increased in NICD4–ER transduced Gen2.2 cells when NICD4 and TLR were co-activated (Figure 6C-D). In contrast, synergistic effects on CD40 and HLA-DR expression were not observed, possibly because TLR mediated activation stimulated maximal expression already (data not shown). Expression of both IFN-α and IFN-β mRNAs was reduced when NICD4 was activated together with TLR7 (Figure 6E-F).

Collectively these data suggest that Notch4 positively affects the maturation of pDC, and qualitatively alters the response to TLR by promoting production of TNF-α on the one hand, while possibly impairing the IFN response.
Figure 5: Notch4 activation regulates TNF-α expression and maturation of pDCs. (A) Gen2.2 transduced with NICD4~ER or EV were cultured in the presence of 4HT or DMSO. Flow cytometric analysis of GFP^DAPI^ cells showing the percentage of CD80^+^ cells at day three. (B) Flow cytometric analysis of Gen2.2 cells transduced and cultured as in panel A. Shown are mean percentages ± SD of six independent experiments (**P<.01). (C) Flow cytometric analysis of CD80 protein expression on Gen2.2 cells transduced and cultured as in panel A. Transduced Gen2.2 are depicted as NICD4~ER (square symbol) or EV (round symbol) and cultured in medium for indicated time in the presence of 4HT (black symbols) or DMSO (open symbols). Shown are mean percentages of CD80^+^ cells of two independent experiments ± SD. (D) Flow cytometric analysis of Gen2.2 cells transduced and cultured as in panel A. Shown are the relative MFIs ± SD for CD40, CD86 and HLA-DR of three independent experiments.
NICD4–ER-transduced Gen2.2 cells augment alloreactive T cell proliferation

To test whether the mature phenotype of NICD4–ER-transduced Gen2.2 cells had functional consequences for their ability to activate T cells, we performed in vitro allogeneic T cell stimulation assays. Gen2.2 cells were transduced with either NICD4–ER or control EV and after sorting cultured for three days in the presence of 4HT or DMSO followed by co-culture with allogeneic CD4+ T cells for six days. T cell proliferation was measured by loss of CellTrace Violet using flow cytometry. As a positive control T cells were polyclonally stimulated with anti-CD3/CD28 beads, which robustly induced T cell proliferation (Figure 7A–B). Control EV-transduced Gen2.2 cells were unable to induce T cell proliferation. In contrast, NICD4–ER Gen2.2 cells pre-treated with 4HT for two days clearly induced T cell proliferation (Figure 7A–B), in line with their mature phenotype. Collectively, our findings suggest that activation of the Notch4 pathway in pDCs in the absence of TLR agonists is sufficient to induce the proliferation of T cells.

DISCUSSION

Here, we show that Notch4 is expressed on human pDCs, but not on resting lymphocytes, and has an instructive role in pDC development and function. Ectopic NICD4 expression in HPCs impaired in vitro pDC development likely resulting from increased apoptosis induction. Stimulation of primary pDCs with recombinant Notch ligands increased apoptosis, but also upregulated CD40 expression on viable cells possibly reflecting an effect on terminal differentiation. We confirmed and extended these results using the pDC model cell line Gen2.2. NICD4 overexpression in Gen2.2 cells induced apoptosis conceivably due to increased expression of BID and HES1. Moreover, we observed increased TNF-α secretion by NICD4-expressing Gen2.2 cells compared to control cells. This, together with the finding that antagonizing TNF-α impaired Gen2.2 maturation adds to the notion that NICD4 may indirectly regulate pDC maturation. Consequently, NICD4-transduced Gen2.2 cells had an increased ability to induce T cell proliferation. Notably, this was independent of TLR ligation, although NICD4 did synergize with TLR activation to further increase pDC maturation. Collectively, our results identify Notch4 as a novel player in the regulatory network of molecules controlling pDC development and function, which may influence the outcome of a pDC-mediated immune response.

Expression of Notch1 and Notch2 on IL-3 or R848-stimulated human pDCs was reported, but Notch function in pDCs remains poorly understood. Studies on the function of Notch4 in the immune system are even more limited. Notch4-deficient mice were generated, but effects...
Figure 6: NICD4 synergizes with TLR7 agonist to induce maturation of pDCs. (A) Flow cytometric analyses of EV (filled histograms) or NICD4~ER (line histograms) transduced Gen2.2 cells cultured in the presence of TLR7 ligand R848 (100ng/ml) plus 4HT or DMSO. Cells were analyzed after one day for expression of CD80. Isotype control stainings are depicted in light grey histograms. (B) Gen2.2 cells transduced and treated as in panel A. Shown are the relative MFIs ± SD for CD80 of three independent experiments (**P<.01). (C-D) QPCR analysis of IL-6 mRNA levels in Gen2.2 cells transduced with either NICD4~ER or EV and cultured in the presence of R848 for four hours in the presence of 4HT or DMSO. Expression in EV cells and addition of 4HT was set to 1. Shown is the relative mRNA expression ± SD of qPCR measured in triplicate of a representative experiment of two independent experiments. Samples were normalized to the average of GAPDH, β-actin and HPRT expression. (E-F) QPCR analysis of IFN-α and IFN-β mRNA levels in Gen2.2 cells transduced with either NICD4~ER or EV and cultured in the presence of R848 for four hours in the presence of 4HT or DMSO. Expression in EV cells and addition of 4HT was set to 1. Shown is the mean mRNA expression ± SD of qPCR measured in triplicate of a representative experiment of two independent experiments. Samples were normalized to the average of GAPDH, β-actin and HPRT expression.
**Figure 7: NICD4 transduced Gen2.2 cells induce CD4+ T cell proliferation.** (A) Flow cytometric analysis of T cell proliferation induced by Gen2.2 cells transduced with EV or NICD4-ER vectors that were sorted and pre-activated with 4HT or DMSO for 48 hours. Pre-activated Gen2.2 cells were irradiated and co-cultured with freshly isolated CellTrace Violet labeled allogeneic CD4+ T cells (ratio Gen2.2:CD4+ T cell 1:1). After six days CD3+ T cells were analyzed for the expression of CellTrace Violet. The percentages of proliferated T cells are depicted in the lower left quadrant (CellTraceVioletloCD3+). CD4+ T cells activated with anti-CD3/CD28 beads are shown as a positive control for proliferation as compared to CD4+ T cells cultured in medium only. Numbers in plots represent percentages of cells that fall in the indicated quadrant. Shown is one representative experiment of three. (B) Cells were analyzed as in panel A. Shown are the mean percentages ± SD of CellTraceVioletloCD3+ T cells of two independent T cell donors.
on pDC development were not examined. Notch1 is not required for pDC differentiation in the mouse, but RBP-J deficient mice lacking all Notch activity, have increased pDC numbers consistent with a pro-apoptotic role of Notch. Interestingly, accumulated pDC numbers were found in synovial tissues of RA patients likely as a result of increased generation of pDC progenitor cells in the bone marrow. This, together with the notion that in RA Notch4 was identified as a susceptibility gene possibly resulting in reduced Notch4 expression, is in agreement with a role of Notch4 in controlling pDC numbers and in line with our results that NICD4 overexpression blocks pDC development.

Overexpression of NICD4 induced maturation, but also apoptosis of Gen2.2 cells. The induction of apoptosis, but not maturation, could be recapitulated by ectopic HES1 expression in Gen2.2. Ectopic NICD4 or HES1 was shown to induce apoptosis in AML samples and cell lines and a variety of B cell malignancies as well, which was attributed to reduced BCL2 levels. This contrasts with our observations as BCL2 levels were unaltered in NICD4-transduced Gen2.2 cells. Instead, NICD4 upregulated expression of the pro-apoptotic factor BID possibly underlying the induction of apoptosis in Gen2.2 cells. In agreement with the observation that TNF-α induced BID, but not other pro-apoptotic genes in human pDCs, a model appears that NICD4 controls apoptosis through its induction of TNF-α. Further, as HES1 overexpression did not induce maturation of Gen2.2 cells, we propose that NICD4 contributes to HES1-dependent and -independent regulatory pathways to drive apoptosis versus maturation of pDCs, respectively.

Though primary pDCs do not proliferate, at least not in vitro in response to cytokines nor in vivo, NICD4 expression in Gen2.2 cells inhibited their proliferation. Obviously, Gen2.2 cells are leukemic cells that acquired as of yet unknown mutations driving continuous cell growth. NICD4-transduced Gen2.2 expressed reduced levels of C-MYC, which is involved in cell cycle progression. Hence, NICD4 may control Gen2.2 proliferation through direct downregulation of C-MYC. Further investigations are warranted to decipher how NICD4 downregulates C-MYC in Gen2.2 cells as C-MYC is generally considered to be up-regulated by Notch at least in T-ALL. Taken together, it is tempting to speculate that Gen2.2 leukemic cells have actively downregulated Notch4 thereby increasing C-MYC levels compared to primary pDCs to avoid tumor suppressor gene activity.

TLR7/9 signaling in pDCs depends on canonical NF-κB activity regulating cytokine production and maturation. Strikingly, NICD4-activated pDCs produced TNF-α and displayed a mature phenotype independent of TLR ligation. Given that Notch regulates NF-κB activity in T cells, initially we hypothesized that Notch4 may similarly do this in pDCs. However, we did not detect increased levels of phosphorylated-RelA/p65 after Notch4 activation (data not shown). While we cannot exclude that Notch4 controls noncanonical p52/RelB NF-κB signaling as observed in Hodgkin-and-Reed-Sternberg cells, in pDCs TNF-α expression was demonstrated to be regulated by canonical NF-κB activity. Alternatively, Notch4 may either directly regulate gene expression of TNF-α or increase TNF-α secretion.

Notch ligands are pleiotropically expressed not only on T cells and DCs, but also on epithelial and endothelial cells. Hence, it is difficult to pinpoint at which stage in an immune response Notch is engaged on pDCs. As in time NICD4 induced apoptosis of pDCs, this possibly reflects terminal
two

Notch4 activation regulates pDC functions

differentiation and resolution of the pDC-induced immune response. Collectively, our data add value to understand how development and function of pDCs is regulated at the molecular level.

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AUTHORSHIP

Contributions: L.C.M.J designed research, performed experiments, analyzed data and wrote the manuscript; J.J.K, K.B, M.N, W.D, R.S.Z, E.W.T-K, C.H, performed experiments D.A. E.C.d.J and C.H.U analyzed data, B.B designed research, analyzed data and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Figure 1. Ectopic expression of NICD4 in Gen2.2 cells induces HES1 expression. HES1 mRNA levels in Gen2.2 cells transduced with either NICD4–ER (square symbol) or EV (round symbol) and cultured in medium for indicated time in the presence of 4HT (black symbols) or DMSO (open symbols). Analysis of mRNA levels by qPCR. Expression of HES1 in EV transduced cells at 0 hour was set to 1. Samples were normalized to the average expression of GAPDH, β-actin and HPRT. Shown are mean values ± SD of relative expression of two independent experiments measured in triplicate.

Supplementary Figure 2. Ectopic expression HES1 in Gen2.2 cells increases early apoptosis. Gen2.2 cells were transduced with either HES1 or EV and cultured for three days. Cells were analyzed by flow cytometry and for expression of AnnexinV and DAPI. Shown is one representative experiment out of two.

Supplementary Figure 3. Expression of co-stimulatory molecules is not regulated by HES1. Gen2.2 cells were transduced with either EV or HES1 (GFP), NICD4–ER and EV (YFP). After three days of culture in the presence or absence of 0.5µM 4HT or DMSO DAPI- cells were analyzed by flow cytometry for expression of CD86, CD40 and CD80. Shown is a representative experiment of three independent experiments. RCN, relative cell number.
Supplementary Figure 4. Ectopic expression of NICD4 in Gen2.2 cells induces CD40 expression. CD40 mRNA levels in Gen2.2 cells transduced with either NICD4-ER (square symbol) or EV (round symbol) and cultured in medium for indicated time in the presence of 4HT (black symbols) or DMSO (open symbols). Analysis of mRNA levels was done by qPCR. Expression of EV at 0 hour was set to 1. Samples were normalized to the average expression of GAPDH, β-actin and HPRT. Shown are mean values ± SD of relative expression of two independent experiments measured in triplicate.

Supplementary Figure 5. Ectopic expression of NICD4 in Gen2.2 cells induces TNF-α expression. TNF-α mRNA levels in Gen2.2 cells transduced with either NICD4-ER (square symbol) or EV (round symbol) and cultured in medium for indicated time in the presence of 4HT (black symbols) or DMSO (open symbols). Analysis of mRNA levels was done by qPCR. Expression of EV at 0 hour was set to 1. Samples were normalized to the average expression of GAPDH, β-actin and HPRT. Shown are mean values ± SD of relative expression of two independent experiments measured in triplicate.

Supplementary Table 1: Primer sequences. Sequences of primers used for qPCR.

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<th>Primers</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<td>Notch4</td>
<td>CAGCCCAGTGGGTATCTCTG</td>
<td>GTTGTGACAGGGTTGGGACT</td>
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<td>C-MYC</td>
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<td>HES1</td>
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<td>β-actin</td>
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