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TLR3 Signaling in Human BDCA-3 Dendritic Cells Results in the Formation of Several ILT3 and ILT4 Populations

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By

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Submitted in partial fulfillment of the requirements for the Degree of Doctoral Philosophy in Molecular Bioscience from the Department of Biology of Seton Hall University

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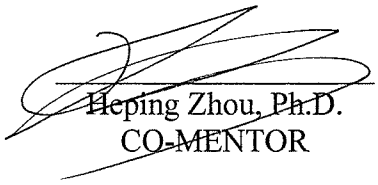

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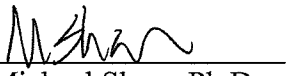

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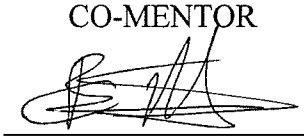

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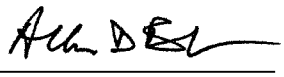

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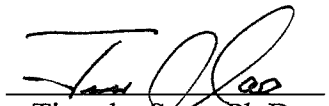

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Abstract

Dendritic cells (DCs) represent a population of innate immune cells that are highly efficient at promoting immune responses. DCs are capable of presenting antigens to both CD4 and CD8 T cells. DC presentation and interaction with T cells can result in either immune stimulation or tolerance. This study intends to phenotype a rare subset of DCs found in the human blood and is distinguishable by the expression of various surface markers including: lineage markers (Lin), HLADR, CD1c, and CD141 or BDCA-3. Stimulating BDCA-3 DCs with Poly I:C, a toll-like receptor (TLR) 3 agonist, resulted in the up-regulation of various canonical activation markers such as CD40, CD80, and CD86 as well as immunoglobulin-like transcript (ILT) 3 and 4 as measured by flow cytometry. ILTs are novel surface molecules with implicated inhibitory functions and are selectively expressed by APCs, such as DCs. The surface induction of ILT3 and ILT4 occurred in both time- and dose-dependent manner. The up-regulation of ILT3 and ILT4 within the BDCA-3 subset of DCs resulted in the unexpected formation of various subsets of BDCA-3 DCs expressing: $ILT3^{-}$ $ILT4^{-}$, $ILT3^{-}$ $ILT4^{+}$, $ILT3^{+}$ $ILT4^{-}$, and $ILT3^{+}$ $ILT4^{+}$. Due to limited numbers of cells, we focused our efforts to determine the biological differences between $ILT3^{-}$--$ILT4^{-}$ and -$ILT4^{+}$ BDCA-3 DCs after Poly I:C stimulation. This study will show that these two populations of BDCA-3 DCs differ in their cytokine secretion profile, genomic signature, and their ability to prime allogenic naïve T cells.

Introduction

The immune system consists of an intricate network of cells that are capable of detecting both foreign and selfantigens. The process of eliminating foreign antigens and the ability to tolerate self-antigens occur within both innate and adaptive immunity. Innate immunity is shared by all multi-cellular organisms and is considered to be a non-specific first line of defense. Components of innate immunity include epithelial barriers, phagocytes, dendritic cells (DCs), natural killer (NK) cells, and the complement system. Detection of microbes through innate immunity is achieved by the use of pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs). PRRs detect foreign substances, but may also detect self-components as danger signals in the presence of infection, inflammation, or other cellular stress (Mogensen, 2009). Adaptive immunity evolved in vertebrates as a means to detect and form a memory response against microbes that have evaded the innate response. There are two types of adaptive immunity, humoral and cell-mediated. Each type of adaptive immunity has the unique ability to achieve a memory of the response, thus subsequent re-infections can be quickly eliminated. Cross-talk occurs between both branches of the immune system to orchestrate both the elimination of microbes as well as the induction of a memory response. This cross-talk is mediated through cells known as antigen presenting cells (APCs), which include B cells, monocytes, macrophages, and DCs. DCs are considered the primary APCs for the activation of naïve T cells. The discovery of human splenic DCs in 1973 by Ralph Steinman and Zanvil Cohn has led to an ever expanding identification of novel DC subsets (Steinman and Cohn, 1973). APCs have

the unique ability to process both intracellular and internalized proteins and display peptides on major histocompatibility complex (MHC) molecules. MHC molecules can be divided into two classes, MHC I and MHC II molecules. All nucleated cells express the MHC class I molecules whereas MHC class II molecules are only expressed by APCs (Ting and Trowsdale, 2002, Hewitt, 2003). T cells are capable of detecting peptides displayed on both MHC molecules through the T cell receptor (TCR). Cytotoxic $CD8^+$ T cells have a TCR that recognizes peptides presented on MHC class I molecules while $CD4^+$ TCR recognizes peptides bound to MHC class II molecules (Ting and Trowsdale, 2002, Hewitt, 2003). The extraordinary genetic diversity that contributes to the specificity of MHC molecules coupled with the numerous genetic rearrangements involved in the production of diverse TCR repertoires has enabled the immune system to keep up with the ever so changing microbial, viral and tumor environment (Robins et al., 2009).

DCs have been identified to populate various organs including the skin, lung, intestinal tract, liver, kidney, and blood. They can be sub-divided by their cellular precursors, migratory capacity, and whether or not they exhibit a DC phenotype in a steady-state condition. In the broadest sense, one can classify DCs as conventional or non-conventional. Conventional DCs exhibit classical DC function and are derived from a common DC progenitor (CDP) and can be further divided into migratory and lymphoid DCs. On the other hand, non-conventional DCs, including plasmacytoid DCs (pDCs), are derived from a pre-DC population along with monocyte-derived DC subsets and are found in various peripheral organs (Kushwah and Hu, 2011). The identification of the

various DC populations is facilitated by the surface markers expressed on the cell membrane called clusters of differentiation (CD).

This study focuses on human blood DC subsets. Blood DCs are rare leukocytes making up approximately 0.16-1.63% of total peripheral blood leukocytes (Haller Hasskamp et al., 2005). Human blood DCs consist of three distinct subsets characterized by their differential expression of three surface markers, termed blood dendritic cell antigens (BDCA), namely BDCA-1 (CD1c), BDCA-2 (CD303), and BDCA-3 (CD141). Each marker selectively identifies cDCs, pDCs, and $CD141^{+}$ cDCs respectively. cDCs are primarily responsible for bacterial clearance and cross-presentation to CD8 T cells (Kushwah and Hu, 2011). pDCs are involved in viral elimination and upon stimulation produce large amounts of type I interferons (Swiecki and Colonna, 2010).

Each subset of blood DCs expresses different levels of environment sensing receptors to detect PAMPs called Toll-like receptors (TLRs). Currently, through genomic analysis, ten human TLRs have been identified. TLRs 1, 2, 4-6 and 11 physically reside on the surface of the cell membrane, while TLRs 3, 7-9 are localized to the endosomal compartments. All TLRs, except for TLR3, signal through the adaptor protein MyD88 or through a MyD88 independent pathway, which results in NF-κB activation. TLR3 and TLR4 signal through the TRIF-dependent pathway. The activation of NF-κB ultimately influences the genes encoding various cytokines. TLR signaling through different DC subsets results in a unique cytokine secretion profile (Kawai and Akira, 2010). TLR1 has been shown to be involved in detecting microbial lipoproteins potentially by associating

with TLR2 (Takeuchi et al., 2002). The natural ligand for TLR1 however still remains elusive. TLR2 has been shown to recognize a wide variety of bacterial cell wall components through heterodimerization with either TLR1 or TLR6 (Kadowaki et al., 2001). TLR3 recognizes double-stranded RNA typically generated by actively replicating viruses and upon activation results in the production of type I Interferons (Alexopoulou et al., 2001). The natural ligand for TLR4 is lipopolysaccharide (LPS) from gram-negative bacteria which upon activation leads to the induction of pro-inflammatory cytokines (Lu et al., 2008). TLR5 has been shown to specifically interact with flagellin from both gram-negative and gram-positive bacteria. Activation of TLR5 results in the production of TNF-α (Hayashi et al., 2001). TLR6 activation typically occurs when the receptor heterodimerizes with TLR2. Lipopeptides have been shown to heterodimerize TLR2 with TLR6 signaling through the MyD88 adaptor protein and activation of NF-κB (Takeuchi et al., 1999, Farhat et al., 2008). TLR7 activation was initially discovered through interactions with small anti-viral imidazoquinoline-derived compounds such as imiquimod and resiquimod (R-848). Further studies revealed that the natural ligand for TLR7 is single strand viral RNA. More recently, studies have shown that bacterial RNA can also be detected by TLR7 (Mancuso et al., 2009). TLR7 activation results in the secretion of type one interferons, IFN-α, by pDCs (Hemmi et al., 2002, Diebold et al., 2004). TLR8 is both genetically and functionally similar to TLR7 as recent studies have shown that TLR7 can detect bacterial RNA. Similar studies have shown that TLR8 can detect bacterial RNA released within phagosomal vacuoles. Activation of TLR8 results in the release of type one interferons, specifically IFN-β (Cervantes et al., 2012).

Interestingly, TLR8 is involved in TLR to TLR cross-talk. Studies have shown that human TLR8 activation inhibits the activation of TLR7 and 9 (Wang et al., 2006). TLR9 has been shown to selectively detect unmethylated CG dinucleotides present in microbial DNA. Un-methylated CG dinucleotides (CpG) are not common in the vertebrate genome and have been shown to be immunostimulatory through TLR9 recognition (Bauer et al., 2001, Rutz et al., 2004).

TLRs are mainly expressed on APCs including monocytes, dendritic cells, and B cells. In the blood, human BDCA-1 and BDCA-3 cDCs selectively express TLRs 2-6, 9 and 3, respectively, and produce large amounts of IL-12 during antibacterial and antiviral responses (Jongbloed et al., 2010, Gupta et al., 2013). In contrast, human BDCA-2 pDCs express TLR7 and TLR9 and have the ability to produce a large amount of type 1 interferons (IFNs) in antiviral immune responses (Colonna et al., 2004). There are species-specific differences in the expression of TLRs across human and murine leukocytes. Mouse pDCs express TLRs 3,4,7-9 while human pDCs mainly express TLRs 7 and 9. Mouse myeloid DCs express TLR9 while human myeloid DCs do not (Ketloy et al., 2008). Due to the rarity of primary blood DCs, many studies rely on monocyte derived dendritic cells (moDCs), which can be generated in large quantities *in vitro* by exposing monocytes to various growth factors such as IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994). Although useful, moDCs are phenotypically and functionally distinct from freshly isolated primary DCs. MoDCs express TLR9 and CD209 while blood $CD11c^{+}$ DCs do not. Blood DCs generate greater T lymphocyte proliferative responses as well as Th1 effectors compared to moDCs (Osugi et al., 2002, Hoene et al.,

2006). Therefore, care must be taken interpreting data derived from moDCs and the translatability of those findings to primary blood DCs.

DC TLR engagement by their respective ligands leads to the up-regulation of various canonical activation markers, including but not limited to CD40, CD80, and CD86. CD40 is a member of the TNF receptor family. Activation of CD40 by its receptor CD40 ligand (CD40L) results in DC cytokine secretion, co-stimulatory molecule migration to the cell surface, and enhanced antigen presentation (Elgueta et al., 2009). CD80 and CD86 provide co-stimulatory signals to T cells to enhance their proliferation, cytokine secretion, and maturation status (Lanier et al., 1995). Following DC activation, a naïve T cell that encounters a DC through TCR-MHCI/II interaction can be primed toward a particular phenotype. The outcome of such a priming event is dependent on the maturation status and the type of DC a naïve T cell encounters in the lymphoid tissue. CD4 T cell priming by DCs can yield various T helper (Th) phenotypes that exhibit a unique cytokine secretion profile. Th1 T cells predominately secrete IFN-ɣ and are typically involved in autoimmune disease pathogenesis and host defense against intracellular pathogens. Th2 T cells secrete IL-4 and IL-5 and are associated with atopic diseases and are involved in the expulsion of extracellular parasite elimination. Th17 cells produce IL-21 and IL-17 and are involved in extracellular bacterial and fungi elimination, as well as certain autoimmune disease. Finally T regulatory cells secrete TGF-β and IL-10 and are involved in immune tolerance (Zhu and Paul, 2008).

Along with pro-inflammatory signals, DCs are also capable of promoting tolerogenic responses. cDCs and pDCs under certain environmental conditions have been reported to prime naïve T cells into T regulatory cells (Tregs) (Kushwah and Hu, 2011). Recently, a subset of DCs has been shown to express the immunoglobulin-like transcript (ILT) receptors. ILTs, such as ILT3 and ILT4, have been demonstrated to be expressed on monocytes and DCs. The cytoplasmic region of ILT molecules contains a putative immuno-receptor tyrosine-based inhibitory motif, suggesting an inhibitory function of ILT receptors. Consistent with the proposed inhibitory function, ILT3 has been shown to induce immunosuppression, including T cell anergy, regulatory T cell (Treg) induction, and reduced allo-stimulatory capacity (Chang et al., 2002, Manavalan et al., 2003). ILT4 expression has also been implicated in the role of DCs to prime Tregs, however its prevalence within the literature is quite limited (Manavalan et al., 2003).

The current study focuses on the phenotypic and functional characterization of the BDCA-3 subset of blood DCs. Each subset of blood DCs expresses different levels of TLRs. Transcriptional profiling of various TLR receptors within a particular DC subset and responsiveness to multiple TLR agonists suggests two hypotheses (A) on per cell basis BDCA-3 DCs express multiple TLRs or (B) there may exist various BDCA-3 DC subsets each with a unique TLR expression. The recent discovery of a human $XCR1^{+}CD141^{+}$ DC subset expressing TLR3 within the conventional DC population supports the latter hypothesis (Bachem et al., 2010). This discovery raises the possibility that multiple yet-to-be-identified populations of BDCA-3 DCs may exist. We intend to show that stimulating primary human blood BDCA-3 DCs with Poly I:C, a TLR3

agonist, results in the formation of various populations identifiable by inhibitory receptors ILT3 and ILT4. These populations will be phenotypically assessed for genomic differences as well as their ability to secrete cytokines and prime T cells. Finally, the current study contributes to the overall understanding of various human DC subsets.

Materials and Methods

i. Isolation and Culture of Human Cells.

Total blood leukapheresis from healthy anonymous donors was purchased from Research Blood Components LLC (Brighton, MA). Total peripheral blood mononuclear cells (PBMCs) were isolated after lysis of red blood cells. Cells were first washed in PBS containing 0.5% human serum albumin. Next, red blood cells were lysed in ACK buffer at 37^{o}C for 10 minutes. Cells were then spun down at 300g for 5 minutes and resuspended in StemCell wash buffer. Total DCs were first enriched using the Human Myeloid DC Enrichment Kit (StemCell Technologies, Vancouver, BC) according to the manufacturer's instructions. Enriched DCs were then stained antibodies including lineage markers (Lin) (BD Bioscience, San Jose, CA), HLADR (BD Bioscience, San Jose, CA), CD1c (Biolegend, San Diego, CA), CD11c (Miltenyi, San Diego, CA), CD123 (BD Bioscience, San Jose, CA), and CD141 (Miltenyi, San Diego, CA). Labeled cells were sorted on a BD FACS ARIA II (BD Biosciences, San Jose, CA). pDCs were sorted based on the expression of cell surface markers, such as Lin^{-}, $CD123^{+}$, and $HLADR^{+}$. cDCs were sorted based on cell surface markers such as Lin^{-}, CD123dim, $HLADR^{+}$, $CD1c^{+}$, and $CD11c^{+}$. BDCA-3 DCs were sorted based on cell surface markers as Lin^{-}, CD123dim, $HLADR^{+}$, $CD1c^{-}$, and $CD141^{+}$. The purity of collected pDCs, cDCs, and BDCA-3 DCs was consistently greater than 98% based on post sort analysis. The post sort purity was determined by acquiring a sorted sample.

ii. Real time polymerase chain reaction for TLR gene expression (mRNA).

Total RNA was extracted from a total of $1x10^6$ freshly purified pDCs, cDCs and BDCA-3 DCs utilizing the RNeasy Plus Mini kit (Qiagen, Valencia, CA). The RNA was reversely transcribed to cDNA utilizing SuperScript VILO (Invitrogen, Grand Island, NY). TLRs 1-10 expression was analyzed using Applied Biosystem's TaqMan Gene Expression Master Mix and primer/probes. PCR parameters were 50 ^oC for 2min, followed by 95 ^oC for 10min proceeding to 40 cycles of 95 ^oC for 15 s and 60 ^oC for 1 min. RPLPO was used as an internal control. Taqman assays were performed on a BioRad Real-Time PCR System CFX384 (Biorad, Hercules, CA). To determine the relative expression of each gene, the $2^{-\Delta\Delta Ct}$ approach (ΔCq method) was employed (Livak and Schmittgen, 2001).

iii. Gene array experiments.

Purified BDCA-3 DCs were cultured in complete XVIVO-15 (5% human serum (Sigma) + 1% Pen/Strep) (Invitrogen, Grand Island, NY) media containing 10 μg/mL Poly I:C at 37^oC for 18 hours. Cells were washed and sorted by the expression of ILT3 and ILT4 (R&D Systems, Minneapolis, MN). Total RNA was extracted from $ILT3^-$ $ILT4^-$, $ILT3^+$ $ILT4^-$, $ILT3^+$ $ILT4^+$ and $ILT3^-$ $ILT4^+$ BDCA-3 DCs utilizing the RNeasy Plus Mini kit (Qiagen, Valencia, CA). RNA was frozen and sent to the Boston University MicroArray Core for further processing. Briefly, all procedures were performed at Boston University Microarray Resource Facility as described in GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Santa Clara, CA),

Nugen Ovation Pico WTA System User Guide, Nugen WT-Ovation ExonModule User Guide and Nugen Encore Biotin Module User Guide (Nugen, San Carlos, California).

iv. Microarray Analysis.

Affymetrix GeneChip Human Gene 1.0 ST CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average (RMA) (Irizarry et al., 2003) in the Affy package (version 1.36.1) (Gautier et al., 2004) included within in the Bioconductor software suite (version 2.12) (Gentleman et al., 2004) and an Entrez Gene-specific probeset mapping (version 17.0.0) from the Molecular and Behavioral Neuroscience Institute (Brainarray) at the University of Michigan (Dai et al., 2005). Array quality was assessed by computing Relative Log Expression (RLE) and Normalized Unscaled Standard Error (NUSE) using the affyPLM Bioconductor package (version 1.34.0). Principal Component Analysis (PCA) was performed using the prcomp R function with expression values that were unadjusted or were adjusted for donor (by creating linear models using the lmFit function in the limma package (version 3.14.4), treating donor as a fixed effect) and had then been normalized across all samples to a mean of zero and a standard deviation of one. Linear mixed-effects modeling and the associated analysis of variance were carried out using the anova.lme function in the nlme package (version 3.1-97). Pairwise differential gene expression was assessed by performing Student t tests on the coefficients of linear models created using lmFit, correcting for donor as a fixed effect. Correction for multiple hypothesis testing was accomplished using the Benjamini-Hochberg false discovery rate (FDR). All microarray

analyses were performed using the R environment for statistical computing (version 2.15.1).

v. Identification of Differentially Expressed Genes (DEG).

For comparative analysis, general linear models for microarray data were performed for probe sets present on the microarray to identify probe sets that were differentially expressed between the groups, based on moderated t-statistics. Probe sets with a 1.5-fold change and a P value less than 0.05 were considered biologically significant. Principal Component Analysis (PCA) was then performed. PCA is a mathematical transform that collapses the variance between samples across a set of large set of variables (here, all ~20,000 genes on the array) into a much smaller set of variables called Principal Components (PCs). These "meta-variables" are arranged such that PC1 explains the most variance in the data, followed by PC2, etc. PCA was performed using all genes across all samples, either before or after adjusting the expressions for donor (using a simple linear model), and plots were made of PC1 vs. PC2

vi. Blood DC TLR activation assay and Poly I:C time course.

After confirming the post sort purity of >98%, $1x10^5$ BDCA-1, BDCA-2, and BDCA-3 DCs were plated in 96 well v-bottom plates in complete X-VIVO 15 media (Lonza, Allendale, NJ). TLR agonists, PAM3CSK4, Poly I:C, LPS, Flagellin, Resiquimod, and CpG2216 (Invivogen, San Diego, CA) were added at a final concentration of 3µg/mL. Plates were incubated at 37^{o}C for 18 hours. Cells were then prepared as stated in the Flow Cytometry section. Plots show the mean of the fold induction from unstimulated

cells calculated using PRISM v6.0, error bars represent SEM. Time course: $1x10^6$ Purified BDCA-3 DCs were added to a FACS tube in complete X-VIVO media. An aliquot of cells was removed and stained as stated in the Flow Cytometry section to acquire an unstimulated sample. Poly I:C was then added at a final concentration of 10µg/mL and cells were incubated at 37°C. An aliquot of cells was removed every hour, stained, and acquired for the next 8 hours. After 18 hours, a final aliquot was taken and cells were stained as stated in the Flow Cytometry section.

vii. BDCA-3 DC activation assay followed by ILT4 sorting.

After confirming the post sort purity of >98%, $1x10^5$ BDCA-3 DCs were plated in 96 well v-bottom plate in complete X-VIVO 15 media (Lonza, Allendale, NJ) (5% human serum, 2mM L glutamine and 1x penicillin/streptomycin). TLR agonists, Poly I:C and LPS, (Invivogen, San Diego, CA) were added at a final concentration of 10µg/mL. Plates were incubated at 37°C for 18hrs. Cells were then harvested and stained with ILT4 (R&D Systems, Minneapolis, MN). After staining, BDCA-3 DCs were sorted based on the expression of ILT4 for subsequent analysis.

viii. Cytokine production assays.

Immediately following sorting, total BDCA-3 DCs were added to a FACS tube containing Poly I:C at a final concentration of 10µg/mL in complete X-VIVO 15 media. BDCA-3 DCs were incubated at 37°C for 18 hours to allow for the formation of ILT4 positive cells. Stimulated BDCA-3 DCs were then re-sorted by their expression of ILT4 directly into complete X-VIVO 15 media without further stimulation. $2x10^4$ cells in

200µL complete X-VIVO 15 media were added to a 96 well plate and incubated for 18 hours at 37^{o}C. Supernatants were harvested and cytokine profiles were assayed with the ProcartaPlex Human Cytokine/Chemokine/Growth Factor Panel (eBiosciences, San Diego, CA) on a Bioplex 200 System running Bioplex Manager Version 6. Statistics were performed by running a two-tailed paired student t-test in GraphPad Prism version 6.0. Results with a p value < 0.05 were considered significant. Intracellular FACS staining of BDCA-3 DCs was performed as follows. Following sorting, total BDCA-3 DCs were added to a FACS tube containing Poly I:C at a final concentration of 10µg/mL in complete X-VIVO 15 media. BDCA-3 DCs were incubated at 37^{o}C for 18 hours to allow for the formation of ILT4 positive cells. Golgistop was then added for 6 hours. Cells were then washed and surface stained with ILT3, ILT4 (R&D Systems, Minneapolis, MN), CD141 (Miltenyi, San Diego, CA). Following surface staining, cells were stained with a viability stain that persists through fixation, Live/Dead (Life Technologies, Waltham, MA) as per the manufacturer's protocol. Finally, cells were intracellularly stained with IFN-γ (BioLegend, San Diego, CA), IL-4 (eBiosciences, San Diego, CA), IL-10 (BD, San Jose, CA), IL-13 (BD, San Jose, CA), IL-5 (BioLegend, San Diego, CA), and TNF-α (BioLegend, San Diego, CA). Data was collected using a BD LSRII and analysis was performed with FlowJo Software V9.7 (Treestar, Ashland, OR).

ix. *In vitro* priming of naïve CD4^{+} and CD8^{+} T cells.

An aliquot of total PBMC was enriched using Human Pan T cell Pre-Enrichment Kit (StemCell Technologies, Vancouver, BC) for the preparation of allogenic naïve CD4^{+}

and $CD8^+$ T cells. Total $CD4^+$ and $CD8^+$ T cells were stained and FACS sorted on a BD FACS ARIA II. Naïve T cells were designated as $CD25^-$, $CD127^+$, $CD62L^+$, and CD49dlow. BDCA-2 and BDCA-3 DCs were isolated as stated previously. Bulk BDCA-2 and BDCA-3 DCs were incubated at 37°C for 18 hours with 3µg/mL CpG2216 or 10µg/mL Poly I:C respectively. After TLR stimulation, BDCA-3 DCs were sorted into pure populations of $ILT4^+$ and $ILT4^-$. BDCA-2 DCs were washed of CpG2216 prior to the addition of allogenic T cells. Allogenic naïve $CD4^+$ and $CD8^+$ T cells were incubated with allogenic BDCA-2 and BDCA-3 DCs ($ILT4^+$ and $ILT4^-$) at a 1:5 DC to T cell ratio at 37°C in complete X-VIVO 15 media. After 7 days, primed T cells were harvested and analyzed for cell surface phenotype as well as intracellular staining.

x. Flow Cytometry.

BDCA-1, BDCA-2, and BDCA-3 DC activation status was assessed using surface stain markers CD40, CD80, CD86, CCR7, ILT3, and ILT4 from BD Biosciences and R&D Systems. Cells were stained with fluorescent antibodies for 30 minutes on ice, washed twice with BD FACS staining buffer (DPBS contains 2% FBS and 0.09% sodium azide) and then acquired on a BD LSR II. Mean fluorescence intensity and cell percentages were determined by FlowJo V9.7 (Treestar, Ashland, OR). Cytokine production by day 7 primed T cells was assessed by intracellular cytokine staining after initial staining with Cell Trace violet to detect proliferation (Life Technologies, Waltham, MA). Day 7 primed T cells were stimulated with PMA (50ng/mL; EMD Millipore, Billerica, MA) and Ionomycin (1µM; Sigma, St. Louis, MO) in the presence of Golgi

Stop (1 µl/mL; BD Bioscience, San Jose, CA) for 6 hours. After incubation, cells were first surface stained with antibodies to CD25 (BD, San Jose, CA) and HLAG (BioLegend, San Diego, CA), 30 minutes on ice. Next, cells were cultured using Live/Dead stain (Life Technologies, Waltham, MA) according to manufacturer's protocol. Finally, cells were stained intracellularly with antibodies against IL-4 (eBiosciences, San Diego, CA), IL-5 (eBiosciences, San Diego, CA), IL-10 (eBiosciences, San Diego, CA), IL-13 (BD, San Jose, CA) and IFN-γ (BD, San Jose, CA). Data was collected using BD LSRII and analysis was performed with FlowJo Software V9.7 (Treestar, Ashland, OR).

xi. EC50 calculations.

All reported EC50 were calculated using the following:

The 4-parameters logistic model has been chosen to fit standard dose-response curves:

$$Y = a + \frac{c}{(1 + \exp(-b(\ln X - d)))}$$

a is the lower asymptote; can be constrained by the user (Bottom).

b is the slope at the inflexion point of the curve (Slope) (eg: Hill's Slope or n-Hill).

c is the difference between upper and lower asymptote (Delta) ; cannot be constrained directly but via the constraint on upper asymptote (Top) which is "a+c".

d is the logarithm of the concentration estimated at inflexion point (CE50 relative); this parameter cannot be constrained.

Results were obtained using the 4-parameter logistic model according to Ratkovsky and Reedy (Ratkowsky and Reedy, 1986). The adjustment was obtained by non-linear regression using the Levenberg-Marquardt algorithm in SAS v9.2 software.

Results

i. Blood dendritic cells express a wide range of TLRs

Isolating sufficient numbers of highly enriched pDCs and cDCs for functional assays requires a large number of input PBMCs. pDCs and cDCs only make up approximately 0.1-0.3% of total PBMCs in healthy individuals, respectively (Rovati et al., 2008). A two-step enrichment process was devised to facilitate the isolation of sufficient quantities of both cDCs and pDCs from a leukapheresis pack. First, total blood DCs were enriched by negative selection, followed by flow cytometry sorting of the enriched DC fractions for pDCs (Lin^-, $CD123^+$, $HLADR^+$), cDCs (Lin^-, $CD123^-$, $HLADR^+$, $CD1c^+$, $CD11c^+$), and BDCA-3 cDCs (Lin^-, $CD123^-$, $HLADR^+$, $CD1c^-$, $CD141^+$), (Fig. 1).

Previous studies suggested that primary cDCs and pDCs exhibit distinct patterns of toll-like receptor (TLR) expression (Kadowaki et al., 2001) as compared to *in vitro* moDCs (Chang et al., 2000, Osugi et al., 2002). We therefore wanted to confirm, as previously reported, the TLR expression profile of highly purified pDC and cDC populations to ascertain their phenotype prior to TLR agonist profiling. qPCR on the sorted primary blood DCs revealed that cDCs expressed a wide range of TLRs including TLR1, TLR2, TLR4, and TLR10. In contrast, pDCs mainly expressed TLRs 7 and 9, and BDCA-3 cDCs expressed mainly TLR1, TLR3, and TLR10 (Fig. 2). Given that BDCA-3 cDCs expressed a limited repertoire of TLRs, we focused our initial efforts to characterize the BDCA-3 cDC response to Poly I:C, a TLR3 agonist.

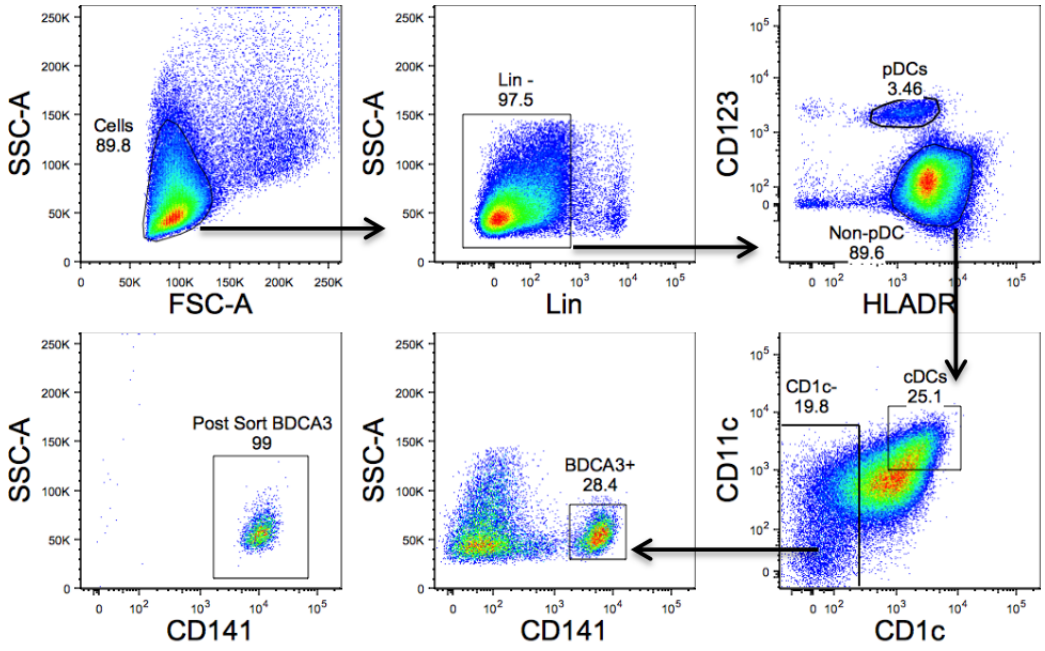


Figure 1: Blood DC gating strategy for FACS sorting. Leukapheresis packs were lysed of red blood cells and then depleted for DC enrichment. pDCs were sorted based on the expression of cell surface markers Lin^{-}, $CD123^{+}$, $HLADR^{+}$. cDCs were sorted based on cell surface markers as Lin^{-}, $CD123^{dim}$, $HLADR^{+}$, $CD1c^{+}$, and $CD11c^{+}$. BDCA-3 DCs were sorted based on cell surface markers as Lin^{-}, $CD123^{dim}$, $HLADR^{+}$, $CD1c^{-}$, and $CD141^{+}$. Figure is representative of one donor out of four. (Colletti et al., 2016)

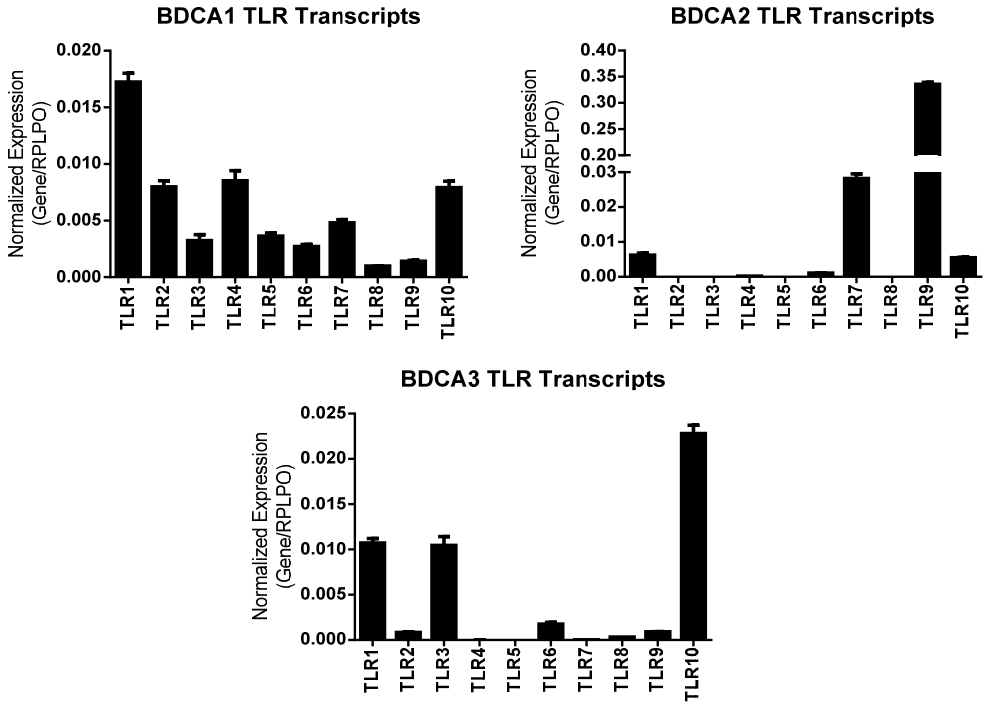


Figure 2: Blood DC subsets express different levels of TLRs.
RNA extracted from sorted pDCs, cDCs, and BDCA-3 DCs was reverse transcribed for qPCR. To determine the relative expression of each gene of interest, genes were normalized to RPLPO, and the $2^{-\Delta\Delta Ct}$ approach (ΔCq method) was utilized. Data represent four donors. Error bars represent SEM. (Colletti et al., 2016)

ii. Blood DCs are activated by TLR agonists:

Blood DCs were stimulated with various TLR agonists and assessed for activation by flow cytometry. DC activation with TLR agonists as measured by activation markers CD40, CD80, and CD86 correlated with the corresponding TLR transcript profile of each DC. To normalize the magnitude of activation, we calculated fold induction from baseline. The mean fluorescence intensity (MFI) from stimulated cells was divided by the MFI of unstimulated cells to yield a fold induction of activation. BDCA-1 DCs were activated by Poly I:C (TLR3) and Resiquimod (TLR7/8), BDCA-2 DCs were activated by Resiquimod and CpG2216 (TLR9), and BDCA-3 DCs were activated by Pam3CSK4 (TLR1/2), Poly I:C, and Resiquimod (Fig. 3).

We decided to further evaluate the response of BDCA-3 cDCs following Poly I:C, stimulation. Stimulation of BDCA-3 cDCs with Poly I:C resulted in activation of these DCs as indicated by the induction of canonical DC-maturation markers, such as CD40 and CD80/86 as well as inhibitory receptors ILT3 and ILT4 (Fig. 4A) after 18 hours. CD40, CD80, and CD86 after stimulation with Poly I:C are uniformly upregulated. Unlike other blood DC subsets (e.g. pDCs), ILT3 and ILT4 up-regulation revealed several populations of BDCA-3 DCs (Fig. 4B). The appearance of these populations ($ILT3^-$ $ILT4^-$, $ILT3^-$ $ILT4^+$, $ILT3^+$ $ILT4^-$, and $ILT3^+$ $ILT4^+$) of BDCA-3 cDCs prompted us to further investigate their biology.

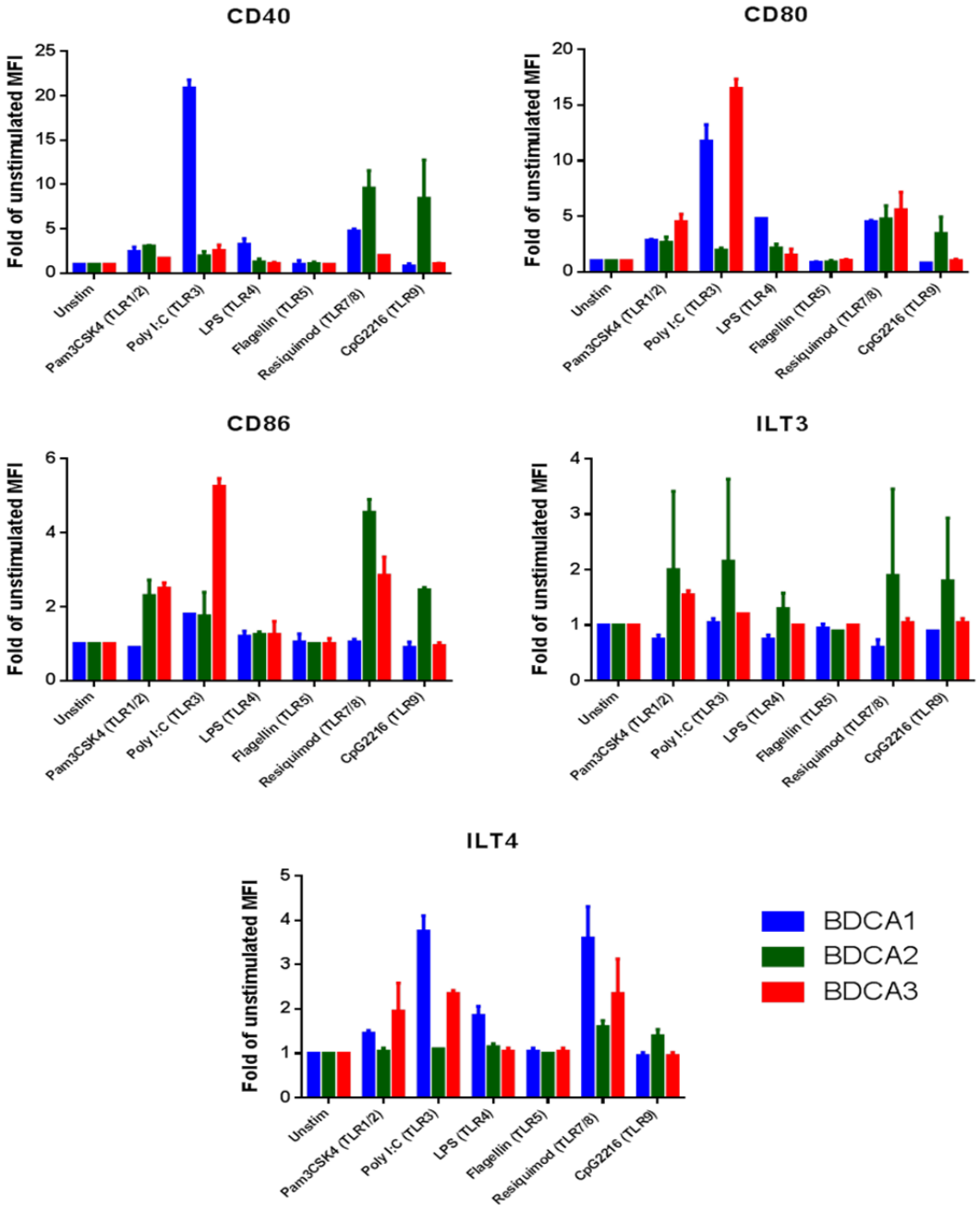
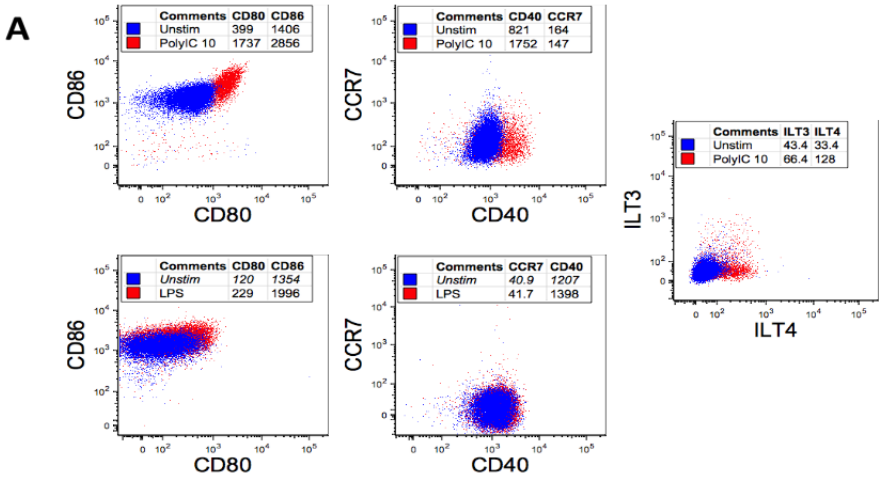


Figure 3: Blood DCs activated by TLR agonists up-regulate activation markers. BDCA-1, BDCA-2, and BDCA-3 DCs were stimulated with TLR 1-10 agonists at 3µg/mL for 18 hours. DC activation status was assessed using surface stain markers CD40, CD80, CD86, CCR7, ILT3, and ILT4. Values represent a fold of the unstimulated MFI. Data represents the average of three single donors, error bars represent SEM.



Activation Marker	Fold of Unstim	Standard Deviation
CD40	2.12	0.02
CD80	8.19	5.43
CD86	3.01	1.38
ILT3	1.38	0.21
ILT4	4.11	0.39
CCR7	1.24	0.49

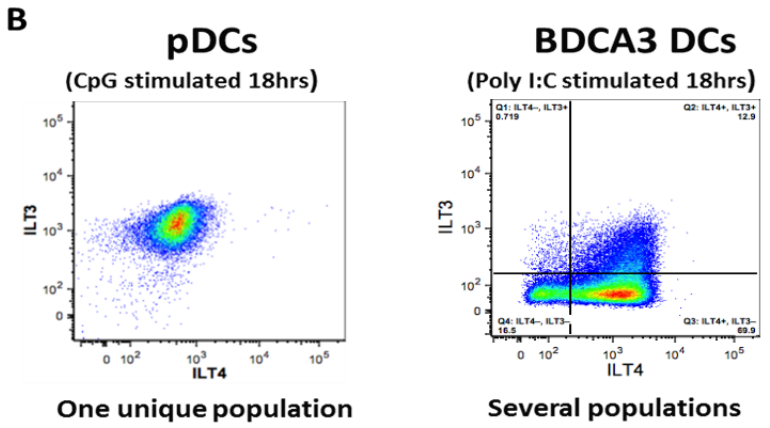
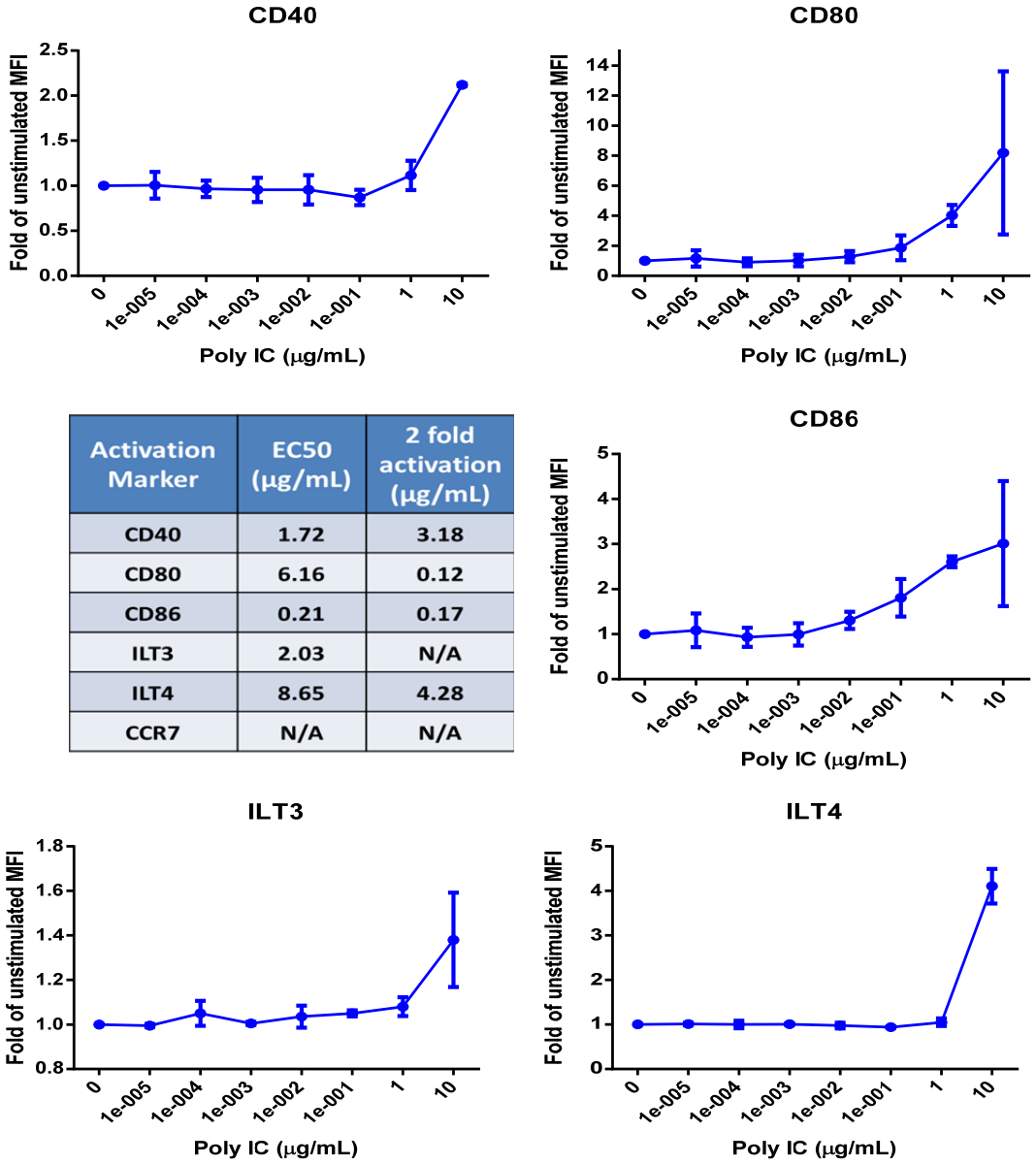


Figure 4: BDCA-3 DC stimulation with Poly I:C up-regulates inhibitory receptors ILT3 and ILT4. A) BDCA-3 DCs were stimulated with Poly I:C and LPS at 10µg/mL for 18 h. Expression of CD80, CD86, CCR7,CD40, ILT3, and ILT4 comparing pre- and post-stimulation, blue dots represent unstimulated cells and red dots identify cells stimulated with TLR agonist, with values representing mean fluorescent intensity (MFI). B) pDCs stimulated with CpG2216 at 3µg/mL and BDCA-3 DCs stimulated with Poly I:C at 10µg/mL for 18 hours results in a DC subset specific pattern of the up-regulation of ILT3 and ILT4 (Colletti et al., 2016).

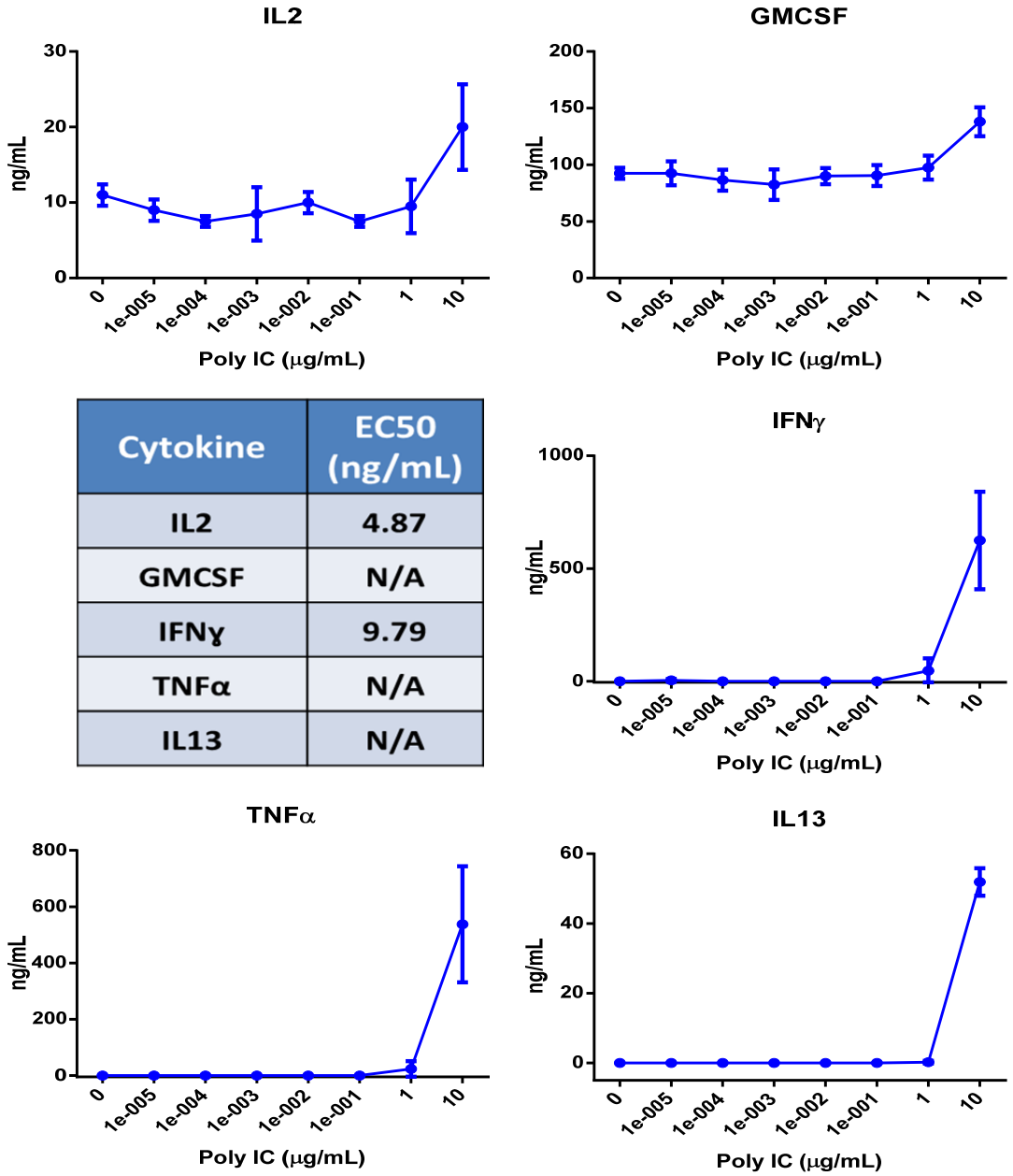
iii. Poly I:C activation of BDCA-3 DCs occurs in a dose dependent manner:

The induction of activation markers measured on BDCA-3 DCs after stimulation with Poly I:C occurred in a dose dependent manner. DCs were considered activated when they achieved an MFI that was two-fold above the unstimulated value at 18 hours post stimulation. Two-fold induction for each of the activation markers was calculated: CD40 [3.2µg/mL], CD80 [0.12µg/mL], CD86 [0.17µg/mL], ILT4 [4.3µg/mL], while ILT3 did not achieve two fold inductions at 18 hours at all doses of Poly I:C tested (Fig. 5)**.** Measurable cytokine production by BDCA-3 DCs after stimulation with Poly I:C was detected at higher concentrations than those required to induce activation marker detection by flow cytometry (Fig. 6)**.**



Activation Marker	EC50 (μg/mL)	2 fold activation (μg/mL)
CD40	1.72	3.18
CD80	6.16	0.12
CD86	0.21	0.17
ILT3	2.03	N/A
ILT4	8.65	4.28
CCR7	N/A	N/A

Figure 5: Poly I:C dose response stimulation of BDCA-3 DC activation markers. BDCA-3 DCs were isolated and then stimulated with varying doses of Poly I:C for 18 hrs at 37 ^{o}C. Cells were the stained with the corresponding activation marker and acquired on an FACS Fortessa. Fold of the unstimulated values were calculated by dividing the concentration of interest's MFI by the unstimulated MFI. EC50 curves and the concentrations at which 2 fold inductions occurred were calculated as stated in Materials and Methods section. Data represents the mean of three single donors, error bars represent SEM.

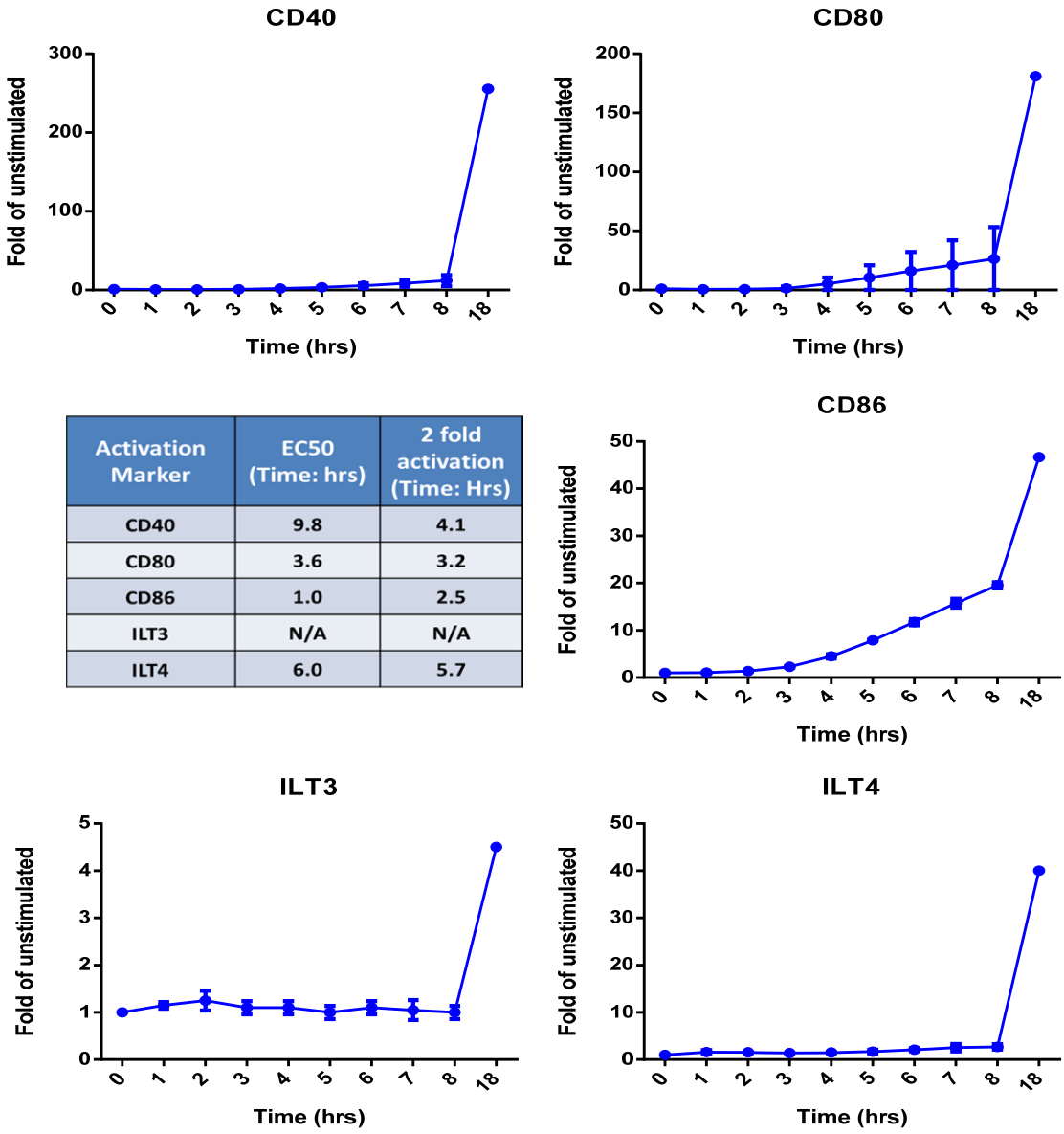


Cytokine	EC50 (ng/mL)
IL2	4.87
GMCSF	N/A
IFNɣ	9.79
TNFα	N/A
IL13	N/A

Figure 6: Poly I:C dose response on BDCA-3 DC cytokine secretion.
BDCA-3 DCs were isolated and then stimulated with varying doses of Poly I:C for 18hrs at 37^{o} C. DC cell supernatants were analyzed for cytokine levels using the human ProcartaPlex Human Cytokine/Chemokine/Growth Factor Panel from eBioscience. EC50 curves were calculated as stated in Materials and Methods section. Data represents three single donors, error bars show SEM. TNF-α and IL13 EC50's were not calculated as they did not fit the curve fitting software.

iv. Poly I:C activation of BDCA-3 DCs occurs in a time dependent manner :

BDCA-3 DCs stimulated with Poly I:C at 3µg/mL resulted in minimal up-regulation of both ILT3 and ILT4 after 18 hours, in order to detect a robust induction of the activation markers of interest, we used a dose of 10µg/mL for further experimentation. 10µg/mL required several hours of stimulation prior to the detection of activation markers to occur (Fig. 7). CD40, CD80, and CD86 achieve detectable two-fold activation at 4.1, 3.2, and 2.5 hours respectively. The detection of ILT4 was further delayed, achieving a two-fold activation measurement after 5.7 hours. ILT3 induction was also delayed as compared to the activation marker induction,. ILT3 up-regulation was detectable only after 8 hours of stimulation. The detection of activation markers occurred prior to the detection of the inhibitory receptors ILT3 and ILT4 as demonstrated by the time required to induce a two-fold activation from baseline.



Activation Marker	EC50 (Time: hrs)	2 fold activation (Time: Hrs)
CD40	9.8	4.1
CD80	3.6	3.2
CD86	1.0	2.5
ILT3	N/A	N/A
ILT4	6.0	5.7

Figure 7: Poly I:C time course of BDCA-3 DC activation marker up-regulation. Purified BDCA-3 DCs were added to a FACS tube in complete X-VIVO media. An aliquot of unstimulated cells was removed and stained as stated in the Flow Cytometry section prior to the addition of Poly I:C. Poly I:C was then added at a final concentration of 10µg/mL and cells were incubated at 37°C. An aliquot of cells was removed every hour, stained, and then acquired. Cells were stained as stated in the Flow Cytometry Materials and Methods section. Data represents the mean of 3 single donors, error bars show SEM. ILT3 EC50 and 2 fold activation was not calculated, curves did not fit the software.

v. ILT3 and ILT4 BDCA-3 DC populations exhibit similar activation marker induction:

To discount the possibility that the various ILT populations ($ILT3^{-}ILT4^{-}$; $ILT3^{+}ILT4^{-}$; $ILT3^{-}ILT4^{+}$; $ILT3^{+}ILT4^{+}$) arose due to differential levels and/or threshold of activation, the maturation status of each of the ILT populations was examined. As shown in Figure 8A, all ILT populations had similar surface expression of DC-maturation associated markers. To address the specificity of the inductive signal for generating these various populations of BDCA-3 cDCs, the TLR4 agonist LPS, was added to purified BDCA-3 cDC cultures for 18 hours. As shown in Figure 8B and consistent with the lack of TLR4 transcripts in BDCA-3 cDCs, TLR4 triggering did not promote the up-regulation of ILT3 and ILT4. These data suggests that the inductive signal driving the development of these populations, designated by the expression of ILT3 and ILT4 on BDCA-3 cDCs is not due to the *in-vitro* culturing conditions but rather specific to TLR3-mediated signaling.

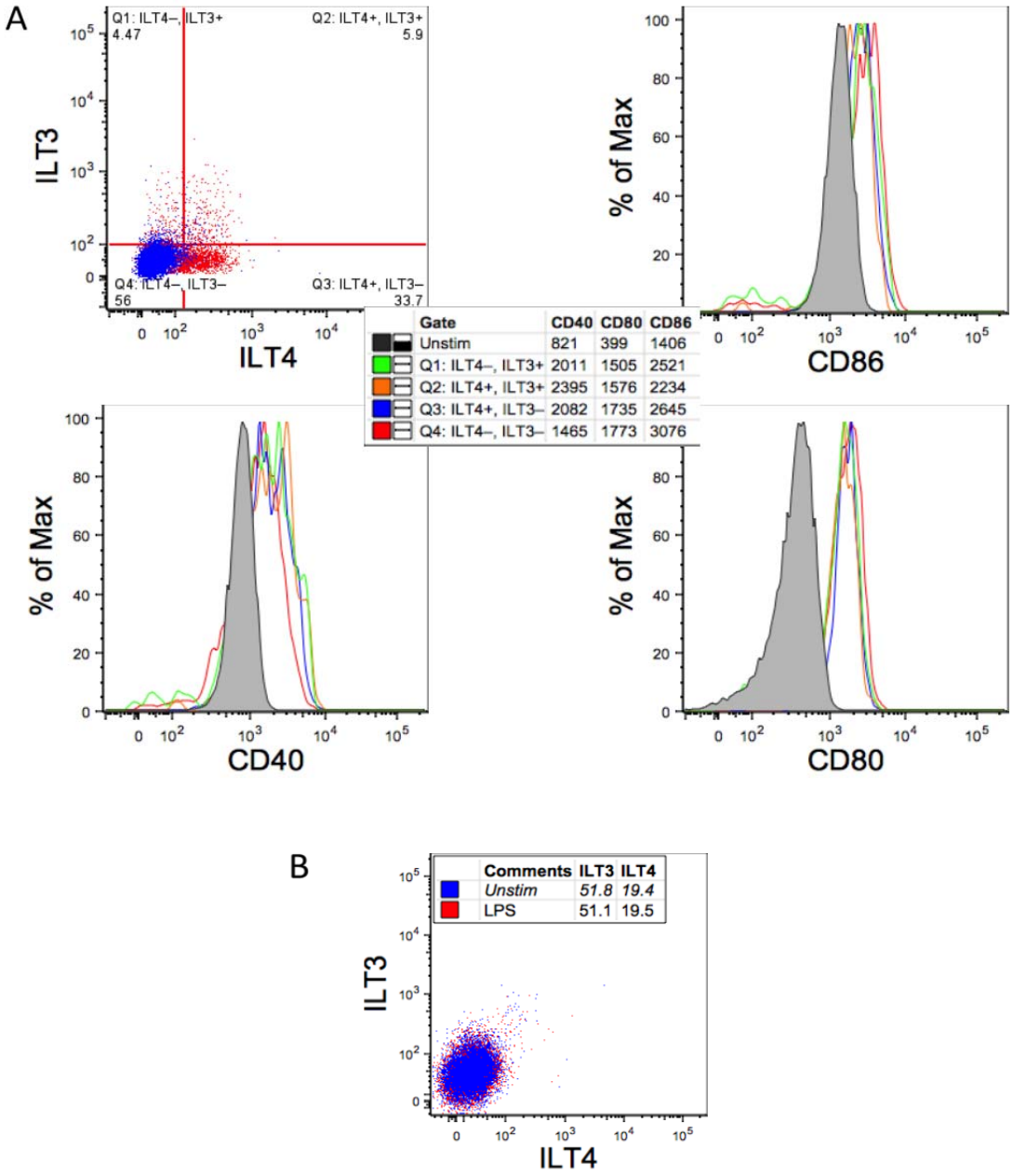


Figure 8: Poly I:C stimulated BDCA-3 DC ILT3 and ILT4 populations are similar in their expression of activation markers CD40, CD80, and CD86. A) BDCA-3 DCs were stimulated with 10µg/mL Poly I:C or B) 3µg/mL LPS for 18 hours at 37°C. ILT3 and ILT4 population's CD40, CD80, and CD86 expression was compared by MFI. Plots representative of one donor of 4 (Colletti et al., 2016).

vi. ILT4$^+$ and ILT4$^-$ BDCA-3 DCs have unique cytokine profiles:

Following stimulation of BDCA-3 DCs with Poly I:C, the emergence of several populations designated by their expression of ILT3 and ILT4 receptors (ILT3$^-$ ILT4$^-$, ILT3$^+$ ILT4$^-$, ILT3$^+$ ILT4$^+$, ILT3$^-$ ILT4$^+$), which have suggested inhibitory effects, prompted us to investigate whether these populations of the TLR3-induced DCs also exhibit differential cytokine production. Due to limited cell numbers in the ILT3$^+$ILT4$^+$ population, we focused our initial investigative efforts on the cytokine secretion profile of the ILT4-expressing DCs (Fig. 9). To determine the level of cytokine secretion amongst the ILT4$^-$ vs ILT4$^+$ populations, bulk-sorted BDCA-3 DCs were stimulated with Poly I:C for the induction of ILT4$^{+/-}$ cells. After 18 hours, ILT4$^-$ and ILT4$^+$ populations were FACs-sorted and re-cultured overnight in the absence of further TLR stimulation. As demonstrated in Figure 10, multiplex cytokine analysis of the cultured supernatant revealed quantitative and qualitative differences in the cytokine secretion potential between the ILT4$^+$ and ILT4$^-$ populations. ILT4$^-$ cells are unique in their capacity to produce IFN-ɣ and IP-10, while ILT4$^+$ cells are poised for TNF-α, IL-12p70, and IL-6 production. To confirm the unique cytokine-secreting profiles between ILT4$^+$ and ILT4$^-$ cells, we performed intracellular cytokine staining (ICS) by stimulating BDCA-3 DCs for 18 hours with Poly I:C and assessed cytokine secretion by each population (Fig. 11). Consistent with the cytokine analysis, ICS analysis reveal that ILT4$^-$ BDCA-3 DCs are capable of producing IFN-ɣ and low levels of TNF-α, conversely, ILT4$^+$ BDCA-3 DCs produced exclusively high levels of TNF-α and undetectable levels of IFN-ɣ (Fig. 11).

Taken together, the data thus far suggested that ILT4^{-} and ILT4^{+} BDCA-3 DCs are phenotypically and functionally unique.

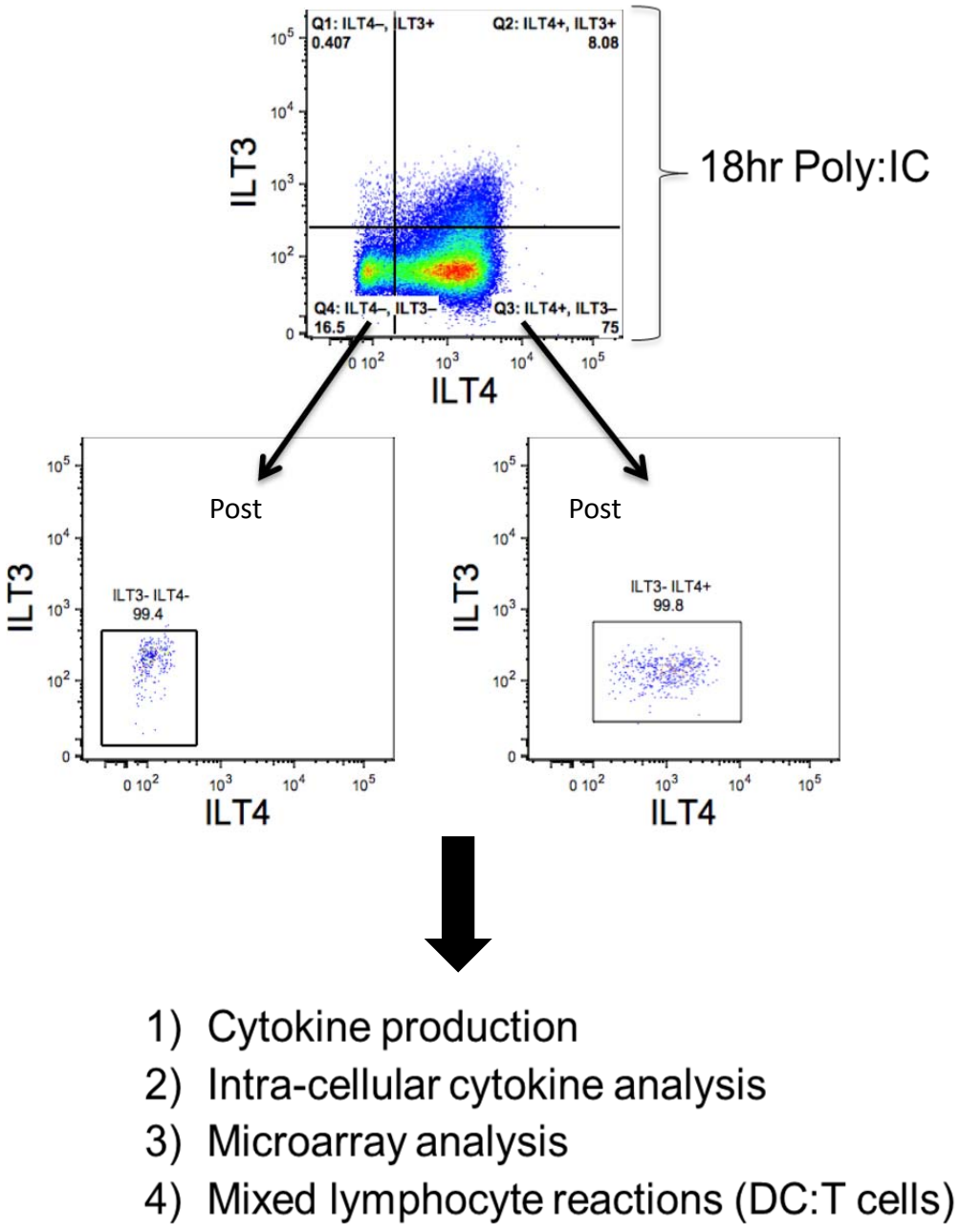


Figure 9: Experimental design of BDCA-3 DC phenotyping.
Purified BDCA-3 DCs were stimulated with 10µg/mL Poly I:C for 18 hours at 37°C. DCs were then stained with ILT3 and ILT4 and sorted. Purified cells were phenotyped by various cellular and molecular assays. Data were representative of one donor (Colletti et al., 2016).

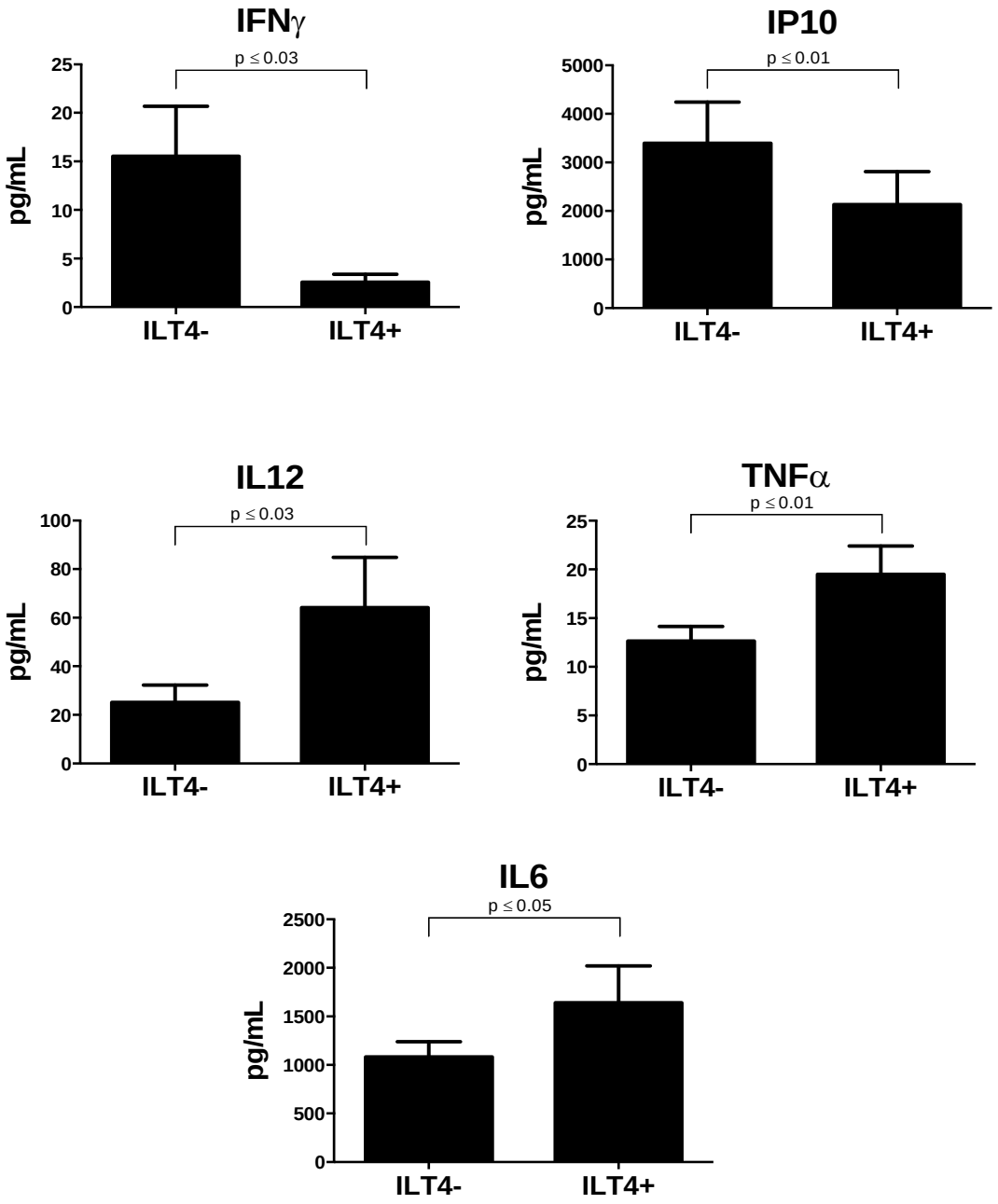


Figure 10: Multiplex cytokine analysis of ILT4^{-} vs ILT4^{+} BDCA-3 DCs.
BDCA-3 cDCs were cultured with Poly I:C for 18 h and then sorted into ILT4^{-} and ILT4^{+} populations. Cells were then plated without further stimulation for 18 hours. Supernatants were assayed for cytokine and chemokine content by luminex analysis. P-values generated using two-tailed student's paired t-test (95% confidence interval). Graphs represent four single donors. Error bars represented SEM (Colletti et al., 2016).

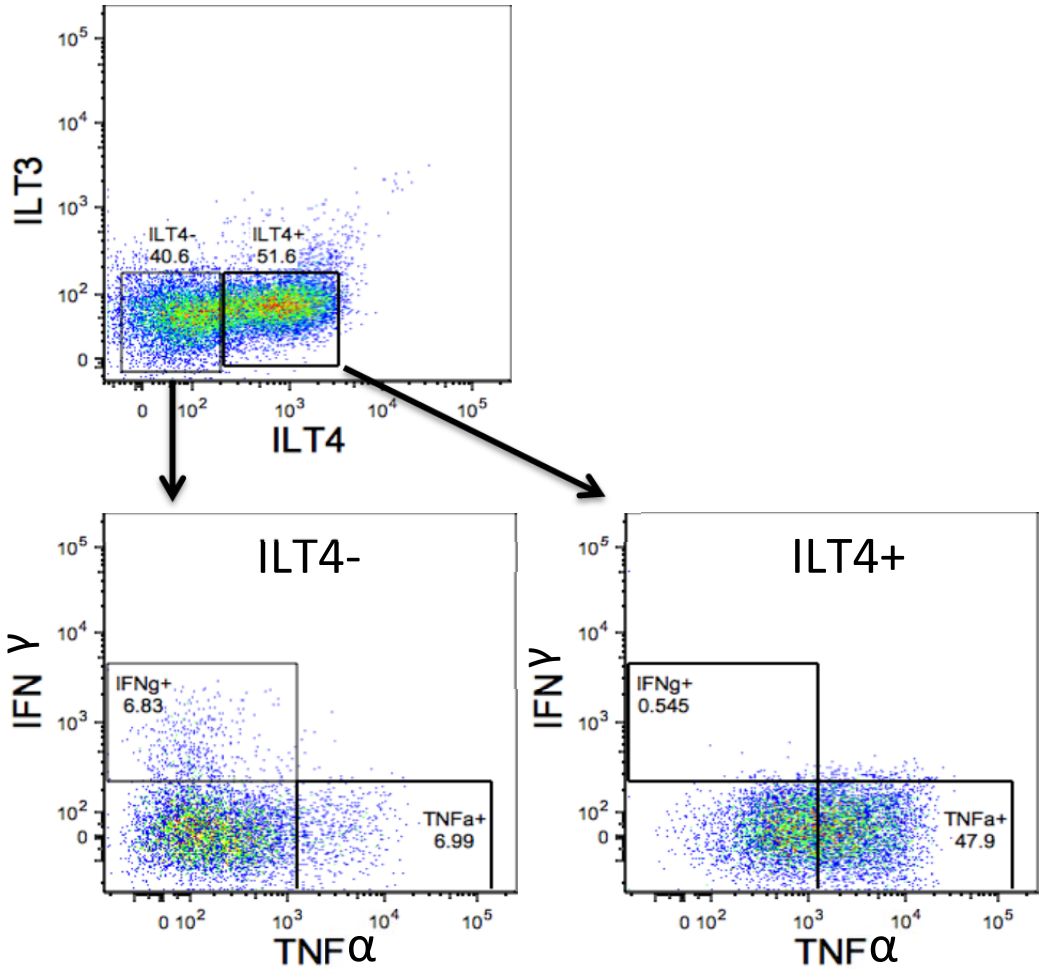
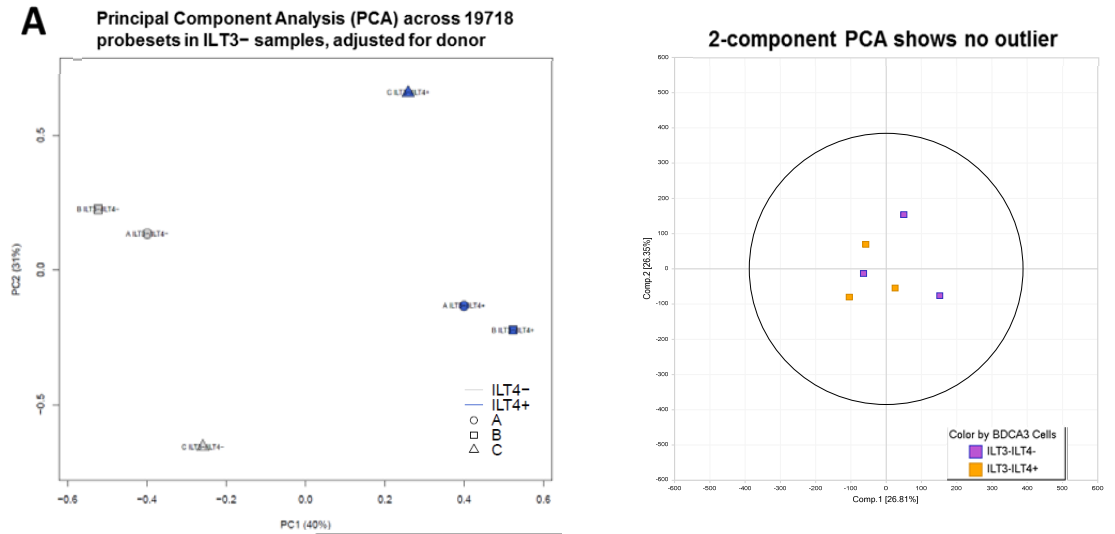


Figure 11: Intracellular cytokine analysis of ILT4$^-$ vs ILT4$^+$ BDCA-3 DCs. BDCA-3 cDCs were stimulated with Poly I:C for 18 h, Golgistop was then added for 6 hours. Cells were surface stained with ILT3, ILT4, and CD141 and then intracellularly stained with IFN-γ and TNF-α. Data showing intracellular staining were representative of one donor out of four (Colletti et al., 2016).

vii. ILT4$^+$ and ILT4$^-$ BDCA-3 DCs have unique genomic signatures:

To better understand whether the ILT4$^+$ and ILT4$^-$ populations represent DC populations with unique characteristics, transcriptional profiling was performed on ILT4$^+$ vs ILT4$^-$ BDCA-3 DCs after Poly I:C stimulation. General linear models for microarray data were performed for probe sets present on the microarray to identify probe sets that are differentially expressed between the groups, based on moderated t-statistics. Probe sets with a 1.5-fold change and a P value less than 0.05 were considered significant. Although our analysis revealed unique gene signatures for the ILT4$^+$ vs ILT4$^-$ populations following stimulation, we were not able to identify unique surface markers to faithfully distinguish between the two populations. A 3D plot generated by principle component analysis (PCA) with OmicSoft ArrayStudio across all probe sets revealed that ILT4$^+$ cells are most dissimilar from ILT4$^-$ cells (Fig. 12A). The ILT4$^-$ population revealed up-regulated genes involved in T cell stimulation, in particular IFN-ɣ, which was consistent with multiplex cytokine and ICS analysis (Fig. 10-11). Finally, the ILT4$^+$ population revealed the up-regulation of two inhibitory receptors, ILT4 and ILT6. The ILT4 expression of the ILT4$^+$ population was confirmed by real-time PCR analysis (Fig. 12B). The microarray analysis of the ILT4$^+$ population showed up-regulation of the TNF-α gene, these results were all consistent with both multiplex cytokine analysis and FACS intracellular staining (Fig. 10-11). Collectively, the functional and genomic analysis of ILT4$^+$ and ILT4$^-$ cells suggested that these cells are distinct DC populations found within the broader BDCA-3 DC subset.



Upregulated Genes in ILT4+	Gene Symbol	Fold Change
HLA Class I antigen receptor (ILT6)	LILRA3	11.0
Inhibitory receptor (ILT4)	LILRB2	2.9
T cell activator	IL12B	2.4
Leukocyte activator	IL1B	2.3
Myeloid Triggering Receptor	TREM1	1.9
Acute Inflammation (MIP1α)	CCL3	1.6
Tumor Necrosis Factor	TNF	1.5
Interleukin 10	IL10	-1

Upregulated Genes in ILT4-	Gene Symbol	Fold Change
Type II Interferon	IFNG	14.2
Immune Activation	CD48	2.8
Enhances T cell activation	CD84	2.3
HLA Class II Gamma Chain	CD74	1.4

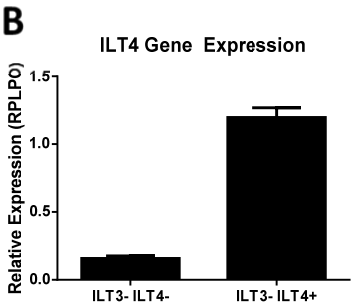


Figure 12: Microarray analysis of ILT4^{+} vs ILT4^{-} BDCA-3 DCs.
Genomic profiling of ILT4^{-} vs. ILT4^{+} was performed using GeneChip Human Gene 1.0 ST arrays. Principal component analysis (PCA) was computed using OmicSoft ArrayStudio, and a plot was generated to show the relative clustering of ILT4^{-} and ILT4^{+}. ILT4^{-} and ILT4^{+} populations were compared to each other by t-test with a threshold set for a fold change >1.5 and a P-value <0.05. ILT4 gene expression was confirmed by qPCR. (Data shown are one representative donor out of four, error bars represented SEM) (Colletti et al., 2016).

viii. BDCA-3 DCs prime naïve CD4 T cells towards a Th1 phenotype:

To characterize the ability for BDCA-3 DCs to prime naïve T cells, we compared their priming potential with pDCs (BDCA-2 DCs). To this end, BDCA-2 and BDCA-3 DCs were stimulated with CpG2216, a TLR9 agonist, and Poly I:C, respectively. Following stimulation, both activated DC subsets were co-cultured with allogenic sorted naïve $CD4^+$ T cells at a DC to T cell ratio of 1 to 5. On day 7 post priming, the phenotype of the resultant $CD4^+$ T cells was assessed by flow-cytometry for the secretion of prototypic Th1- and Th2-associated cytokines. Interestingly, both DC subsets primed naïve CD4 T cells towards different Th phenotypes. pDCs stimulated with a TLR9 agonist resulted in the priming of T cells towards a Th2 phenotype, mainly secreting IL-4 (6%) and IL-10 (33%). BDCA-3 DCs stimulated with Poly I:C resulted in the priming of T cells towards a Th1 phenotype, secreting mostly IFN-ɣ (75%) (Fig. 13). To further investigate the ability for BDCA-3 DCs to prime naïve T cells, we decided to compare the priming capabilities of $ILT4^-$ vs $ILT4^+$ BDCA-3-DCs following Poly I:C stimulation.

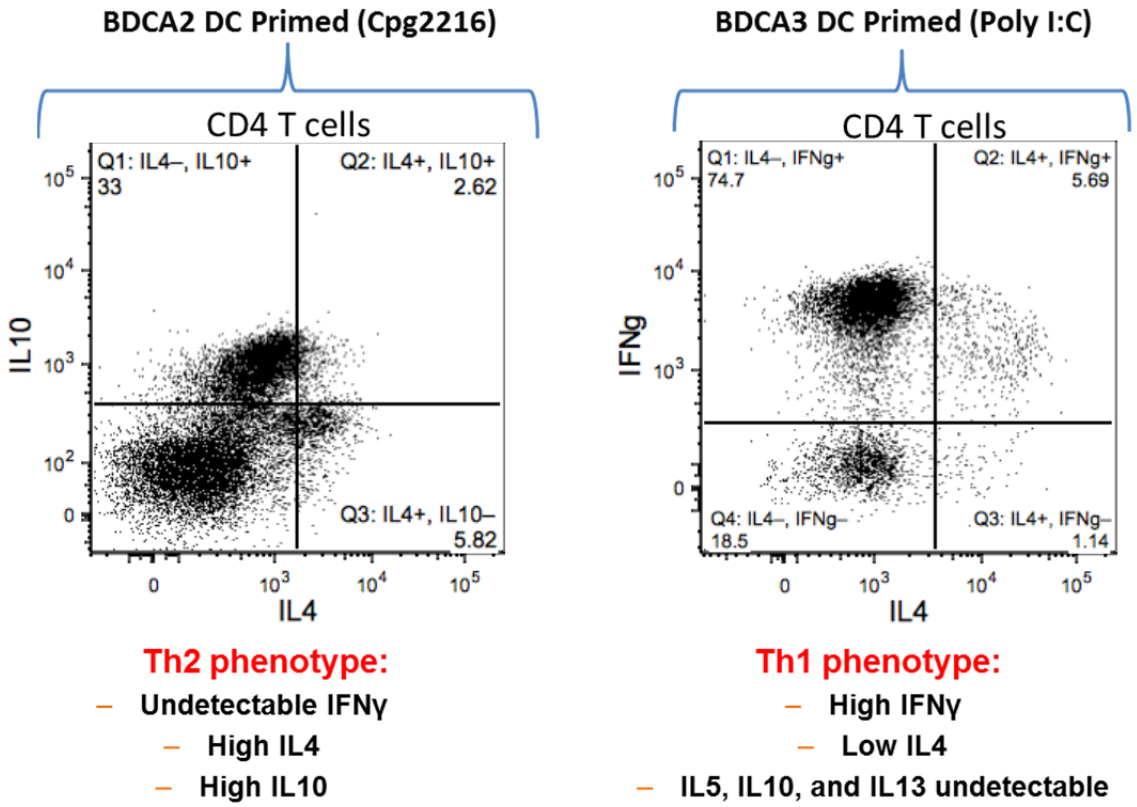


Figure 13: BDCA-2 and BDCA-3 DC priming of naïve CD4 T cell outcomes. Bulk BDCA-2 and BDCA-3 DCs were incubated at 37°C for 18 hours with 3µg/mL CpG2216 or 10µg/mL Poly I:C respectively. After TLR stimulation, BDCA-3 DCs were sorted into pure populations of $ILT4^+$ and $ILT4^-$. BDCA-2 DCs were washed of CpG2216 prior to the addition of allogenic T cells. Allogenic naïve $CD4^+$ and $CD8^+$ T cells were incubated with allogenic BDCA-2 and BDCA-3 DCs ($ILT4^+$ and $ILT4^-$) at a 1:5 DC to T cell ratio at 37°C in complete X-VIVO 15 media. After 7 days, primed T cells were harvested and analyzed for intracellular cytokine staining. Data represented one donor out of three.

ix. ILT4$^-$ and ILT4$^+$ BDCA-3 DCs differ in their ability to prime allogenic naïve T cells:

The differences in cytokine secretion and transcriptional profiles of ILT4$^-$ and ILT4$^+$ BDCA-3 DCs implied that these DC populations may have distinct T cell priming potential. To address this possibility we assessed the ability of each ILT4 population to prime naïve CD4$^+$ and CD8$^+$ T cells. To this end, BDCA-3 DCs were first stimulated with Poly I:C and subsequently sorted to high purity into ILT4$^-$ and ILT4$^+$ populations. The sorted ILT4 populations were then co-cultured with allogenic sorted naïve CD4$^+$ and CD8$^+$ T cells at a DC to T cell ratio of 1 to 5. T cells were then stained with Cell Trace prior to the mixed lymphocyte reaction (MLR) to assess the level of their proliferation. Proliferation with Cell Trace is detected by a dilution of the incorporated dye within the cell membrane following subsequent cellular divisions. On day 7 post priming, the phenotype of the resultant CD4$^+$ and CD8$^+$ T cells was assessed by flow-cytometry for the secretion of prototypic Th1- and Th2-associated cytokines, such as IFN-ɣ and IL-4, respectively. Both ILT4$^+$ and ILT4$^-$ DCs demonstrated the capacity to prime both naïve CD4$^+$ and CD8$^+$ T cells as demonstrated by the dilution of Cell Trace dye (Figs. 14-15). More importantly, both DC populations preferentially primed naïve CD4$^+$ and CD8$^+$ T cells towards IFN-ɣ Th1 cells (Fig. 14-15). IL-4 producing cells were also observed, but at a much lower frequency than IFN-ɣ producing cells. Despite the ability to prime naïve CD4$^+$ and CD8$^+$ T cells towards a Th1 phenotype, ILT4$^+$ DCs appeared to be less efficient at promoting Th1 induction as compared to ILT4$^-$ DCs (Figs. 14-15). CD4$^+$ T

cell activation remained largely intact, while the priming of $CD8^+$ T cells was impaired by as much as 53% when co-cultured with $ILT4^+$ DCs (Figs. 14-15).

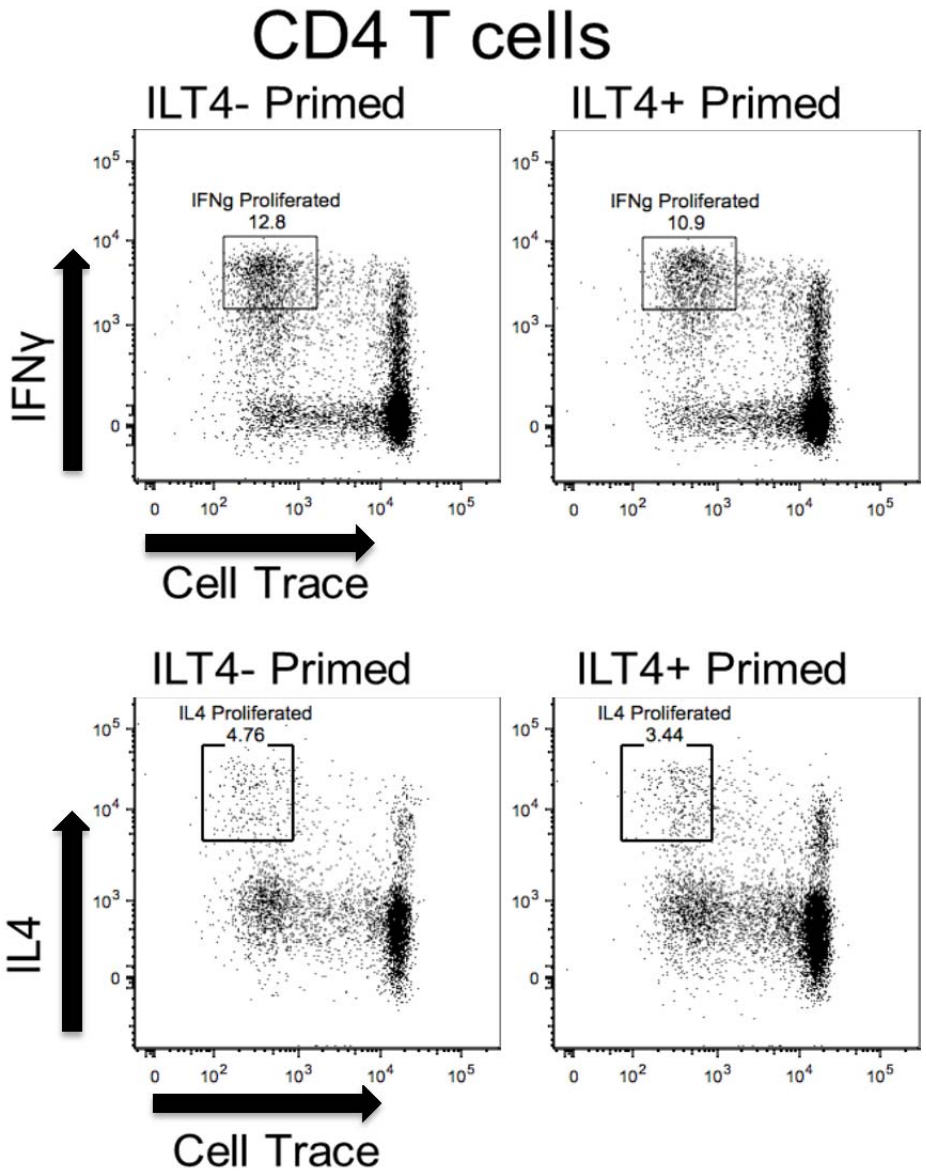


Figure 14: ILT4^{-} and ILT4^{+} BDCA-3 DC mixed lymphocyte reactions (MLRs). BDCA-3 cDCs were cultured with Poly I:C for 18 h. Cells were then sorted into ILT4^{-} and ILT4^{+} populations and incubated with naïve CD4 allogenic T cells (CD25^{-}, CD127^{+}, CD62L^{+}, CD49d^{low}). After 7 days, the resultant T cells were assessed for surface marker and intracellular cytokine expression. Dot plots are gated on live CD3^{+} and CD4^{+} T cells. Data shown were one representative donor out of four (Colletti et al., 2016).

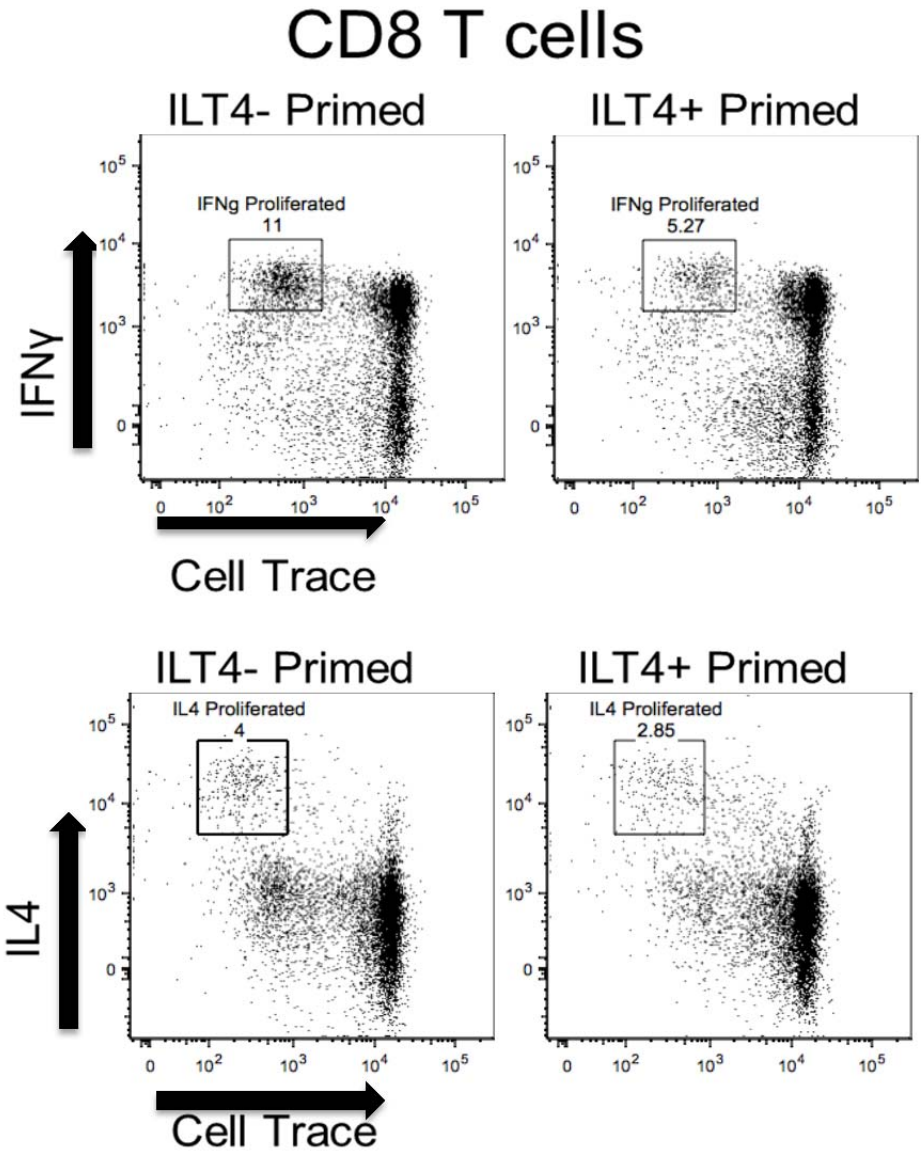


Figure 15: ILT4^{-} and ILT4^{+} BDCA-3 DC mixed lymphocyte reactions (MLRs). BDCA-3 cDCs were cultured with Poly I:C for 18 h. Cells were then sorted into ILT4^{-} and ILT4^{+} populations and incubated with naïve CD8 allogenic T cells (CD25^{-}, CD127^{+}, CD62L^{+}, CD49d^{low}). After 7 days, the resultant T cells were assessed for surface marker and intracellular cytokine expression. Dot plots are gated on live CD3^{+} and CD8^{+} T cells. Data shown were one representative donor out of four (Colletti et al., 2016).

x. ILT4$^+$ BDCA-3 DCs impaired T cell priming is IL-10 independent:

To examine the ILT4$^+$ DC impaired T cell priming, we examined the expression of HLA-G, a known ligand for the ILT4 receptor (Ristich et al., 2005). Cell surface expression of HLA-G has been implicated in the induction of tolerogenic functions in various physiological and pathological settings (Jurisicova et al., 1996, Rouas-Freiss et al., 1997, Paul et al., 1998, Braud et al., 1999). Assessment of the T cell expression of HLA-G after priming with ILT4$^+$ and ILT4$^-$ DCs revealed that CD8$^+$ T cells primed in the presence of ILT4$^+$ DCs showed an approximately 9 fold increase in HLA-G expression as compared to CD8$^+$ T cells co-cultured with ILT4$^-$ DCs (Fig. 16A). On the other hand, there was no observable induction of HLA-G expression on CD4$^+$ T cells co-cultured with either ILT4$^+$ or ILT4$^-$ DCs. The observed impaired T cell priming ability of ILT4$^+$ DCs could be the result of active inhibition by suppressor CD8$^+$ T cells expressing HLA-G. To this end, we investigated the level of IL-10, a known anti-inflammatory cytokine secreted by various cell types, including T regulatory cells (O'Garra et al., 2004), in our DC primed T cell cultures. As shown in Figure 16B, we found similar levels of T cell-derived IL-10 in both ILT4-positive and –negative DCs primed T cell cultures. Taken together this data suggests a potential IL-10-independent mechanism of dampening DC priming capabilities.

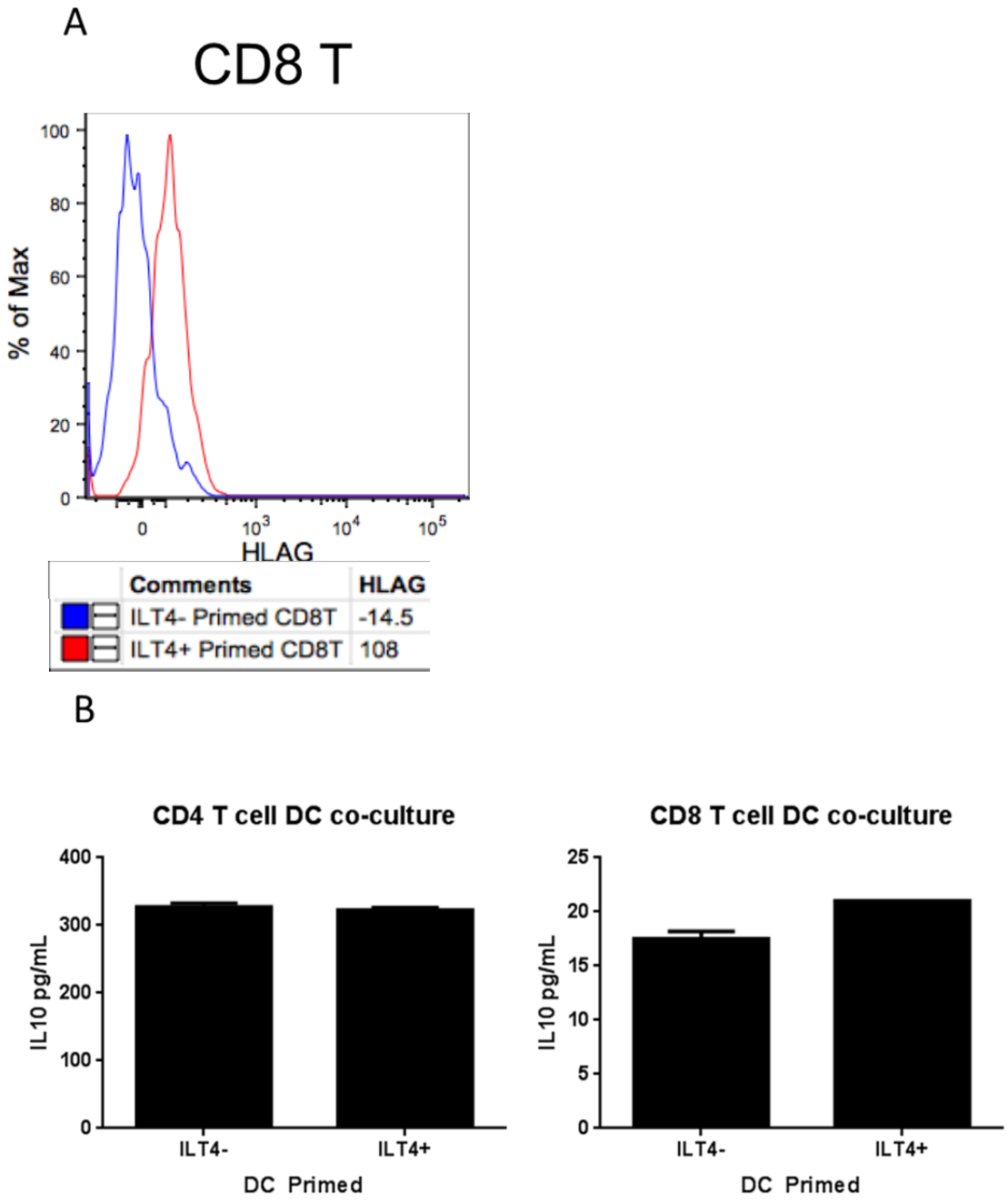


Figure 16: ILT4$^-$ and ILT4$^+$ BDCA-3 DC MLR CD8 T cell HLAG expression and IL-10 supernatant levels.
A) MLR CD8 T cells were stained with HLAG after 7 days of ILT4$^-$ or ILT4$^+$ BDCA-3 DC priming. B) CD4 and CD8 T cell MLR supernatants were harvested after 7 days and assessed for the level of IL-10. Error bars show SEM. (Colletti et al., 2016)

Discussion:

Prior to profiling human blood DC stimulation with TLR agonists, we characterized their TLR expression pattern. Genomic transcript analysis of human blood DC TLR expression revealed that different DC subsets expressed different levels of TLRs (Fig. 2). BDCA-1 DCs expressed a wide range of TLRs while BDCA-2 expression was restricted to mainly TLR 7 and 9. BDCA-3 DCs mainly expressed TLR3 which is consistent with previous studies (Hornung et al., 2002). We also observed that BDCA-3 DCs expressed high levels of TLR10, which suggests functional TLR10 protein. The role that TLR10 plays in the immune response as well as the ligand remains to be elucidated. Transcript expression of various TLR receptors and responsiveness to multiple TLR agonists suggests two hypotheses (A) on per cell basis BDCA-3 DCs express multiple TLRs or (B) there may exist various BDCA-3 DC subsets each with a unique TLR expression. The recent discovery of a human $XCR1^{+}CD141^{+}$ DC subset expressing TLR3 within the conventional DC population supports the latter hypothesis (Bachem et al., 2010). This discovery raises the possibility that multiple yet-to-be-identified populations of BDCA-3 DCs may exist.

Currently, human blood DCs can be identified and purified based on various surface expression markers. BDCA-1 DCs can be isolated by the absence of lineage marker expression (Lin^{-}), $HLADR^{+}$, $CD1c^{+}$, and $CD11c^{+}$. BDCA-2 DCs identified by Lin^{-}, $HLADR^{+}$, and $CD123^{+}$. BDCA-3 DCs identified by Lin^{-}, $HLADR^{+}$, $CD1c^{-}$, and $CD141^{+}$. In the present study, we demonstrated that stimulation of BDCA-1, 2, and 3

with various TLR agonists induced the expression of canonical markers associated with DC activation/maturation, such as CD40, CD80, and CD86 (Fig. 3). This activation with TLR agonists correlated with the TLR transcript profile of each DC subset, for example, BDCA-2 DCs showed high levels of TLR 7 and 9 gene expression and upon stimulation with CpG2216 (TLR9 agonist) showed a 10 fold increase in CD40 and a 3 fold increase in CD86 (Figs. 2-3). Poly I:C stimulation of BDCA-3 DCs induced the expression of CD40, CD80, CD86, ILT3, and ILT4 as detected by flow cytometry (Fig. 4A). The induction of BDCA-3 activation markers occurred in a dose dependent manner and was specific for each activation marker. CD80 achieved a 2 fold increase in MFI at concentrations as low as 0.12ug/mL of Poly I:C, while CD40 required approximately 3.18 μg/ml Poly I:C (Fig. 5). Multiplex cytokine analysis revealed that BDCA-3 DCs secrete cytokines at higher concentrations of Poly I:C than is required for the up-regulation of activation markers (Fig. 5-6). Measurable levels of IFN-ɣ and TNF-α were detectable after stimulation of BDCA-3 DCs with 0.1μg/mL Poly I:C (Fig. 6) whereas CD86 up-regulation occurs after stimulation with 0.01μg/mL Poly I:C (Fig. 5).

Unlike the activation markers CD40, CD80, and CD86, the expression pattern of the ILT receptors allowed for the distinction of various populations, namely $ILT3^{-}$ $ILT4^{-}$, $ILT3^{+}$ $ILT4^{-}$, $ILT3^{-}$ $ILT4^{+}$, and $ILT3^{+}$ $ILT4^{+}$, within the total BDCA-3 DC population. The various ILT populations observed after Poly I:C stimulation of BDCA-3 DCs is unique to this particular blood DC subset. Stimulation of BDCA-2 DCs with CpG2216, a TLR9 agonist, resulted in the uniform up-regulation of ILT3 and ILT4 (Fig. 4B). ILT3 and ILT4 are surface proteins of the immunoglobulin superfamily, which have been

demonstrated to be expressed on monocytes and DCs. The cytoplasmic region of ILT molecules contains a putative immunoreceptor tyrosine-based inhibitory motif, suggesting an inhibitory function of ILT receptors. Consistent with the proposed inhibitory function, ILT3 has been shown to induce immunosuppression, including T cell anergy, regulatory T cell (Treg) induction, and reduced allo-stimulatory capacity (Chang et al., 2002, Manavalan et al., 2003). We further investigated the expression of activation markers within each ILT sub-population as well as the time required for receptor up-regulation. CD40, CD80, and CD86 are rapidly up-regulated and detectable after just 2 hours of stimulation with 10μg/mL Poly I:C. The inhibitory receptor ILT4 was further delayed in its up-regulation and was measurable by flow cytometry after 5 hours of stimulation with 10μg/mL Poly I:C (Fig. 7), ILT3 detectable only after 8 hours of stimulation. This delay in inhibitory receptor expression may contribute to an immune dampening process after a rapidly occurring immune response. Each described ILT population shows similar expression of CD40, CD80, and CD86, this lead us to hypothesize the potential for functional differences among these various ILT expressing DC populations (Fig. 8).

Previous studies have generated BDCA-3 DCs from monocytes in the presence of various growth factors and cytokines. In the presence of IL-10, these DCs, termed DC-IL10, expressed high levels of ILT receptors, namely ILT2 and ILT3 (Velten et al., 2004). These *in vitro* derived DCs retained immunosuppressive functions, including IL10 production and the ability to generate $CD4^+$ Tregs (Velten et al., 2004). BDCA-3 DCs have also been found outside of the blood. $ILT3^+$ expressing BDCA-3 DCs have

been identified in the dermis of the human skin. These dermal DCs were shown to produce IL-10, induce T cell hyporesponsiveness, and were able to inhibit skin inflammation by inducing Tregs (Chu et al., 2012). In addition to ILT3 expression on BDCA-3 DCs, ILT4 was detected by gene array analysis following Poly I:C stimulation (Balan et al., 2014). The various ILT populations we detected after Poly I:C stimulation on primary BDCA-3 DCs occur in varying cell frequencies. $ILT4^{-}ILT3^{+}$ and $ILT4^{+}ILT3^{+}$ cell numbers post Poly I:C stimulation cell were consistently limited so we focused our studies on profiling $ILT4^{+/-}$ $ILT3^{-}$ BDCA-3 DCs. We first sorted $ILT4^{-}$ and $ILT4^{+}$ BDCA-3 DCs after Poly I:C stimulation and compared their transcriptional profiles by microarray. Complementing the detection of ILT4 by flow cytometry, the expression of ILT4 transcript was increased in the $ILT4^{+}$ BDCA-3 DC microarray samples. To further validate this ILT4 expression, we performed Taqman analysis on $ILT4^{+}$ BDCA-3 DCs after sorting. $ILT4^{+}$ DCs expression of ILT4 was 5 fold higher than the $ILT4^{-}$ isolated DCs (Fig. 12B). ILT6 expression was also highest in the $ILT4^{+}$ expressing DCs (Fig. 12A). ILT6, unlike other ILT members, lacks a transmembrane domain and is a soluble receptor (Torkar et al., 2000). As immune modulators, the heightened expression of ILT4 and ILT6 may work in synergy to modulate T cell priming in $ILT4^{+}$ DC MLR reactions (Fig. 14-15). ILT6 has been implicated as both an immune-stimulatory or anti-inflammatory protein with a reduced affinity for HLA-G as compared to ILT4 (An et al., 2010, Ryu et al., 2011, Low et al., 2013). ILT6 may compete with ILT4 to maintain an ongoing immune response in the presence of HLA-G. The $ILT4^{+}$ BDCA-3 DCs also expressed higher levels of transcripts for interleukin-12 beta-subunit (IL-12b/IL-12p40)

and tumor necrosis factor α (TNF-α) (Fig. 12A). IL-12b, a shared subunit for both bioactive IL-12p70 and IL-23, may promote the generation of either T-helper 1 (Th1) and/or Th17 cells. IL-12p70 was also greater in expression within the $ILT4^+$ BDCA-3 DCs. TNF-α is a well-studied pro-inflammatory cytokine. TNF-α is known for its ability to induce systemic inflammation and the acute phase reaction. TNF-α regulates the expansion and survival of $CD4^+$ and $CD8^+$ T cells and has been implicated in the progression of various diseases (Beutler et al., 1985, Locksley et al., 2001, Gaur and Aggarwal, 2003). A migratory chemokine, CCL3, was also produced exclusively by the $ILT4^+$ BDCA-3 DCs (Fig. 16A). CCL3 has been shown to enhance the differentiation, migration, and effector functions of $CD8^+$ T cells (Trifilo et al., 2003). Based upon the $ILT4^+$ BDCA-3 DC cytokine profile detected by microarray analysis, they may function to provide a link between innate and adaptive immunity. On the contrary, the $ILT4^-$ BDCA-3 DCs showed mostly type II interferon (IFN-ɣ) (Fig. 12A). The microarray genomic expression of IFN-ɣ was further confirmed at the protein level by both intracellular FCS and multiplex cytokine analysis (Fig. 10-11). IFN-ɣ plays several roles in both the innate and adaptive immune response. Studies show that it is involved in anti-viral, -bacterial, and -tumor biology (Ikeda et al., 2002, Perry et al., 2005, Hermant and Michiels, 2014). IP-10 levels were also elevated in the supernatant of cultured $ILT4^-$ BDCA-3 DCs. IP-10 secretion is intricately linked to the output levels of IFN-ɣ production. As a migratory chemokine, IP-10 is known to induce chemotaxis of numerous leukocytes (Taub et al., 1993, Jinquan et al., 2000). Along with cytokines, the $ILT4^-$ BDCA-3 DCs also express various surface marker immune activators including

CD48, CD84, and CD74 (Fig. 12A). CD84 is expressed mainly on monocytes, macrophages, granulocytes, and DCs and is involved in leukocyte activation (Sintes et al., 2010). CD74 regulates the class II major histocompatibility complex (MHC) proteins in APCs. The induction of such activation markers may partially contribute to their ability to more efficiently prime naïve $CD4^+$ T cells (Fig. 14) (Beswick and Reyes, 2009). The discovery of various populations of $BDCA3^+$ DCs after stimulation with a TLR3 agonist lead us to hypothesize that these populations could be delineated by a newly discovered subset expressing XCR1 (Bachem et al., 2010). $XCR1^+$ $BDCA3^+$ DCs have been shown to have a high capacity for cross-presentation and have the ability to both acquire and present necrotic antigens (Bachem et al., 2010, Balan et al., 2014). Our microarray analysis did not reveal any differences in the expression of XCR1 within the $ILT4^-$ and $ILT4^+$ populations, suggesting that their expression is independent of the expression of XCR1. Through genomic profiling of these two different ILT expressing DCs, we have shown that they express different immunological surface markers as well as have unique cytokine transcript profiles.

DCs have the ability to prime naïve T cells towards various T-helper cell phenotypes. The outcome of T cell priming is influenced by many factors. The DC subset as well as the maturation status of the DC during the priming event can lead to different T helper outcomes (Langenkamp et al., 2000, Kushwah and Hu, 2011). We have shown that BDCA-2 DCs stimulated with a TLR9 agonist results in the priming of naïve CD4 T cells towards a Th2 phenotype, while BDCA-3 DCs simulated with a TLR3 agonist primes naive CD4 T cells towards a Th1 profile (Fig. 13). DCs of the same

lineage may also differ in their priming capacity based upon their physical location. Studies have shown that skin BDCA-3 DCs secrete high levels of IL-10 and induce T regulatory cells (Chu et al., 2012). High ILT3 expression on BDCA-3 DCs has been previously shown to be involved in the impairment of allo-stimulatory priming of naïve T cells (Velten et al., 2004). We have shown in our study that BDCA-3 DCs expressing high levels of ILT4 also have a reduced allo-stimulatory capacity that is more prominent for $CD8^+$ T cells (Fig. 15). The difference in the capacity of the ILT expressing BDCA-3 DCs to prime T cells might be linked to the activity of the ILT4 receptor. A natural ligand of the ILT4 receptor is a non-classical class I heavy chain MHC molecule, HLA-G (Colonna et al., 1998). HLA-G has been previously shown to inhibit *in vitro* allogenic MLRs and can be expressed on T cells (Riteau et al., 1999, Le Friec et al., 2003). We tested for the expression of HLA-G on primed T cells after the MLR with BDCA-3 DCs. $ILT4^+$ DCs that primed $CD8^+$ T cells skewed their polarization towards a Th1 phenotype and induced the expression of HLA-G (Fig. 20). HLA-G has been associated with the induction of IL-10 producing T suppressor cells (Carosella et al., 2011, Raker et al., 2015). We tested the levels of IL-10 in the resulting supernatants of our allogenic MLRs and did not detect any differences between the cultures containing $ILT4^-$ or $ILT4^+$ DCs (Fig. 16B), suggesting an IL-10 independent means of attenuation. These results are consistent with a previous study indicating that $HLAG^+$ T cells present within whole PBMCs do not mediate suppression through IL-10 secretion (Feger et al., 2007). The induction of HLA-G on primed T cells with a distinct DC population may suggest an

immuno-suppressive mechanism that could potentially prevent the overstimulation of the adaptive branch of the response.

In the present study, we have shown that a purified population of BDCA-3 DCs upon stimulation with Poly I:C, rapidly up-regulates canonical activation markers CD40, CD80, and CD86 which leads to the secretion of various cytokines. This up-regulation of activation markers occurs in both a dose and time dependent manner. Stimulating purified BDCA-3 DCs with 10μg/mL Poly I:C for 18 hours resulted in the expression of inhibitory receptors ILT3 and ILT4. The increased expression of ILT3 and ILT4 was not uniform as was detected with various activation markers and does not correlate to the recently discovered expression of XCR1. ILT4 expressing BDCA-3 DCs have a unique cytokine secreting and genomic profile as compared to ILT4 negative BDCA-3 DCs. Allogenic CD8 T cell priming is attenuated upon co-culture with $ILT4^+$ BDCA-3 DCs as compared to $ILT4^-$ BDCA-3 DCs. CD8 T cells primed by $ILT4^+$ BDCA-3 DCs showed expression of HLA-G which could account for the impaired priming during the MLR. Taken together, these populations of BDCA-3 DCs may work to mediate various aspects of an immunological response.

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