Transcriptional patterns associated with BDCA3 expression on BDCA1$^+$ myeloid dendritic cells

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Abstract

Myeloid dendritic cells, including BDCA3^{hi} DCs and BDCA1$^+$ DCs (hereafter dubbed DC1 and DC2 for clarity), play a pivotal role in the induction and regulation of immune responses. Interestingly, a fraction of DC2 also express low to intermediate levels of BDCA3. It is unknown whether BDCA3$^+$ DC2 also share other traits with DC1 that are absent in BDCA3$^-$ DC2 and/or whether BDCA3 expression renders DC2 functionally distinct from their BDCA3-lacking counterparts. Here, we used expression analysis on a predefined set of immunology-related genes to determine divergence between BDCA3-positive and BDCA3-negative DC2 and their relation to *bona fide* BDCA3^{hi} DC1. Results showed that mRNA fingerprints of BDCA3$^+$ DC2 and BDCA3$^-$ DC2 are very similar, and clearly distinct from that of DC1. Differences in mRNA expression, however, were observed between BDCA3$^+$ DC2 and BDCA3$^-$ DC2 that pointed toward a more activated status of BDCA3$^+$ DC2. In line with this, higher steady state maturation marker expression and TLR-induced maturation marker expression and inflammatory cytokine production by BDCA3$^+$ DC2 were observed. This dataset provides insight into the relationship between myeloid DC populations and contributes to further understanding of DC immunobiology.

INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells located in peripheral blood, lymphoid and non-lymphoid tissues, where they sample the environment for invading pathogens. Recognition of foreign antigens induces activation of DCs, which is associated with upregulation of several surface receptors and production of cytokines that enable DCs to migrate to secondary lymph nodes and to activate T cells and other immune cells.[1] The human DC family comprises several subsets, including BDCA2$^+$BDCA4$^+$ plasmacytoid DCs, BDCA1$^+$ myeloid DCs, also referred to as DC2, and BDCA3^{hi} CLEC9A$^+$XCR1$^+$ (DC1) myeloid DCs.[2] The markers BDCA1, BDCA2, BDCA3 and BDCA4 are generally used to classify human DC. However, BDCA3 is not exclusively expressed by DC1, but also at intermediate levels by other DCs, such as DC2 in blood,[3,4] and even more so by DC2 in lymphoid tissues[5] and nonlymphoid tissues.[6]

BDCA3, also known as CD141 or thrombomodulin, is a cell surface-expressed transmembrane glycoprotein. On vascular endothelial cells, BDCA3 is known for its anticoagulant activity, and has been described to have an anti-inflammatory function as it blocks proinflammatory proteins and activates anti-inflammatory proteins and inhibitors of the complement system.[7,8] However, the exact function of BDCA3 expression on immune cells is not completely understood. Although DC2 have been shown to represent a subset that is distinct from DC1,[6,9] it is possible that the expression of BDCA3 endows them with distinct functional properties or indicates a closer relation to DC1. Here, we compared the expression signature of predefined immune-related genes of BDCA3$^+$ DC2, BDCA3$^-$ DC2 and DC1, and investigated the presence of differences between BDCA3$^+$ and BDCA3$^-$ DCs within the DC2 population. Results showed that although BDCA3$^+$ DC2 and BDCA3$^-$ DC2 are highly comparable and distinct from *bona fide* DC1, BDCA3-positive DC2 have a more activated status

than DC2 that do not express BDCA3, and produce higher levels of cytokines upon TLR activation.

RESULTS AND DISCUSSION

Immunology-related gene expression patterns of BDCA3$^+$ DC2 are similar to those of BDCA3$^-$ DC2

To assess how BDCA3$^+$ DC2 relate to their BDCA3-negative counterpart, DC populations shown in Figure 1a were sorted and expression of a set of 579 preselected immunology-related genes was analyzed by multiplex gene expression analysis (for complete list of obtained gene expression values, see Supplementary table 1). BDCA3 expression on DC2 was highly variable between donors and ranged from 41% to 85% (Figure 1a, Supplementary figure 1). Of note, purity of magnetic bead and flow isolated DC populations was above 98% and populations were free of non-DC contaminants (Supplementary figure 2). Sample clustering and principal component analysis revealed a high level of overlap between the immunology-related gene expression profile of DC1, BDCA3$^-$ DC2 and BDCA3$^+$ DC2 (Figure 1b and c). BDCA3$^+$ DC2 and BDCA3$^-$ DC2, however, appeared far more comparable to each other than to DC1 (Figure 1b and c). Although BDCA3$^-$ DC2 and BDCA3$^+$ DC2 were found to be very similar, some minor differences in immunology-related gene expression levels were observed (Figure 1b).

Comparison of DC1 and BDCA3$^+$ DC2

In total, 10 genes showed a trend toward differential expression between BDCA3$^+$ DC2 and BDCA3$^-$ DC2 by *t*-testing, but the difference was significant for none after applying multiple testing correction (Figure 1d). To test the hypothesis that BDCA3$^+$ DC2 may resemble DC1 more than BDCA3$^-$ DC2 does, we investigated whether these 10 genes were also higher expressed in *bona fide* DC1. Interestingly, nine of those genes were also differentially expressed between BDCA3$^-$ DC2 and DC1 (up in BDCA3^{hi} DCs: SLAMF7, CD83, SRC, CXCR3, and down: CD99, STAT5A, PRKCD, CD4, IRF4), indicating that BDCA3 expression on DC2 is accompanied by a trend for higher expression of other genes also highly expressed by DC1 (Figure 2a). A complete list of genes differentially expressed between DC1 and BDCA3$^-$ DC2 is provided in Supplementary table 2.

Gene Set Enrichment Analysis can be used to test a broad spectrum of genes on whether the differences in expression pattern observed between two cell types (e.g. DC1 and DC2) resembles the pattern observed between two other cell types (e.g. BDCA3$^+$ and BDCA3$^-$ DC2). For this analysis two gene sets containing the top 50 genes higher and lower expressed in DC1 compared to BDCA3$^-$

DC2 were generated by simple fold-change calculation (genes included in each set are indicated in Supplementary table 3). As expected, the DC1-overexpressed gene set contained the DC1 hallmark genes TLR3, BATF3 and XCR1,[10] and antigen (cross)-presentation-related genes (HLA, PSMB, TAP; DC1 > DC2, Supplementary table 3). The DC2-overexpressed gene set, in contrast, included the pathogen recognition receptor TLR4 for bacterial recognition and ITGAM, the alpha subunit of the MAC-1 integrin heterodimer that facilitates phagocytosis (DC2 > DC1, Supplementary table 3).[11–13]

When applying Gene Set Enrichment Analysis to visualize the distribution of these two sets of 50 genes on the immune-related genes ranked on expression difference between BDCA3$^+$ DC2 compared to BDCA3$^-$ DC2, we observed that the majority, but not all, of the DC1 over-expressed gene set was also higher expressed in BDCA3$^+$ DC2 relative to DC2 lacking BDCA3 (Figure 2b). Enrichment of the gene set in BDCA3$^+$ DC2, however, was not overwhelming (normalized enrichment score 1,6; false discovery rate *q*-value 0.285, Figure 2b). Genes lower expressed in DC1 were also, more often than not, lower in BDCA3-positive DC2, but the negative enrichment (i.e. depletion) of this gene set was even less apparent (normalized enrichment score −1.5; false discovery rate *q*-value 0.925, Figure 2c).

Finally, the expression of several established hallmark genes of either DC1 or DC2 was assessed for each gene individually. For these key genes, BDCA3-positive DC2 behaved comparably to their BDCA3-lacking counterparts (Figure 2d and e). Importantly, BDCA3$^+$ DC2 and BDCA3$^-$ DC2 expressed equal levels of the transcription factors IRF8 and BATF3, which together have been shown to be expressed in and to regulate differentiation into DC1.[9,14–16] Furthermore, BDCA3$^+$ DC2 expressed more IRF4, which has been associated with differentiation into DC2.[17,18] These results suggest that even though BDCA3$^+$ DC2 may resemble DC1 slightly more than do their BDCA3$^-$ counterparts, this seems not to be an effect of these fate-determining transcription factors. In line with this, BDCA3$^+$ DC2 did not express any transcripts for XCR1, which was highly abundant in DC1 and is considered the hallmark gene for DC1 (Supplementary table 1 and Figure 2).[3] Additionally, DC1 marker CLEC9A, which was not one of the immune-related genes measured by RNA multiplex, was absent on BDCA3$^+$ DC2 by flow cytometry (Supplementary figure 2).

BDCA3$^+$ DC2 have a higher cytokine-producing capacity than BDCA3$^-$ DC2

To get more insight into the DC1 genes that were also higher in BDCA3$^+$ DC2 and to derive functional
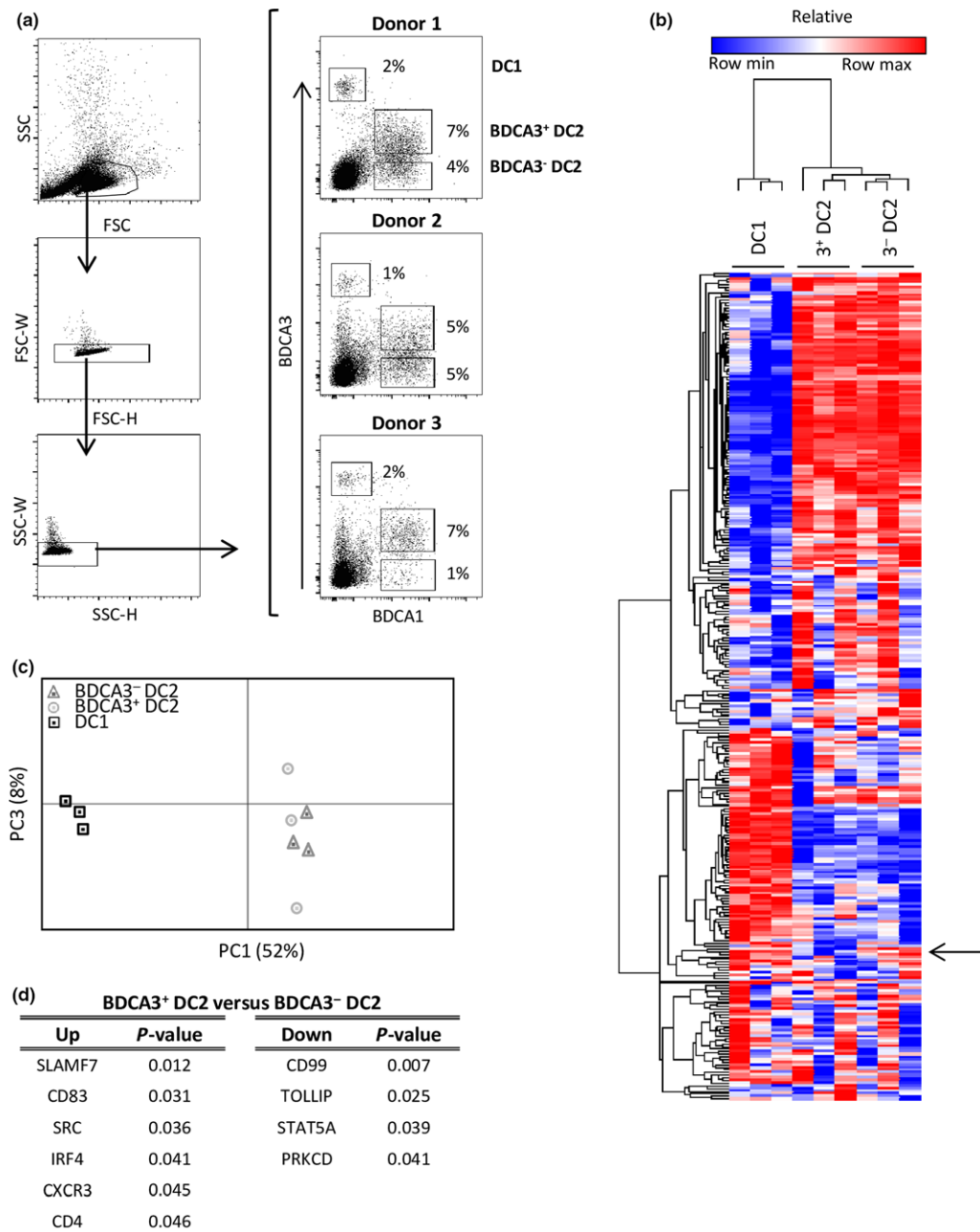
Figure 1. Gating strategy and expression profiles of immunology-related genes. **(a)** Peripheral blood mononuclear cells were isolated from healthy controls and enriched for DCs using a Dynabeads Human DC Enrichment kit. Duplicates were excluded based on size, and DC populations were subsequently identified as BDCA3^{hi} cells (DC1), BDCA3$^+$BDCA1$^+$ cells (BDCA3$^+$ DC2) and BDCA3$^-$BDCA1$^+$ cells (BDCA3$^-$ DC2). Representative results of three different donors in three different experiments are shown. **(b-d)** mRNA isolated from DC populations of 2 or 3 different donors sorted in three different experiments was pooled per sample and gene expression levels were measured using the NanoString nCounter system. **(b)** Log2 expression levels of genes that were expressed in at least one of the three DC subtypes (expression level > 50) are shown in a heatmap. Hierarchical clustering was performed using Pearson correlation. The black arrow indicates differential gene expression between BDCA3$^+$ DC2 and BDCA3$^-$ DC2. **(c)** Principal component analysis was performed using the MeV software, based on genes expressed in at least one of the three DC subtypes (expression level > 50). PC1 accounts for 52% and PC3 for 8% of the total variance. Each point represents a sample, classified by DC type. **(d)** Genes differentially expressed in BDCA3$^+$ DC2 as compared to BDCA3$^-$ DC2, $P < 0.05$ by an unpaired Student's t-test not corrected for multiple testing (MR). Bonferroni corrected α-value = 0.00016, rendering none of these genes significant after MT correction.
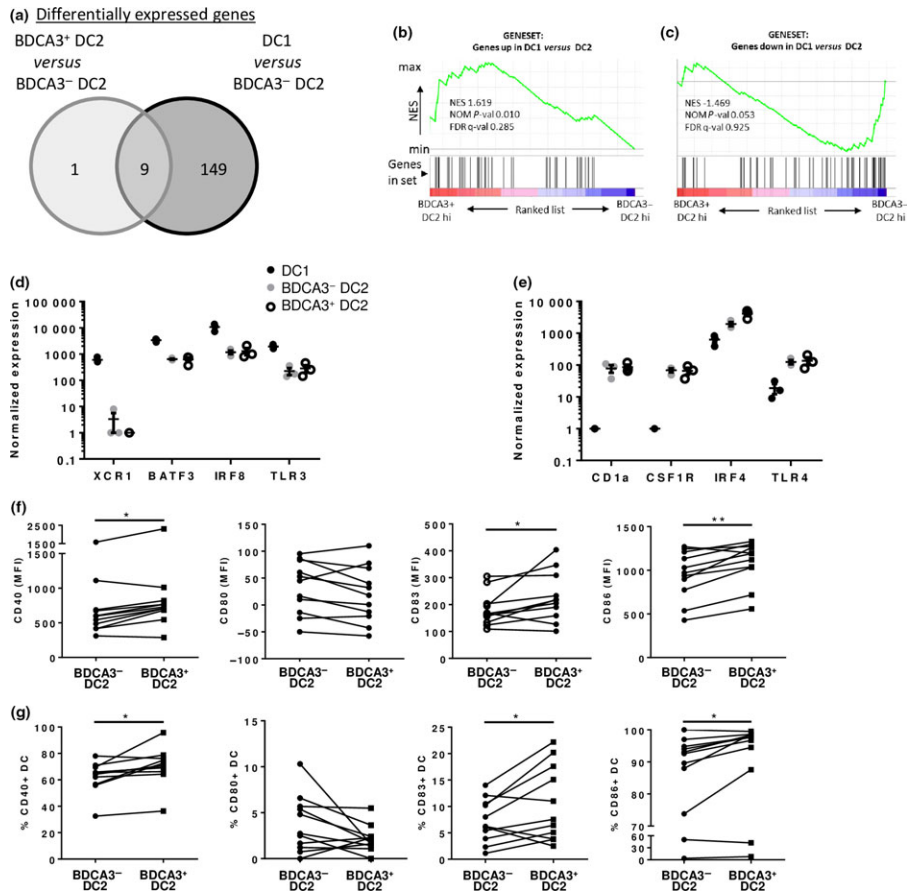
Figure 2. Analysis of DC population gene signatures and activation status. **(a–e)** mRNA isolated from DC populations of 2 or 3 different donors sorted in three different experiments was pooled per sample. Gene expression levels were measured using the NanoString nCounter system. **(a)** The Venn diagram represents the number of genes significantly differentially expressed between the indicated DC populations ($P < 0.05$, unpaired t-test). **(b, c)** Enrichment plots of Gene Set Enrichment Analysis performed using the DC1-overexpressed gene set, DC2-overexpressed gene set (Supplementary table 3) and a ranked list containing genes ranked by fold-change in expression values between BDCA3$^+$ DC2 compared to BDCA3$^-$ DC2. For statistical analysis, the gene sets were complemented by the curated C2 gene sets published on software.broadinstitute.org to properly determine the normalized enrichment score, nominal P-value (NOM P-val) and false discovery rate. **(d, e)** Normalized expression of hallmark genes of DC1 **(d)** or DC2 **(e)** by the different DC populations. **(f, g)** Mean fluorescence intensity **(f)** and percentage of cells expressing the indicated markers **(g)** were measured on freshly isolated DC by flow cytometry. Data of six independent experiments, performed with 11 different donors, are shown. *$P < 0.05$, **$P < 0.01$, paired Student's t-test.

implications, we inspected more closely the genes higher expressed in these two cell populations compared to BDCA3$^-$ DC2. Enhanced expression of (I) SLAMF7,[19] a CD2 family receptor expressed by mature DCs; (II) CD83, a DC maturation marker; (III) SRC,[20] a protein tyrosine kinase which is activated following engagement of many different classes of cellular receptors; (IV) IRF4,[21] a transcription factor associated with induction of a Th2 response by DCs and (V) the chemokine receptor CXCR3; as well as reduced expression of (VI) TOLLIP,[22] a ubiquitin-binding protein that negatively regulates TLR-mediated signaling, suggested a more activated status and possibly differential cytokine production by BDCA3$^+$ DC2 compared to BDCA3$^-$ DC2. To further investigate this,

activation marker expression on freshly isolated cells, and maturation and cytokine-producing capacity after *in vitro* TLR stimulation, were assessed. Directly after isolation, CD40, CD83 and CD86 expression, but not CD80 expression, was significantly higher on BDCA3$^+$ DC2 compared to BDCA3$^-$ DC2, confirming a more activated status of these cells (Figure 2f and g). Furthermore, upon culture with or without TLR ligands polyI:C, R848 and LPS, CD83 and CD86 expression was more enhanced on BDCA3$^+$ DC2 compared to BDCA3$^-$ DC2 (Figure 3a). Although CD80 was not differentially expressed on freshly isolated DC, CD80 was augmented on BDCA3$^+$ DC2 compared to BDCA3$^-$ DC2 upon culture (Figure 3a). The opposite we observed for CD40 that was differentially
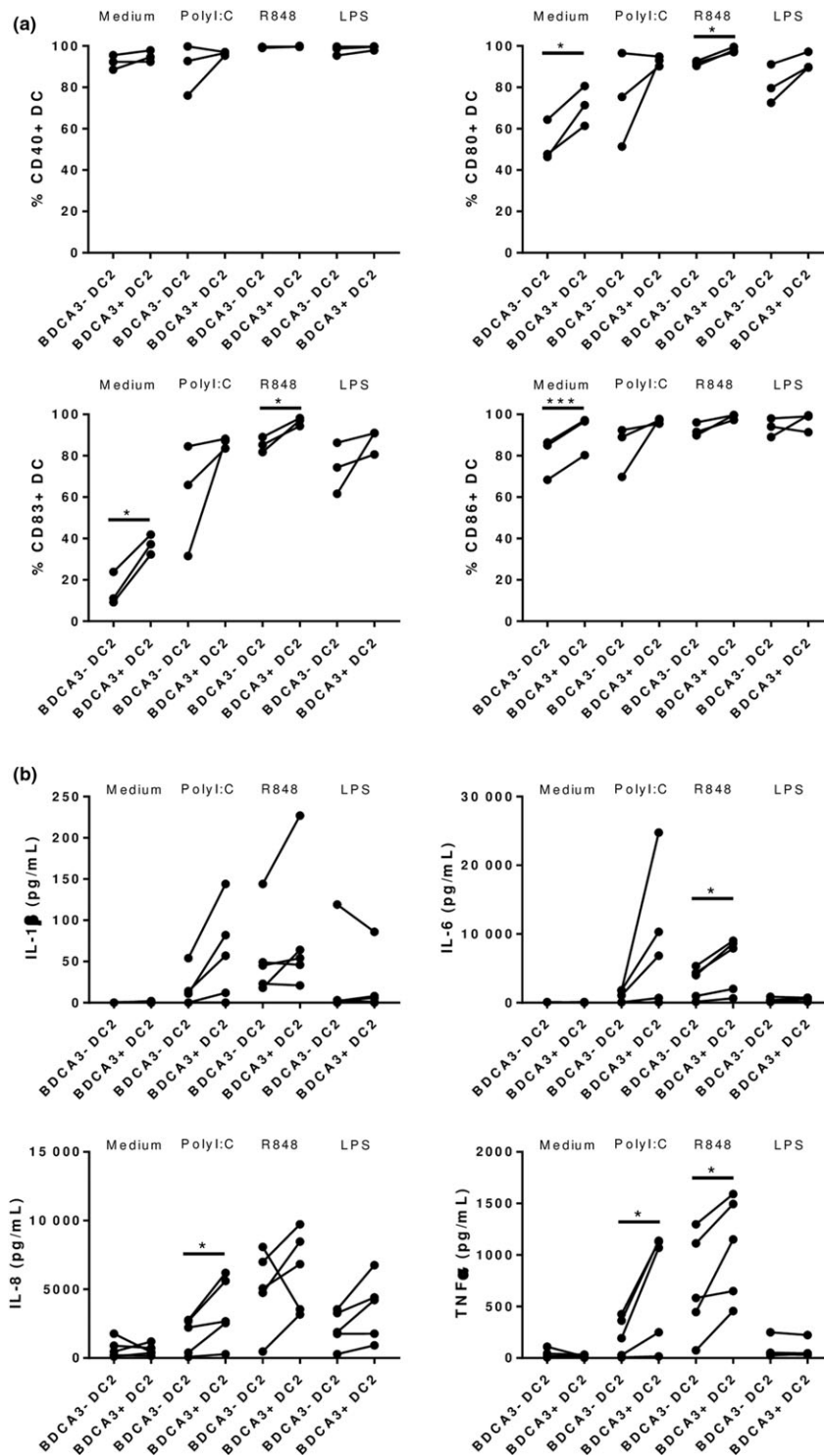
Figure 3. Maturation marker expression and cytokine production upon stimulation. BDCA3$^+$ DC2 and BDCA3$^-$ DC2 were FACSorted from Peripheral blood mononuclear cells enriched for DCs and stimulated for 24 h with or without polyI:C, R848 or LPS. **(a)** Expression of CD40, CD80, CD83 and CD86 was measured by flow cytometry. Percentage of cells expressing the indicated markers is shown. Data of two independent experiments, performed with three different donors, are shown. *$P < 0.05$, ***$P < 0.001$, paired Student's *t*-test. **(b)** Mean ± s.e.m. cytokine levels as determined by ELISA ($n = 5$). Data are pooled from two independent experiments, performed with five different donors. *$P < 0.05$, paired Student's *t*-test.

expressed on fresh DCs, but not upon culture in the presence or absence of TLR ligation (Figure 2f and g, Figure 3a). Furthermore, upon stimulation with polyI:C or R848, IL-1β, IL-6, IL-8 and TNF-α production was consistently higher for BDCA3$^+$ DC2 compared to BDCA3$^-$ DC2 (Figure 3b). TLR3, TLR4 or TLR8 expression levels were not significantly different between BDCA3$^+$ DC2 and BDCA3$^-$ DC2, indicating that a difference in TLR levels was not the underlying cause for the observed differences (Figure 2d and e, Supplementary table 1). Together, our data indicate that BDCA3$^+$ DC2 have an enhanced activation status, reflected by a higher costimulatory molecule expression and cytokine-producing capacity as compared to their BDCA3$^-$ counterparts.

The association between BDCA3 expression *in vivo* and enhanced DC activation and cytokine production is in line with an increase in BDCA3 expression on pDCs and DC2 upon maturation of these cells *in vitro*.[23] In addition, BDCA3 expression is upregulated on monocyte-like cells upon TNF-α and IL-1β stimulation,[24] and also associated with increased IFN-λ-producing capacity by CD34$^+$-derived DCs.[25] BDCA3 is also higher expressed on DC2 in peripheral tissues compared to DC2 in peripheral blood.[6] Previously, functional comparison of BDCA3$^+$ and BDCA3$^-$ monocyte-derived DC (moDC) showed that BDCA3$^+$ moDC induced a more strongly Th2-polarized response compared to BDCA3$^-$ moDC.[26] As BDCA3 has been described to exhibit direct and indirect anti-inflammatory functions,[7,8,27] it is tempting to speculate that although BDCA3 is expressed on more activated DCs, BDCA3 may play a role in dampening and/or balancing proinflammatory responses.

The gene expression dataset presented here is valuable for the study of DC immunobiology, as it provides useful information regarding phenotypic, functional and developmental characteristics of the different DC subpopulations and the relationship between the marker BDCA3 and cell function. Nevertheless, further research is required to determine the exact function of BDCA3 expression on DC2 or other DC subsets.

METHODS

Cell isolation

Peripheral blood mononuclear cells were isolated from heparinized peripheral blood samples or buffy coats from healthy donors using Ficoll density gradient centrifugation. The study was approved by the medical ethical committee of the Erasmus MC University Medical Center and donors gave written informed consent before blood donation. Cells were enriched for DCs using Dynabeads (Life Technologies AS, Oslo, Norway) and DC subsets were isolated based on BDCA1, BDCA3 and BDCA4 expression by FACSorting.

Flow cytometric analysis

For phenotypic analysis, cells were labeled with fluorochrome-conjugated antibodies recognizing BDCA3/CD141 (Miltenyi Biotec, Leiden, The Netherlands); BDCA1/CD1c (Miltenyi Biotec and Biolegend UK Ltd, London, UK), BDCA4/CD304 (Biolegend UK Ltd), CD3, CD11c, CD14, CD19, CD56, HLA-DR, CD40, CD80, CD83 (all eBiosciences, Fisher Scientific, Landsmeer, The Netherlands), CD86 (BD Horizon), CLEC9A (ITK Diagnostics, Uithoorn, The Netherlands) and CD11b (Becton Dickinson BV, Breda, The Netherlands).

Gene expression analysis using Nanostring nCounter system

RNA was extracted using an RNAeasy Micro kit or a Nucleospin RNA II kit for samples containing $<5 \times 10^5$ or $>5 \times 10^5$ cells, respectively. RNA concentration and integrity was assessed using a Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). Human immunology-related genes were profiled using the nCounter GX Human Immunology v2 kit Gene Expression CodeSet (Nanostring Technologies, Seattle, WA, USA). Gene expression levels were normalized according to 15 housekeeping genes. Gene clustering analysis was performed using GENE-E software (http://www.broadinstitute.org/cancer/software/GENE-E/). Principal component analysis was performed using MeV (4.9.0) software http://www.tm4.org/mev.html. Gene set enrichment analysis was performed using Gene Set Enrichment Analysis software.[28]

DC activation and cytokine secretion

FACSorted DCs were stimulated with or without polyI:C (20 μg mL^{-1}; Invivogen, Toulouse, France), LPS (200 ng mL^{-1}; Invivogen) or R848 (1 μg mL^{-1}; Enzo Life Sciences, Brussels, Belgium) for 24 h at 37°C in the presence of 10 ng mL GM-CSF (Enzo Life Sciences). The levels of secreted human IL-1β, IL-6, IL-8 and TNF-α were measured in the supernatant using ELISA (eBioscience).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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