Identification of human peripheral blood monocyte derived pro-inflammatory dendritic cells

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I List of abbreviations

aa	amino acids
APC	Antigen Presenting Cell
BDCA	Blood Dendritic Cell Antigen
BM	Bone Marrow
CBA	Cytokine Bead Array
CCL	Chemokine (CC) motif Ligand
CCR	Chemokine (CC) Receptor
CD	Cluster of Differentiation
cDC	conventional Dendritic Cell
CDP	Common Dendritic Cell Precursors
CLEC	C-type Lectin
CpG	DNA-oligonucleotide containing unmethylated CpG motifs
cor	correlation coefficient
CTL	Cytotoxic T Lymphocyte
CXCR	Chemokine (CXC) motif Receptor
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-specific intracellular adhesion molecule 3-grabbing non-integrin
dH_2O	deionized water
DNA	Deoxyribonucleic Acid
DPI	Diphenyleneiodonium
dsDNA	double stranded Deoxyribonucleic Acid
dsRNA	double stranded Ribonucleid Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Flow Assisted Cytometric Cell Sorting
Fc	crystallisable Fragment
FcR	crystallisable Fragment Receptor
FSC	Forward Scatter
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GO	GeneOntology
H_2O_2	Hydrogen peroxide
HLA	Human Leukocyte Antigen
IFN	Interferon
IκB	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor
IKKe	Inhibitor of Kappa light polypeptide gene enhancer in B-cells, Kinase epsilon
IL	Interleukin
IL-1R	Interleukin-1 Receptor
IL-1RA	Interleukin-1 Receptor Antagonist
imoDC	immature monocyte derived Dendritic Cell
iNOS	inducible Nitric Oxide Synthase
IRAK	Iinterleukin-1 Receptor-Associated Kinase
IRF	Interferon Regulatory Factor
mM	millimolar
MACS	Magnetic Activated Cell Separation
mAb	monoclonal Antibody

MB	Microbeads
MCM	Monocyte-Conditioned Media
MDP	Macrophage/DC Precursor
MHC	Major Histocompatibility Complex
mmoDC	mature monocyte derived Dendritic Cell
moDC	monocyte derived Dendritic Cell
MP	Myeloid Precursor
MyD88	Myeloid Differentiation primary response gene 88
NAC	N-Acetylcysteine
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B-cells
NGS	Normal Goat Serum
NK cell	Natural Killer cell
NO	Nitric Oxygen
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
pDC	plasmacytoid Dendritic Cell
PI	Propidium Iodide
PMA	Phorbol-12-Myristat-13-Acetat
PRR	Pattern Recognition Receptor
RPMI	Roswell Park Memorial Institute medium
RA	Rheumatoid Arthritis
RNA	Ribonucleid Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Standard Error of the Mean
SSC	Side Scatter
ssRNA	single stranded Ribonucleid Acid
TAK	Transforming growth factor beta Activated Kinase
TBK1	TANK-Binding Kinase 1
TCR	T Cell Receptor
T_H cell	T Helper cell
Tip-DC	TNFα/iNOS producing Dendritic Cell
TIR	Toll/Interleukin-1 Receptor
TIRAP	Toll-Interleukin 1 Receptor domain containing Adaptor Protein
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor alpha
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
TRAF	Tumor Necrosis Factor Receptor-Associated Factor
TRIF	Toll/Interleukin-1 Receptor *domain* containing adapter inducing Interferon-β

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1 Summary

Several years ago, the definition of human blood dendritic cells (DCs) described by HLA-DR, CD4, CD11c and CD123 was extended by the discovery of blood dendritic cell antigens (BDCA) 1-4. BDCA1$^+$ (CD1c$^+$) DCs, however, were not homogenous as they contained a substantial subpopulation carrying the monocyte marker CD14. BDCA1$^+$CD14$^+$ cells showed a marked phenotypic convergence with CD14$^+$CD16$^-$ monocytes and it was unclear whether this population belonged to DCs or monocytes and what functions they had.

This work describes for the first time BDCA1$^+$CD14$^+$ pro-inflammatory DCs (pro-iDCs) present in blood under steady state conditions. Although pro-iDCs shared phenotypic characteristics of CD14$^+$CD16$^-$ monocytes, they expressed markers of activated DCs such as HLA-DR, CD40 and CD54 but showed reduced expression of CXCR4, which is involved in monocyte hemostasis. This was in line with a strong DC function of *ex vivo* pro-iDCs as observed by the induction of allogeneic T cell proliferation, which is a hallmark of DCs. Pro-iDCs secreted high amounts of pro-inflammatory and polarizing cytokines already in the steady state, which was due to an enhanced susceptibility to reactive oxygen species and resultant autocrine IL-1β stimulation. Pro-iDC derived inflammatory cytokines matured conventional DCs (cDCs) and promoted T cell proliferation. Additionally, they secreted significant amounts of IL-12 and IL-23 and were superior in priming IFNγ$^+$IL-17$^+$ T cells when compared to BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes.

BDCA1$^+$CD14$^+$ cells resembling blood pro-iDCs were present in samples from patients suffering from psoriasis, dermatomyositosis and inflamed halo nevus, while lacking in healthy donor's skin. The presence in inflamed but lack in healthy skin indicated that BDCA1$^+$CD14$^+$ cells were not tissue resident but recruited to sites of inflammation to promote immune responses. This is in line with mouse Ly6C^{hi} monocytes, orthologs of human CD14$^+$CD16$^-$ monocytes, that give rise to inflammatory DCs during inflammation. However, a detailed translation from the murine into the human system has not been succeeded, yet. In order to shed light on the developmental relationship between pro-iDCs and monocytes, pro-iDCs were compared to CD14$^+$CD16$^-$ monocytes, cDCs and *in vitro* generated moDCs on a whole genome level. Transcriptome comparison between pro-iDCs and the other subsets indicated a monocytic origin, as numbers of genes differentially expressed were much smaller between pro-iDCs and monocytes than between pro-iDCs and other DC subsets. However, functional annotation analysis of genes upregulated in pro-iDC over monocytes revealed a DC specific signature,

whereas all monocyte specific genes were significantly higher expressed in monocytes compared to pro-iDCs. In addition, genes differentially regulated between pro-iDCs and monocytes were overall regulated in the same way by blood cDCs and *in vitro* generated moDCs, indicating an ongoing development of pro-iDCs from monocytes towards DCs. This developmental concept was supported as $CD14^+CD16^-$ monocytes cultured under inflammatory conditions gained BDCA1 expression that correlated with a strong capacity to induce T cell proliferation.

In summary, this work identifies a novel subset of pro-inflammatory DCs presumably of monocytic origin in peripheral blood present under steady state conditions. Pro-iDCs secreted vast amounts of pro-inflammatory cytokines and induced highly inflammatory T_H17 cells. As pro-iDCs showed a high similarity with $BDCA1^+CD14^+$ cells found *in situ* only during inflammation and the recently described inflammatory DCs in inflamed tissues, pro-iDCs can be regarded as immediate precursors of inflammatory DCs. Thus, in respect of an obvious monocytic origin and a presumably inflammatory DC fate, pro-iDCs may constitute the first proof for moDC differentiation occurring in humans *in vivo*.

2 Zusammenfassung

Vor einigen Jahren wurde die Definition von dendritischen Zellen (DZ) des Blutes neben den Markern HLA-DR, CD4, CD11c und CD123 mit der Entdeckung der Blut dendritischen Zell Antigene (BDCA) 1-4 ergänzt. BDCA1$^+$ (CD1c$^+$) DZ hingegen waren nicht homogen, da ein wesentlicher Anteil den monozytären Marker CD14 exprimierte. BDCA1$^+$CD14$^+$ Zellen zeigten eine deutliche phänotypische Übereinstimmung mit CD14$^+$CD16$^-$ Monozyten und es war unklar, ob diese Subpopulation DZ oder Monozyten angehörten und ob sie mit individuellen Funktionen ausgestattet waren.

Diese Arbeit beschreibt zum ersten Mal die Existenz von BDCA1$^+$CD14$^+$ pro-inflammatorischen DZ (pro-iDZ) im Blut unter homöostatischen Bedingungen. Obwohl pro-iDZ phänotypische Eigenschaften von CD14$^+$CD16$^-$ Monozyten aufwiesen, exprimierten sie Marker aktivierter DZ wie HLA-DR, CD40 und CD54 und zeigten eine verringerte Expression des Markers CXCR4, der an der Homöostase von Monozyten beteiligt ist. Das stimmte mit DZ Funktionalität von *ex vivo* pro-iDZ überein: Sie induzierten eine starke allogene T Zell Proliferation, die ein Kennzeichen von DZ ist. Pro-iDZ sezernierten bereits im homöostatischen Zustand große Mengen an pro-inflammatorischen und polarisierenden Zytokinen, die einer erhöhten Suszeptibilität gegenüber reaktiven Sauerstoff Spezies und einer daraus resultierenden autokrinen IL-1β Stimulation geschuldet waren. Die pro-inflammatorischen Zytokine, die von Pro-iDZ sezerniert wurden, maturierten konventionelle DZ (kDZ) und unterstützten T Zell Proliferation. Zusätzlich sezernierten sie erhebliche Mengen an IL-12 und IL-23 und waren bei der Polarisierung von IFNγ$^+$IL-17$^+$ T Zellen BDCA1$^+$CD14$^-$ DZ und CD14$^+$CD16$^-$ Monozyten überlegen.

Pro-iDZ ähnliche BDCA1$^+$CD14$^+$ Zellen wurden nur in Hautproben von Patienten die an Psoriasis und Dermatomyositis litten oder ein entzündetes Halonävus trugen gefunden, während sie in Hautproben von gesunden Spendern fehlten. Die Anwesenheit in entzündeter und Abwesenheit in gesunder Haut deutete darauf hin, dass BDCA1$^+$CD14$^+$ Zellen nicht gewebsständig sind, sondern in entzündete Gewebe rekrutiert werden, um möglicherweise eine Immunreaktion zu fördern. Die Rekrutierung ist vergleichbar mit den zu CD14$^+$CD16$^-$ Monozyten orthologen murinen Ly6C^{hi} Monozyten, die unter entzündlichen Bedingungen zu inflammatorischen DZ differenzieren. Eine detaillierte Übertragung vom murinen in das humane System ist bisher jedoch noch nicht gelungen. Um Aufschluss über die entwicklungsbedingte Verwandtschaft zwischen pro-iDZ und Monozyten zu erhalten wurden in dieser Arbeit pro-iDZ mit CD14$^+$CD16$^-$ Monozyten, kDZ des Blutes und *in vitro* generierte

aus Monozyten differenzierte DZ (moDZ) auf Genom-Ebene miteinander verglichen. Die Analyse des Transkriptoms zwischen pro-iDZ und den anderen DZ-Typen deutete auf einen monozytären Ursprung der pro-iDZ hin, da die Anzahl der differentiell exprimierten Gene zwischen pro-iDZ und Monozyten viel kleiner waren als zwischen pro-iDZ und den anderen DZ-Typen. Eine funktionelle Annotations-Analyse von Genen die in pro-iDZ höher reguliert waren als in Monozyten deckte eine DZ spezifische Gen-Signatur auf, wohingegen alle Monozyten-spezifischen Gene deutlich höher in Monozyten als in pro-iDZ reguliert waren. Zusätzlich waren die meisten der Gene in der gleichen Weise in den kDZ des Blutes und den *in vitro* generierten moDZ wie in den pro-iDZ reguliert, was auf eine beginnende Entwicklung der pro-iDZ von Monozyten zu DZ hindeutet. Diese *in silico* sich abzeichnende Entwicklung wurde zusätzlich unterstützt indem gezeigt werden konnte, dass $CD14^{+}CD16^{-}$ Monozyten, die unter inflammatorischen Bedingungen kultiviert wurden, Expression des DZ Marker BDCA1 erlangten, welche mit der Induktion von T Zell Proliferation korrelierte.

Zusammengefasst identifiziert diese Arbeit pro-inflammatorische DZ unter homöostatischen Bedingungen im peripheren Blut mit wahrscheinlich monozytären Ursprung. Pro-iDZ sezernierten große Mengen an pro-inflammatorischen Zytokinen und induzierten hochentzündliche T_H17 Zellen. Da pro-iDZ eine hohe Ähnlichkeit mit $BDCA1^{+}CD14^{+}$ Zellen, die nur in entzündeten Gewebe zu finden waren und starke Ähnlichkeit mit den erst kürzlich beschriebenen inflammatorischen DZ in entzündeten Geweben aufzeigten, können pro-iDZ als direkte Vorläuferzellen von inflammatorischen DZ angesehen werden. Ihr offensichtlicher Ursprung aus $CD14^{+}CD16^{-}$ Monozyten deutet darauf hin, dass pro-iDZ das Bindeglied zwischen Monozyten und inflammatorischen DZ sind und somit den ersten *in vivo* Beweis für eine im Menschen stattfindende moDZ Differenzierung darstellen könnte.

3 Introduction

3.1 Innate Immunity

The immune system protects its host from pathogenic invaders including bacteria, viruses, parasites and fungi and eradicates host malignant cells. Pathogens that successfully penetrated physical and chemical barriers, e.g. skin or mucosa of the respiratory airway and gastrointestinal tracts, are confronted with a line of conserved, innate defense mechanisms. Antimicrobial proteins including defensins and the complement cascade[1] inhibit extracellular pathogen growth[2-4] and allow opsonization for uptake by phagocytic cells. Effector cells with high phagocytic activity including neutrophil granulocytes, monocytes and mast cells are attracted chemotactically to the site of inflammation where they become activated, take up and destroy pathogens or pathogen infected host cells[5,6]. Leukocytes sense conserved pathogen associated molecular patterns (PAMPs) and host danger associated molecular patterns (DAMPs) with a broad range of germ-line encoded pattern recognition receptors (PRRs), which mediate rapid cellular activation[7,8]. Activated leukocytes secrete vast amounts of soluble mediators including chemokines, interleukins (ILs), interferons (IFNs) and cytotoxic molecules that contribute to leukocyte attraction and activation or cause cell damage and thereby mediate pathogen clearance. Cells dying from infection or violence secrete nucleic and cytosolic constituents into the extracellular space where they act as DAMPs to alert its host[9]. Known DAMPs include nucleic acids[10,11], chromatin associated proteins[12], ATP[13] and uric acid[14]. Recognition by specialized receptors can initiate and maintain sterile immune responses[15]. Common PAMPs are of bacterial, viral, fungal or parasitic origin and like DAMPs, they are recognized by vesicular and surface bound germ-line encoded Toll-like receptors (TLRs) 1-13 or cytosolic PRRs including DAI, RIG-I, MDA-5, PKR or AIM2, NOD1, NOD2. However, only 9 TLRs are known as functional receptors in humans[16,17]. The inflammasome constitutes another tightly regulated PRR complex. It controls the secretion of the highly inflammatory cytokines IL-1β and IL-18. The inflammasome consists of several components and is triggered by a plethora of stimuli leading to the activation of caspase-1 that cleaves immature pro-IL-1β and pro-IL-18 to functional cytokines[18,19]. Several inflammasomes are known and named after the receptor recognizing the PAMP or DAMP[20,21]. The AIM2 inflammasome recognizes microbial or host aberrant dsDNA[22,23], NLRP1 and NLRP4 inflammasomes are activated by bacteria and its toxins[24-27], whereas the most investigated

NLRP3 inflammasome is activated by crystallized endogenous molecules, microbial products and reactive oxygen species (ROS)[28-34]. ROS are thought to be a main trigger of NLRP3 inflammasome activation and are induced in response to various stress signals[35]. Figure 1 displays TLRs and IL-1R/IL-18R localization and signaling pathways and table 1 provides an overview of TLRs and their respective ligands.

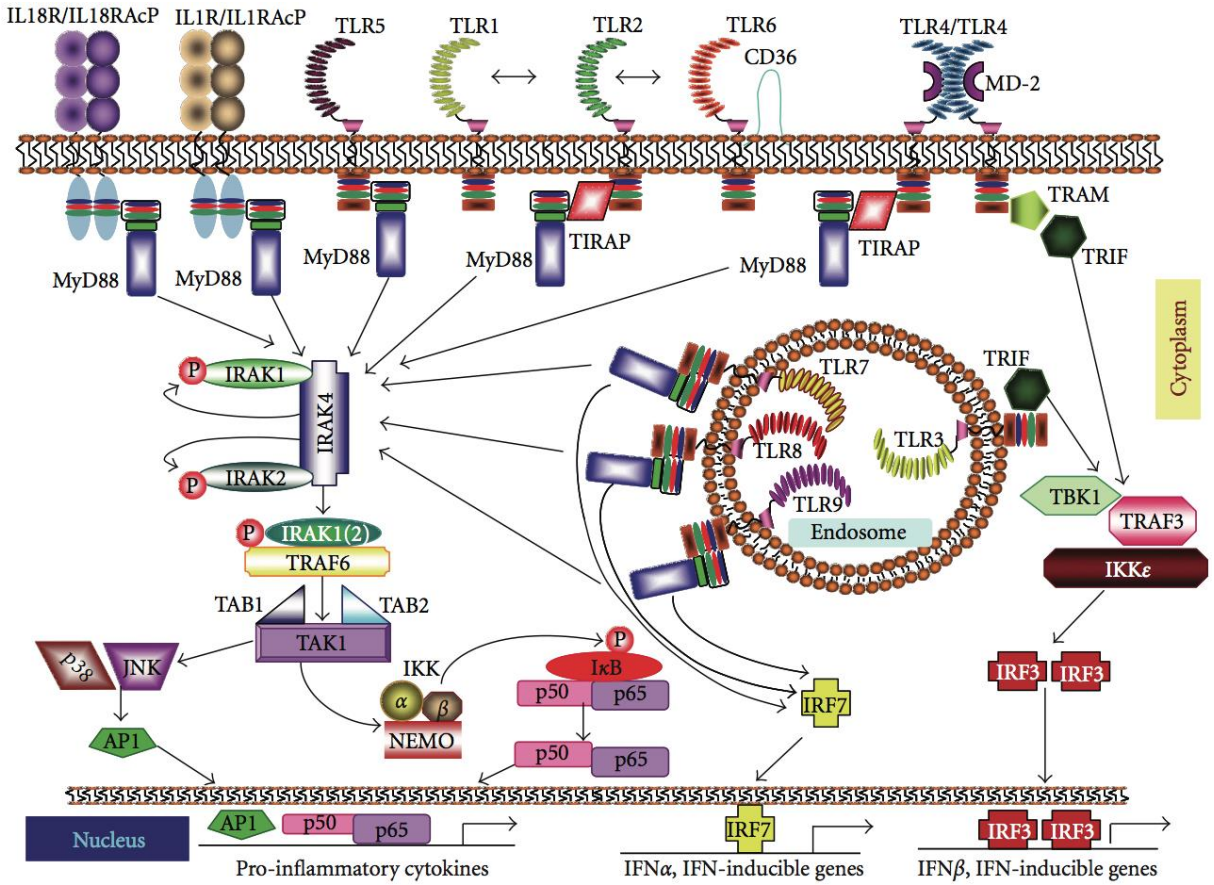


Figure 1 Localization and signaling pathways of IL-1R/IL-18R and TLRs
Upon ligation with IL-1, IL-18 or respective Toll-like receptor (TLR) ligands, signaling is mediated via the TIR domains associated with each receptor. IL-1R/IL-18R and all TLRs signal via MyD88 adaptor protein except for TLR3 and in case of TLR2 and TLR4, TIR and MyD88 are bridged by TIRAP. TLR3 and TLR4 employ TRIF besides MyD88 signaling that leads over TBK1/TRAF3 and IKKe to the nuclear localization of IRF3 inducing transcription of IFN-β and IFN-inducible genes. MyD88 downstream events involve the phosphorylation of IRAK1/IRAK2 by IRAK4, allowing the association of IRAK1/2 with TRAF6. This complex activates the kinase TAK1 that promotes NF-kB (p50/p65) via IkB phosphorylation and AP-1 translocalization to the nucleus and subsequent transcription of pro-inflammatory cytokines. Besides MyD88 dependent IRAK4 signaling, vesicular TLR7, TLR8 and TLR9 triggering leads to nuclear localization of IRF7 and promotes IFNα and IFN-inducible gene transcription. (Figure taken from Loiarro et al.[36])

TLRs	Ligands	Synthetic ligand	Pathogen
TLR 1/2	Triacyl lipopeptides	Pam3CSK4	Bacteria[37,38]
TLR 2/6	Diacyl lipopeptides	FSL-1	Mycoplasma [39]
TLR 3	Double stranded RNA	Poly I:C	Viruses[40]
TLR 4	Lipopolysaccharide (LPS), bacterial HSP60	LPS	Gram-negative bacteria [41]
TLR 5	Flagellin	Flagellin	Bacteria[42]
TLR 7	Single stranded RNA (ssRNA)	R837, Loxoribin	Viruses[43]
TLR 8	ssRNA	R848	Viruses[43]
TLR 9	Unmethylated CpG-DNA oligonucleotides	CpG-DNA	Bacteria, viruses[44]
TLR 10	Unknown	Unknown	Unknown[45]

Table 1 TLRs and their respective natural and synthetic ligands with occurrence in pathogens
Reviewed by Akira et al.[46]

Soluble mediators do not only attract and modulate the function of innate immune cells, they also play a major role in lymphocyte recruitment and activation. They are usually secreted in response to PRR triggering. IFNα decreases the susceptibility of host cells to intracellular infections by the induction of antiviral gene expression[47,48]. Chemokines attract effector leukocytes to sites of inflammation and control trafficking of naïve lymphocytes within lymphoid organs[49]. For an organized immune response, leukocytes are able to communicate by the secretion and reception of interleukins and can modulate the cellular activation state[50]. IFNs activate natural killer cells (NK cells)[51] to eradicate virus or bacteria infiltrated cells. NK cells lyse cells with an altered surface especially including reduced levels of human leukocyte antigen (HLA) molecule expression[52,53]. HLA class I molecules are important for the presentation of cell derived molecules to lymphocytes, which eradicate cells presenting non-self molecules. Thus rendering MHC class I levels is a common escape mechanism of cellular pathogenic infection[54,55] and a sign of malignant transformation[56-58]. Activated phagocytes as eosinophil granulocytes or monocytic cells are able to secrete nitric oxide (NO)[59,60], reactive oxygen species (ROS)[61-64] and cytotoxic proteins[65] into the extracellular space to promote bacterial and parasitic killing[66]. However, pathogens are equipped with a number of mechanisms and a naturally high mutation rate to escape the recognition and eradication by innate immunity.

3.2 Adaptive immunity

In contrast to the immediately activated innate immune system that is responding to a limited number of conserved structures, adaptive immunity requires more time for an educated, antigen specific reaction. Similar to innate immune defense mechanisms, adaptive immunity comprises both, a humoral response executed by antibody secreting B cells and a cellular response mediated by T cells that recognize cell bound antigens. In contrast to NK cells that detect the absence of HLA molecules, T cells specifically recognize host cell derived or endocytosed antigens loaded onto HLA molecules, allowing the determination between host and foreign[1]. Antigens presented in context of HLA molecules are proteins processed to short peptides. T cells use highly polymorphic and per cell uniform T cell receptors (TCRs)[67,68] that enables the host to respond to a nearly unlimited number of antigens. TCRs are unlike PRRs not germ-line encoded and their recognition is not restricted to a limited number of structures. Instead, they are transcribed from randomly and locally rearranged parts of the genome, resulting in any number of TCRs with different specificities[69]. Clonal selection during development eradicates T cells that recognize host antigens and thereby favors detection of foreign, pathogen associated or host mutated antigens found in malignant cells[70-72]. Like TCRs, B cell receptors (BCRs), which are basically surface bound antibodies, are encoded by rearranged parts of the genome and B cells undergo clonal selection to avoid recognition of self, a cause of autoimmunity[73]. Cytotoxic $CD8^+$ T lymphocytes (cTLs) are similar in function to NK cells, but eradicate target cells antigen specifically after recognition of peptides predominantly loaded onto HLA class I molecules[74]. By contrast, $CD4^+$ helper T cells (T_H cells) primarily detect peptides on HLA class II molecules[75] and generally do not lyse the cells that present foreign antigen. Instead, T_H cells activated by antigen recognition orchestrate complex immune responses at the site of inflammation involving the recruitment and activation of effector cells[76]. In order to meet the requirements to support the eradication of different types of pathogens, T_H cell responses can be adapted by the polarization into different helper subsets dependent on the cytokine environment during their priming. Intracellular pathogens mainly induce IL-12 dependent T_H1 responses[77], whereas extracellular parasitic, bacterial and fungal infections cause T_H2 and T_H17 polarization, in a milieu containing IL-4 and IL-13 or IL-1β, IL-6, IL-23 and TGF-β[78-81], respectively. In order to strengthen the dominance of an established T_H cell type response, cytokines also augment a specific polarization by inhibiting deviating T_H cell polarizations. For example, TGF-β is thought to inhibit T_H1 cell polarization

and therefore to favor T_H17 differentiation[82], whereas IL-12 induces T_H1 cells and inhibits T_H2 polarization[83]. The defined set of cytokines secreted by polarized T_H cells support specific immune functions that are required to eradicate distinct pathogens. IFNγ, mainly secreted by T_H1 cells, promotes NK cell mediated lysis of cells infected with intracellular pathogens, increases phagocytic and lysosomal activity in macrophages to degrade ingested material and enhances the cellular antigen presentation machinery[84]. T_H2 cells predominantly secrete IL-4, IL-5 and IL-13 and thereby activate granulocytes to secrete cytotoxic mediators[85,86] that eradicate parasites present in the extracellular space. IL-17 produced by T_H17 cells[87] induces the secretion of inflammatory cytokines and chemokines from host cells to enhance inflammation and to attract effector cells including neutrophil granulocytes to the site of inflammation[88] and thus supports fungal and bacterial clearance[89,90]. In addition, activated follicular helper T cells (T_{FH} cells) provide the necessary support for B cells to secrete antigen specific antibodies[91] that play a major role in the neutralization of pathogens or their toxic metabolites present in the extracellular space[1]. Once the host has encountered a pathogen, it is "remembered" by the adaptive immune system for an immediate counteraction. A minor population of activated T and B cells undergoes memory cell differentiation and thereby establishes a long lasting antigen specific memory[92]. In contrast to TCRs consisting of an α and β chain[93] and recognizing classical HLA molecules, lipid antigens on non-classical CD1 molecules are recognized by T cells that are equipped with γ/δ TCRs (γ/δ T cells)[94,95]. In addition, γ/δ T cells are suspected to be a major producer of IL-17 during inflammation[96]. Finally, in order to initiate clonal activation and subsequent proliferation of antigen specific T and B cells, matured professional antigen presenting cells (APCs) are required.

3.3 Dendritic cells as linkers of innate and adaptive immunity

Dendritic cells (DCs) are professional APCs with continuous endocytotic activity in the immature state and high T cell stimulatory capacity after maturation. DCs were originally identified by Ralph Steinman and Zanvil Cohen in 1973[97]. The heterogeneous DC populations originate from bone marrow $CD34^+$ hematopoietic stem cells. While in mouse further development stages towards DC including a myeloid progenitor (MP), macrophage/DC precursor (MDP) and common DC precursors (CDP) are described, in humans a similar development is anticipated, however, human DC ontogenesis remains to be elucidated[98,99].

Human DCs can be subdivided into Flt3L dependent BDCA2$^+$CD123$^+$ plasmacytoid DCs (pDCs) and conventional DCs (cDCs)[100]. cDCs are comprised of tissue resident and migratory DCs[101]. The cDC lineage is determined by the expression of transcription factors including IRF4[102-105], BATF3[106-108] and ZBTB46[109,110]. Whereas IRF4 and BATF3 are also expressed in lymphoid cells[111,112], ZBTB46 has recently been described to be a master transcription factor for DCs[109,110]. Blood contains pDCs and myeloid cDCs, which consist of BDCA1$^+$ DCs and BDCA3$^+$CLEC9a$^+$ DCs, whereas the skin contains epidermal CD207$^+$ Langerhans cells and dermal CD14$^+$ DCs and BDCA1$^+$ DCs[113]. A pivotal task of DCs is the continuous uptake of extracellular material and its presentation to T cells. They are equipped with a number of cell surface bound receptors including scavenger receptors, c-type lectins (CLEC) and Fc receptors (FcRs) to sense and take up antigens[114]. The intracellular antigen degradation machinery is, unlike to other phagocytes, assigned for preserving small protein fragments, peptides consisting of up to 17 amino acids (aa) that are loaded onto HLA molecules allowing for TCR dependent T cell recognition[115]. In direct antigen presentation, class I HLA-molecules (HLA-A, B and C) bind intracellular derived peptides of 8-12aa in length[116], whereas HLA class II molecules (HLA-DP, DQ and DR) are loaded with extracellular derived peptides with a length of 13-17aa[117]. The endocytosed material is strictly monitored for foreign PAMPs or host DAMPs by a broad range of PRRs[118-120]. As a consequence of PAMP recognition by respective TLRs or by IL-1 receptor (IL-1R) triggering, subsequent signaling as depicted in figure 1 leads to a complex signaling cascade finally resulting in the maturation of DCs[121]. In the course of maturation, DCs migrate to lymphatic tissues where they act as potent stimulators of antigen specific T cell responses[122]. Lymphatic tissues are highly organized structures that are continuously frequented by leukocytes to facilitate the encounter of cognate APCs and T cells[123]. In contrast to immature DCs, maturation includes a strong enhancement of the antigen presenting machinery activity and thereby forces a predominant loading of high amounts of HLA molecules with DAMP or PAMP associated antigens[124,125]. Since the entire T cell priming process including the localization and interaction with a cognate APC requires a persistent antigen presentation over a period of time, the half-life of surface HLA molecules on matured DCs is strongly increased[126]. A second signal besides TCR mediated antigen loaded HLA molecule recognition on the APC is required for T cell activation. It ensures an antigen specific T cell response focused on structures associated with an alert signal and thereby avoiding immune responses directed against self-antigens[127,128]. PAMP experienced DCs

upregulate the surface expression of T cell stimulatory and regulatory molecules including CD40, CD83[129] and the B7-family members (CD80, CD86, CD273, CD274, CD275, CD276 and VTCN1)[130] during maturation. Furthermore, stimulation of PRRs in DCs leads to the secretion of inflammatory, polarizing and regulatory cytokines including IL-1β, IL-6, TNFα, IL-12, IL-13, IL-23 and IL-10[131-135]. In the rare event of recognition of the antigen presented by matured DCs, the T cell undergoes rapid proliferation. DCs critically determine the type of T_H cell polarization as these secrete certain sets of cytokines in response to various PAMPs. Activated T cells start to produce IL-2 and IL-2 receptor (CD25, CD122 and CD132 subunits) surface expression[136,137] and thereby mediate their own clonal expansion[138]. Hence, CD25 upregulation is regarded as a T cell activation marker. In the case antigen is recognized on immature DCs that have not undergone pathogen induced activation and thereby lack co-stimulatory molecules, antigen specific peripheral T cell tolerance is induced[139]. Tolerance includes the induction of apoptosis and anergy or differentiation into regulatory T cells (T_{regs}), which dampen immune responses[140]. However, upon failure of tolerance induction, immune responses directed against host antigens can be established resulting in self-destructive autoimmunity. Thus, DCs critically determine the initiation and outcome of an immune response and several subsets have been ascribed specialized properties in this manner. A functional specialization can be suggested due to distinct expression PRR patterns in different DC subsets[141,142]. $BDCA1^+$ DCs are equipped with many TLRs and respond to a wide range of PAMPs with maturation and unique cytokine expression[143] that establishes a T cell polarizing and inflammatory environment, whereas pDCs are specialized in secreting vast amounts of antiviral type I IFN in response to TLR7 and TLR9 stimulation with nucleic acids[144]. $BDCA3^+$ DCs are superior in antigen cross-presentation[107,143,145], a process in that extracellular derived peptides are presented on HLA class I instead of class II molecules[146]. Cross-presenting DCs thereby provide the basis for cTL responses against intracellular pathogens[147]. Besides DCs differentiated from hematopoietic precursors at steady state, it is known that murine DCs can be derived from monocytes *in vivo* and *in vitro.* In humans it is verified that monocyte differentiate towards DC (moDC) *in vitro* and various protocols have been described.

3.4 Human monocyte derived DCs

In vitro generated moDCs are widely used as a valuable tool and as a supplement for the rare and hard to isolate blood DCs, as moDCs are available in large numbers and can be generated with great plasticity[148]. A prototypic protocol used to induce human moDCs involves GM-CSF and IL-4 for differentiation from monocytes towards immature moDCs[149]. For moDC maturation, immature moDC were initially treated with monocyte cell culture derived supernatants termed monocyte-conditioned media (MCM)[150]. Later, Jonuleit et al.[151] specified the cytokines contained in MCM required for moDC maturation as a cocktail of IL-1β, IL-6 and TNFα. He additionally applied PGE_2 as an enhancer of the aforementioned cytokines. The resulting DCs gained inflammatory gene expression[152] and a strong T cell proliferative capacity[153,154]. However, although PGE_2 induces CCR7 expression, a receptor mediating homing to lymphatic tissues, it directs moDCs regulatory, as so treated cells attract and activate T_{regs}[155] instead of naïve T cells[156] and show decreased IL-12 and increased regulatory IL-10 secretion[152,157]. Instead, LPS and IFNγ are commonly used for inducing the secretion of inflammatory and T_H1 polarizing cytokines and enhanced antigen presentation to $CD4^+$ and $CD8^+$ T cells[158]. Another maturation method using TNFα/CD40L favors T_H2 responses and decreases the ability to activate cTL responses[159]. By contrast, IFNα accomplishes both, monocyte differentiation and maturation and enables so treated monocyte derived IFN-DCs to cross-present and prime antigen specific $CD8^+$ T cell responses[160]. In addition to soluble mediators, cell-cell contact also drives the differentiation towards moDCs, as described for activated NK cells, macrophages and NKT cells[161-163]. Contact with $CD8^+$ T cells leads to monocyte differentiation towards TNFα and iNOS producing DCs (Tip-DCs)[164]. Thus, monocytes differentiate into DCs with high plasticity including the expression of different sets of cytokines and surface markers, which depends on the presence of particular environmental factors. The high availability and plasticity of monocytes has made them targets with extensive use in cellular therapy approaches in malignant diseases. *In vitro* differentiated and tumor antigen loaded moDCs from monocytes were used to prime patients' adaptive immune systems to eradicate neoplasms[165-167]. Despite the well-investigated human *in vitro* moDC model, a direct translation to the *in vivo* situation is commonly suspected but coherent evidence is missing. However, similar to moDCs generated *in vitro*, some studies report the existence of monocyte related DCs *in vivo*[168-173].

3.5 Inflammatory DCs

In contrast to naturally occurring DCs, inflammatory DCs are of monocytic origin and only found during inflammation[174]. In mice, it has been well established that inflammatory DCs differentiate Flt3L independently[175-177] from Ly6C^{hi}CD11b^{+}CX3CR1^{low} (Ly6C^{hi}) monocytes under inflammatory conditions *in vivo* and *in vitro*. Ly6C^{hi} monocytes traffic CCR2 dependently from the bone marrow (BM) to the blood stream[178]. During inflammation, they migrate to inflamed tissues and differentiate into CD11c expressing DCs[179,180] where they become the dominant inflammatory cell type[179-183]. Subsequently they acquire the ability to migrate to draining lymph nodes and become the main population in the T cell areas[173,184]. They may act as potent antigen specific T cell stimulators of direct- and cross-presented antigens[185]. At the site of inflammation, some inflammatory DCs mainly act through innate defense mechanisms including TNFα/iNOS production and are therefore referred to as Tip-DCs. Tip-DCs are directly involved in parasitic clearance of *Trypanosoma brucei*[186], *Listeria monocytogenes*[187], *Brucella melitensis*[188] in a TNFα and NO dependent way. Inflammatory DCs can also determine the polarization of T_H cells depending on the immunological environment present during inflammatory DC differentiation. In HSV-2 and *Listeria monocytogenes* infection, inflammatory DCs restimulate and prime T_H1 responses[172,189], they induce T_H2 responses mediated by house dust mite allergen[190] and T_H17 cells against the fungus *Aspergillus fumigates*[182]. In contrast to the involvement in host protective immune responses, inflammatory DCs have also been linked to experimental models of autoimmune diseases including allergic asthma[175,190], rheumatoid arthritis[191], colitis[192,193], psoriasis[194] and experimental autoimmune encephalomyelitis (EAE)[195,196], an animal model for human multiple sclerosis.

Until now, inflammatory DCs are identified by co-expression of several monocyte and DC specific markers and transcription factors as a single marker remains to be identified. As monocytes give also rise to macrophages[197,198] and show phenotypic convergence with inflammatory DCs, analysis is complicated as a major challenge lies in the distinction of both cell types[199]. However, macrophages differ in their biologic functions from DCs. In contrast to inflammatory DCs, macrophages are mostly tissue resident and show a more anti-inflammatory role, which has been addressed to them by their strong ability to secrete IL-10[199,200]. They rather degrade ingested material and are limited in antigen presentation. In contrast to DCs, they show a low T cell stimulatory capacity and rarely migrate to lymphoid

organs[201]. Mouse inflammatory DCs express monocytic surface markers including CD115, F4/80 and CD11b as well as DC markers such as CD11c and high levels of MHC II, and CD40, CD86[202] and CD64[174,203]. The expression of the transcription factor ZBTB46, which has recently been described to be specific for mouse and human DCs[173], clearly affiliates them with DCs.

To date, very limited data describes the phenotype and function of human inflammatory DCs. Similar to murine counterparts, the presence of human inflammatory DCs was found to be restricted to inflamed tissues and inflammation associated lymphatic organs. They shared hallmarks of activated DCs and like *in vitro* generated moDCs, inflammatory DCs appeared with varying phenotypes and functions.

Inflammatory dendritic epidermal cells (IDECs) were first described in the skin of lesional atopic eczema, psoriasis vulgaris and atopic dermatitis, while lacking in non-lesional atopic eczema and healthy skin. They were defined by the expression of CD1a, CD11b, CD11c, FcεR1α, high levels of HLA-DR and CD36 and were distinct from skin resident Langerhans cells by the lack of Birbeck granules[168,169]. IDECs are thought to be related to T_H1 and T_H2 cell responses, because they were involved in the autoimmune skin disease atopic dermatitis, which is initiated and maintained by a T_H2 and T_H1 response, respectively[204]. Another type of inflammatory DCs, Tip-DCs, is present during inflammation and predominantly linked with the presence of T_H1/T_H17 cells. They were discovered in psoriatic lesions, an inflammatory autoimmune disease of the skin, by the expression of HLA-DR, CD11c and TRAIL and lack of BDCA1[171,205,206]. Their contribution to autoimmune inflammation was addressed to TNFα and NO secretion and priming of T_H1 and T_H17 immune responses[171]. Recently, another group identified inflammatory DCs in rheumatoid arthritic synovial fluids and tumor ascites and in the spleen from patients suffering from gastric cancer, while lacking in healthy donor's tonsils, non-invaded lymph nodes from untreated breast cancer patients and in the spleen from pancreatic cancer patients. They were characterized by the co-expression of FcεR1α, the monocytic markers CD14 and CD11b, DC markers including BDCA1, CD206, high levels of HLA-DR and a strong T cell proliferative capacity. Their inflammatory potential was underlined by the induction of T_H17 responses mediated by the secretion of IL-1β, IL-6, IL-23 and TGF-β. In contrast to monocytes and macrophages, these inflammatory DCs expressed the DC specific transcription factor ZBTB46[170].

The authors of these studies suggested a monocytic origin of the respective inflammatory DCs. Their idea is supported as mouse $Ly6C^{hi}$ monocytes, orthologs of human $CD14^+CD16^-$

monocytes[207], give rise to inflammatory DCs. However, the origin of inflammatory DCs remains to be identified.

3.6 The aim of this study

Mouse monocytes differentiate under pathogen encounter towards inflammatory DCs, which become numerically the main DC population in inflammatory diseases. They act as key players in protective as well as in self-destructive autoimmunity. As the beginning understanding of human inflammatory DCs in diseases seem to parallel murine observations, further understanding of human inflammatory DCs can offer valuable opportunities for intervention in a multitude of inflammatory diseases.

In humans, three blood DC populations comprising pDCs, BDCA1$^+$ DCs and BDCA3$^+$ DCs are described. BDCA1$^+$ DCs, however, are not homogenous, since a substantial population expresses the monocytic marker CD14. This BDCA1$^+$CD14$^+$ cell subset shares properties of both, monocytes and DCs and may constitute an intermediate development stage between both cell types. This study aims to characterize BDCA1$^+$CD14$^+$ cells on phenotypic, functional and whole genome level.

4 Material and Methods

4.1 Antibodies and staining

Up to 10^7 cells were stained in CliniMACS buffer (Phosphate Buffered Saline (PBS)/5mM Ethylenediaminetetraacetic acid (EDTA); Miltenyi Biotec) supplemented with 0.5%AB-serum (Lonza) and respective antibodies in a total volume of 110µl for 10min at 4°C in the dark. Afterwards, cells were washed in 1ml buffer/0.5% AB-serum and resuspended in 100-300µl CliniMACS/0.5% AB-serum for flow cytometric analysis. All antibodies used including clone names are listed below:

BDCA1-PE (AD5-8E7), BDCA1-APC (AD5-8E7), BDCA3-APC (AD5-14H12), BDCA3-PE (AD5-14H12), CD3-Vioblue (BW264/56), CD4-FITC (VIT4), CD4-Viogreen (VIT4), CD11b-PE (M1/70.15.11.5), CD11c-PE (MJ4-27G12), CD14-FITC (TÜK4), CD16-PE (VEP13), CD19-APC (LT19), CD25-PE (3G10), CD32-PE (2E1), CD36-PE (AC106), CD40-PE (HB14), CD45RA-PE (T6D11), CD45RO-PE (UCHL1), CD64-PE (10.1.1), CD68-PE (Y1/82A), CD80-PE (2D10), CD83-PE (HB15), CD86-PE (FM95), HLA-DR-PE (AC122), FcεR1α-PE (CRA1), CX_3CR1-PE (2A9-1), CXCR4-PE (12G5), CLA-PE (HECA-452), CD172a-PE (REA144), CD163-PE (GHI/61.1), CD206-PE (DCN228), CD209-PE (DCN47.5), CD275-PE (MIH12), CD276-PE (FM276), SLAN-PE (DD-1), CLEC9a-PE (8F9), IL-6-PE (MQ2-13A5), IL-10-APC (JES3-9D7), TNFα-APC-Vio770 (cA2), IFNγ-FITC (45-15), IL-17-APC (CZ8-23G1) (all Miltenyi Biotec, dilution: 1:11), CD54-APC (HA58) (dilution: 1:10), HLA-ABC-FITC (DX17, dilution: 1:50), CD1a-PE (HI149, dilution: 1:10) (BD Pharmingen), CCR5 (45531, 20µg/ml), CCR2-PE (48607) (dilution 1:10, both R&D Systems), CD274-PE (MIH1) (eBioscience)

Mouse isotype controls included: IgG1-PE (IS5-21F5), IgG1-APC (IS5-21F5), IgG2a-PE (S43.10), IgG2a-APC (S43.10), IgG2b-PE (IS6-11E5.11) and IgG2b-APC (IS6-11E5.11) (all Miltenyi Biotec)

Rat isotype controls included: IgG1-APC (eBRG-1) and IgG1-PE (eBRG-1; both eBioscience)

Secondary antibodies: anti-mouse IgG2ab-PE (X-57, dilution 1:11; Miltenyi Biotec)

4.2 Cell isolation

4.2.1 PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated from buffy coats (Klinikum Dortmund) or from leukapheresis products (Klinikum Köln). Buffy coats were separated into three 50ml canonical tubes (BD Falcon) and filled with PBS/EDTA buffer to a total volume of 35ml. The cell suspension was carefully transferred to a new tube containing 15ml Ficoll-Paque Plus or Pancoll (GE Healthcare, PAN Biotec, respectively) without disturbing the resulting overlaying phases. After centrifugation at 445g for 35min, the white leukocyte layer was removed into a new 50ml tube and filled with PBS/EDTA buffer to a total volume of 50ml. The cells were centrifuged twice, subsequently at 300g and 200g for 15min and supernatants were replaced by 50ml CliniMACS buffer and with 10ml CliniMACS buffer/0.5% AB-serum, respectively. Leukapheresis products were initially separated into eight 50ml tubes and contents of two 50ml tubes were combined by each subsequent step. The cells were stored at 4°C over night and yielded $5x10^8$ PBMCs from buffy coats and $4\text{-}10x10^9$ PBMC from leukapheresis products on average.

4.2.2 BDCA1$^+$ DC isolation

CD19 depleted BDCA1$^+$ DCs were isolated from PBMC using the CD1c (BDCA-1)$^+$ Dendritic Cell Isolation Kit according to manufacturer's protocol.

4.2.3 BDCA3$^+$ DC isolation

BDCA3$^+$ DCs were isolated from PBMC using the CD141 (BDCA-3) Microbead Kit according to manufacturer's protocol.

4.2.4 pDC isolation

pDCs were isolated from PBMC using the Plasmacytoid Dendritic Cell Isolation Kit II according to manufacturer's protocol.

4.2.5 Naïve CD4$^+$ T cell isolation

CD45RA$^+$CD45RO$^-$CD4$^+$ T cells were isolated from PBMC using the Naïve CD4$^+$ T Cell Isolation Kit II according to manufacturer's protocol.

4.2.6 Monocyte isolation

$CD14^+CD16^-BDCA1^-$ monocytes were isolated from PBMCs using the Monocyte Isolation Kit II according to manufacturer's protocol. To deplete contaminating $BDCA1^+$ cells, the first incubation with antibodies deviated from the protocol in that PBMCs were incubated with the monocyte-biotin-antibody cocktail additionally spiked with anti-BDCA1-biotin antibodies in a 1:5 ratio. The resulting $CD14^+$ cells were further purified using CD14-microbeads and subsequently separated using two LS-columns according to manufacturer's protocol.

4.2.7 Fluorescence Assisted Cell Sorting

Magnetically purified $BDCA1^+CD19^-$ cells as described in 4.2.2 were labeled with BDCA1-PE and CD14-FITC antibodies and stored in RPMI1640 (Miltenyi Biotec)/5% AB-serum pre-coated 5ml polystyrene round-bottom tubes (BD Falcon) on ice. $BDCA1^+$ cells were further flow sorted into $BDCA1^+CD14^-$, $BDCA1^+CD14^{int}$ and $BDCA1^+CD14^+$ cells. Dead cells were discriminated by propidium iodide uptake and excluded by appropriate gating according to Figure 3. During sorting, cells were continuously kept at 4°C. Cell sorting was performed on a BD FACS Vantage SE at the Institute of Genetics (Cologne) and generally yielded purities of >98%.

4.2.8 Flow cytometric analysis

Cells were acquired on a MACSQuant analyzer and evaluated with MACSQuantify or FlowJo versions 10.0.5 and 10.0.6.

4.2.9 Calculation of absolute $BDCA1^+$ cell numbers from whole blood

In order to obtain absolute $BDCA1^+CD14^-$ DC and $BDCA1^+CD14^+$ pro-iDC cell numbers from healthy donor's whole blood, blood average BDCA1 expressing cell numbers were obtained from literature[208] and multiplied with the individual ratios of $BDCA1^+CD14^-$ DCs / $BDCA1^+CD14^+$ pro-iDCs (Figure 4). Equivalent cell numbers from RA patients were obtained by the calculation of the arithmetic product of absolute cell numbers of $BDCA1^+CD19^-$ cells from the unseperated cells and ratios of $BDCA1^+CD14^-$ DCs / $BDCA1^+CD14^+$ pro-iDCs.

4.3 Cell culture and treatment

After isolation, dendritic cells and monocytes were cultured in RPMI1640 supplemented with 5% AB-serum (Lonza), 1x Penicillin/Streptomycin and 2mM L-glutamin. For cultures containing T cells, medium was additionally supplemented with 1x non-essential amino acids and 1x

sodium-pyruvate (all Gibco). All cells were incubated in 96 flat bottom well plates at 37°C, 5% CO_2 and 100% humidity.

4.3.1 Cell treatment for microarray experiments

BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ pro-iDCs were magnetically and subsequently flow sorted as described in 4.2.2 and 4.2.7. Monocytes were magnetically enriched as described 4.2.6. Resulting CD14$^+$CD16$^-$ monocytes were labeled with BDCA1-PE and CD14-FITC and flow sorted to obtain CD14$^+$CD16$^-$BDCA1$^-$ monocytes. 2x10^5 cells were lysed in RA1 lysis buffer (Macherey-Nagel) and stored at -70°C. In order to generate immature and mature monocyte-derived DCs, monocytes were isolated from PBMC using CD14-microbeads and subsequent separation with LS and MS magnetic columns according to manufacturer's protocol (Miltenyi Biotec). 10^7 monocytes were cultured in 10ml Mo-DC differentiation medium (Miltenyi Biotec) in T75 flasks (Becton, Dickinson). Medium was exchanged on day 4. On day 7, cells were washed and 5x10^5 cells were lysed in RA1 lysis buffer and stored at -70°C. The remaining cells were cultured for 3 more days in 10ml Mo-DC maturation medium (Miltenyi Biotec) in T75 flasks. After a washing step, 5x10^5 cells were lysed as described above. RNA quality assessment based on 18S to 28S ribosomal subunit degradation was evaluated using the Agilent 2100 Bioanalyzer expert software 3 (Agilent Technologies). Resulting RNA Integrity Numbers (RIN), based on a numbering system from 1 (mostly degraded) to 10 (perfectly intact), are depicted in table 2. Samples with a RIN >6 are regarded as acceptable.

Sample No.	BDCA1$^+$CD14$^+$ pro-iDC	CD14$^+$CD16$^-$ BDCA1$^-$ monocyte	imoDC	mmoDC	BDCA1$^+$CD14$^-$ DC	BDCA3$^+$CD14$^-$ DC
1	9.0	7.3	9.6	9.3	9.0	8.0
2	8.8	8.9	9.6	8.6	9.7	7.1
3	9.1	8.5	9.7	8.5	8.6	7.4
4	10.0	8.7	9.8	9.6	-	-

Table 2 RNA quality of samples included in microarray analysis
Numbers indicate RNA Integrity Numbers (RIN) on a total scale from 1 (mostly degraded) to 10 (mostly intact) obtained from the Agilent 2100 Bioanalyzer expert software. (imoDC, immature monocyte derived dendritic cell; mmoDC, mature monocyte derived dendritic cell)

4.3.2 Generation of APC supernatant and analysis of cytokine secretion

5x10^4 BDCA1$^+$CD14$^-$CD19$^-$ DCs, BDCA1$^+$CD14^{int}CD19$^-$ cells, BDCA1$^+$CD14$^+$CD19$^-$ pro-iDCs, CD14$^+$CD16$^-$BDCA1$^-$ monocytes and BDCA3$^+$CD14$^-$ DCs were cultured in 200µl medium (see 4.3) for 24h in presence or absence of 10µg/ml Pam3CSK4 (Invivogen), 10µg/ml Poly I:C (Sigma), 100ng/ml LPS (Sigma), 100ng/ml Flagellin, 1µg/ml FSL-1, 5µg/ml R837, 5µg/ml R848

(all Invivogen), 5µg/ml CpG-B (Metabion). 1µg/ml double stranded DNA motif poly A:T was transfected with 0.5µg/ml Lipofectamin 2000 (both Invitrogen) according to manufacturer's protocol. To analyze the influence of erythrocytes on spontaneous cytokine secretion, autologous erythrocytes were co-cultured with $BDCA1^+CD14^+$ pro-iDCs at 50% hematocrit. Erythrocytes from density gradient centrifugation were washed three times in 1x PBS by centrifugation at 300g for 10min. To block the antioxidant system, erythrocytes were treated with 50mM aminotriazole (Sigma) and 0.4mM H_2O_2 (Sigma) at 37°C for 1h. After a subsequent washing step, the gluthatione peroxidase was inhibited by treatment with 0.2mM mercaptosuccinate (Sigma) at 37°C for 1h. To remove chemicals used during treatment, erythrocytes were washed thoroughly by 3 subsequent washing steps. To analyze the influence of reactive oxygen species on spontaneous cytokine secretion, $BDCA1^+CD14^+$ pro-iDCs were treated with 20µM diphenyleneiodonium (DPI) and 20mM N-acetylcysteine (NAC, both Sigma). To analyze the influence of IL-1R signaling on spontaneous cytokine secretion, $BDCA1^+CD14^+$ pro-iDCs were treated with 4µg/ml IL-1β neutralizing antibody (BD Pharmingen, clone: AS10) and 10µg/ml IL-1R antagonist (IL-1RA, Miltenyi Biotec). As an isotype control, 4µg/ml functional grade SIINFEKL antibody (Miltenyi Biotec, clone: 25-D1.16) was applied. Cell free supernatants were collected and kept at -70°C. Assessment of cytokine amounts was performed using cytokine bead arrays (Bender MedSystems and Miltenyi Biotec), IL-1β ELISA (Biolegend) and IL-23 ELISA (eBioscience) according to manufacturer's instructions.

4.3.3 Maturation of APCs using APC derived supernatants

Cell supernatants of 24h cultured $BDCA1^+CD14^-CD19^-$ DCs, $BDCA1^+CD14^+CD19^-$ pro-iDCs and $CD14^+CD16^-BDCA1^-$ monocytes were collected as described in 4.3.2 and added in a 1:5 ratio to sorted $BDCA1^+CD14^-CD19^-$ DCs, $BDCA3^+CD14^-$ DCs and $CD14^+CD16^-BDCA1^-$ monocytes. After 24h, cells were stained for CD40-APC, CD80-APC, CD83-APC and CD86-FITC and analyzed using flow cytometry.

4.3.4 Mixed leukocyte reaction

Sorted $BDCA1^+CD14^-CD19^-$ DCs, $BDCA1^+CD14^+CD19^-$ pro-iDCs or $CD14^+CD16^-BDCA1^-$ monocytes were serially diluted starting from $2.5x10^4$ to 781 cells and stimulated with 5µg/ml R848 or left untreated. $5x10^4$ naïve $CD4^+$ T cells (for isolation see 4.2.5) were added to each well and cultured for 6 days. Cells were stained for CD3-Vioblue, CD4-FITC and $CD3^+CD4^+$ cell numbers were assessed by flow cytometry.

4.3.5 Anti-CD3/anti-CD28 bead supported cytokine mediated T cell proliferation

Culture supernatants of R848 treated or unstimulated BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ pro-iDCs were generated as described in 4.3.2. Naïve CD4$^+$ T cells were stained with the proliferation dye Violet Dye (Invitrogen) according to manufacturer's protocol.

5x10^4 Violet Dye$^+$ T cells were stimulated with 2.5 x10^4 CD3/CD28 beads (Treg suppressor inspector, Miltenyi Biotec) that induced a sub-proliferative stimulation in presence or absence of 25µl supernatants resulting in 100µl total volume. After 84h of cultivation, proliferation was evaluated by flow cytometry. In some experiments, neutralizing antibodies recognizing IL-1β (BD Pharmingen, clone: AS10), IL-6 (clone: MQ2-13A5) and TNFα (clone: cA2; both Miltenyi Biotec) or respective isotype controls were used. Supernatants were incubated with 8µg/ml anti-IL-1β, 20µg/ml anti-IL-6 or 20µg/ml anti-TNFα at 37°C for 2h prior to addition to T cell cultures. The resulting concentrations for the culture period were 2µg/ml anti- IL-1β, 5µg/ml anti-IL-6 and 5µg/ml anti-TNFα. Isotype controls in the same order included, anti-mouse H-2K^b-SIINFEKL (mouse IgG1, clone: 25-D1.16), anti-mouse CLEC9a (rat IgG1, clone: 8F9) and a humanized anti-human IgG1 isotype antibody (all Miltenyi Biotec) and were applied in the same concentrations as the cytokine neutralizing antibodies. In some experiments, recombinant cytokines including 33ng/ml IL-1β, 100ng/ml IL-6 and 100ng/ml TNFα were used instead of APC derived supernatants.

4.3.6 Intracellular cell staining

10^5 magnetically enriched BDCA1$^+$ cells or CD14$^+$ monocytes were cultured for 6h in presence of absence of 10µg/ml Pam3CSK4 and for the final 4h cellular export was blocked by the presence of 0.4µg/ml Brefeldin A (Miltenyi Biotec) or analyzed for cytokine expression straight after isolation. For staining, cells were kept in approx. 20µl medium and a cocktail of BDCA1-PE, CD14-FITC, IL-10-APC, TNFα-APC-Vio770 or BDCA1-APC, CD14-FITC, IL-6-PE or respective isotype controls were applied for 10min to the cells. Subsequently, incubation with 25µl InsideFix for 20min followed by 20µl 10x InsidePerm for 10min was applied. Cells were washed with 100µl 1x InsidePerm and centrifuged at 300g for 10min. Supernatants were removed and cells were resuspended in CliniMACS buffer/0.5% AB-Serum prior to analysis by flow cytometry. All steps were performed at RT and included initial 2 min shaking at 450rpm. All materials were obtained from Miltenyi Biotec.

4.3.7 T cell polarization assay

Flow sorted BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ pro-iDCs (see 4.2.7) and CD14$^+$CD16$^-$BDCA1$^-$ monocytes (see 4.2.6) were cultured at a concentration of 5x10^4 cells in 100µl and stimulated with 10µg/ml Pam3CSK4, 10µg/ml Poly I:C, 100ng/ml LPS or 5µg/ml R848 or left unstimulated for 24h. 1x10^5 naïve CD4$^+$ T cells were added to each well and cultured for 11 days. On days 4 and 7, cells were splitted and supplemented with 4ng/ml IL-2 (Miltenyi Biotec). On day 9, cells were washed and kept deprived of IL-2. On day 11, cells were restimulated using 1µg/ml Ionomycin and 50ng/ml PMA (both Sigma) for 7h. During the last 5h, 2µg/ml Brefeldin A were added and cells were subsequently stained with CD3-Vioblue, CD4-Viogreen, IL-17-APC, IL-10-PE and IFNγ-FITC following fixation and permebilization. Cells were acquired on a MACSQuant analyzer and analyzed using FlowJo 10.0.6.

4.3.8 Statistical analysis

Paired students' t tests (with a 95% confidence interval), ANOVA with Bonferoni post-tests were performed using Prism 4.0 and 6.0 (GraphPad Software). P-values were defined as *p < 0.05; **p < 0.01; and ***p < 0.001.

4.3.9 May-Giemsa/Grünwald staining

1x10^5 purified BDCA1$^+$CD14$^-$CD19$^-$ DCs, BDCA1$^+$CD14$^+$CD19$^-$ pro-iDCs and CD14$^+$CD16$^-$BDCA1$^-$ monocytes (see 4.2.7) were immobilized on microscopy glass slides using cytospin technique. Briefly, 150µl cell suspension was applied to a reservoir that was mechanically adhered to the slide. After centrifugation at g for min, the reservoir was carefully removed and the slide was dried for 15min at RT. The slide was covered with 2.5ml May-Grünwald solution for 3min, carefully washed with dH_2O, covered with 5ml Giemsa (Merck Millipore) solution for 15min and washed again. Slides were dried overnight at RT and images were taken on a Zeiss Axiostar plus microscope at 100x magnification using a Canon G7 camera. Image processing was performed using Graphic Converter software (Lemke Software GmbH, version 8.5.1).

4.4 Patients

Blood samples from 8 patients suffering from rheumatoid arthritis (RA) at a mean age of 51 years ± 13.6 years (SD) were analyzed for frequencies of BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ pro-iDCs. Samples were analyzed as described in chapter 7.2.9. 7 individuals were seropositive for rheumatoid factor and/or anti-cytoplasmic antibodies (ACPA). The criteria of the American

College of Rheumatology (ACR) (revised in 2010) and the European League Against Rheumatism (EULAR)[209,210] were fulfilled by all patients. Patients were negative for active tuberculosis and hepatitis and none had severe co-morbidities. Local ethics committees approved this study. The Department of Rheumatology and Clinical Immunology at the Charité Universitätmedizin Berlin randomly provided patients who agreed to these studies by a written informed consent.

4.5 MELC

The method to prepare images showing multiple parameters using MELC technology has been described by Eckhardt et al.[211] In brief, a cryotome (Leica CM 3050S, Leica, Wetzlar, Germany) was used to prepare tissue sections of 5 µm. Sections were incubated in aceton (Carl Roth) for 10 min at -20 °C and air-dried for 5 min. To avoid unspecific binding of antibodies, the sections were incubated in PBS (PAA, Pasching, Austria) for 5 min and subsequently in 5% NGS/PBS (Dako, Hamburg, Germany) for 30 min at RT. 24 fluorochrome-labeled antibodies and propidium iodide were used. Samples were analyzed on a Leica DM IRE2 using a 20x lens at a numerical aperature of 0.7 (Leica Microsystems, Wetzlar, Germany). A 900 x 900nm^2 area corresponded to 1024 × 1024 pixels. To allow the acquisition of multiple parameters, samples were stained with fluorochrome labeled antibodies, washed, acquired on the microscope with a cooled CCD camera for image acquisition (Apogee KX4, Apogee Instruments) and bleached using the excitation wavelength in repetitive cycles. Automated image processing was performed with a software provided by the former company MelTec GmbH. Images were aligned to a preciseness of ±1 pixel and illumination faults were corrected using flat-field correction.

4.6 In silico methods

4.6.1 RNA preparations and hybridization

RNA from the DC and monocyte samples was extracted using NucleoSpin RNA II (Macherey-Nagel) according to manufacturer's protocol. For amplification, 25ng of each sample RNA were used and subsequently labeled with Cy3 using the Low Input Quick Amp Labeling Kit (Agilent Technologies) according to manufacturer's protocol. Labeled RNA samples were hybridized to an Agilent Whole Human Genome Oligo Microarray (8x60K; Agilent Technologies) for 17h at 65°C and washed with buffer 1 at RT and buffer 2 at 37°C for 1min each and 30s with acetonitrile at RT. Fluorescent signal intensities were quantified employing the Microarray Scanner System (G2505C, Agilent Technologies) and resulting image files were processed using the Agilent Feature Extraction Software (FES 10.7.3.1).

4.6.2 Microarray analysis

Rosetta Resolver software (Rosetta, Inpharmatics, LLC.) was used to obtain raw intensity data from Feature Extraction output files. R/Bioconductor software suites (R version 2.13.1., http://www.R-project.org/[212], http://www.bioconductor.org/[213]) were used for all calculations including background correction among intensity values (Figure 2A) and quantile normalization[214] between arrays (Figure 2B). Signals were considered reliable if p≤0.01 (Rosetta error model[215]). Expression profiles were analyzed by correlation analysis on normalized $\log_2$ intensities and correlation coefficients were displayed in hierarchically clustered matrices using Euclidean distance and average linkage[216]. Statistical differences were evaluated using ANOVA- and Tukey post-hoc tests for multiple group comparisons on normalized $\log_2$ signal intensities, which were subsequently corrected for multiple testing according to Benjamini and Hochberg[217]. Genes were considered differentially expressed between groups of 2 cell populations if the median expression values differed ≥2 fold, p-values ≤0.01 and Tukey p-values ≤0.01 between test and reference group.

As prerequisite for considering a gene as differential expressed between 2 groups, only groups were allowed that had reliable signal intensities for all reporters in the group with higher expression (Rosetta p≤0.01). The data is publicly available via the NCBI Gene Expression Omnibus[218] under the GEO Series accession number GSE54094.

4.6.3 Trend analysis

Genes differentially expressed between BDCA1^{+}CD14^{+} pro-iDCs and CD14^{+}CD16^{-}BDCA1^{-} monocytes (see tables 3 and 4; p-value ≤ 0.01, Tukey p-value ≤0.01, and fold change ≤-2 (green circles) or ≥2 (red circles)) were considered for trend analysis. Correlation coefficients were obtained from pairwise comparisons of median $\log_2$ signal intensities from BDCA1^{+}CD14^{+} pro-iDCs against CD14^{+}CD16^{-}BDCA1^{-} monocytes, BDCA1^{+}CD14^{-} DCs, BDCA3^{+}CD14^{-} DCs, imoDCs and mmoDCs, respectively.

4.6.4 Expression profiles for specific markers of dendritic cells and monocytes

Genes with higher expression in BDCA1^{+}CD14^{+} pro-iDCs over CD14^{+}CD16^{-} monocytes were analyzed for annotation enrichment using TreeRanker software (Miltenyi Biotec). After subtraction of the expected background, enriched terms were evaluated using Fisher's exact test and subsequently corrected for multiple testing[217]. Annotations were obtained from sources as described by Barral et al.[219] These typically included GeneOntology (GO, www.geneontology.org), signaling pathway membership, sequence motifs, literature keywords and cell-specific marker genes. Genes selected according to significant enrichments in DC or monocyte specific markers were hierarchically clustered (Euclidean distance, complete linkage method[220]) and displayed in a heatmap image using TM4 suite[221,220] (MeV 4.8.1).

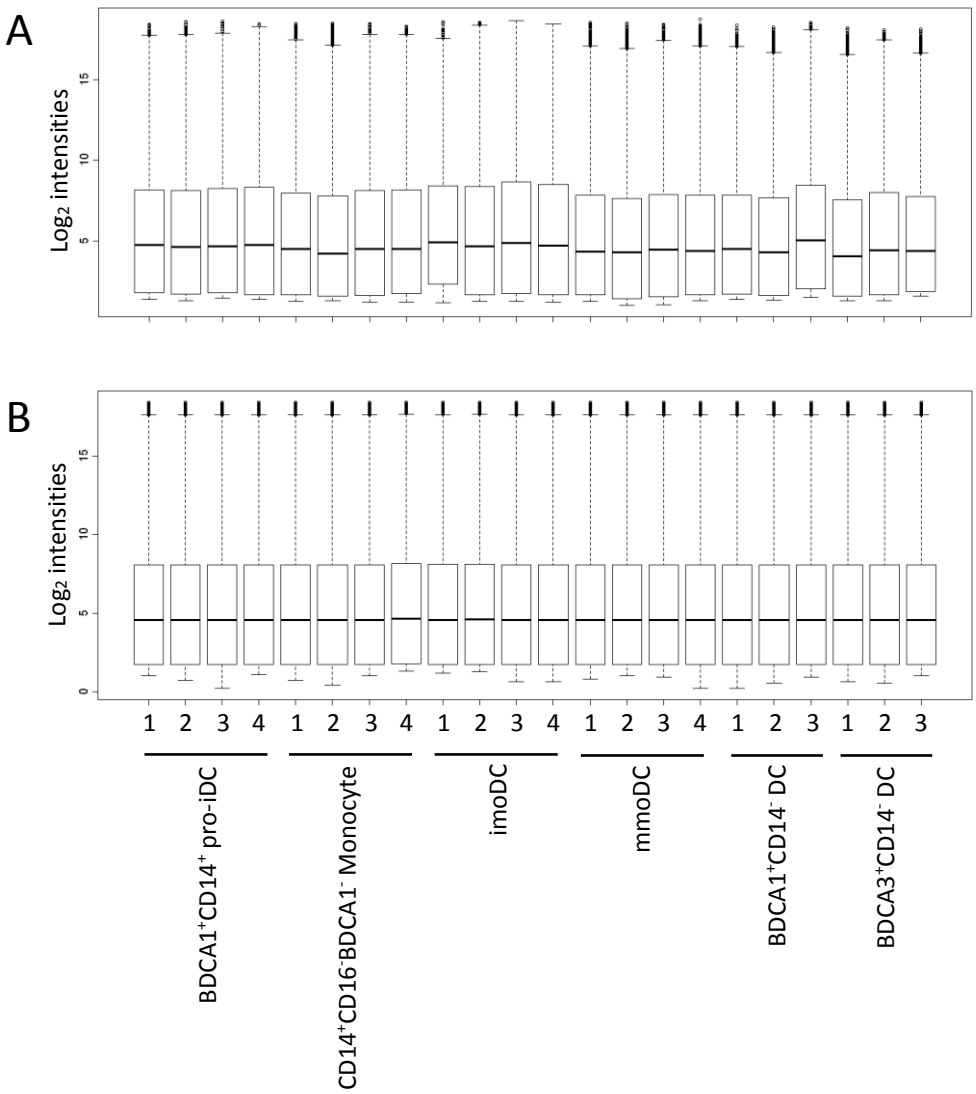


Figure 2 Boxplots of log_2 transformed signal intensities

Boxplots of raw (A) and quantile normalized (B) log_2 intensities of indicated samples obtained from Human Genome Oligo Microarrays 8X60K (Agilent Technologies). The mark within the box indicates the median, the lower and upper boundaries represent the lower and upper quartile, respectively. The whiskers indicate 1.5 fold of the interquartile range and circles indicate outliers. (imoDC, immature monocyte derived dendritic cell; mmoDC, mature monocyte derived dendritic cell)

5 Results

5.1 Phenotypic characterization of the BDCA1$^+$CD14$^+$ cell subset

A decade ago, the expression of BDCA1 (CD1c) was described on CD3$^-$, CD16$^-$, CD19$^-$, CD56$^-$, CD11c$^+$ and HLA-DR$^+$ myeloid dendritic cells (DCs) in human peripheral blood[222]. However, BDCA1$^+$ DCs were phenotypically not homogenous, as a substantial proportion of the cells expressed the monocyte marker CD14 at different levels, indicating similarities to monocytes. To date the CD14-expressing BDCA1$^+$ cells were not further characterized so it remained unexplored whether these cells belong to the DC-lineage or rather resemble monocytes. Within the CD14$^+$ fraction two different subsets could be identified, one expressing high levels of CD14 (Figure 3 gate C), termed BDCA1$^+$CD14$^+$ cells and an additional subset that expressed intermediate levels of CD14, termed BDCA1$^+$CD14^{int}, covering the expression range between the CD14$^-$ and CD14$^+$ subset (Figure 3, gate B). In the present work the BDCA1$^+$CD14$^+$ cell subset is characterized. As a first step to shed light on the cellular affiliation, expression levels of various cell surface markers on BDCA1$^+$CD14$^+$ cells were compared to BDCA1$^+$CD14$^-$ DCs and monocytes. Due to the low expression level of BDCA1 on the BDCA1$^+$CD14$^+$ cells a direct gating on BDCA1$^+$CD14$^+$ cells and BDCA1$^-$CD14$^+$ monocytes was not feasible. For this reason, BDCA1$^+$ and BDCA1$^-$ cells were separated by magnetic cell sorting (MACS) prior to the flow cytometric analysis. To do so, a two-step strategy was chosen. In the first step human peripheral blood mononuclear cells (PBMCs) were depleted for B cells, which partly also express BDCA1, using CD19 conjugated magnetic beads (Miltenyi Biotec). In the second step the CD19$^-$ fraction was separated in a BDCA1$^+$ fraction containing the CD14$^-$ (Figure 3, gate A) and CD14$^+$ subsets (Figure 3, gate B + C) and a BDCA1$^-$ fraction containing CD14$^+$CD16$^-$ monocytes (Figure 3, gate D). Peripheral blood BDCA1$^+$ cells from the enriched fraction (the sum of cells contained in gates A + B + C) and magnetically purified CD14$^+$CD16$^-$ monocytes (Figure 3, gate D) were then analyzed for the expression of further cell surface markers (Figure 4). In experiments in which single BDCA1$^+$ populations were required, CD19$^-$BDCA1$^+$ cells were flow sorted into BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14^{int} and BDCA1$^+$CD14$^+$ cells (Figure 4, gates A, B and C). Expression levels of these markers were then compared between the BDCA1$^+$ subsets and CD14$^+$CD16$^-$ monocytes. The chemokine receptor CCR5, which is involved in tissue inflammation[223], was highly expressed on BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$ monocytes compared to BDCA1$^+$CD14$^-$ DCs whereas CLA, which mediates homing to the skin[224], was

strongly upregulated on BDCA1$^+$CD14$^-$ DCs. CCR2, CCR7 and CX_3CR1 showed similar expression levels on all three subsets. In contrast, expression of CXCR4, a chemokine receptor that was reported to maintain the long-term CD4 and CD14 expression in monocytes/macrophages but not DCs[225], clearly segregated BDCA1-expressing cells from CD14$^+$CD16$^-$ monocytes. CXCR4 was expressed at comparable levels on BDCA1$^+$CD14$^+$ cells and BDCA1$^+$CD14$^-$ DCs and significantly lower when compared to CD14$^+$CD16$^-$ monocytes. BDCA1$^+$CD14$^+$ cells showed the most activated phenotype as depicted by the expression of molecules involved in T cell stimulation: HLA-ABC, CD40, CD54 and CD86 were slightly higher regulated on BDCA1$^+$CD14$^+$ cells compared to BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes. High expression levels of HLA-DR, which is a hallmark of dendritic cells[226,227], was found 8-fold upregulated on BDCA1-expressing cells when compared to CD14$^+$CD16$^-$ monocytes. CD80, CD83, CD274, CD275 and CD276, also involved in DC-T cell interaction[130,228-230] were almost not detectable on all subsets tested. Fc receptors including CD32 and CD64 were higher expressed on BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$ monocytes compared to BDCA1$^+$CD14$^-$ DCs, whereas all subsets lacked CD16. In contrast, FcεR1α was regulated in an opposite way, being highest expressed on BDCA1$^+$CD14$^-$ DCs. All cells were almost negative for the macrophage marker[231] CD68, SLAN-DC marker[232] SLAN, macrophage mannose receptor (CD206) and Langerhans cell marker[233] CD1a. BDCA1$^+$CD14$^+$ cells expressed the highest levels of CD163, a marker predominantly expressed on monocytes and macrophages[234]. Several surface markers showed a gradual increase of expression levels from BDCA1$^+$CD14$^-$ DCs to BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$ monocytes. Molecules including CD11b, the scavenger receptor CD36[235] and the inhibitory receptor CD172a[236,237] followed this pattern. As indicated in Figure 4, the BDCA1 expressing CD14$^-$ and CD14$^+$ cells might be present at different numbers. The comparison of the frequencies of BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ cells from whole blood in the BDCA1 enriched PBMC fraction revealed a ratio of 1:3.8. In contrast, the percentages from perfused bone marrow cells, which were analyzed in the same way, showed an inverted ratio of 2.3:1 (Figure 5). Taking together, except for few markers, the expression levels of markers on BDCA1$^+$CD14$^+$ cells was between the levels observed for BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes. BDCA1$^+$CD14$^+$ cells thereby showed stronger phenotypic similarity to CD14$^+$CD16$^-$ monocytes, however, expression levels of several markers were reminiscent of activated APCs, indicating that these cells may represent a developmental stage between monocytes and DCs. This was also reflected by morphological appearance of flow sorted BDCA1$^+$CD14$^+$

cells, which combined characteristics of both monocytes (lobulated nuclei) and DCs (short cell processes; Figure 6).

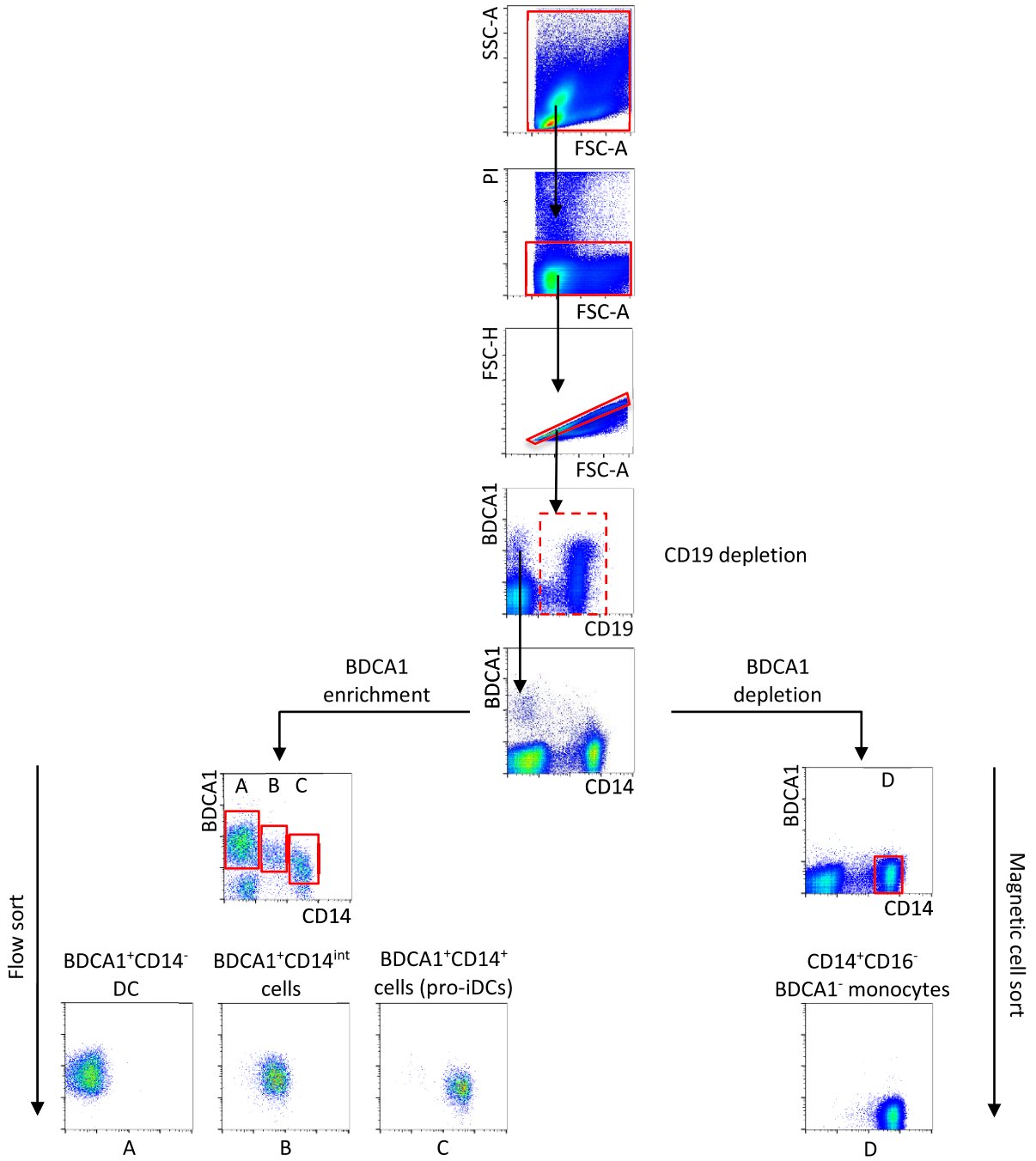


Figure 3 Separation strategy for CD19$^-$BDCA1$^+$ cells and CD14$^+$CD16$^-$BDCA1$^-$ monocytes from PBMCs
In order to isolate CD19$^-$BDCA1$^+$ cells and CD14$^+$CD16$^-$BDCA1$^-$ monocytes, B cells were magnetically depleted from PBMCs using CD19-microbeads (MB). Cells were further separated into CD19$^-$BDCA1$^+$ and CD19$^-$BDCA1$^-$ cells using BDCA1-MB for magnetic separation (gates A-C). The latter fraction was further magnetically separated into CD14$^+$CD16$^-$ monocytes using CD14-MB (Gate D). Cells were stained for CD14, CD19 and BDCA1. Single viable cells from the BDCA1 enriched fraction were flow sorted into BDCA1$^+$CD14$^-$ DC (gate A), BDCA1$^+$CD14^{int} cells (gate B) and BDCA1$^+$CD14$^+$ cells (gate C).

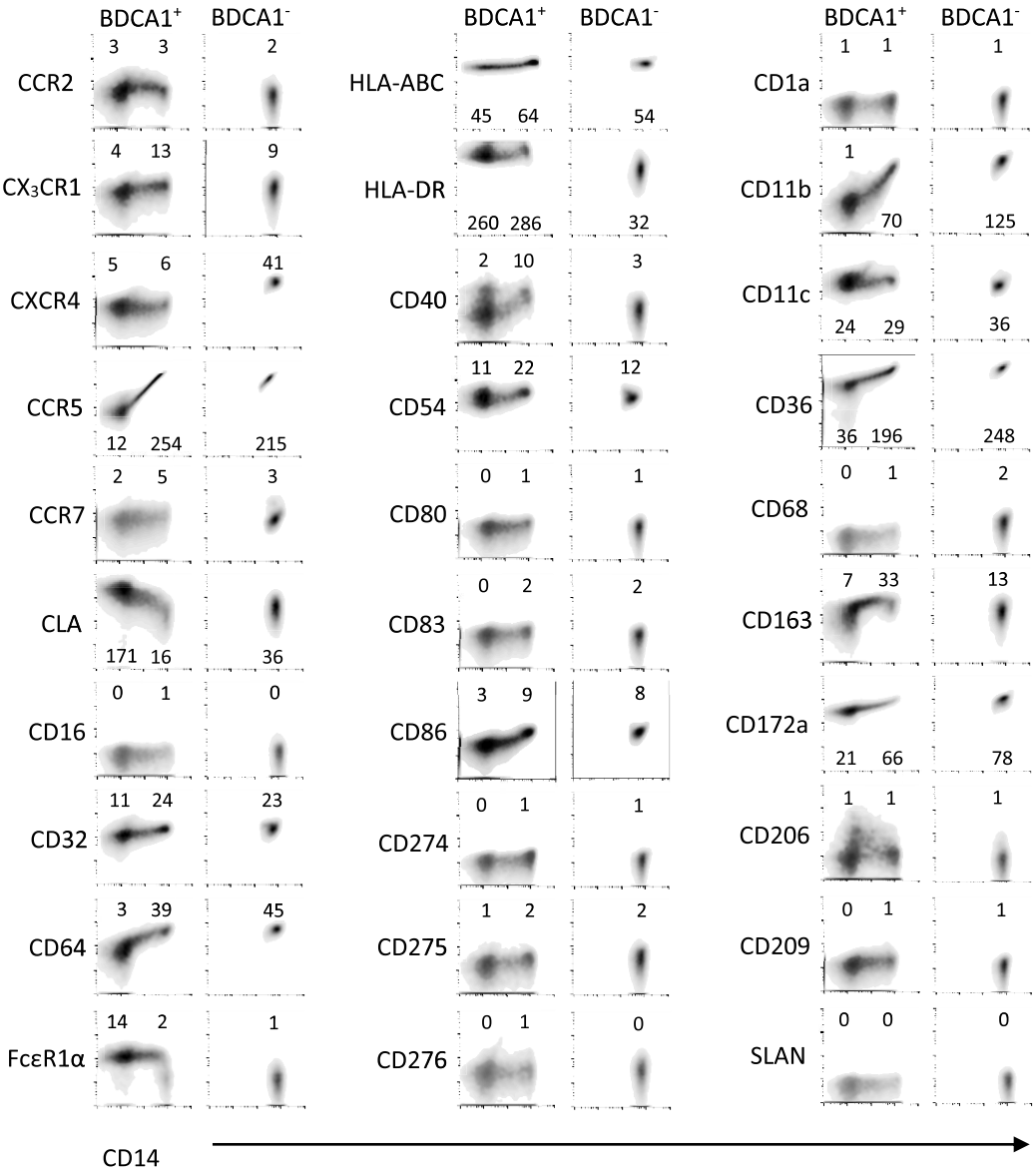


Figure 4 Expression analysis of different cell surface markers on BDCA1$^+$CD14$^-$ DC, BDCA1$^+$CD14^{int} cells, BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$BDCA1$^-$ monocytes

BDCA1$^+$ cells and CD14$^+$CD16$^-$BDCA1$^-$ monocytes were magnetically enriched as described in figure 3 and stained for different cell surface markers. Numbers in each plot represent average median values from 5 donors of indicated markers.

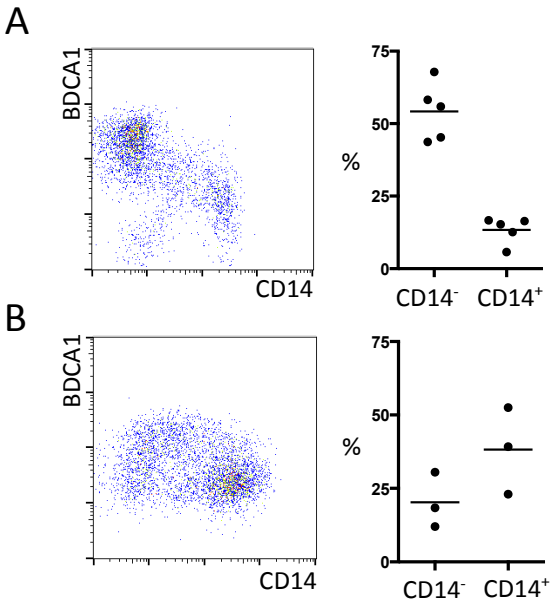
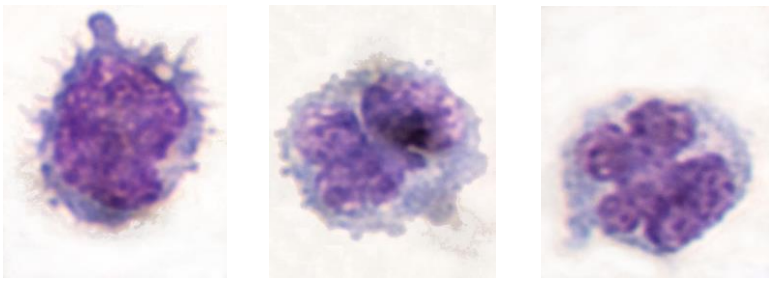


Figure 5 Frequencies of BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ cells in the BDCA1 enriched fraction from human blood and perfused bone marrow cells
The representative density plot shows magnetically enriched BDCA1$^+$ cells from PBMC (A) and perfused bone marrow cells (B). Percentages of BDCA$^+$CD14$^-$ DCs (CD14$^-$) and BDCA1$^+$CD14$^+$ cells (CD14$^+$) are indicated in the chart. One representative dot plot is shown.



BDCA1$^+$CD14$^-$ DC | BDCA1$^+$CD14$^+$ cell | BDCA1$^-$ Monocyte

Figure 6 Light microcopy images of flow sorted *May-Grünwald/Giemsa* stained BDCA1$^+$CD14$^-$ DC, BDCA1$^+$CD14$^+$ cell and BDCA1$^-$CD14$^+$BDCA1$^-$ monocyte from human blood

5.2 BDCA1$^+$CD14$^+$ cells produce high amounts of inflammatory cytokines

By phenotypic analysis, BDCA1$^+$CD14$^+$ cells appeared to be very similar to monocytes (Figure 4), however, some markers implied characteristics of activated professional APCs. In addition, elevated expression levels of BDCA1 pointed to an affiliation with BDCA1$^+$ DCs. For further characterization beyond phenotype, levels of secreted cytokines from BDCA1$^+$CD14$^+$ cells were analyzed after TLR and cytosolic DNA PRR stimulation and compared to CD14$^+$CD16$^-$ monocytes, BDCA1$^+$CD14$^-$ DCs and BDCA3$^+$CD14$^-$ DCs. Freshly isolated BDCA1$^+$CD14$^+$ cells diverged from the other APCs by the secretion of vast amounts of the inflammatory cytokines IL-1β, IL-6 and TNFα and significant levels of IL-10, IL-12 and IL-23 already in the absence of stimulation (Figure 7 A). None of the other *ex vivo* cell subsets analyzed produced cytokines without stimulation. To compare activated APCs in terms of cytokine secretion, a TLR8 ligand, R848 was applied to all subsets. TLR8 is a germ line encoded intracellular PRR sensing viral infection[43] and expressed in BDCA1$^+$CD14$^-$ DCs, BDCA3$^+$CD14$^-$ DCs[143], CD14$^+$CD16$^-$ monocytes and moDCs[238]. After R848 stimulation, all cell types responded with cytokine production, dominated by IL-6 and TNFα, except for BDCA1$^+$CD14$^+$ cells, which principally secreted IL-1β. Compared to CD14$^+$CD16$^-$ monocytes, BDCA1$^+$CD14$^-$ DCs and BDCA3$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells revealed a pro-inflammatory character by an over 10 fold higher inflammatory IL-1β secretion, indicating a stronger inflammasome activity. In addition, R848 stimulation lead to decreased levels of regulatory IL-10 when compared to untreated BDCA1$^+$CD14$^+$ cells (Figure 7 B). BDCA1$^+$CD14$^+$ cells increased in secretion of T_H1 and T_H17 cell promoting cytokines IL-12 and IL-23 and thus were comparable to cDCs rather than to CD14$^+$CD16$^-$ monocytes, as these produced lower levels of IL-12 and no IL-23. BDCA3$^+$CD14$^-$ DCs did not produce any IL-1β but were similar to BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ cells by the secretion of higher levels of IL-23.

To distinguish cells by means of responsiveness to various pathogenic structures, a panel of TLR agonists was applied to all cell subsets and analyzed for cytokine secretion. Amounts of secreted cytokines of PRR triggered cells were set in relation to untreated cells. Whereas CD14$^+$CD16$^-$ monocytes, BDCA1$^+$CD14$^-$ DCs and BDCA3$^+$CD14$^-$ DCs induced cytokine secretion following stimulation, BDCA1$^+$CD14$^+$ cells basically only altered the expression profile following Pam3CSK4 treatment by reduction in IL-10 and IL-23 levels and R848 stimulation as already described (Figure 8).

Except for Flagellin and CpG-B stimulation, any TLR stimulation resulted in a homogenous cytokine induction in monocytes for most cytokines, whereas IL-23 was not detected after FSL-1 and R837 treatment. TNFα and IL-6 were highly induced, whereas IL-1β, IL-10 and IL-12 induction was lower and at comparable levels. As monocytes are equipped with TLR5[239] that recognizes Flagellin, the lack of cytokine induction was unexpected. Intracellular poly A:T delivery had no effect as treatment with the transfection reagent alone induced similar levels of cytokines. In line with a broad expression of TLRs in BDCA1$^+$ DC[143], all TLR agonists except for CpG-B led to induction of cytokines. In contrast to the inflammatory cytokine profile of BDCA1$^+$CD14$^+$ cells, the response from BDCA1$^+$CD14$^-$ DCs was dominated by regulatory IL-10 induction followed by TNFα, IL-6, IL-1β and IL-12, whereas IL-23 was only induced by poly I:C and R848 stimulation. However, BDCA1$^+$CD14$^+$ cells resembled rather BDCA1$^+$CD14$^-$ DCs and BDCA3$^+$CD14$^-$ DCs than CD14$^+$CD16$^-$ monocytes, since cytokine secretion was principally induced by R848 stimulation, whereas CD14$^+$CD16$^-$ monocytes responded to any TLR stimulation with homogenous levels of cytokines. Moreover, induction of the polarizing cytokines IL-12 and IL-23 was mainly induced by R848 in BDCA3$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ cells, whereas IL-12 was reduced or not observable in both cell types after Pam3CSK4, when compared to most other stimulations. BDCA1$^+$CD14$^-$ DCs were responsive to poly A:T stimulation, as this induced the highest levels of IL-23 besides TNFα, IL-1β, IL-6 and IL-10, implying the presence of functional cytoplasmatic dsDNA receptors. The amount of cytokines induced in BDCA3$^+$CD14$^-$ DCs after stimulation with TLR agonists was the lowest when compared to the other APCs tested. TNFα and IL-6 were present after every treatment and surprisingly CpG-B induced significant levels of IL-6 and IL-23, which can be addressed to DNA receptors apart from TLR9, as BDCA3$^+$CD14$^-$ DCs do not express TLR9[143]. In contrast to BDCA1$^+$CD14$^-$ DCs, IL-23 was induced by Pam3CSK4 and R837 besides R848 in BDCA3$^+$CD14$^-$ DCs. Latter induced the highest levels of cytokines and exclusively triggered IL-10 and IL-12 secretion (Figure 8). By comparison, every subset could be ascribed a unique cytokine expression profile following TLR stimulation. However, despite the differences, BDCA1$^+$CD14$^+$ cells rather resembled BDCA1$^+$CD14$^-$ DCs in responsiveness to TLR stimulation than monocytes, as latter showed very similar responses to basically all TLR agonists and former were principally triggered by R848 stimulation. However, a major difference lies in the types of secreted cytokines in between BDCA1$^+$CD14$^+$ cells and BDCA1$^+$CD14$^-$ DCs, as BDCA1$^+$CD14$^+$ cells predominantly secreted inflammatory cytokines whereas BDCA1$^+$CD14$^-$ DCs showed high

induction of IL-10. BDCA3$^+$CD14$^-$ DCs generally responded with low levels of cytokines but were like BDCA1$^+$CD14$^+$ cells and BDCA1$^+$CD14$^-$ DCs and unlike CD14$^+$CD16$^-$ monocytes most responsive to R848 stimulation. However, the high secretion of cytokines from untreated BDCA1$^+$CD14$^+$ cells and low responsiveness to TLR ligands was surprising and indicated an already TLR like activation, which required further investigation.

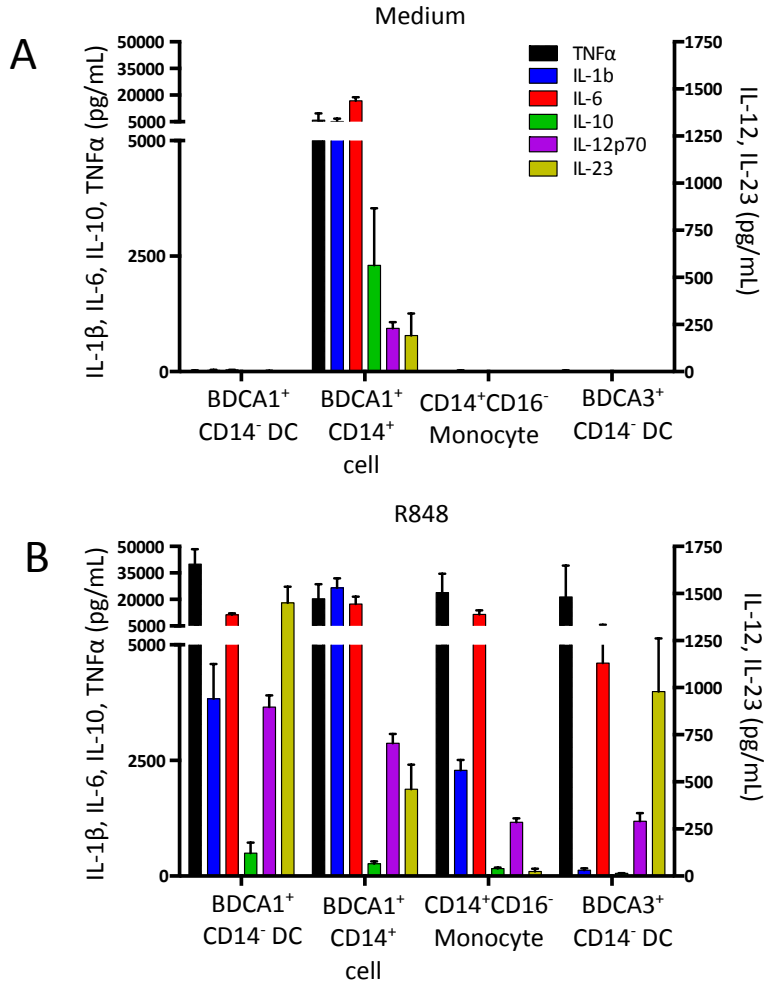


Figure 7 *Ex vivo* BDCA1$^+$CD14$^+$ cells secrete high amounts of inflammatory and polarizing cytokines
Flow sorted BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells, CD14$^+$CD16$^-$BDCA1$^-$ monocytes and BDCA3$^+$CD14$^-$ DCs were cultured for 24h in the absence (A) or presence (B) of R848. Cell free supernatants were harvested and analyzed for cytokine levels of IL-1β, IL-6, TNFα, IL-10, IL-12 and IL-23 by cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA) (n=3). Error bars represent SEM.

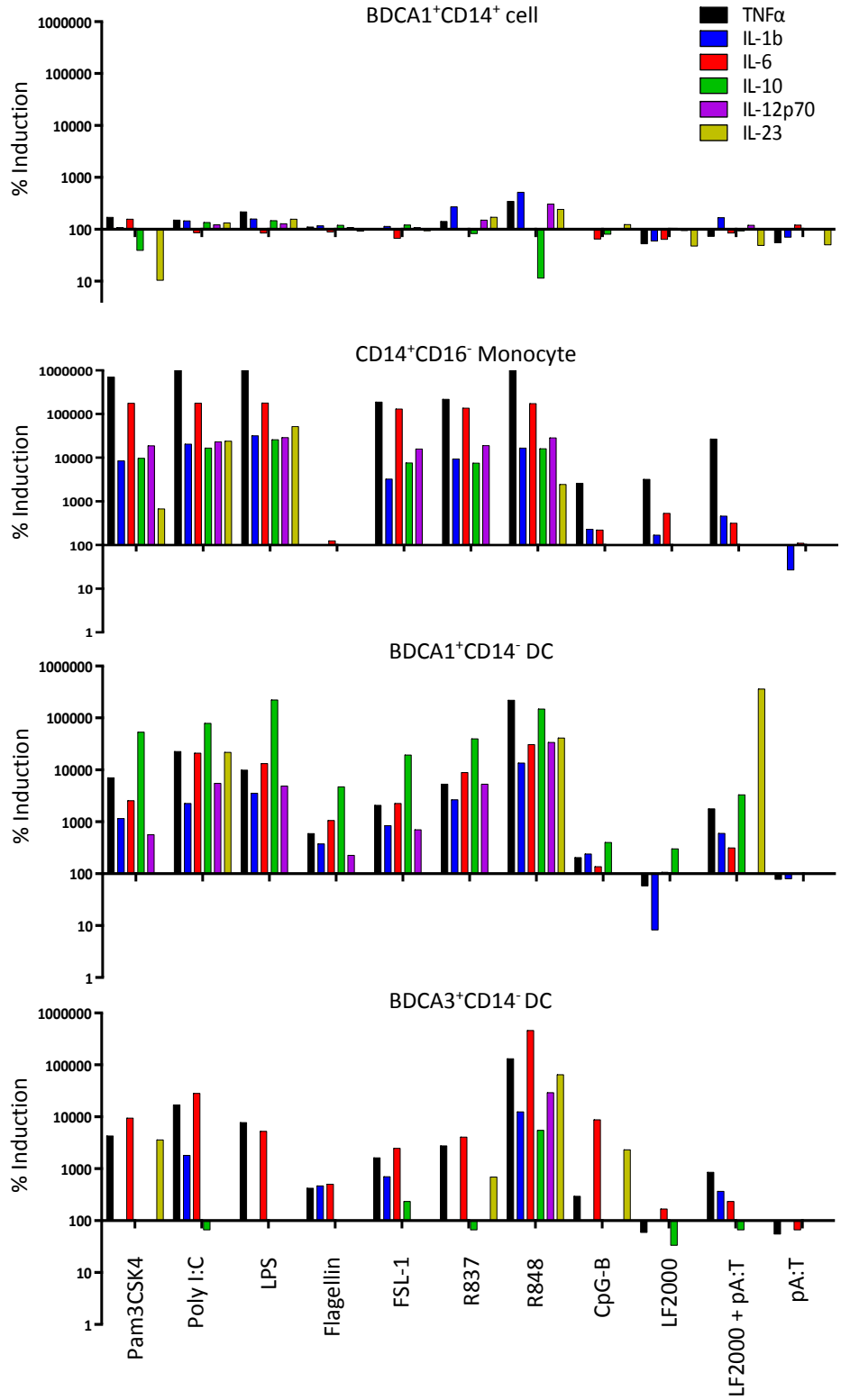


Figure 8 Cytokine secretion profiles of pattern recognition receptor (PRR) stimulated APCs
Flow sorted BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells, CD14$^+$CD16$^-$ monocytes and BDCA3$^+$CD14$^-$ DCs were cultured in the presence of different TLR agonists and cytosolic delivered poly A:T sequence dsDNA using Lipofectamin 2000 (pA:T + LF2000). After 24h of stimulation, cell free supernatants were collected and cytokine levels of IL-1β, IL-6, TNFα, IL-10, IL-12 and IL-23 were analyzed by cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA). Shown is the percent change of secreted cytokines of stimulated cells relative to cells cultured in medium alone (n=3).

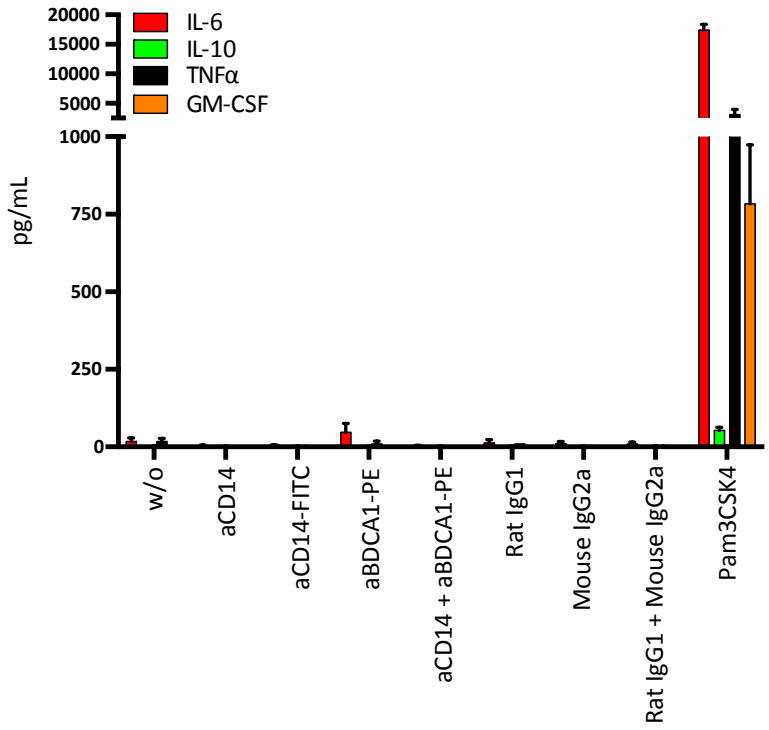


Figure 9 Magnetically enriched BDCA1$^+$CD19$^-$ cells do not secrete cytokines without stimulation
Magnetically enriched BDCA1$^+$ cells from CD19 depleted PBMCs were cultured in the presence or absence of indicated antibodies or Pam3CSK4 (n=5). After 24h, cell free supernatants were collected and cytokine levels of IL-6, TNFα, IL-10 and GM-CSF were analyzed by cytometric bead array (CBA). Error bars represent SEM.

5.3 Spontaneous cytokine release is due to ROS and appears after flow sorting

The spontaneous release of cytokines from flow sorted BDCA1$^+$CD14$^+$ cells was a surprising observation and as this was not reported from any other primary blood cell type, the underlying mechanisms were investigated. After magnetic isolation, the *ex vivo* BDCA1$^+$ cell fraction including BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14^{int} cells and BDCA1$^+$CD14$^+$ cells did not produce significant amounts of cytokines (Figure 9) as it was the case for flow sorted BDCA1$^+$CD14$^+$ cells (Figure 7). In addition, none of the antibodies used for flow sorting of BDCA1$^+$CD14$^+$ cells was able to trigger cytokine secretion. The spontaneous release of cytokines after flow sorting from BDCA1$^+$CD14$^+$ cells was unique, as both flow sorted, BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14^{int} cells did not release significant amounts of cytokines without appropriate stimulation (Figure 7 and 10). To address the question, whether the high amounts of cytokines from BDCA1$^+$CD14$^+$ cells were due to the release of pre-synthesized cytokines by cell necrosis occurring during cell culture, freshly isolated BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$ monocytes were stained intracellularly for TNFα and IL-6 (Figure 11).

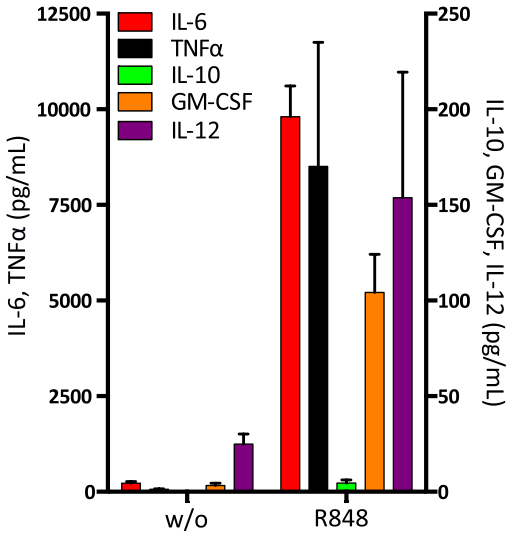


Figure 10 Flow sorted BDCA1^{+}CD14^{int} cells do not secrete cytokines without stimulation
Flow sorted BDCA1^{+}CD14^{int} cells (population shown in figure 4, gate B) from magnetically enriched BDCA1^{+}CD19^{-} cells were cultured in the presence or absence of R848 (n=5). After 24h, cell free supernatants were harvested and cytokine levels of IL-6, TNFα, IL-10, GM-CSF and IL-12 were analyzed by cytometric bead array (CBA) (n=5). Error bars represent SEM.

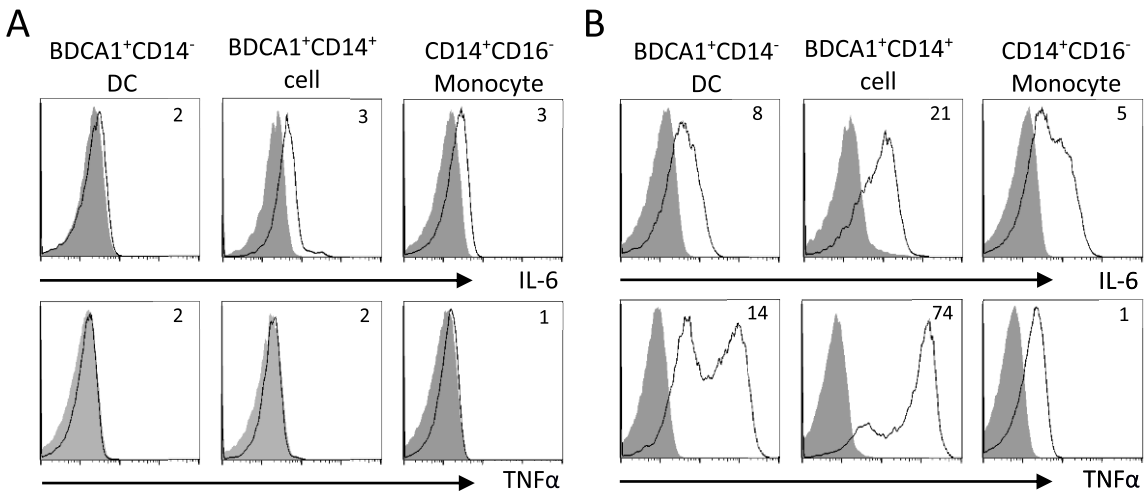


Figure 11 Flow sorted BDCA1^{+}CD14^{+} cells do not contain pre-synthesized cytokines but respond strongly to TLR stimulation
Magnetically isolated BDCA1^{+}CD19^{-} cells containing BDCA1^{+}CD14^{-} DCs and BDCA1^{+}CD14^{+} cells, and CD14^{+}CD16^{-} BDCA1^{-} monocytes magnetically enriched from the BDCA1^{-} fraction from PBMCs were stained for intracellular IL-6 and TNFα before (A) and after 6h culture in the presence of R848 (B) (n=5). Numbers in the histograms indicate median intensity values of a representative experiment.

None of the tested and freshly isolated populations contained any cytokines. Thus cytokines were produced *de novo* when triggered with appropriate PRR agonists (Figure 11). However, the capacity to respond quickly with the secretion of large amounts of cytokines was enhanced in BDCA1^{+}CD14^{+} cells as these accumulated the highest amounts of IL-6 and TNFα when

compared to BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$BDCA1$^-$ monocytes after 6h of culture in the presence of R848. (Figure 11 B). In addition, as mRNA levels of all tested cytokines were comparable in BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells, CD14$^+$CD16$^-$ monocytes and BDCA3$^+$CD14$^-$ DCs, levels of transcripts in BDCA1$^+$CD14$^+$ cells could not explain the rapid and strong cytokine secretion (Figure 12).

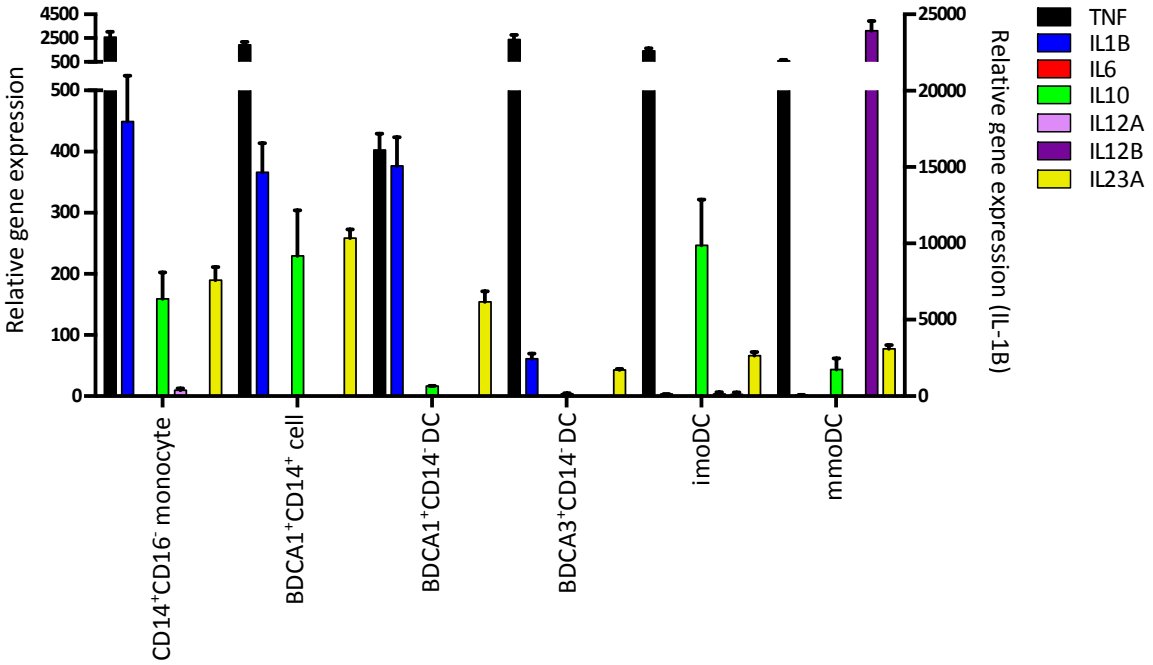


Figure 12 mRNA levels of selected cytokines from non-cultured BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells, CD14$^+$CD16$^-$BDCA1$^-$ monocytes and BDCA3$^+$CD14$^-$ DCs
Relative gene expression levels of indicated cytokines were obtained from normalized whole genome microarray experiments as described in detail in the material and methods section. Samples included flow sorted BDCA1$^+$CD14$^-$ DCs, BDCA3$^+$CD14$^-$ DCs (n=3), CD14$^+$CD16$^-$BDCA1$^-$ monocytes, BDCA1$^+$CD14$^+$ cells and *in vitro* generated immature and mature monocyte derived DCs (imoDC, mmoDC) (n=4). Error bars represent SEM.

BDCA1$^+$CD14$^+$ cells only secreted cytokines in the absence of PRR stimulation after flow sorting (Figure 7). As another candidate mechanism for the spontaneous release of cytokines, the role of reactive oxygen species (ROS) in cytokine production was determined. ROS are able to induce cytokine production[240,241] and flow sorting increases cellular susceptibility to ROS[242]. Thus spontaneous cytokine secretion of flow sorted BDCA1$^+$CD14$^+$ cells could be due to an enhanced susceptibility to ROS induced cytokine secretion. The involvement of ROS appeared feasible since BDCA1$^+$CD14$^+$ cells produced the highest amounts of ROS compared to BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes within 6h cell culture (Figure 13).

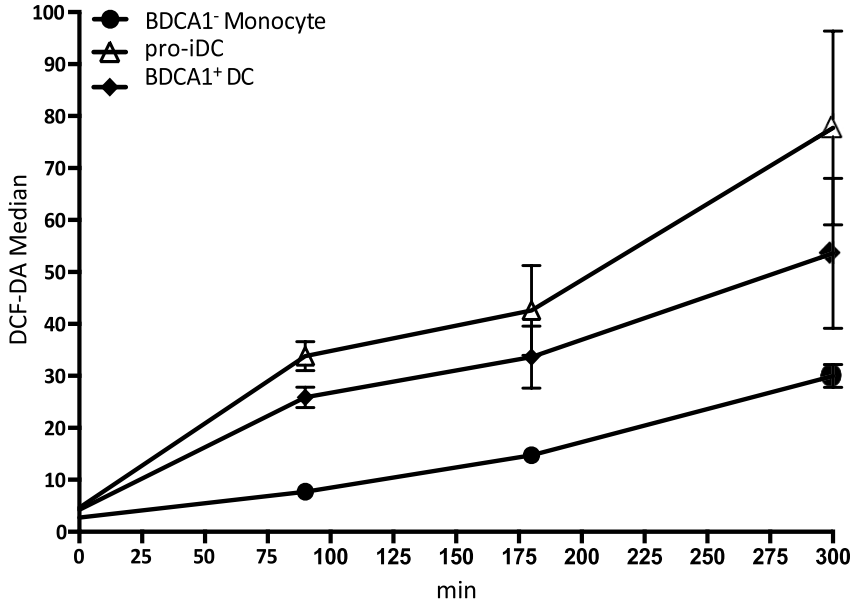


Figure 13 BDCA1^{+}CD14^{+} cells accumulate the highest amounts of reactive oxygen species (ROS)
Magnetically enriched BDCA1^{+} cells and CD14^{+}CD16^{-}BDCA1^{-} monocytes from the BDCA1^{-} fraction of CD19 depleted PBMC were cultured up to 6h. At depicted time points, ROS were measured by the addition of 2',7'-dichlorodihydrofluoresceindiacetate (DCF-DA) 30min prior to assessment by flow cytometry (n=5). Error bars represent SEM.

To elucidate the role of ROS for spontaneous cytokine release, pharmacological ROS inhibitors were applied to cultures of BDCA1^{+}CD14^{+} cells. Diphenyl-iodonium (DPI) was used to inhibit ROS production[243]. Its pharmacological effect comprises the inhibition of ROS generating enzymes including NADPH oxidase, NO synthase, xanthine oxidase[244], and mitochondrial complex I[245]. Indeed, inhibition of cellular ROS generation using DPI[246] abolished IL-1β and IL-6 and downregulated TNFα, GM-CSF and IL-10 secretion (Figure 14). However, DPI may also directly inhibit mRNA transcription of inflammatory cytokines[241]. Therefore, another chemical agent, a scavenger of already produced ROS, N-acetylcysteine (NAC)[247,248] was applied. NAC was similar efficient to DPI in inhibiting cytokine release compared to DPI. As TLR signaling requires ROS[249], treatment with NAC and DPI almost abolished the secretion of R848 induced inflammatory cytokines.

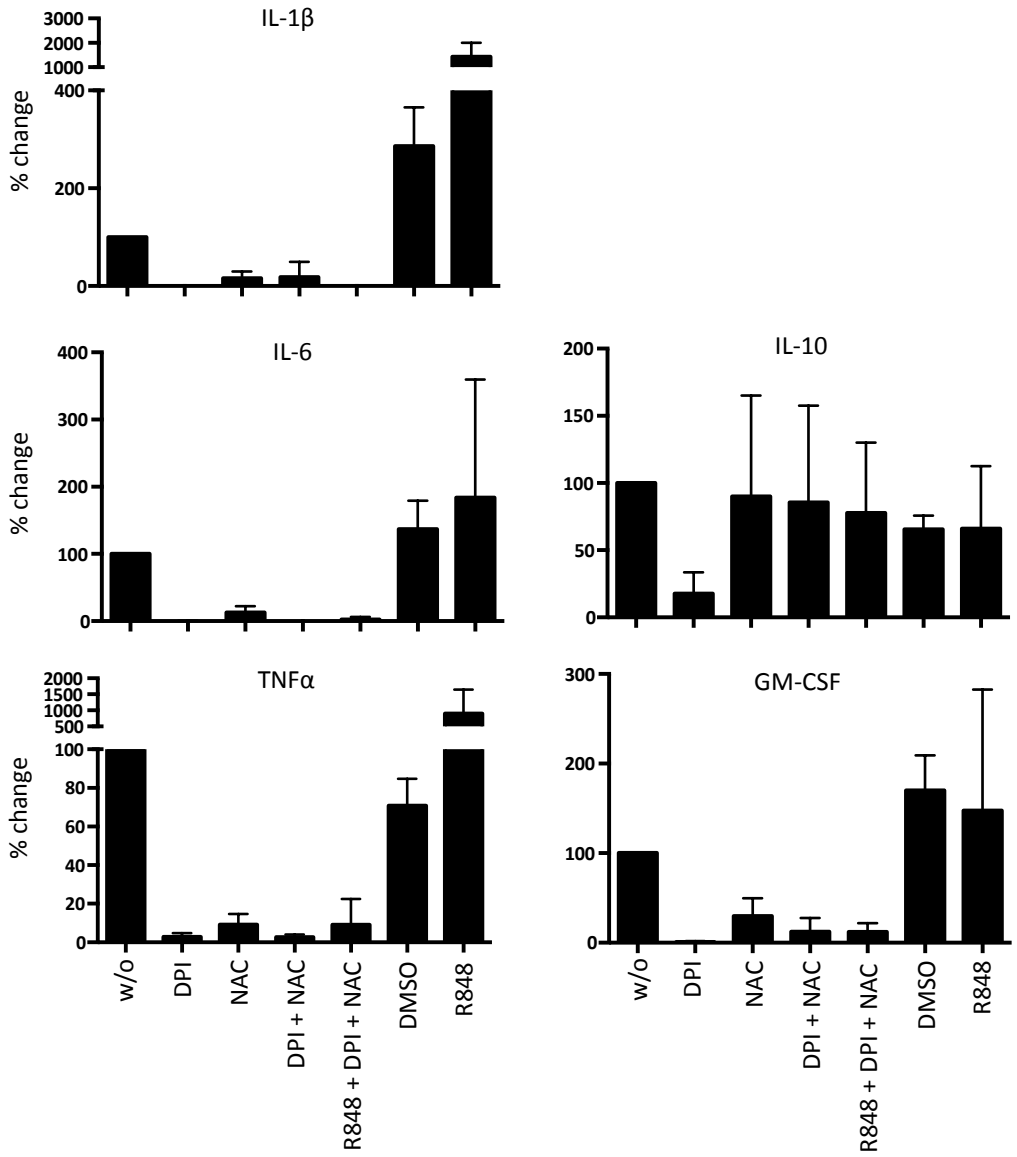


Figure 14 Spontaneous release of cytokines from BDCA1$^+$CD14$^+$ cells is caused by reactive oxygen species (ROS) Flow sorted BDCA1$^+$CD14$^+$ cells were cultured in the presence or absence of R848 and pharmacological ROS inhibitors diphenylene-iodide (DPI) and N-acetylcysteine (NAC). As DPI was dissolved in DMSO, it was included as a control. After 24h, cell free culture supernatants were collected and cytokine levels of IL-6, TNFα, IL-10, GM-CSF and IL-1β were analyzed by cytometric bead array (CBA) and enzyme-linked immunosorbent assay (ELISA), respectively. Percent changes normalized to untreated cells are shown (n=5). Error bars represent SEM.

IL-1β was reported to promote cytokine secretion in mononuclear cells[250,251]. Thus, a possible role for IL-1β induced cytokine secretion was determined for BDCA1$^+$CD14$^+$ cells. After IL-1R blocking using IL-1R antagonist (IL-1RA) flow sorted BDCA1$^+$CD14$^+$ cells secreted reduced amounts of IL-6, TNFα, GM-CSF and IL-10 (Figure 15).

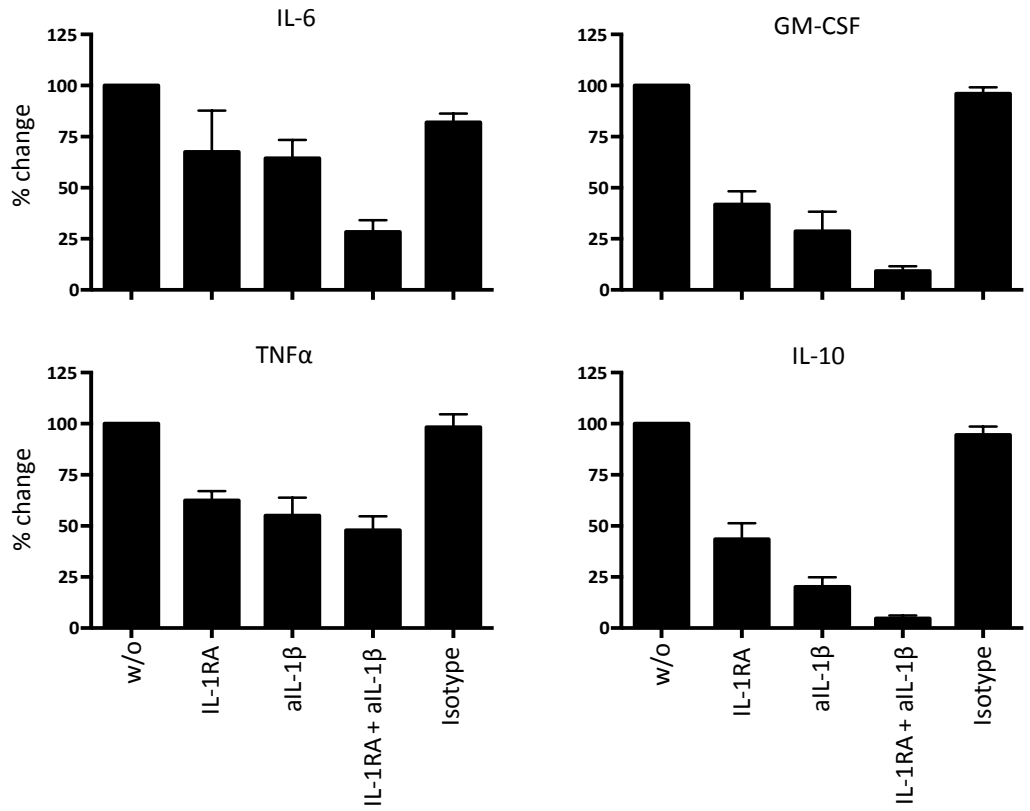


Figure 15 Inflammatory cytokines and IL-10 secretion by BDCA1$^+$CD14$^+$ cells is in part induced by IL-1β
Flow sorted BDCA1$^+$CD14$^+$ cells were cultured in the presence or absence of IL-1 receptor antagonist (IL-1RA), neutralizing IL-1β antibody (aIL-1β) or a respective isotype control. After 24h, cell free supernatants were collected and cytokine levels were analyzed by cytometric bead array (CBA). Percent changes normalized to untreated cells are shown (n=5). Error bars represent SEM.

When IL-1β neutralizing antibodies were additionally applied, levels of cytokines further declined. However, cytokine secretion was not completely abolished in the presence of IL-1RA or neutralizing IL-1β antibody.

As BDCA1$^+$CD14$^+$ cells appear to be prone to ROS generation or susceptible for ROS during cellular stress and ROS mediate the spontaneous release of cytokines, an inhibitory mechanism in blood is likely to exist, as TNFα secretion in blood can induce a health threatening septic shock[252]. Cytokine secretion in response to PAMP in SLAN DCs is inhibited by the presence of erythrocytes, mediated by CD47 binding to CD172a on DCs[236]. In order to check for a similar inhibitory role, pro-iDCs were co-cultured with erythrocytes in the presence or absence of TLR ligands. However, erythrocytes rendered the CD172a expressing (Figure 4) BDCA1$^+$CD14$^+$ cells regulatory by enhanced IL-10 secretion but inflammatory cytokines were

not affected throughout all treatments (Figure 16). Even the decrease of IL-10 after R848 stimulation (Figure 7) was subverted in the presence of erythrocytes.

Thus, these data indicate an enhanced capacity for ROS generation and increased ROS susceptibility of BDCA1$^+$CD14$^+$ cells, which became effective here after flow sorting leading to inflammatory cytokine secretion *in vitro*.

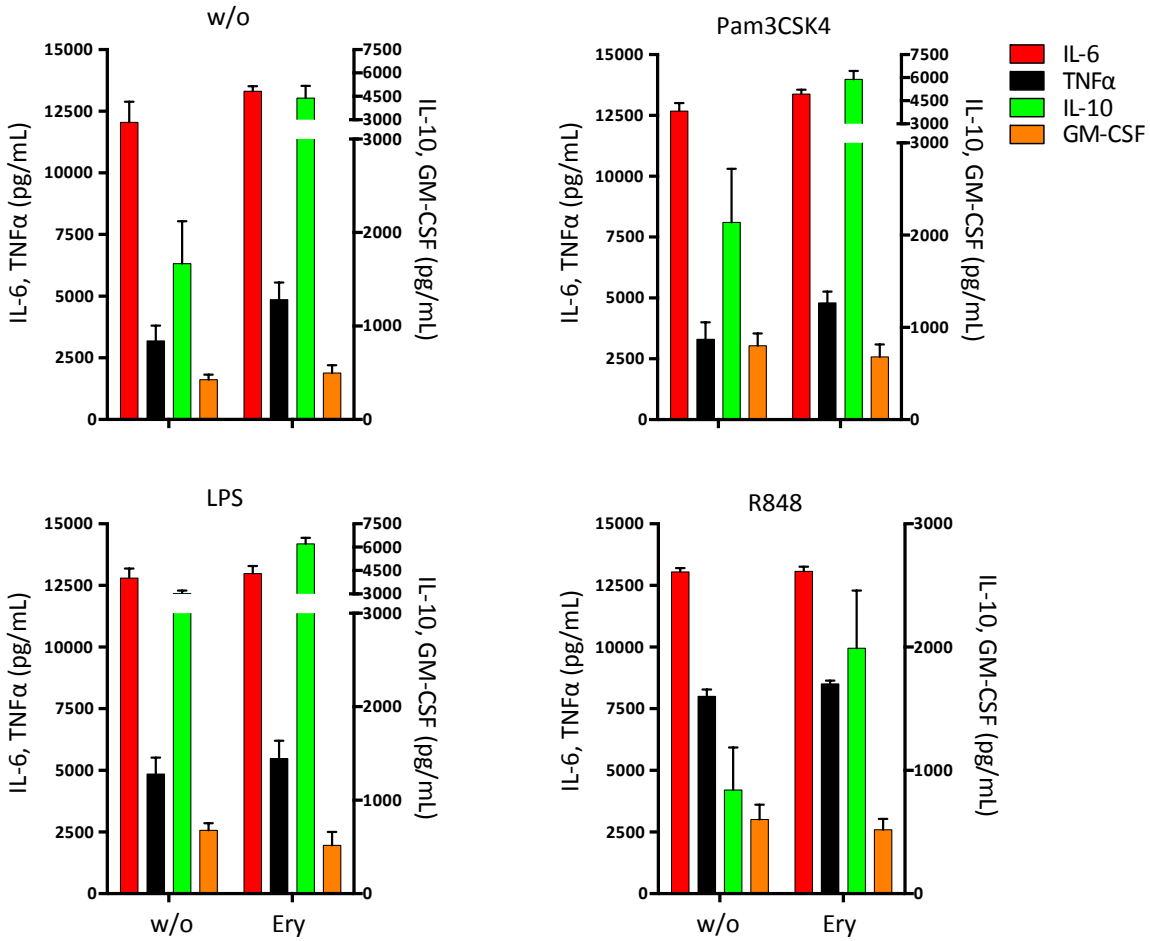


Figure 16 Erythrocytes increase IL-10 production in BDCA1$^+$CD14$^+$ cells *in vitro*
Flow sorted BDCA1$^+$CD14$^+$ cells were cultured in the presence or absence of autologous, antioxidant system and gluthatione peroxidase inactivated erythrocytes (Ery) in 1000 fold excess. After 24h, cell free supernatants were collected and indicated cytokine levels were analyzed by cytometric bead array (CBA) (n=5). Error bars represent SEM.

5.4 BDCA1$^+$CD14$^+$ cells induce T_H17 cell responses

The cytokines secreted by BDCA1$^+$CD14$^+$ cells including IL-1β, IL-6, IL-23 and IL-12 were described to prime and maintain T_H17 and T_H1 cells[78,79,81]. In order to elucidate their polarization capacity, BDCA1$^+$CD14$^+$ cells were co-cultured with allogeneic naïve CD4$^+$ T cells and compared to BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes (Figure 17). According to the spontaneous release of inflammatory and polarizing cytokines, already unstimulated BDCA1$^+$CD14$^+$ cells were effective in priming T_H17 cells. In line with enhanced IL-1β secretion after LPS and R848 stimulation (Figure 4), the percentage of IL-17 producing T cells induced by BDCA1$^+$CD14$^+$ cells slightly increased (Figure 17 A, B). In contrast, BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes required TLR mediated stimulation for T_H17 induction. However, BDCA1$^+$CD14$^+$ cells were superior in priming T_H17 cells, since frequencies of IL-17 producing T cells were generally lower with BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes (Figure 17 B). BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes were similar in T_H17 cell induction throughout all stimulations. All tested subsets induced high frequencies of IFNγ$^+$ T cells even in the absence of stimulation (Figure 17 C). CD14$^+$CD16$^-$ monocytes predominantly induced regulatory IL-10 producing T cells and most significantly after stimulation with TLR agonists (Figure 18 D). According to the induction of high frequencies of T_H17 cells, BDCA1$^+$CD14$^+$ cells were superior in inducing IL-17$^+$ T cells co-producing IFNγ when compared to BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes (Figure 17 E), which was reported be to due to IL-1β[253] and is in line with their superior IL-1β secretion. Among several APCs, the highly inflammatory IFNγ$^+$IL-17$^+$ T cells were reported to be primarily induced by human inflammatory DCs[170] and associated with several autoimmune diseases[171,254-256]. CD14$^+$CD16$^-$ monocytes were superior in inducing IL-10$^+$ T cells co-producing IFNγ (Figure 17 F), which were associated with protection against collateral immune damage but are also involved in chronic infection due to the failure of pathogen elimination[257]. Induction of IL-17$^+$IL-10$^+$ T cells was not observed from any subset (Figure 17 G). Although highly similar in phenotype, BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$ monocytes differ in function: BDCA1$^+$CD14$^+$ cells on the one hand are highly inflammatory cells evinced by their superior capacity to induce IL-17$^+$ T cells whereas CD14$^+$CD16$^-$ monocytes on the other hand are rather regulatory by mainly inducing IL-10$^+$ T cells.

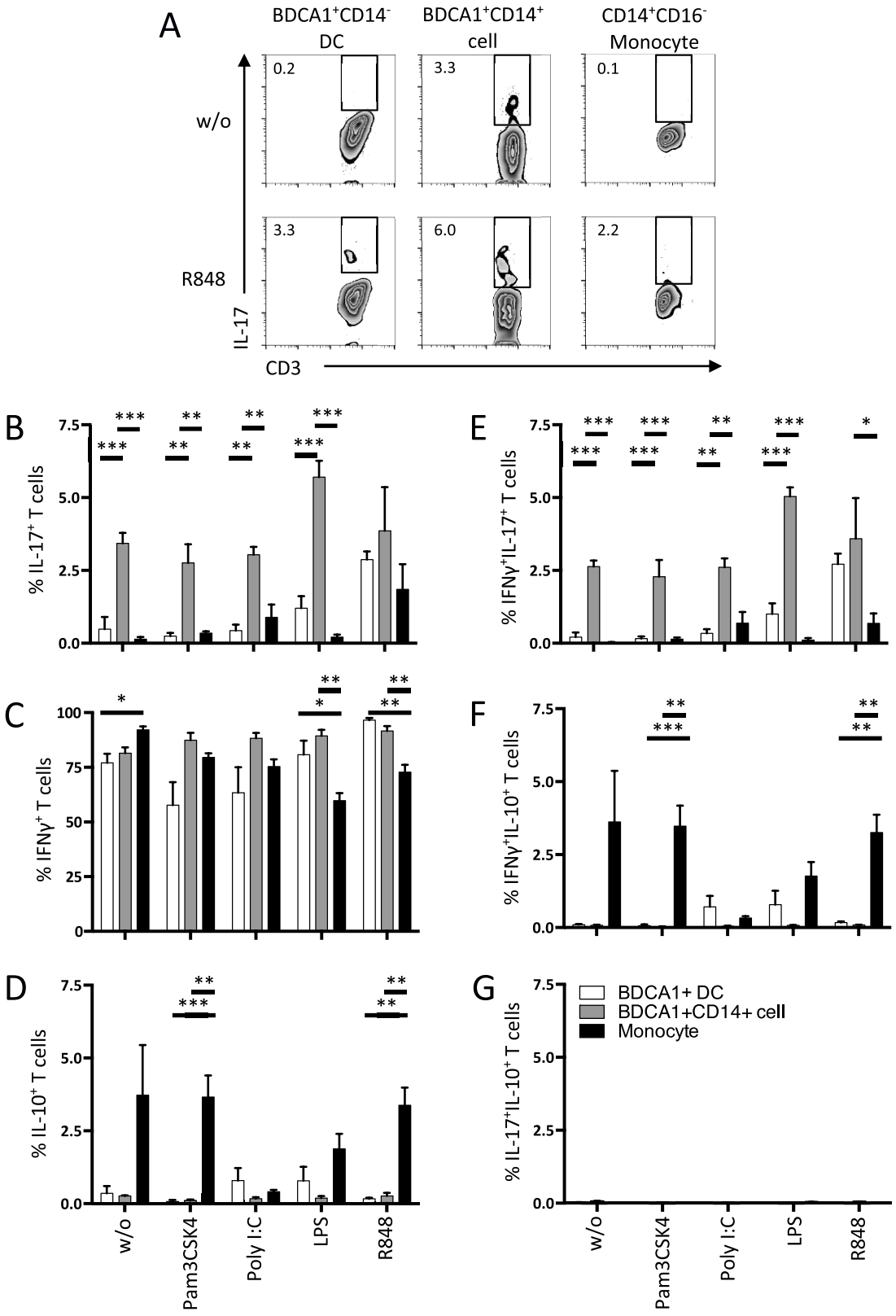


Figure 17 BDCA1$^+$CD14$^+$ cells induce IL-17$^+$ T cells

Flow sorted BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$BDCA1$^-$ monocytes were co-cultured with naïve CD4$^+$ T cells for 11 days in the presence or absence of depicted TLR agonists. T cells were restimulated using PMA/Ionomycin and analyzed for intracellular IL-17, IFNγ and IL-10 expression (A-D). Average percentages of IL-17 (B), IFNγ (C) and IL-10 (D) secreting T cells from 5 experiments are shown. Frequencies of T cells co-expressing of IFNγ/IL-17 (E), IFNγ/IL-10 (F) and IL-17/IL-10 (G) were assessed using boolean 'combination gates'. Error bars represent SEM.

5.5 BDCA1$^+$CD14$^+$ cells functionally resemble DCs

To further distinguish BDCA1$^+$CD14$^+$ cells on functional level from either monocytes or DCs, BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$ monocytes were compared for T cell stimulatory capacity. Therefore, expression levels of molecules involved in T cell activation[130] including CD40, CD80, CD83, CD86, HLA-ABC and HLA-DR were analyzed after treatment with a panel of TLR agonists and intracellularly delivered dsDNA (Figure 18). BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ cells increased expression levels of CD40, CD80 and HLA-ABC the most after treatment with Poly I:C, LPS and R848. CD83 was upregulated by all TLR agonists except for CpG-B in BDCA1$^+$CD14$^-$ DCs. CD83 expression levels on BDCA1$^+$CD14$^+$ cells followed the same pattern although with reduced intensity. While CD86 was mainly induced after Poly I:C, LPS and R837 on BDCA1$^+$CD14$^-$ DCs, induction on BDCA1$^+$CD14$^+$ cells was restricted to LPS stimulation. HLA-DR expression was unaltered throughout all stimulations in all cells types. Intracellular dsDNA stimulation enhanced expression of co-stimulatory molecules and HLA-ABC the most in BDCA1$^+$CD14$^+$ cells and BDCA1$^+$CD14$^-$ DCs when compared to CD14$^+$CD16$^-$ monocytes. Thus, BDCA1$^+$CD14$^+$ cells and BDCA1$^+$CD14$^-$ DCs similarly share the ability to mature upon stimulation with TLR agonists, whereas CD14$^+$CD16$^-$ monocytes remain immature.

Since *ex vivo* BDCA1$^+$CD14$^+$ cells showed an enhanced expression of the T cell stimulatory molecules HLA-DR, CD40, CD54 and CD86 (Figure 4) their function was tested in an allogeneic T cell co-culture (Figure 19). Compared to BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes, BDCA1$^+$CD14$^+$ cells induced the strongest T cell proliferation, which is a hallmark of dendritic cells[258]. However, after treatment with R848, BDCA1$^+$CD14$^-$ DCs were superior compared to BDCA1$^+$CD14$^+$ cells, while monocytes did not induce T cell proliferation at both conditions. The reduced capacity to induce T cell proliferation of R848 stimulated BDCA1$^+$CD14$^+$ cells despite increased levels of co-stimulatory molecules (Figure 18) could be addressed to a reduced viability after R848 stimulation (Figure 20). By contrast, the same stimulation increased survival of BDCA1$^+$CD14$^-$ DCs and probably supported the co-stimulatory molecule dependent T cell proliferative capacity (Figure 19). Thus, BDCA1$^+$CD14$^+$ cells were similar to DCs rather than to monocytes in function by regulation of stimulatory molecules and induction of T cell proliferation. Their inflammatory properties were reminiscent of inflammatory DCs found in inflamed tissues[174], however, in contrast to blood BDCA1$^+$CD14$^+$ cells, inflammatory DCs were only found during inflammation[170,171,193]. Thus, due to their similarity to inflammatory DCs[174]

and pro-inflammatory properties but constant presence in blood, BDCA1$^+$CD14$^+$ cells are therefore further on referred to as pro-inflammatory DCs (pro-iDCs).

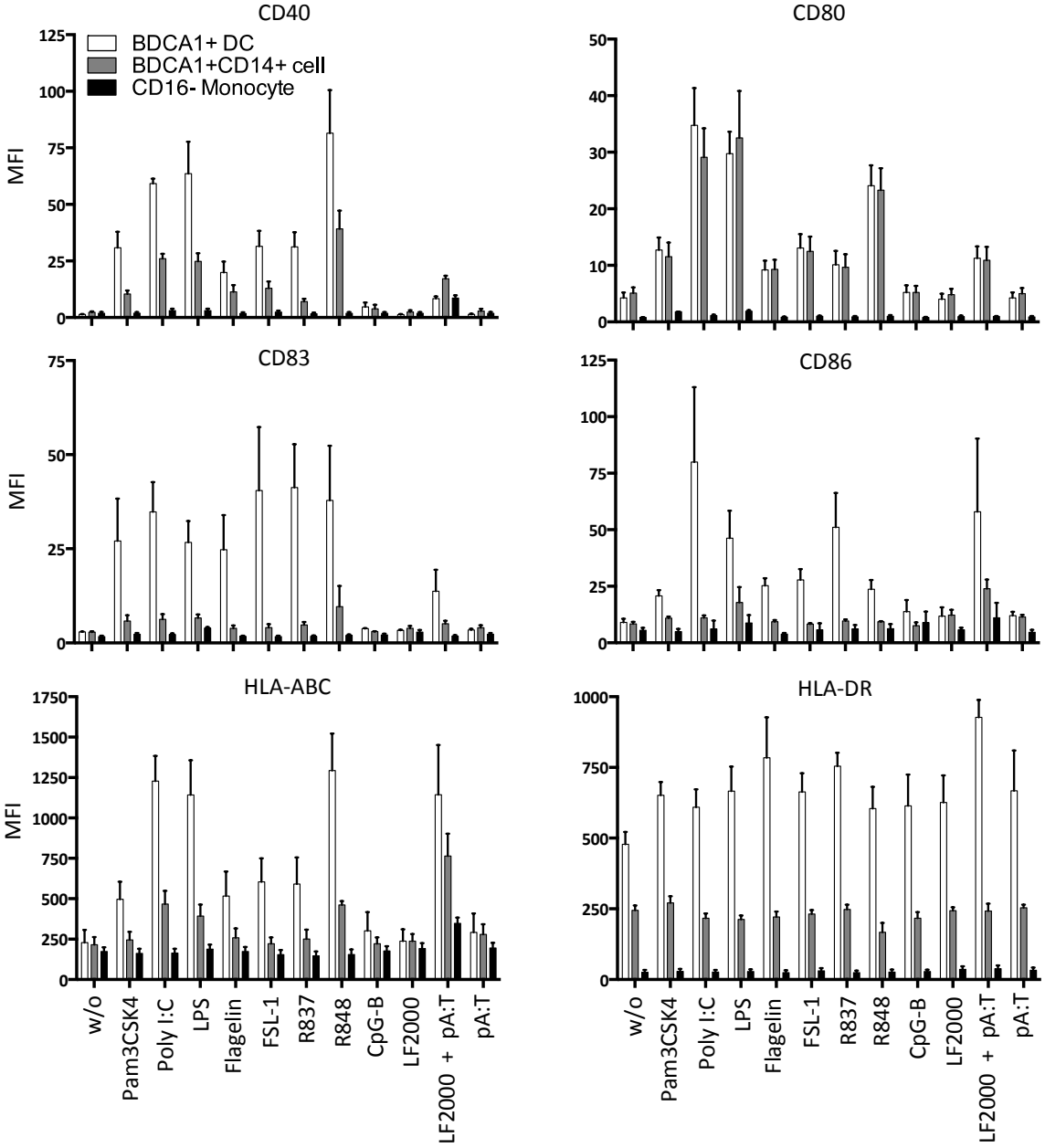


Figure 18 BDCA1$^+$CD14$^+$ cells are similar to BDCA1$^+$CD14$^-$ DCs in terms of T cell stimulatory molecule expression Magnetically enriched BDCA1$^+$CD14$^-$ DCs, BDCA1$^+$CD14$^+$ cells and CD14$^+$CD16$^-$BDCA1$^-$ monocytes from CD19 depleted PBMC were cultured in the presence of depicted TLR agonists and cytosolic delivered poly A:T dsDNA using Lipofectamin 2000 (pA:T + LF2000). After 24h, expression levels of CD40, CD80, CD83, CD86, HLA-ABC and HLA-DR were assessed by flow cytometry. Shown are average median fluorescence intensities of 3 experiments. Error bars represent SEM.

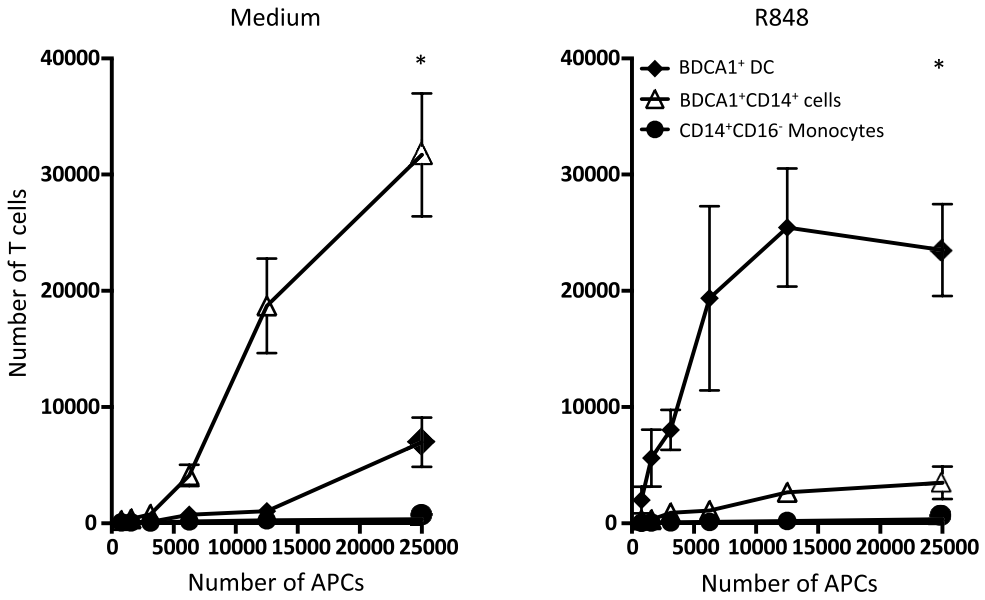


Figure 19 *Ex vivo* BDCA1$^+$CD14$^+$ cells are potent inducers of T cell proliferation
Flow sorted BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ cells and magnetically enriched CD14+CD16$^-$BDCA1$^-$ monocytes were serially diluted and co-cultured with 25000 allogeneic naïve CD4$^+$ T cells in the presence or absence of R848. After 6 days, numbers of T cells according to CD3 expression were assessed using flow cytometry (n=5). Error bars represent SEM.

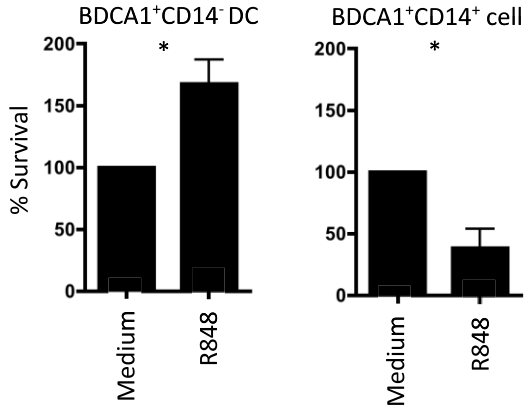


Figure 20 R848 stimulation reduces survival of BDCA1$^+$CD14$^+$ cells
Flow sorted BDCA1$^+$CD14$^-$ DCs and BDCA1$^+$CD14$^+$ cells were cultured in the presence or absence of R848. After 24h, numbers of viable cells were assessed using flow cytometry according to propidium iodide (PI). Average percent changes normalized to untreated samples from 5 experiments are shown. Error bars represent SEM.

5.6 Cytokines secreted by *ex vivo* pro-iDCs drive APC maturation *in trans*

Pro-inflammatory cytokines have extensive effects on the immune system including induction of maturation of professional APCs[151,259,260]. An early study by Jonuleit et al.[151] demonstrated that cytokines including IL-1β, IL-6 and TNFα are able to induce maturation and augment the T cell stimulatory capacity of DCs differentiated from monocytes. Since *ex vivo* pro-iDCs produce vast amounts of pro-inflammatory cytokines that partly resembled the cocktail used by Jonuleit et al., the impact of pro-iDC derived culture supernatants on APC maturation was assessed.

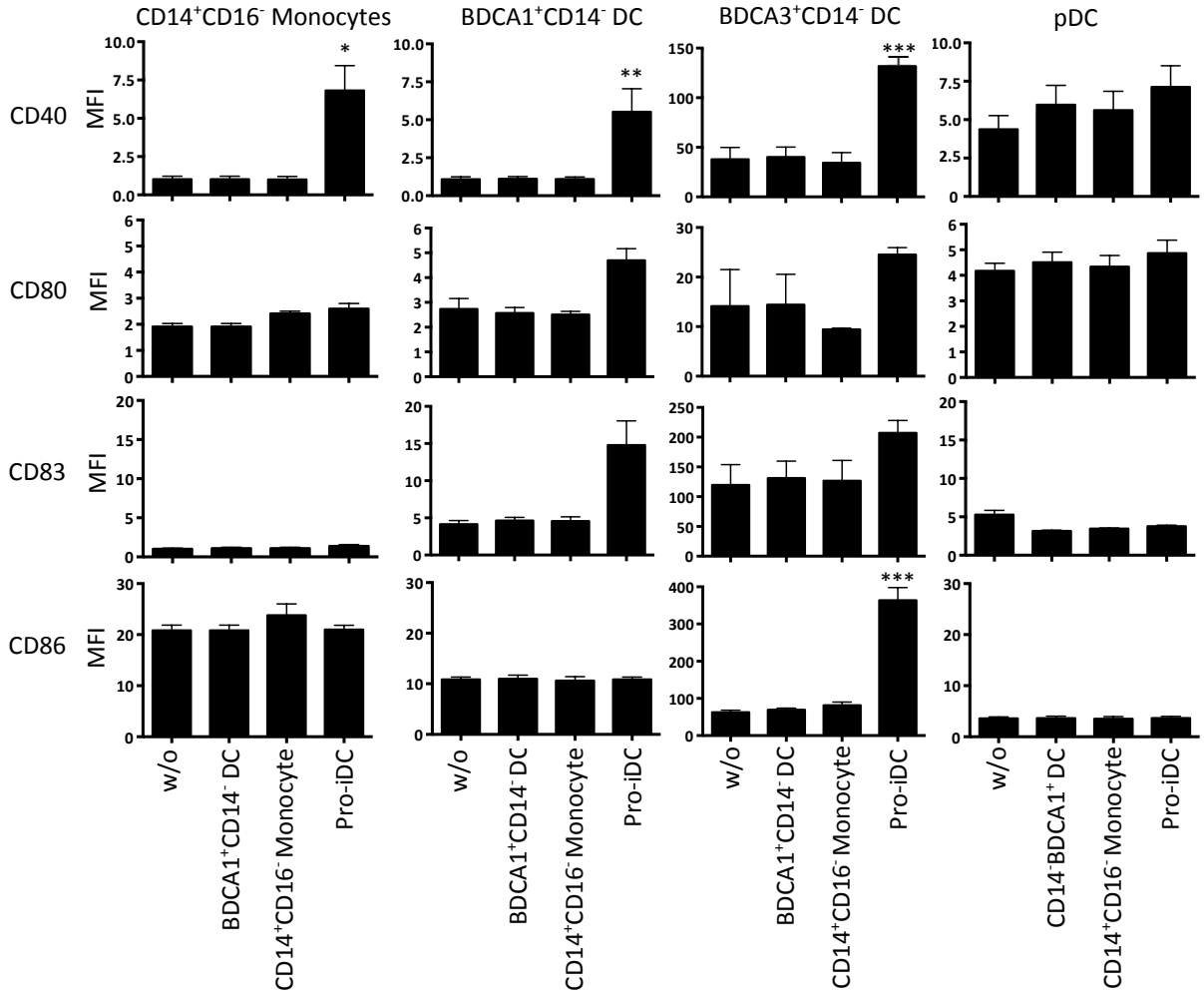


Figure 21 Pro-iDC derived culture supernatants induce maturation of conventional DCs
Magnetically isolated BDCA1$^+$CD14$^-$ DCs, BDCA3$^+$CD14$^-$ DCs, plasmacytoid DCs (pDCs) and CD14$^+$CD16$^-$BDCA1$^-$ monocytes of CD19 depleted PBMC were cultured in the presence or absence of culture supernatants derived from BDCA1$^+$CD14$^-$ DCs, CD14$^+$CD16$^-$BDCA1$^-$ monocytes and pro-iDCs, which were cultured for 24h without stimulation. Afterwards, cells were analyzed for expression levels of CD40, CD80, CD83 and CD86 by flow cytometry. Average median fluorescence intensities (MFI) of 5 experiments are shown. Error bars represent SEM.

To check for the maturation capacity of APC derived soluble mediators, $CD14^+CD16^-$ monocytes, $BDCA1^+CD14^-$ DC, $BDCA3^+CD14^-$ DC and pDCs were cultured in presence or absence of cell-free supernatants derived from 24h cultured pro-iDCs, $CD14^+CD16^-$ monocytes and $BDCA1^+CD14^-$ DCs or left unstimulated for 24h (Figure 21). Subsequently, APCs were analyzed for the expression of maturation markers CD40, CD80, CD83 and CD86. Whereas supernatants from $BDCA1^+CD14^-$ DCs and $CD14^+CD16^-$ monocytes showed no effect, pro-iDC derived supernatants induced the strongest maturation in cDCs. Compared to $BDCA1^+CD14^-$ DCs, $BDCA3^+CD14^-$ DCs had an about 5-20-fold higher basal level of the tested molecules and the same ratio was kept after culture with pro-iDC derived culture supernatants. While $BDCA1^+CD14^-$ DCs upregulated CD40, CD80 and CD83, $BDCA3^+CD14^-$ DCs additionally upregulated CD86. In contrast, $CD14^+CD16^-$ monocytes upregulated CD40 expression only and pDCs were unresponsive.

Since only mature APCs are able to induce a significant T cell proliferation[261,262], upregulation of maturation markers on APCs were correlated with their T cell stimulatory capacity (Figure 22).

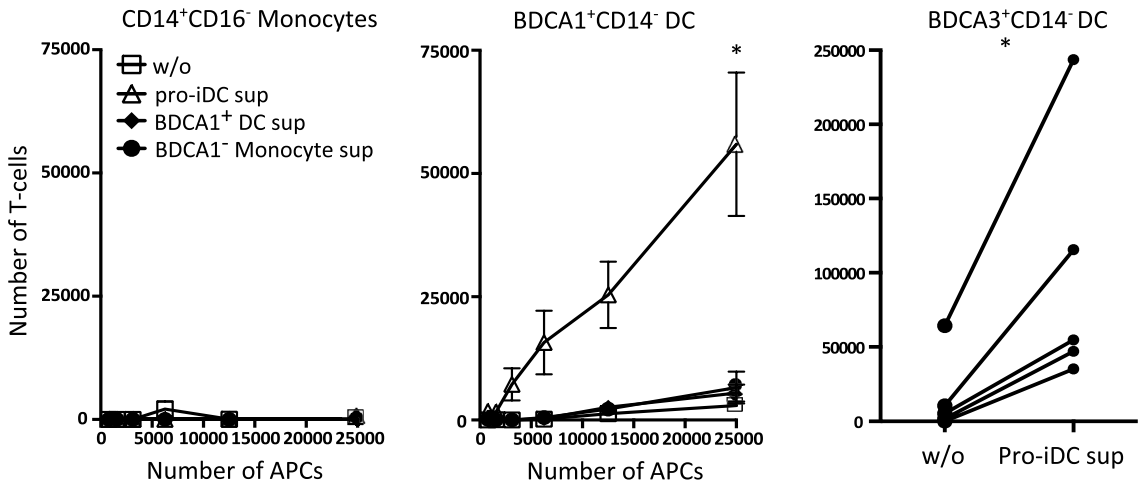


Figure 22 cDCs matured with pro-iDC derived supernatants induce naïve $CD4^+$ T cell proliferation
APC-derived supernatant (sup) treated and flow sorted $BDCA1^+CD14^-$ DCs and magnetically enriched $CD14^+CD16^-$ $BDCA1^-$ monocytes were co-cultured with 25000 allogeneic naïve $CD4^+$ T cells as described in figure 23. Due to low numbers obtainable from PBMC, an average number of $6.3x10^3$ $BDCA3^+CD14^-$ DCs were cultured in the presence of pro-iDC derived supernatant or left untreated for 24h and subsequently co-cultured with 25000 T cells. Resulting numbers of T cells according to CD3 expression were assessed by flow cytometry after 6 days (n=5). Error bars represent SEM.

In an allogeneic T cell co-culture, pro-iDC derived supernatants treated BDCA1$^+$CD14$^-$ DCs and BDCA3$^+$CD14$^-$ DCs gained a strong capacity to induce T cell proliferation, whereas other supernatants or untreated APCs failed to do so. In line with their inability to upregulate co-stimulatory molecules (Figure 21), CD14$^+$CD16$^-$ monocytes did not promote T cell growth. Since BDCA3$^+$CD14$^-$ DCs could not be obtained in similar high numbers as BDCA1$^+$CD14$^-$ DCs from peripheral blood, they were co-cultured at an average number of 6.3x10^3 cells and only treated with pro-iDC derived supernatants or left unstimulated. In accordance with the higher expression of co-stimulatory molecules before and after treatment with pro-iDC derived supernatants, BDCA3$^+$CD14$^-$ DCs induced a stronger T cell proliferation when compared to BDCA1$^+$CD14$^-$ DCs.

5.7 Inflammatory cytokines secreted by *ex vivo* pro-iDCs support T cell proliferation

As inflammatory cytokines including IL-1β, IL-6 and TNFα promote T cell growth[263-267] and as these were found in large amounts in culture supernatants from *ex vivo* pro-iDCs (Figure 7), their influence on T cell proliferation was analyzed. Naïve T cells require TCR and co-stimulatory molecule stimulation for proliferation. To assess the impact of cytokines, an experimental setting was necessary in which the requirement for TCR and co-stimulation was given but at an intensity that was below the threshold for productive T cell expansion. To achieve this, T cells were cultured in the presence of beads coupled to low amounts of antibodies recognizing CD3 and CD28, which are part of the TCR and co-stimulatory molecules[268]. At this setting, T cells cultured in the presence of beads alone basically failed to proliferate (Figure 23, medium). In line with the high levels of inflammatory cytokines found in supernatants from freshly isolated and 24h cultured pro-iDCs (Figure 7 A), naïve CD4$^+$ T cells cultured in the presence of pro-iDC derived supernatants induced a considerable T cell proliferation when compared to BDCA1$^+$CD14$^-$ DC derived supernatants (Figure 23 A). As TLR stimulated BDCA1$^+$CD14$^-$ DCs secreted similar amounts of inflammatory cytokines when compared to *ex vivo* pro-iDCs (Figure 7), supernatants from R848 treated BDCA1$^+$CD14$^-$ DCs and untreated pro-iDCs induced a strong T cell proliferation at comparable levels. To determine the impact of single cytokines contained in pro-iDC derived supernatants on T cell proliferation, IL-1β, IL-6 and TNFα were blocked using neutralizing antibodies in a subsequent experiment (Figure 23 B). Neutralization of IL-6 and followed by TNFα decreased the extent of

T cell proliferation the most, whereas IL-1β neutralization had only a minor impact. Remarkably, IL-1β, IL-6 and TNFα present in pro-iDC derived supernatants exclusively drove T cell proliferation as combined neutralization of these cytokines abrogated proliferation to the basal level. The proliferative capacity was specific to the cytokines, as respective isotype antibody controls did not influence the intensity of T cell proliferation. The individual impacts of IL-1β, IL-6 and TNFα on T cell proliferation were confirmed in an experimental setting using recombinant cytokines. These induced T cell proliferation at the same magnitude as neutralization of these cytokines from pro-iDC derived supernatants decreased T cell proliferation (Figure 23 C). Combined treatment with IL-1β, IL-6 and TNFα induced the strongest T cell division, whereas single treatment with TNFα weakly increased proliferation and IL-1β alone had no effect.

Until now, the superior capability of *ex vivo* pro-iDCs to induce the strong T cell proliferation as seen in the allogeneic co-culture (Figure 19, medium) could not be explained, as the expression of co-stimulatory molecules did not highly differ from freshly isolated (Figure 4) or 24h cultured BDCA1^{+}CD14^{-} DCs (Figure 18). Instead, the influence of the cytokines secreted by pro-iDCs may explain their major T cell proliferative capacity.

T cells were activated at the same time by pro-iDC derived cytokines measured by CD25 expression (Figure 23 D). TNFα activated T cells the most, as its neutralization mediated the strongest decline in CD25^{+} T cells. Neutralization of IL-6 slightly increased the percentage of CD25 expressing T cells. Taken together, pro-iDCs may support immune responses *in trans* by the secretion of pro-inflammatory cytokines that augments APC activation and T cell proliferation.

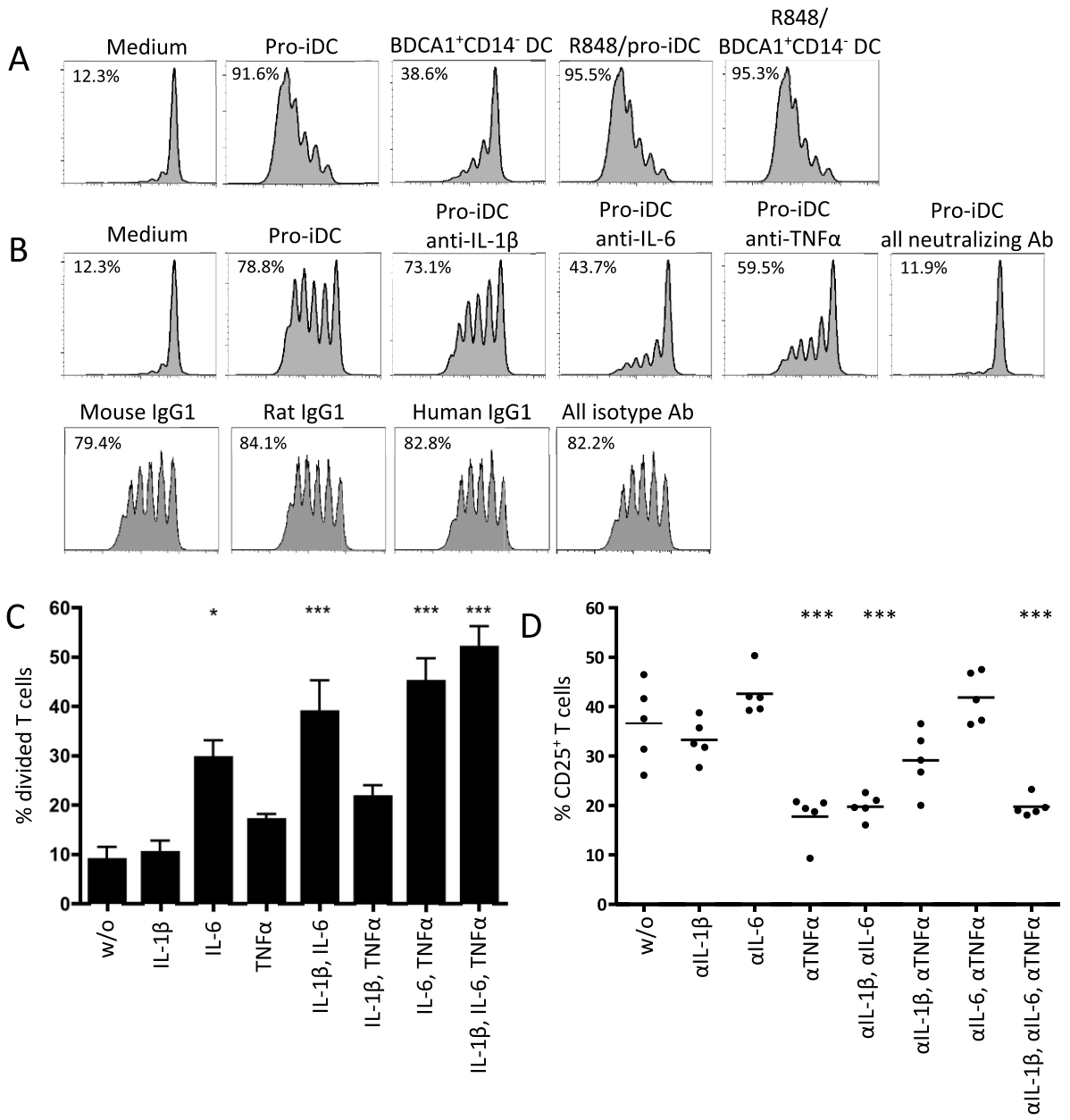


Figure 23 Pro-iDC derived supernatants contain cytokines supporting T cell proliferation
Naïve $CD4^+$ T cells were stained with the proliferation marker violet dye and cultured in the presence of anti-CD3/anti-CD28 beads alone (A, medium) or in the presence of supernatants derived from pro-iDCs or $BDCA1^+CD14^-$ DCs either treated with R848 or left unstimulated during the culture for supernatant generation. After 84h, T cell proliferation was assessed using flow cytometry. Numbers in the histograms indicate the percentage of cells that divided at least once. For some experiments, IL-1β, IL-6 and TNFα were functionally blocked in pro-iDC derived supernatants using neutralizing antibodies (Ab) prior to addition to the T cell culture (B, one representative of 5 individual experiments is shown). The influence on T cell proliferation of the depicted cytokines in B were analyzed in a control experiment using recombinant IL-1β, IL-6 and TNFα in the presence of anti-CD3/anti-CD28 beads. Percentages of cells that divided at least once are shown (C). T cell activation was assessed in the same samples as in B with additional combinations of cytokine neutralizing antibodies by determination of the frequencies of CD25-expressing T cells (D). Error bars represent SEM. Data shown are obtained from individual 5 experiments.

5.8 Pro-iDCs can be recruited from monocytes

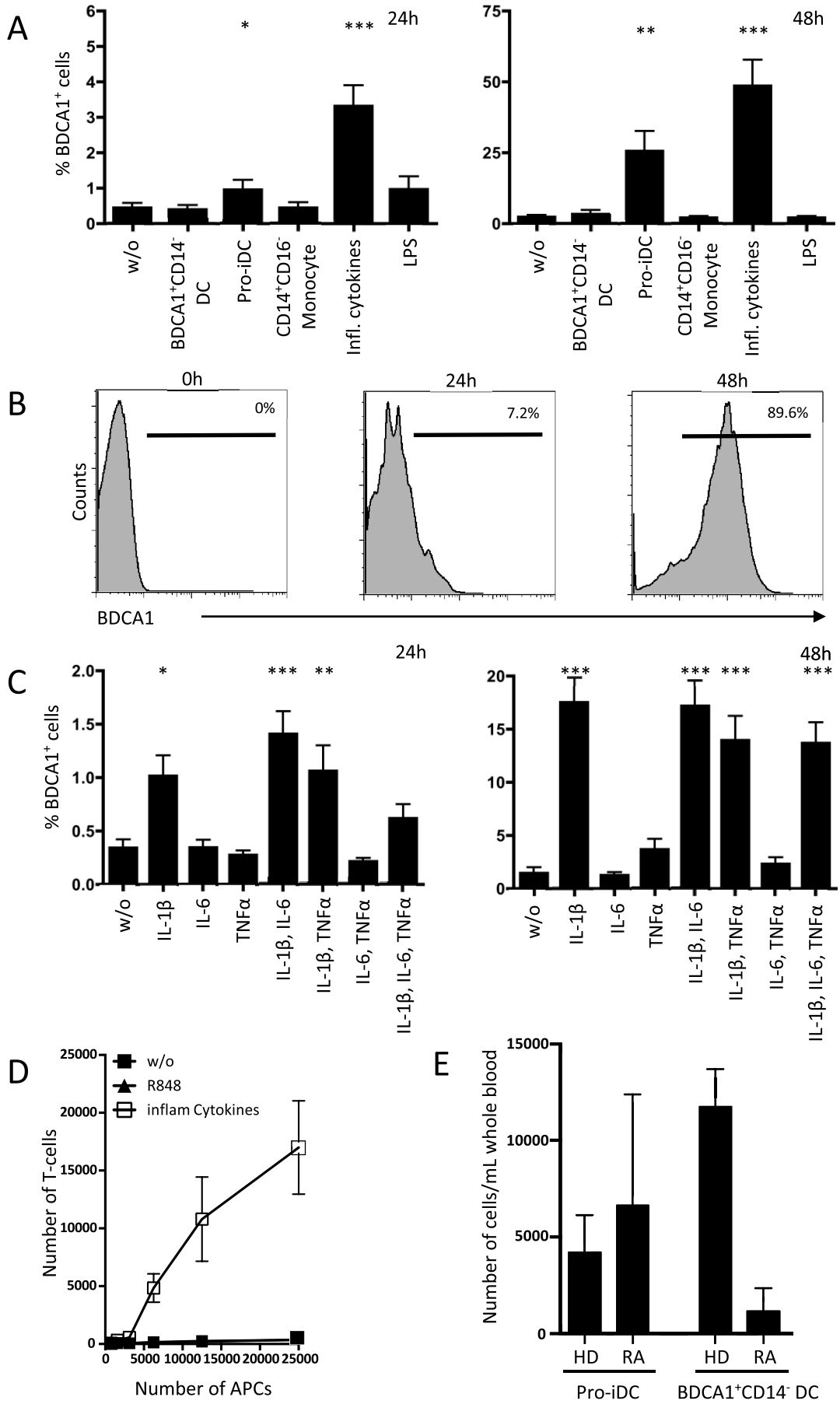
(Collection of blood samples from RA patients was done by PD Dr. Eugen Feist and acquisition was performed by Stefanie Gamradt, both at the Charité, Berlin)

Ly6C^{hi} monocytes, which are murine orthologs of CD14$^+$CD16$^-$ monocytes[207], differentiate towards inflammatory DCs under inflammatory conditions[174]. In humans, the inflammatory DC character of pro-iDCs (Figures 7 and 17) correlates with the mouse model in terms of T cell polarization and cytokine secretion[170]. In addition, the high phenotypic similarity between pro-iDCs and CD14$^+$CD16$^-$ monocytes (Figure 4) suggests a monocytic origin of pro-iDCs. To test this hypothesis, CD14$^+$CD16$^-$ monocytes were cultured for 24h and 48h under inflammatory conditions using stimuli including pro-iDC derived supernatants, a cocktail of recombinant cytokines comprised of IL-1β, IL-6, PGE_2, TNFα, GM-CSF and IL-4 or controls including BDCA1$^+$CD14$^-$ DC and CD14$^+$CD16$^-$ monocyte derived supernatants (Figure 24 A). As pro-iDCs can be identified by BDCA1 expression from monocytes, so cultured CD14$^+$CD16$^-$ monocytes were subsequently analyzed for BDCA1 expression. Pro-iDC derived supernatants and the cocktail of recombinant cytokines induced detectable levels of BDCA1 already after 24h. Almost 50% of the cells expressed significant amounts of BDCA1 after 48h of treatment with the cocktail of recombinant cytokines (Figure 24 B). By contrast, LPS or other cell derived supernatants had no effect. Since pro-iDC derived culture supernatants include high levels inflammatory cytokines and control supernatants from cultured BDCA1$^+$CD14$^-$ DC and CD14$^+$CD16$^-$ monocyte lack these (Figure 7 A), BDCA1 expression on CD14$^+$CD16$^-$ monocytes was suggested to be triggered by pro-inflammatory cytokines. To identify the key cytokines that drive BDCA1 induction on CD14$^+$CD16$^-$ monocytes, cytokines were considered that were present in both, the cocktail and in pro-iDC derived supernatants (Figure 24 C) including IL-1β, IL-6 and TNFα. In line with the requirement of IL-1R mediated signaling for murine inflammatory DC differentiation from monocytes[269,270], IL-1β induced BDCA1 expression on CD14$^+$CD16$^-$ monocytes, while IL-6 and TNFα had no effect. However, IL-1β and IL-6 synergized in inducing up-regulation of BDCA1 in the early phase of differentiation, however, this effect vanished after 48h. To show whether CD14$^+$CD16$^-$ monocytes had differentiated into DCs and not only gained BDCA1 expression alone, DC differentiation can be functionally analyzed by the capacity to induce T cell proliferation[271]. In contrast to R848 treated BDCA1$^-$ or unstimulated CD14$^+$CD16$^-$ monocyte controls, inflammatory cytokine activated CD14$^+$CD16$^-$ BDCA1$^+$ cells significantly increased the number of allogeneic naïve CD4$^+$ T cells (Figure 24 D).

Thus, under inflammatory conditions $CD14^+CD16^-$ monocytes not only acquired a phenotype resembling pro-iDCs by BDCA1 expression but had also developed into DCs, capable of inducing proliferation of T cells. So far, no studies could show the recruitment and DC differentiation in humans *in vivo*. As an approach to analyze whether pro-iDC differentiation under inflammatory conditions occurs *in vivo*, numbers of $BDCA1^+CD14^-$ DCs and pro-iDCs from peripheral blood were assessed in patients suffering from rheumatoid arthritis (RA) and compared to equivalent numbers of healthy donors (Figure 24 E). As DC migration from blood to tissues was shown to be associated with RA and results in a lower abundance in blood[272,273], $BDCA1^+CD14^-$ DCs from RA patients showed reduced numbers when compared to healthy donors. The same reason might hamper the acquisition of pro-iDCs and therefore assessed numbers can be considered lower than numbers of actually differentiated cells. In addition, the cell count might be influenced by chemotaxis mediated by the chemokine receptor CCR5 that is involved in leukocyte migration to RA synoviums[274]. Since pro-iDCs express over 20 fold higher levels of CCR5 when compared to $BDCA1^+CD14^-$ DCs (Figure 4), numbers of pro-iDCs were expected to be decreased in blood obtained from RA patients. However, numbers of pro-iDCs were slightly increased in RA patients compared to healthy donors. This indicates an ongoing recruitment and differentiation from monocytes towards pro-iDCs already in peripheral blood *in vivo*.

Figure 24 (next page) $BDCA1^+CD14^+$ cells with a strong DC function can be induced from $CD14^+CD16^-BDCA1^-$ monocytes

$CD14^+CD16^-BDCA1^-$ monocytes were treated with culture supernatants derived from $BDCA1^+CD14^-$ DCs, pro-iDCs, $BDCA1^-CD14^+CD16^-$ monocytes, LPS and a cocktail of inflammatory cytokines containing IL-1β, IL-6, TNFα, GM-CSF, IL-4 and PGE_2. After 24h and 48h, frequencies of BDCA1 expressing cells were assessed by flow cytometry (A). One representative example from inflammatory cytokine treated cells is shown as a histogram plot (B). Monocytes were treated with IL-1β, IL-6 and TNFα or combinations thereof and BDCA1 induction was assessed after 24h and 48h (C). To assess DC function, $CD14^+CD16^-BDCA1^-$ monocytes were cultured in the presence of R848, the cocktail of inflammatory cytokines mentioned above or medium alone for 24h, washed intensively and co-cultured with allogeneic naïve $CD4^+$ T cells (D). After 6 days, numbers of T cells according to CD3 expression were assessed using flow cytometry (n=5). To evaluate whether pro-iDCs were recruited during inflammation, numbers of $BDCA1^+CD14^-$ DCs and $BDCA1^+CD14^+$ pro-iDCs were determined in peripheral blood from 8 patients suffering from rheumatoid arthritis (RA) and compared to healthy donors. For the calculation of absolute cell numbers refer to material and methods (E). Error bars represent SEM.


A
% BDCA1+ cells
24h
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w/o
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Pro-iDC
CD14+CD16- Monocyte
Infl. cytokines
LPS
B
0h
24h
48h
0%
7.2%
89.6%
Counts
BDCA1
C
% BDCA1+ cells
w/o
IL-1β
IL-6
TNFα
IL-1β, IL-6
IL-1β, TNFα
IL-6, TNFα
IL-1β, IL-6, TNFα
D
w/o
R848
inflam Cytokines
Number of T-cells
Number of APCs
E
Number of cells/mL whole blood
HD
RA
Pro-iDC
BDCA1+CD14- DC

5.9 Transcriptome analysis indicates dendritic cell character of pro-iDCs

(Microarray raw data processing was performed by Michail Knauel and Figures 26-28 were generated by Dr. Jutta Kollet at Miltenyi Biotec)

By phenotypic analysis, pro-iDCs appeared to be highly related to CD14$^+$CD16$^-$ monocytes but endowed with key DC properties (Figures 4, 7, 18). In addition, CD14$^+$CD16$^-$ monocytes gave rise to pro-iDC resembling BDCA1$^+$CD14$^+$ cells with DC function under inflammatory conditions *in vitro* (Figure 24). In order to elucidate the affiliation of pro-iDCs, the transcriptome of pro-iDCs was compared to that of CD14$^+$CD16$^-$BDCA1$^-$ monocytes, BDCA1$^+$CD14$^-$ DC and BDCA3$^+$CD14$^-$ DC and *in vitro* generated immature and mature monocyte derived DCs (imoDCs and mmoDCs). To analyze the reliability of the microarray analysis, mRNA expression levels of relevant markers were compared to corresponding protein levels: correlation was proven for BDCA1, CD14, HLA-DR, CD40 and CD36 levels (Figures 4 and 25).

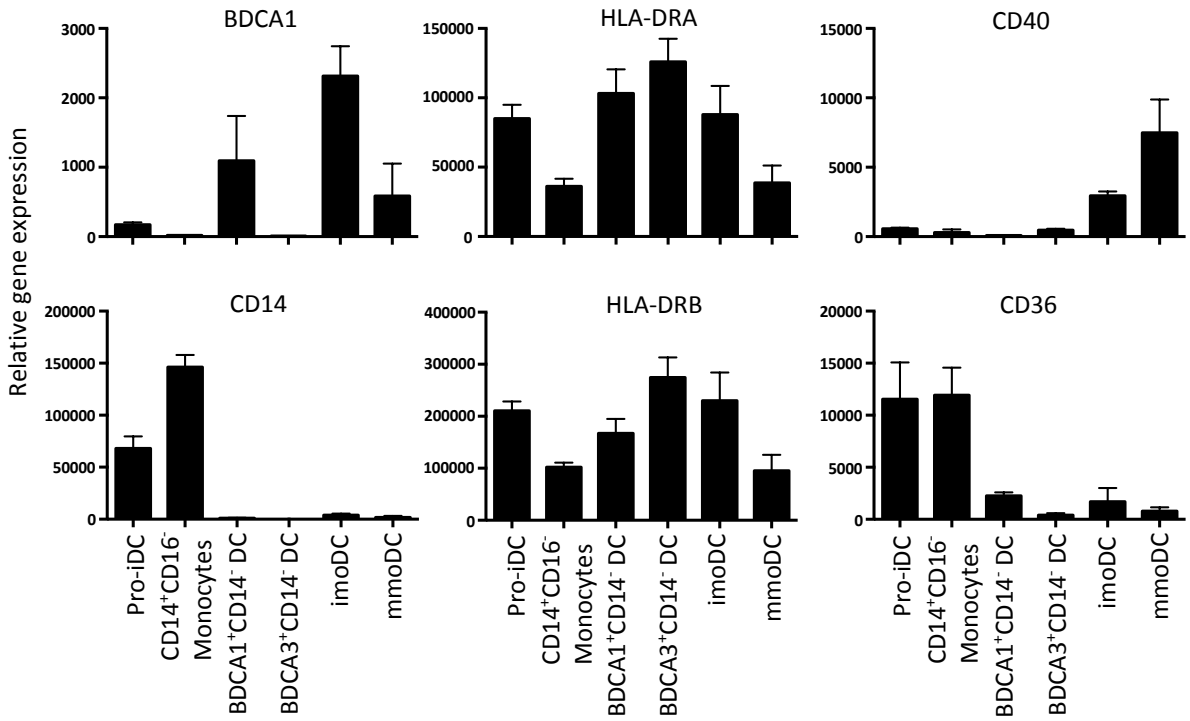


Figure 25 Relative gene expression levels of selected genes correlate with protein expression levels on monocytes and different DC subsets

Data was generated from *ex vivo* samples of flow sorted pro-iDCs, CD14$^+$CD16$^-$BDCA1$^-$ monocytes, BDCA1$^+$CD14$^-$ DCs and BDCA3$^+$CD14$^-$ DCs and *in vitro* generated immature and mature monocyte derived DCs (imoDC, mmoDC). Shown are relative mRNA expression levels from normalized whole genome microarray experiments of the selected genes BDCA1, CD14, HLA-DRA, HLA-DRB, CD40 and CD36. Error bars represent SEM.

In line with surface marker expression (Figure 4), pro-iDCs clearly clustered with CD14$^+$CD16$^-$ BDCA1$^-$ monocytes at whole genome level. BDCA1$^+$CD14$^-$ DCs clustered with BDCA3$^+$CD14$^-$ DCs and the *in vitro* generated imoDCs and mmoDCs were both distantly related to the *ex vivo* cell subsets (Figure 26).

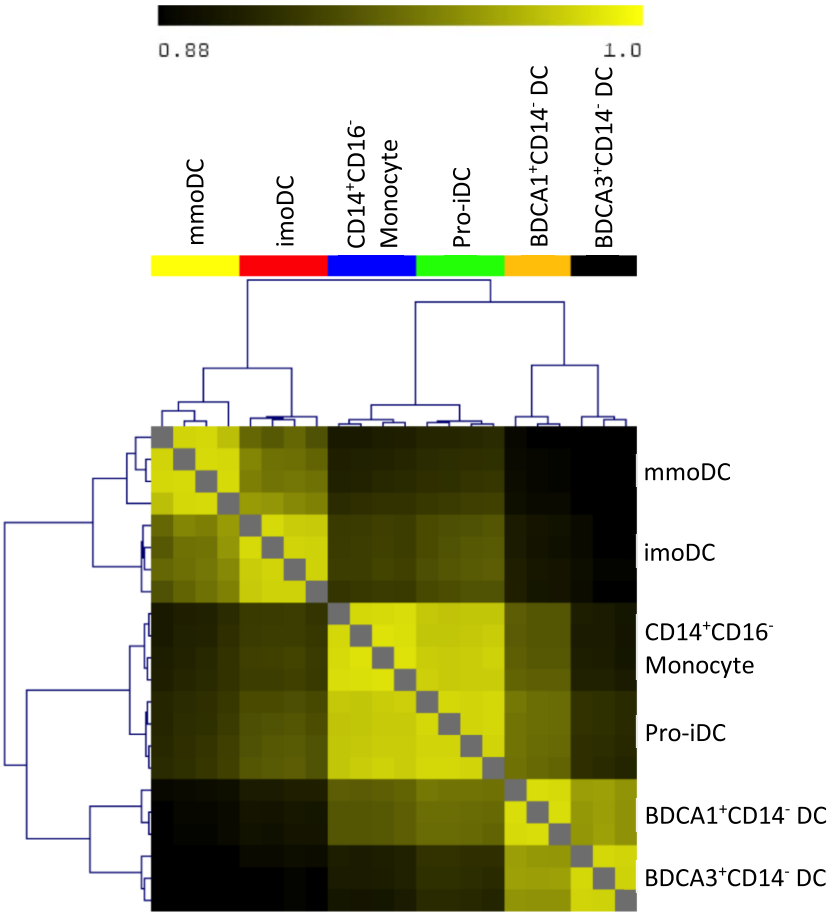


Figure 26 Pro-iDCs are closely related to CD14$^+$CD16$^-$BDCA1$^-$ monocytes on whole genome level
Hierarchical clustering of normalized (log_2) intensities using Euclidean distance metrics of the whole data set. Cell samples included pro-iDCs (green), CD14$^+$CD16$^-$BDCA1$^-$ monocytes (blue), BDCA1$^+$CD14$^-$ DCs (orange) and BDCA3$^+$CD14$^-$ DCs (black) and *in vitro* generated immature monocyte derived DCs (imoDC, red)) and mature moDC (mmoDC, yellow).

For comparison, only 680 genes (2.43% of total genes) were >2 fold differentially expressed (352 genes were overrepresented in pro-iDCs and 328 genes were higher expressed in monocytes) between pro-iDCs and CD14$^+$CD16$^-$BDCA1$^-$ monocytes, whereas 6994 (25.19%), 8610 (31.01%), 6609 (23.80%) and 7526 genes (27.10%) were >2 fold differentially expressed in BDCA1$^+$CD14$^-$ DCs, BDCA3$^+$CD14$^-$ DCs, imoDC and mmoDC, respectively, when compared to CD14$^+$CD16$^-$BDCA1$^-$ monocytes (a full list of reporters differentially regulated between pro-iDCs and CD14$^+$CD16$^-$BDCA1$^-$ monocytes can be found in the appendix in tables 3 and 4). Since pro-iDCs are highly similar to monocytes but endowed with DC functions, a potential

development from monocyte towards DC may be reflected by the 680 differentially expressed genes between CD14$^+$CD16$^-$BDCA1$^-$ monocytes and pro-iDCs. To prove this hypothesis, expression of these genes was correlated each between pro-iDCs and CD14$^+$CD16$^-$ monocytes, BDCA1$^+$CD14$^-$ DC, BDCA3$^+$CD14$^-$ DC, imoDCs and mmoDCs (Figure 27).

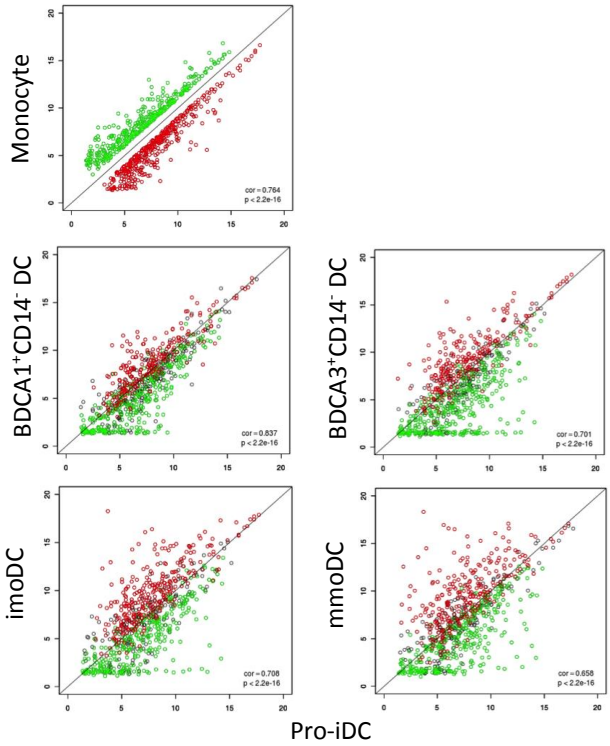


Figure 27 Trend analysis of differentially regulated genes between pro-iDCs and CD14$^+$CD16$^-$BDCA1$^-$ monocytes revealed the highest correlation between pro-iDCs and BDCA1$^+$CD14$^-$ DCs
Log_2 median signal intensities of pro-iDCs of >2 fold differentially regulated genes between pro-iDCs and CD14$^+$CD16$^-$BDCA1$^-$ monocytes were correlated in pair-wise scatter plots with CD14$^+$CD16$^-$BDCA1$^-$ monocytes, BDCA1$^+$CD14$^-$ DCs, BDCA3$^+$CD14$^-$ DCs, imoDCs and mmoDCs. Red dots indicate transcripts stronger expressed in pro-iDCs and genes indicated by green dots are higher expressed in CD14$^+$CD16$^-$BDCA1$^-$ monocytes. Numbers in the plots indicate p-values and correlation coefficients.

Transcripts that were higher expressed in pro-iDCs over CD14$^+$CD16$^-$ monocytes (red circles) were widely higher expressed in all DC subsets tested, whereas transcripts higher regulated in CD14$^+$CD16$^-$ monocytes (green circles) were generally lower expressed in the DC subsets. Among subsets compared to pro-iDCs, the gene expression profile correlated the most with BDCA1$^+$CD14$^-$ DCs (correlation coefficient (cor)=0.837), which was clearly above CD14$^+$CD16$^-$ monocytes (cor=0.764) and followed by imoDC (cor=0.706), BDCA3$^+$CD14$^-$ DCs (cor=0.701) and mmoDCs (cor=0.658). Thus, the regulation of these genes in pro-iDCs pointed to a DC affiliation or an ongoing development towards DC.

To further analyze the DC affiliation of pro-iDCs on a single gene level, differentially expressed genes were subjected to annotation enrichment analysis and resulting gene clusters were analyzed for associations with dendritic cells and monocytes (Figure 28).

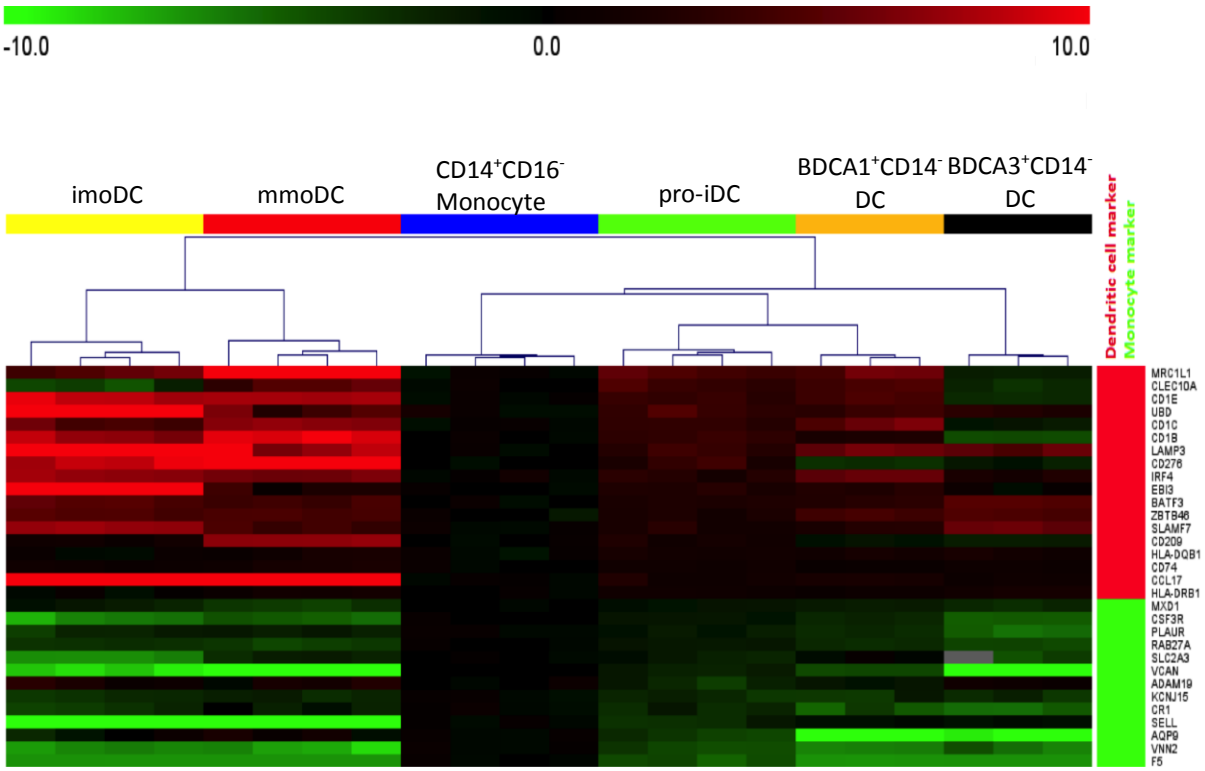


Figure 28 Term enrichment analysis revealed a DC expression profile in pro-iDCs
Genes >2 fold differentially regulated between pro-iDCs and CD14$^+$CD16$^-$BDCA1$^-$ monocytes with significant term enrichment in DC or monocyte specific categories were hierarchically clustered (Euclidean distance, complete linkage). Cell samples included pro-iDCs (green), CD14$^+$CD16$^-$BDCA1$^-$ monocytes (blue), BDCA1$^+$CD14$^-$ DCs (orange) and BDCA3$^+$CD14$^-$ DCs (black) and *in vitro* generated immature monocyte derived DCs (imoDC, red) and mature moDC (mmoDC, yellow). The range of the $\log_2$ sample intensities was centered to monocytes and set to -10 (green) to 10 (red). The adjacent color bar indicates genes associated with dendritic cells (red) and monocytes (green).

Hierarchical clustering of genes associated with these associations revealed a higher similarity to BDCA1$^+$CD14$^-$ DCs than to CD14$^+$CD16$^-$ monocytes. DC specific genes were selectively upregulated in pro-iDCs and the other tested DC subsets, whereas monocyte specific genes were downregulated in pro-iDCs and other DC subsets. Among the analyzed cluster of genes, mRNA levels of transcription factors involved in DC differentiation including *IRF4*[102-105] and *BATF3*[106-108] and the transcription factor *ZBTB46*, recently described as specific for conventional DC lineage[110,275], were >3.5 fold upregulated in pro-iDCs over CD14$^+$CD16$^-$ monocytes. Furthermore, DC specific genes including *LAMP3*[276], *TNFRSF11A*[277], *PLXNA1*[278] and *UBD*[279] were regulated in the same way. Especially genes involved in T cell stimulation (*CD276*)

and antigen presentation including *CD74*, *HLA-DQ*, *HLA-DRA*, *HLA-DRB*, *CD1C (BDCA1)* and *CD1E* and were >2 fold upregulated in pro-iDCs over $CD14^+CD16^-$ monocytes.

The inflammatory character of pro-iDCs was underlined by >5 fold stronger expression of the T_H1 and NK cell attracting and T_H2 inhibitory chemokine *CXCL9*[280-282]. In contrast to the protein level (Figure 4), the transcript of the inflammatory DC marker *MRC1L1 (CD206, mannose receptor)*[169,283] was 21 fold higher regulated in pro-iDCs over $CD14^+CD16^-$ monocytes, while the higher protein expression of monocyte/macrophage marker *CD163* (Figure 4) could not be confirmed on gene level. As protein level regulation commonly lags behind gene expression levels[284], a strongly increased mRNA level of *MRC1L1* supports the hypothesis of an ongoing development towards DC. Genes that were described to be specifically enriched in both, macrophage and DC lineages including *TIFAB*[285], *CLEC10A*[286] and *GAS6*[287,288], were >2 fold stronger expressed in pro-iDCs over monocytes. In addition, genes differentially regulated between pro-iDCs and monocytes did not indicate an affiliation or an ongoing development towards macrophages as these lacked macrophage specific transcription factors including *STAT1*, *STAT3*, *STAT6*[289] and *VENTX*[290] and genes involved in macrophage differentiation *EGR2*, *EMR1*, *MAFB*[291] and macrophage markers *ITGB2*, *LAMP2* and *CD68*[292]. Moreover, *EGR1* that is involved in macrophage differentiation[293] was >2 fold lower expressed in pro-iDCs compared to monocytes. RUNX3 expression positively correlates with long-term expression of CD4/CD14 in monocytes and macrophages[222]. Hence, the >3.5 fold lower expression of *RUNX3* in pro-iDCs compared to $CD14^+CD16^-$ monocytes further indicates a differentiation towards DC. This in line with a decreased CXCR4 protein expression on pro-iDCs versus monocytes (Figure 4), as CXCR4 dependent signaling mediated by CXCL12 leads to increased RUNX3 levels and thereby prevents monocyte differentiation towards DC[225]. The highest regulated genes in pro-iDCs are *C1QA*, *C1QB* and *C1QC* with a 47 to 140 fold higher expression over $CD14^+CD16^-$ monocytes. The translated genes form the heterotrimeric protein C1q, which belongs to the complement factors[294]. As C1q expression is restricted to DCs and lacks in monocytes[295-298], the very high expression of its subunits *C1QA*, *C1QB* and *C1QC* genes further pointed to a differentiation of pro-iDCs towards DCs. In contrast, monocyte specific genes including *VNN2*[299] and *CSF3R*[145,300] were 18 and 2 fold higher regulated in $CD14^+CD16^-$ monocytes over pro-iDCs, respectively. Based on genes that were >2 fold differentially expressed between pro-iDCs and monocytes, an annotation enrichment analysis using the publically available database GeneOntology[301] was performed. Terms that were most enriched in an annotation

analysis based on genes >2 fold higher expressed in pro-iDCs over $CD14^+CD16^-$ monocytes indicated key DC functions[302-304] in pro-iDCs, which included antigen processing/presentation and T cell activation (Figure 29). This set included DC specific genes *CD1B*, *CD1C*, *CD1E*, *HLA-DR*, *HLA-DQ* and *UBD*. Thus, despite the high similarity to monocytes on protein (Figure 4) and whole genome level, upregulation of DC-specific transcription factors, DC-specific cell surface molecules and downregulation of transcripts involved in macrophage differentiation and monocyte maintenance and functional enrichment of genes involved in antigen presentation and T-cell stimulation indicate an ongoing developmental program towards DC.

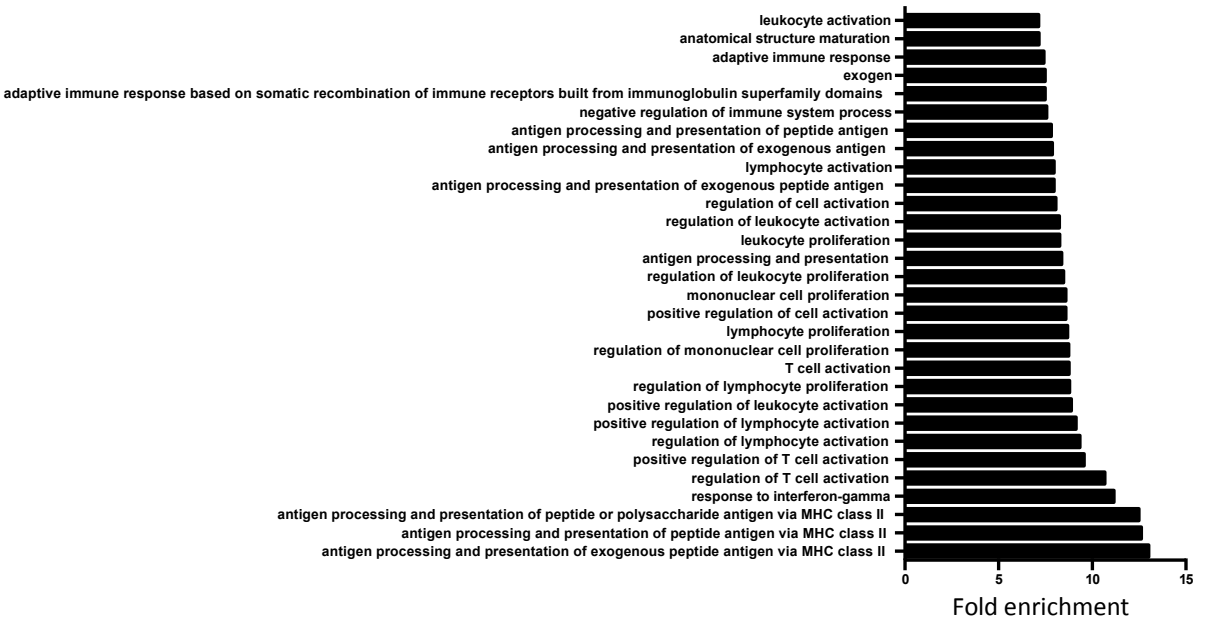


Figure 29 GeneOntology term analysis reveals an enrichment in genes involved in DC function in pro-iDCs
Genes selected from transcripts >2 fold differently expressed between pro-iDCs and $CD14^+CD16^-BDCA1^-$ monocytes were analyzed for term enrichment. Significant enrichments based on the publically available database GeneOntology (GO)[301] in "GO Biological Process" are shown for terms with a minimal gene count >10, a minimal enrichment >7 and $p<10^{-6}$.

5.10 Cells phenotypically resembling pro-iDCs are present in inflamed tissues

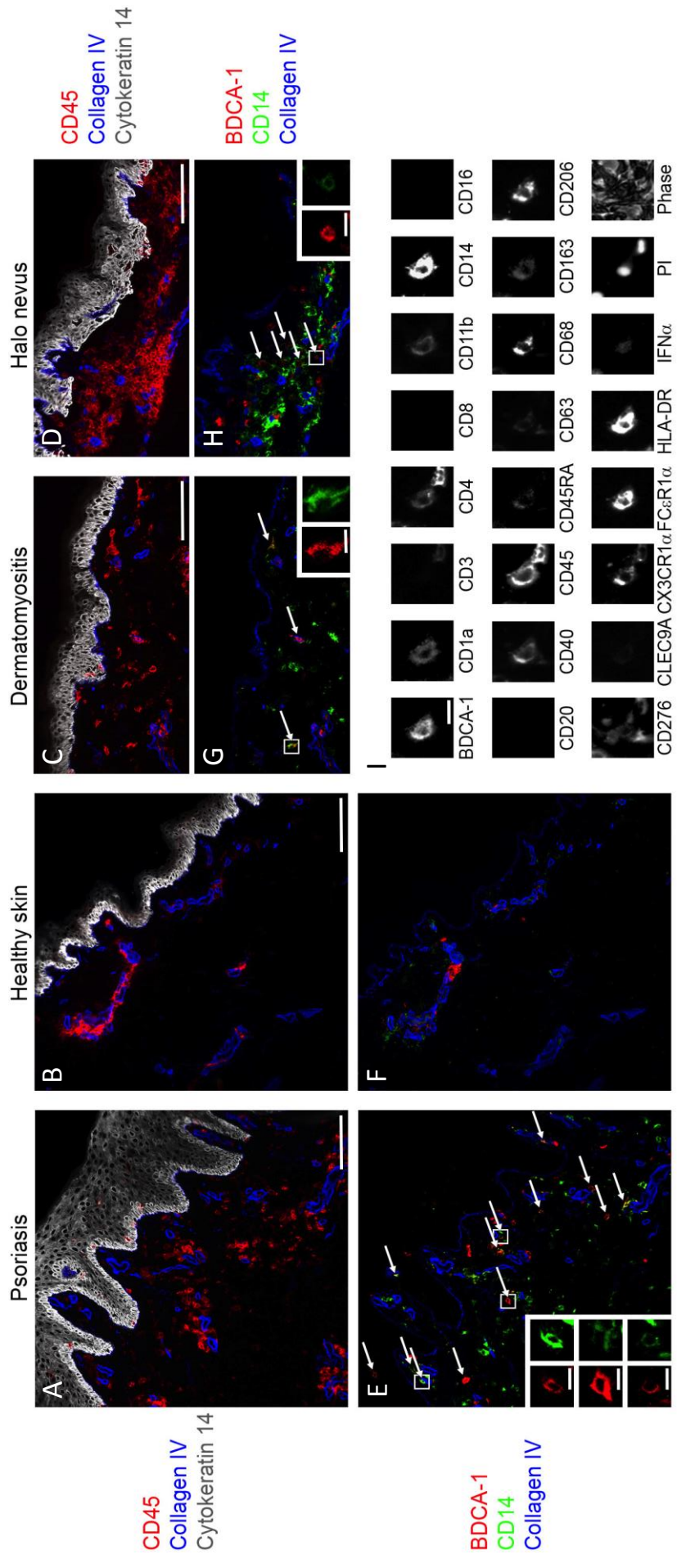
(Data was generated by Christian Ostalecki at the University of Erlangen)

To date, there is no solid data on the characterization of human monocyte derived DCs *in vivo*. However, Ly6C^{hi} monocytes, which are murine counterparts of human CD14^{+}CD16^{-} monocytes[207] migrate to inflamed tissue and differentiate in response to inflammatory factors towards inflammatory DCs[172,173,186]. As to date human inflammatory DCs were only found at the site of inflammation[168-170] and this work suggests pro-iDCs to be precursors of inflammatory DCs. Thus, samples of human inflamed skin were analyzed for the presence of cells with phenotypic characteristics of blood pro-iDCs.

The presence and phenotype of cells present in the skin were analyzed *in situ* on the basis of BDCA1 and CD14 co-expression by making use of an imaging technique (multiple epitope ligand cartography, MELC) that allows the analysis of multiple parameters on the same specimen (Figure 30). Skin samples from individuals suffering from psoriasis, dermatomyositosis and inflamed halo nevus or from healthy donors were analyzed for leukocyte infiltration. All specimens were infiltrated by CD45^{+} leukocytes (Figure 30 A-D, CD45^{+} red). Of note is, that in inflamed skin, cells were abundantly distributed, whereas leukocytes localized strictly around blood vessels in healthy skin. As B cells express also BDCA1 and in addition to CD20, B cells cannot be distinguished from BDCA1^{+} DCs and thus CD20^{+} cells were excluded from the following analysis. Similar to mouse inflammatory DCs[172,173,186], human BDCA1^{+}CD14^{+} cells (arrows) were present in inflamed but not in healthy skin, indicating that these cells are not tissue resident but might be rather recruited to sites of inflammation (Figure 30 E-H). In contrast, in healthy skin only single positive BDCA1^{+}CD14^{-} DC (red) and BDCA1^{-}CD14^{+} cells (green) were detected. For deeper characterization besides CD45, BDCA1 and CD14, skin sections were analyzed for the expression of multiple cell surface markers and cytokines, which have been previously shown to be associated with the pro-iDC phenotype (Figure 30 I). As shown for one representative BDCA1^{+}CD14^{+} cell from psoriatic skin, cells were positive for human inflammatory DC markers BDCA1, CD14, CD1a, CD11b, CD206, FcεR1α and HLA-DR[168-170,305] and also for CD40, CD45, CD68, CX_3CR1. Lower expression was observed for CD45RA, CD63, CD163, CD276 and IFNα. BDCA1^{+}CD14^{+} cells were distinct from T cells, CD16^{+} monocytes, B cells and BDCA3^{+} DCs by the lack of CD3, CD8, CD16, CD20 and CLEC9a expression, respectively. BDCA1^{+}CD14^{+} cells show not only a high similarity with human inflammatory DCs[170], they resemble pro-iDCs isolated from peripheral blood to a high degree.

Thus, the high convergence between pro-iDCs and BDCA1$^+$CD14$^+$ cells found here with high similarities to inflammatory DCs[170] strongly suggests that pro-iDCs may represent the immediate precursor of inflammatory DCs in tissues.

Figure 30 (next page) BDCA1$^+$CD14$^+$ cells present in inflamed tissues resemble inflammatory DCs
Skin samples from healthy donors or patients suffering from psoriasis, dermatomyositis and inflamed halo nevus were analyzed using multiple epitope ligand cartography (MELC), a technology allowing the assessment of multiple parameters on a single sample. The samples show subepidermal skin layers indicated by cytokeratin 14 (grey), blood vessels by collagen IV (blue). Leukocytes (CD45$^+$ cells) are colored red. Scale bar: 100µm (A-D). BDCA1 (red), CD14 (green) and collagen IV (blue) are shown on the same samples. Scale bar: 10µm (E-H). BDCA1$^+$CD14$^+$ cells are highlighted by arrows. A representative BDCA1$^+$CD14$^+$ cell from psoriatic skin is depicted and indicated markers are shown. Scale bar: 50µm (I).


Psoriasis
Healthy skin
Dermatomyositis
Halo nevus
A
B
C
D
E
F
G
H
CD45
Collagen IV
Cytokeratin 14
BDCA-1
CD14
Collagen IV
BDCA-1
CD1a
CD3
CD4
CD8
CD11b
CD14
CD16
CD20
CD40
CD45
CD45RA
CD63
CD68
CD163
CD206
CD276
CLEC9A
CX3CR1α
FCεR1α
HLA-DR
IFNα
PI
Phase

6 Discussion

This work describes a novel BDCA1^{+}CD14^{+} myeloid DC subset with high phenotypic similarity to monocytes, but endowed with a decisive dendritic cell gene signature and function. The cells spontaneously secreted vast amounts of inflammatory cytokines, mediated T_H17 polarization and augmented T cell proliferation. BDCA1^{+}CD14^{+} DCs constitute a yet uncharacterized cell type with inflammatory properties and were therefore termed pro-inflammatory DCs (pro-iDCs).

Although visualized by flow cytometry analysis by BDCA1/CD14 expression for the first time over 10 years ago[222], a detailed characterization of pro-iDCs, especially the cellular affiliation and function remained elusive. The lack of data could be addressed to methodical obstacles that complicated a detailed analysis, including low cell numbers, low expression of the distinguishing dendritic cell marker BDCA1 and thus difficult discrimination from monocytes. Data obtained in this study showed a very high similarity of pro-iDCs with monocytes, however, pro-iDCs differed from monocytes in functions and expression of markers on gene and protein level in a manner that pointed to a DC affiliation. The high overall similarity with monocytes suggested that pro-iDCs have a monocytic origin. The lack of CD16 and SLAN expression on pro-iDCs excluded an affiliation with CD14^{dim}CD16^{+} monocytes or the SLAN DC subset thereof[207]. Instead, pro-iDCs were most similar to the CD14^{+}CD16^{-} monocyte subset, as both cell types expressed comparable levels of CD14 and were only distinguishable from each other by few markers as obtained in flow cytometry. As monocytes have the capability to differentiate into DCs and macrophages[199], we tried to classify pro-iDCs with known DC and macrophage markers to get a first impression on their ontogeny. Pro-iDCs expressed elevated levels of activated DC markers including BDCA1, CD40 and CD54 when compared to monocytes, and high levels of HLA-DR resembling BDCA1^{+}CD14^{-} DCs. In terms of typical macrophage markers[234,292], pro-iDCs lacked CD68 but expressed CD163 at high levels. To gain further insight into the classification besides phenotypic analysis on protein level, a transcriptome expression analysis was performed with pro-iDCs.

As most of the genes of pro-iDCs and CD14^{+}CD16^{-} monocytes were regulated in the same way, genes differentially expressed between these subsets were used to characterize pro-iDCs. A macrophage affiliation was unlikely as genes higher regulated in pro-iDCs over monocytes did neither include the macrophage specific transcripts *VENTX*[290], *STAT1*, *STAT3*, *STAT6*[289] nor known genes involved in macrophage differentiation *EGR1*[293], *EGR2*, *EMR1*, *MAFB*[291]. Instead

the few genes higher regulated in pro-iDCs over monocytes included the DC transcription factors *ZBTB46*[110,275], *IRF4*[102-105] and *BATF3*[106-108] and DC specific genes including *LAMP3*[276], *TNFRSF11A*[277], *PLXNA1*[278] and *UBD*[279]. Trend analysis of differentially expressed genes between pro-iDCs and $CD14^+CD16^-$ monocytes applied to various DC subsets and monocytes revealed the highest correlation between pro-iDCs and $BDCA1^+CD14^-$ DCs. Furthermore, all genes that were higher expressed in pro-iDCs compared to $CD14^+CD16^-$ monocytes were even higher expressed in the cDC and moDC subsets, emphasizing their association with DCs. The same was true for genes lower expressed in pro-iDCs than in $CD14^+CD16^-$ monocytes. Clusters of genes that are known to be part of cellular processes can be associated to terms using the publically available database GeneOntology[301]. Term enrichment analysis based on genes higher expressed in pro-iDCs over monocytes revealed terms including antigen processing/presentation and T cell activation, which are key DC functions[302-304]. Functional evidence on the capacity of pro-iDCs to induce T cell activation and proliferation supported the association of pro-iDCs with DCs, although T cell proliferation was presumably in part dependent on the lowering of the activation threshold by the secretion of inflammatory cytokines. In terms of responsiveness to PRR stimulation, pro-iDCs expressed several co-stimulatory molecules such as CD40 and CD80 in a way resembling $BDCA1^+CD14^-$ DCs and not $CD14^+CD16^-$ monocytes after TLR triggering. In conclusion, phenotype, transcriptome and function suggest that pro-iDCs are an intermediate subset between monocytes and DCs, and it is interesting to speculate that they differentiate from human monocytes *in vivo*.

In mice, evidence of monocyte differentiation into DCs *in vivo* has been established[174]. In contrast to naturally occurring DCs, which are derived from a common DC precursor[199], inflammatory DCs are derived from $Ly6C^{hi}$ monocytes under inflammatory conditions[172,269,270]. In humans, DCs can be generated from monocytes in the presence of inflammatory cytokines *in vitro*[149,151]. Even though moDC differentiation *in vivo* is anticipated[170,171] real evidence and underlying mechanisms are lacking. Although a consistent human precursor has not been identified yet, transcriptome and phenotypical analysis led to the speculation that blood monocytes might give rise to inflammatory DCs[170,171]. It is thus interesting to discuss whether human monocytes give rise to pro-iDCs *in vivo* and which mechanisms may be involved. Based on previous findings[151], we chose to induce monocyte differentiation using a cocktail of IL-4, GM-CSF, IL-6, TNFα, PGE_2 and IL-1β. Indeed, we found that these cytokines induced the acquisition of DC functions including induction of T cell proliferation in differentiated

monocytes. In a number of studies, PGE_2 was shown to have enhancing and modulating effects on DCs in terms of T cell proliferation and cytokine induction rather than a role in inducing DC differentiation[151,306-308]. Also reported by several groups, IL-1β induced the secretion of IL-6 and TNFα from blood mononuclear cells[250,251,309]. Hence, we sought to determine whether IL-1β alone is sufficient to induce monocyte differentiation. Strikingly, we found that IL-1β treated $CD14^+CD16^-$ monocytes started to express the DC marker BDCA1 at comparable levels as pro-iDCs. This is an important finding since $CD14^+CD16^-$ monocytes are orthologs of murine $Ly6C^{hi}$ monocytes that have been reported to differentiate into inflammatory DCs in the presence of inflammatory stimuli. We further found that IL-1β alone triggered the secretion of GM-CSF, TNFα and IL-6 from pro-iDCs. Thus, we believe that the addition of these cytokines into the differentiation cocktail is not necessary as IL-1β stimulation may lead in part to autocrine supply of moDC differentiation factors in pro-iDCs. In line with this, observations from studies in mice point to a pivotal role for IL-1β in DC differentiation from monocytes. For mouse $Ly6C^{hi}$ monocytes, a MyD88 dependent signal, as determined by MyD88-KO animals, was required for the differentiation towards inflammatory DCs and Tip-DCs under inflammatory conditions[180,188,269,270,310]. In a *Brucella melitensis* infection model, a MyD88 dependent signal was required but receptors mediating the signaling were interchangeable, as the absence of a single TLR could be substituted by another TLR, and Tip-DCs were still induced albeit at reduced frequencies[188]. Other studies described the differentiation of monocytes towards inflammatory DCs in a NLRP3 inflammasome and MyD88 dependent way during sterile inflammation induced by alum[269,270]. As NLRP3 inflammasome induction leads to IL-1β and IL-18 secretion, autocrine and MyD88 dependent IL-1R and IL-18R signaling could be considered to be crucial for inflammatory DC differentiation. Of note, IL-1β signaling induces IRF4 expression[311] in humans that is associated with DC differentiation[105]. Although this observation was made in $CD4^+$ T cells, a similar regulation in monocytes is to be expected as IL-1R signaling in both monocytes and T cells employ MyD88 dependent NF-κB and MAPK pathways[312-314]. In contrast to mice, where MyD88 signaling mediated by interchangeable TLRs induced monocyte differentiation into inflammatory DCs[172], human monocytes are less promiscuous. Human monocytes do not differentiate towards DCs in response to TLR7/8 dependent R848 stimulation[315] and according to established protocols, LPS stimulation is not sufficient to drive DC differentiation from monocytes[149,151]. In line with the data presented in this work, MyD88 dependent TLR8 and TLR4 signaling mediated by R848 and LPS were not

sufficient to induce moDC differentiation as shown by the lack of BDCA1 expression and DC function. Instead, IL-1β stimulation of $CD14^+CD16^-$ monocytes induced $BDCA1^+CD14^+$ cells reminiscent of pro-iDCs. Thus, in humans, moDC differentiation may occur *in vivo* already under steady state conditions as indicated by blood resident pro-iDCs obtained from healthy donors. These may be induced by IL-1R signaling, as IL-1β stimulation led to $BDCA1^+$ pro-inflammatory DC differentiation from $CD14^+CD16^-$ monocytes. This hypothesis is emphasized as this work revealed increased numbers of pro-iDCs in blood from patients suffering from RA when compared to healthy donors. It thus indicates that pro-iDCs are mobilized from progenitor cells during inflammation in a way resembling the fate of murine $Ly6C^{hi}$ monocytes. Interestingly, inflammatory DCs recently been described by Segura *et al*.[170] were found in synovial fluids from RA patients. Strikingly, they showed a high similarity to pro-iDCs found in this study, both in terms of phenotype and function but also differed in some aspects. Both subsets were similar in marker expression including BDCA1, CD14, HLA-DR, CD11b, CD172a, and absence of CD16 and CD209. Functionally, both cell types secreted large amounts of inflammatory cytokines, induced T_H17 responses and drove T cell proliferation. However, in contrast to inflammatory DCs found in RA, pro-iDCs lacked FcεR1α and CD206 expression. As IL-4 was described to induce FcεR1α protein expression[316], the expression of FcεR1α on inflammatory DCs found at the site of inflammation and the lack on blood pro-iDCs may be due to factors present only in inflamed tissues. Despite the absence of the CD206 protein, pro-iDCs expressed its transcript at very high levels, which were 21-fold higher than in $CD14^+CD16^-$ monocytes. Post-transcriptional control of *MRC1L1/CD206* translation could inhibit protein expression, which may be reversed by factors only present in inflamed environments. When compared to blood cells, no other population showed a higher convergence with inflammatory DCs than pro-iDCs. As the authors speculated a blood monocyte origin of inflammatory DCs[170] and pro-iDCs are likely to originate from monocytes, pro-iDCs may be regarded as an intermediate development stage between blood monocytes and DCs and an intermediate progenitor to inflammatory DCs found in inflamed tissues. Thus, the existence of pro-iDCs in blood may support their hypothesis that inflammatory DCs found in inflamed tissues originate from blood monocytes[170]. Other types of human inflammatory DCs have been found in different diseases, and despite divergences in phenotype and function, they were all termed inflammatory DCs. Wollenberg et al.[169] described inflammatory dendritic epidermal cells (IDECs) present in several autoimmune diseases including atopic dermatitis

and psoriasis. Among the few markers they analyzed, IDECs did not share the expression of markers with pro-iDCs. However, it is interesting to speculate that both cell types can be related considering that pro-iDCs are precursors of BDCA1$^+$CD14$^+$ cells, which we found in psoriatic skin, since IDECs and BDCA1$^+$CD14$^+$ cells shared the expression of CD1a, CD206 and FcεR1α. Zaba et al.[171,205] further described an inflammatory Tip-DC subset present in psoriatic skin. This subset was also distinct from blood pro-iDCs and were characterized as TNFα$^+$, IL-23$^+$, iNOS$^+$, CD11c$^+$ and BDCA1$^-$. Contrary to pro-iDCs, Tip-DCs did not express BDCA1, and key monocyte/macrophage and DC specific mRNA levels including *CD163*, *CD68*, *CD14, EGR1, VNN2* and *CD1E* were also differently regulated. In contrast to the relationship of pro-iDCs with CD14$^+$CD16$^-$ monocytes found in this work, BDCA1$^-$ Tip-DCs were discussed to be derived from inflammatory CD14$^+$CD16$^+$ monocytes based on transcriptome profiling[207], and might represent a distinct subset of inflammatory DCs. In contrast, another type of inflammatory DCs present in Crohns disease[317], an autoimmune disease affecting the gastrointestinal tract, is similar to blood pro-iDCs and inflammatory DCs from rheumatoid arthritis (RA)[170]. They were described as CD14$^+$, HLA-DR^{hi}, CD172a$^+$, IL-1β$^+$, TNFα$^+$ but lacked BDCA1 expression. Interestingly, all types of the before mentioned inflammatory DCs shared the presence in inflammatory diseases, were absent in healthy tissues and, where analyzed, had a strong capacity to induce T_H1/T_H17 cells. Although all authors speculated a monocytic origin[170,171,317,169], inflammatory DCs isolated from different pathological environments show phenotypic heterogeneity. This is similar to *in vitro* generated moDCs, as monocytes give rise to DCs with various phenotypes and functions dependent on the factors used for their differentiation[148]. In this respect, monocytes may differentiate towards inflammatory DCs with varying phenotypes and functions. However, it is still open whether these cells originate from different or common progenitors.

The synthesis and release of cytokines is a crucial function of DCs to induce defined signaling pathways in target cells. Similar to inflammatory DCs[170,171,317] found in inflamed tissues, pro-iDCs secreted vast amounts of inflammatory cytokines. Whereas the release of cytokines from unstimulated inflammatory DCs isolated from inflamed tissues could be addressed to environmental factors, the spontaneous release of cytokines from blood pro-iDCs was unexpected. A predisposition to strong cytokine secretion could not be observed, since pro-iDCs expressed similar levels of transcripts encoding for cytokines as cDCs and monocytes. Like BDCA1$^+$CD14$^-$ DCs and CD14$^+$CD16$^-$ monocytes, pro-iDCs did not contain pre-synthesized

cytokines that could be released by necrotic cell death during the culture. As BDCA1^{+}CD14^{int} cells were treated in an identical way as pro-iDCs, experimental procedures such as labeling with CD14 and BDCA1 antibodies and general cell handling could be excluded as a trigger for cytokine production. A reason might be a lower tolerance to cellular stress. As flow cytometric cell sorting increases cellular stress and ROS production[242], pro-iDCs could have an increased susceptibility to a ROS mediated release of cytokines. ROS in turn is detected by the NLRP3 inflammasome and leads to the secretion of IL-1β[19]. Indeed, the release of IL-1β could be abolished by the inhibition of ROS generating enzymes and ROS neutralization. In addition, IL-1R signaling mediated by IL-1β could be addressed as an upstream event for the release of further cytokines, as IL-1R blockade and IL-1β neutralization diminished levels of TNFα, IL-6, IL-10 and GM-CSF. Remaining levels of those cytokines might be due to incomplete blocking and the presence of non-neutralized IL-1α, as IL-1α binds to IL-1R as well[318]. Thus, an enhanced susceptibility to ROS may have caused NLRP3 inflammasome activation in pro-iDCs and enabled these cells for a rapid inflammatory response. In addition, it is possible that a spontaneous cytokine production and release by pro-iDCs is controlled by inhibitory signals present in the natural environment. As a consequence, cytokine release would no longer be inhibited after experimental isolation of pro-iDCs from blood. In human blood under non-inflamed conditions, mechanisms have been revealed, which inhibit a spontaneous release of cytokines to prevent a possible TNFα mediated septic shock. Schäkel et al.[236] demonstrated a CD172a dependent inhibitory mechanism for SLAN DCs. Ligation of CD47 present on erythrocytes to CD172a on SLAN DCs significantly inhibited the release of IL-12 and TNFα. Underlying mechanisms might be similar in pro-iDCs, as spontaneous release of cytokines could be inhibited from inflammatory DCs found in Crohns disease by the presence of a CD47 multimer[317]. These cells were described as HLA-DR^{hi}CD172a^{hi}, which intriguingly characterize a group of DCs capable of spontaneous release of cytokines. These DCs include SLAN DCs, inflammatory DCs isolated from RA tissues[170] and notably pro-iDCs as described in this work. We sought to investigate whether a similar anti-inflammatory mechanism in pro-iDCs could prevent spontaneous release of inflammatory cytokines in blood. Indeed, blood erythrocytes enhanced anti-inflammatory IL-10 secretion in pro-iDCs and thereby pointing to an immune dampening function in blood. However, the release of inflammatory cytokines was unaffected and possible inhibitory mechanisms remain to be elucidated.

We found pro-iDCs to be readily capable of secreting high amounts of inflammatory cytokines,

and are consistently present in the blood. Hence, we speculated that they should be quickly responsive for recruitment into inflammatory environments where they execute their function. During inflammation, DCs are thought to leave the blood stream and migrate to inflamed tissues[319]. In order to home to the site of inflammation, DCs require the exposition to chemotactic molecules that can be detected with a number of chemokine receptors[320]. This work demonstrated that pro-iDCs expressed several chemokine receptors including CCR2 and CCR5. CCL2, the ligand for CCR2, was reported to be markedly elevated during inflammation and plays a major role in attraction of monocytes and T cells in various autoimmune diseases such as psoriasis, RA, asthma and uveitis[321]. Besides, $CD14^+$ monocytes upregulated CCR2 expression during atopic dermatitis and psoriasis[322]. Data generated from *in vitro* experiments further indicated that human monocytes and moDCs selectively migrated in response to CCL2[323,324]. In addition, CCR5 mediates leukocyte migration especially to sites of inflammation[223,274] and pro-iDCs expressed the highest levels of CCR5 when compared to $BDCA1^+CD14^-$ DCs. It is interesting to speculate that pro-iDCs are recruited from blood to inflamed tissues in a CCR2 and CCR5 dependent manner during inflammation. Interestingly, this work showed that $BDCA1^+CD14^+$ cells that resemble pro-iDCs were found in inflamed tissues such as psoriatic skin but not in skin from healthy donors.

The release of soluble mediators by APCs has extensive effects on the activation and differentiation of immune cells. The secretion of IL-6 and TNFα from pro-iDCs lowered the activation threshold for T cells to undergo expansion and activation, which is in line with published observations[266,267]. In addition, inflammatory cytokines induced the maturation of blood cDCs but not pDCs, and IL-1β facilitated $CD14^+CD16^-$ monocyte differentiation towards $BDCA1^+CD14^+$ cells. Since ROS play a central role in the induction of cytokine production in macrophages and DCs[325,326], the ROS sensitive pro-iDCs might rapidly support immune responses by the release of vast amounts of inflammatory cytokines at sites of inflammation. This role for pro-iDCs is emphasized as stimulation with the TLR8 agonist R848 lead to the production of the highest amounts of IL-6 and TNFα in pro-iDCs when compared to $BDCA1^+CD14^-$ DCs and $CD14^+CD16^-$ monocytes. As this work shows, the secreted cytokines in turn could augment APC function and promote T cell activation. In contrast to mice, data showing direct implications of inflammatory DCs in disease exacerbation *in vivo* in humans is still missing. However, functional properties of pro-iDCs as shown in *in vitro* experiments in this work may indicate that the ability of pro-iDCs to readily secrete vast amounts of

inflammatory cytokines upon activation could further augment the presence of inflammatory mediators in pathogenic conditions, thereby increase the level of inflammation. This work demonstrated that pro-iDC derived cytokines could mature cDCs but not pDCs. In a study describing DCs in psoriatic and RA, isolated cDCs were characterized as mature whereas pDCs were immature as measured by expression levels of CD80, CD83, CD86 and CD40[327]. These markers were regulated in the same way on cDCs and pDCs treated with mediators released by pro-iDCs as described in this work. As pro-iDC related cells were present in both diseases[168,170] and were described as active cytokine producers, this observation strengthens the hypotheses that pro-iDC related inflammatory cells may contribute *in trans* by cytokine secretion to inflammation and disease progression. Furthermore, the capacity of pro-iDCs to secrete high amounts of inflammatory cytokines including IL-1β, IL-6 and TNFα, and to induce T_H17 cells could contribute to inflammation *in vivo*, as these cytokines and T_H17 cells were commonly found in inflamed tissues[171,328,329]. As described above, this work showed that TNFα and IL-6 were implicated in T cell activation and proliferation. Interestingly, IL-6 was essential for the maintenance of T cell responses in Crohn's disease and ulcerative colitis *in vivo*, which was due to the induction of the cellular anti-apoptotic factors bcl-2 and bcl-xL[330]. Besides, IL-1β was reported to turn IFNγ producing T cells into highly inflammatory IFNγ/IL-17 double-producers[253], which are present in several autoimmune diseases[254-256]. Indeed, psoriasis is dominated by T_H1 and T_H17 polarized T cells[171]. In addition, pro-iDCs were found to secrete IL-23, a cytokine that enables T_H17 cells to release IL-17[331], and which in turn induces pleiotropic inflammatory responses[332,333]. As a consequence, neutrophils, T cells and APCs are recruited to and activated at the site of IL-17 secretion leading to local inflammation[332,333]. IL-17 leads to progression in psoriasis[334], RA and noninfectious uveitis and is underlined in that IL-17 neutralization followed alleviation in severity[335]. Thus, pro-iDC related cells may be involved in several autoimmune diseases and establishment of new analysis methods will shed light on their implications *in vivo*.

7 Conclusion

Inflammatory DCs were shown in a number of studies in mice to be implicated in a multitude of inflammatory diseases with host protective and destructive properties. These reports allowed a detailed description of inflammatory DCs including their cellular origin, recruitment to and impact at inflamed and lymphatic tissues, as well as their crucial impact during inflammation. Inflammatory DCs were recently identified in humans, however a characterization of their origin and differentiation are missing, and their biologic function is far away from being understood. This work demonstrated a novel human DC subset with inflammatory properties originating from $CD14^{+}CD16^{-}$ monocytes and probable precursors of inflammatory DCs found in inflamed tissues. Functional data on pro-iDCs confirmed the immunogenic role of inflammatory DCs by the secretion of inflammatory cytokines, priming of T_H17 responses and APC activation. First studies and data presented here may indicate a similar role of (pro-)inflammatory DCs in several autoimmune diseases. In order to elucidate the pathogenesis of inflammatory diseases and to find medical treatments, a major objective is the comprehension of human moDC differentiation and recruitment *in vivo*. Since $CD14^{+}CD16^{-}$ monocytes as probable precursors of (pro-)inflammatory DCs show a high abundance in blood and could thereby support continuous replenishment of inflammatory DCs, a first goal is to elucidate the role of pro-iDCs *in vivo*. Therefore and to interfere with a possible disease outcome, further understanding of factors controlling the activation and recruitment of pro-iDCs *in vivo* is required.

8 Appendix

Reporter Name	Reporter ID	Entrez Gene ID	Fold-change	Tukey test p-value
C1QB	A_23_P137366	713	140.5072	5.90E-07
C1QC	A_23_P125977	714	65.1864	4.67E-06
C1QA	A_24_P222655	712	47.9181	2.87E-06
ENHO	A_33_P3417626	375704	45.5854	1.71E-07
GFRA2	A_33_P3317580	2675	35.3834	1.73E-04
FPR3	A_33_P3247042	2359	28.6706	3.76E-05
ENHO	A_33_P3405114	375704	27.7420	9.71E-07
SLCO2B1	A_23_P150768	11309	22.4089	2.52E-06
SLC38A1	A_23_P363399	81539	22.3236	4.46E-08
MRC1L1	A_23_P12746	4360	21.4812	1.30E-06
GUCY1A3	A_33_P3269110	2982	20.7063	9.33E-06
SLC38A1	A_24_P261734	81539	20.5562	6.47E-08
GCOM1	A_33_P3423874	100820829	18.6681	9.96E-05
LMNA	A_33_P3311285	4000	16.3589	5.75E-06
CLEC4F	A_23_P380990	165530	16.2684	5.72E-03
ABCB4	A_33_P3336760	5244	16.2009	8.32E-07
DNASE1L3	A_33_P3234202	1776	15.4925	4.80E-05
DKFZP564O0823	A_24_P191781	25849	15.4015	7.86E-04
LMNA	A_23_P34835	4000	15.3642	2.59E-05
LMNA	A_33_P3408762	4000	14.8975	1.56E-05
ADD2	A_33_P3241786	119	14.6416	2.08E-06
CDCP1	A_23_P113613	64866	14.5808	3.49E-06
CTTNBP2	A_23_P215744	83992	14.2808	4.92E-04
A2M	A_23_P116898	2	14.1330	8.00E-06
MRC2	A_33_P3364741	9902	13.4637	4.54E-05
TIFAB	A_33_P3380383	497189	13.4450	3.11E-05
NAV1	A_24_P102880	89796	13.3660	7.29E-06
SLC4A3	A_23_P39647	6508	13.1228	3.67E-05
FBLN2	A_23_P143981	2199	13.0864	1.95E-07
H19	A_24_P52697	283120	12.1510	3.63E-05
LOC651721	A_33_P3560679	100287221	11.7860	1.36E-06
CLEC10A	A_23_P141505	10462	11.5434	1.25E-04
PKIB	A_23_P145529	5570	11.4995	4.15E-04
PTPN13	A_23_P18493	5783	11.4597	3.15E-03
ITGB5	A_23_P166633	3693	11.4161	5.67E-06
GUCY1A3	A_23_P69573	2982	11.2902	6.23E-05
PLVAP	A_23_P56328	83483	11.2472	1.71E-03
C5orf20	A_23_P81441	140947	11.0387	2.94E-04
CD1E	A_23_P310410	913	10.8792	4.68E-07
UBD	A_23_P81898	10537	10.7443	1.52E-03
LOC100128913	A_33_P3294317		10.6738	1.29E-05
STAG3	A_33_P3317058	10734	10.0945	8.94E-06
PTPRH	A_23_P101642	5794	9.7981	3.64E-04

LHX2	A_23_P32165	9355	9.6364	1.88E-03
CLIC2	A_33_P3775848	1193	9.4873	9.52E-09
P2RY6	A_23_P64611	5031	9.4840	1.64E-06
ARHGAP20	A_23_P422933	57569	9.4676	9.42E-06
CD1C	A_33_P3273474	911	9.4512	1.86E-05
CDCP1	A_24_P160401	64866	9.4055	5.65E-06
ENST00000377666	A_23_P371076	11278	9.3827	1.04E-06
HES4	A_33_P3342628	57801	9.2792	1.69E-03
HLA-DOA	A_32_P356316	3111	9.0914	2.93E-08
CD1B	A_23_P351844	910	9.0098	1.32E-06
SETBP1	A_32_P32413	26040	8.5150	4.26E-05
HLA-DPB2	A_32_P505730		8.2907	1.09E-04
LAMP3	A_23_P29773	27074	8.2792	2.76E-03
SASH1	A_24_P658584	23328	8.1965	1.44E-05
SEMA7A	A_23_P106389	8482	7.9723	2.42E-07
S100B	A_23_P143526	6285	7.9118	2.27E-03
SETBP1	A_23_P4551	26040	7.9008	4.13E-05
GPR161	A_23_P303455	23432	7.8218	6.74E-06
LGMN	A_23_P25994	5641	7.7409	6.14E-03
RGL1	A_33_P3240532	23179	7.7355	1.62E-05
NAV1	A_33_P3405068	89796	7.6555	1.36E-06
PLXNA1	A_23_P57667	5361	7.6290	6.76E-08
SCD	A_32_P163858	6319	7.6026	2.45E-05
C1orf195	A_33_P3486059	727684	7.5921	1.88E-08
GAS6	A_23_P146922	2621	7.5084	2.09E-07
GATM	A_23_P129064	2628	7.4307	1.88E-04
BLNK	A_33_P3363637	29760	7.2955	9.94E-05
KLF12	A_23_P354805	11278	7.2904	2.94E-06
GPR126	A_24_P411749	57211	7.2727	3.91E-06
SDS	A_23_P2645	10993	7.1429	8.98E-04
FIGLA	A_33_P3315944	344018	7.1330	3.44E-05
SLAMF8	A_23_P200138	56833	7.1206	3.08E-04
SASH1	A_33_P3209279	23328	7.0812	3.57E-04
TREM2	A_24_P12397	54209	7.0299	1.09E-04
CCR5	A_23_P412321	1234	6.9283	4.11E-04
TNFRSF11A	A_23_P390518	8792	6.9163	4.90E-06
DEPDC6	A_23_P60166	64798	6.7342	2.72E-07
C16orf59	A_23_P26557	80178	6.6668	5.32E-06
FLJ14213	A_23_P116512	79899	6.5842	3.30E-04
UNC5B	A_23_P52336	219699	6.5342	1.75E-03
CD2	A_23_P161076	914	6.2031	5.59E-05
P2RY14	A_24_P165864	9934	6.1029	3.26E-03
CD276	A_33_P3222917	80381	6.0776	2.35E-04
RGS1	A_23_P97141	5996	5.9381	5.09E-05
IRF4	A_23_P214360	3662	5.9340	1.66E-04
CXCL9	A_23_P18452	4283	5.7777	7.20E-04
MSR1	A_33_P3379326	4481	5.7697	3.63E-03

FAM23A	A_33_P3382031	653567	5.7140	2.01E-03
P2RY12	A_23_P143902	64805	5.6923	9.99E-03
UST	A_23_P314145	10090	5.6295	9.19E-10
HIC1	A_23_P129856	3090	5.6022	2.18E-03
PPARG	A_23_P252062	5468	5.5964	1.40E-03
CIB2	A_33_P3308914	10518	5.5732	1.38E-03
A_19_P00323082	A_19_P00323082		5.4774	1.95E-04
FAM190A	A_33_P3343365	401145	5.4604	1.70E-04
APOC1	A_24_P109214	341	5.4208	2.74E-03
ATP1B1	A_23_P62932	481	5.4189	2.37E-04
TMEM51	A_33_P3363560	55092	5.2890	9.52E-07
BLNK	A_24_P64344	29760	5.2561	1.15E-03
PPARG	A_33_P3350726	5468	5.2216	1.91E-03
TDRKH	A_23_P46351	11022	5.1766	1.18E-03
DOCK1	A_23_P45059	1793	5.1676	2.74E-06
FAM167A	A_23_P334955	83648	5.1142	1.18E-05
RYR1	A_23_P78867	6261	5.0806	2.47E-03
HSPB1	A_33_P3287646	3315	5.0613	1.01E-07
GUCY1B3	A_23_P121564	2983	5.0438	4.32E-03
MAF	A_23_P397376	4094	5.0263	1.43E-03
LOC729085	A_23_P69154	729085	5.0176	1.93E-03
CENPE	A_23_P253524	1062	5.0176	4.04E-06
BCL7A	A_24_P203056	605	4.8838	3.55E-06
PLAU	A_33_P3306146	5328	4.8753	5.39E-04
FAM20A	A_32_P108254	54757	4.7502	8.36E-03
DOK7	A_23_P398854	285489	4.7338	5.82E-03
TNFRSF25	A_23_P126844	8718	4.6605	2.21E-03
CD72	A_23_P250245	971	4.6203	4.13E-06
TRH	A_23_P132760	7200	4.5805	6.68E-03
EBI3	A_23_P119478	10148	4.5410	5.33E-03
ADORA1	A_23_P74299	134	4.4383	4.42E-03
MLLT4	A_23_P436353	4301	4.4199	1.68E-04
IL21R	A_24_P227927	50615	4.3939	1.72E-04
TTC7B	A_23_P25974	145567	4.3908	3.98E-04
PRDM1	A_33_P3342081	639	4.3757	4.09E-03
SLC35F2	A_23_P339098	54733	4.3259	7.49E-05
BATF3	A_23_P160720	55509	4.2398	6.90E-06
LOC399804	A_33_P3360525		4.2105	8.71E-04
MCOLN2	A_23_P23639	255231	4.2061	4.78E-03
ADA	A_23_P210482	100	4.2032	4.18E-06
EPS8	A_23_P136347	2059	4.1655	1.88E-04
NDRG2	A_33_P3334515	57447	4.1153	2.36E-03
PRDM1	A_23_P350451	639	4.1025	4.63E-04
CCDC34	A_33_P3313796	91057	4.0869	1.22E-03
SLC39A14	A_23_P59950	23516	4.0855	3.32E-04
MGLL	A_33_P3281795	11343	4.0615	2.39E-04
EPB41L2	A_23_P134109	2037	4.0278	8.52E-07

TLR10	A_33_P3383970	81793	3.9931	7.69E-04
C10orf55	A_33_P3666817	414236	3.9834	5.89E-05
SLC29A4	A_23_P168551	222962	3.9751	1.08E-03
DAB2	A_33_P3338928	1601	3.9627	7.33E-04
ZBTB46	A_33_P3266674	140685	3.9586	3.15E-05
NRARP	A_33_P3337272	441478	3.9408	4.52E-04
SDC3	A_23_P74887	9672	3.9163	1.29E-03
AIG1	A_23_P93431	51390	3.8704	1.42E-05
CD79B	A_23_P207201	974	3.8691	3.88E-03
ANKH	A_24_P303145	56172	3.8530	2.58E-03
NDRG2	A_33_P3383233	57447	3.8437	2.88E-03
DHCR24	A_23_P379475	1718	3.8357	6.16E-04
SPRED1	A_24_P107859	161742	3.8278	6.08E-05
C3orf59	A_32_P117464	151963	3.7711	6.62E-03
BMP2	A_33_P3237150	650	3.7555	7.92E-03
SFTPD	A_32_P107617	6441	3.7503	9.69E-03
IL18BP	A_33_P3228322	10068	3.7451	7.46E-05
ANKH	A_24_P371628	56172	3.7373	7.53E-03
HLA-DPB1	A_33_P3271635	3115	3.7025	1.94E-05
GPR146	A_23_P20035	115330	3.6782	1.80E-04
PTTG1	A_23_P7636	9232	3.6655	2.23E-05
ZBTB46	A_24_P592012	140685	3.6364	1.12E-04
ADAMTS10	A_33_P3223056	81794	3.6301	4.28E-03
AIG1	A_33_P3416563	51390	3.6276	1.40E-05
SEZ6L	A_23_P80242	23544	3.6188	6.99E-03
LOC100131110	A_32_P151782		3.6150	5.72E-03
STMN1	A_33_P3317523	3925	3.6113	4.26E-03
CRYBB1	A_23_P143621	1414	3.6050	4.68E-03
HSPB1	A_33_P3353242	3315	3.5975	1.70E-04
CHST10	A_23_P102351	9486	3.5851	1.31E-03
SLC46A1	A_33_P3235611	113235	3.5751	1.48E-04
A_19_P00323081	A_19_P00323081		3.5665	2.88E-05
RUNX3	A_23_P51231	864	3.5492	3.19E-05
MTSS1	A_23_P347632	9788	3.5345	7.91E-04
CDK6	A_24_P166663	1021	3.5333	3.82E-05
C6orf145	A_24_P272290	221749	3.5101	1.26E-04
UBE2T	A_23_P115482	29089	3.5004	3.03E-04
SMO	A_23_P70818	6608	3.4955	4.35E-03
MGLL	A_24_P226008	11343	3.4762	7.21E-04
OLFML2B	A_23_P137689	25903	3.4762	8.81E-05
AFF3	A_23_P373464	3899	3.4462	3.17E-04
PLA1A	A_24_P294408	51365	3.4176	2.77E-03
C11orf63	A_23_P83751	79864	3.3976	1.02E-03
SEPT8	A_23_P415827	23176	3.3799	7.93E-03
RUNX3	A_33_P3221748	864	3.3753	1.19E-05
OIP5	A_23_P379614	11339	3.3543	1.12E-03
CA11	A_23_P78782	770	3.3253	5.52E-03

TMEM97	A_24_P190168	27346	3.3173	5.52E-05
AIG1	A_33_P3416574	51390	3.3047	1.87E-05
ATP10A	A_24_P215765	57194	3.2966	2.89E-03
MKL2	A_23_P54556	57496	3.2944	4.09E-06
HES1	A_23_P6596	3280	3.2750	9.32E-05
TCEA3	A_23_P34375	6920	3.2445	2.70E-03
CCNE1	A_23_P209200	898	3.2423	1.55E-06
C1orf225	A_24_P734953	388610	3.2243	1.96E-03
TSPYL5	A_23_P417951	85453	3.2154	7.88E-04
EVL	A_23_P140427	51466	3.2076	3.27E-04
PYCR1	A_23_P130194	5831	3.1821	1.27E-03
EFNB1	A_24_P365807	1947	3.1744	2.81E-06
A_19_P00803836	A_19_P00803836		3.1711	2.08E-03
HLA-DQB2	A_23_P19510	3120	3.1602	1.24E-03
HRG	A_23_P321892	3273	3.1340	4.99E-03
RAPH1	A_24_P924862	65059	3.1231	8.20E-03
RAP2A	A_24_P81965	5911	3.1199	8.35E-05
ENST00000374149	A_33_P3341916	170371	3.1091	4.63E-04
FMNL2	A_33_P3310552	114793	3.0951	3.34E-04
CXCL12	A_23_P202448	6387	3.0887	2.13E-03
HLA-DPB1	A_33_P3271651	3115	3.0377	2.94E-06
ORC6L	A_23_P100344	23594	3.0293	6.81E-06
RHOC	A_23_P406424	389	3.0230	8.25E-09
FLJ22795	A_23_P398275	388152	3.0199	4.18E-03
SLAMF7	A_24_P353638	57823	2.9938	1.80E-03
A_33_P3329104	A_33_P3329104		2.9887	1.18E-04
STAT4	A_23_P68031	6775	2.9866	7.98E-03
KIAA0284	A_23_P48550	283638	2.9856	9.84E-03
KDELC2	A_33_P3214849	143888	2.9670	2.10E-03
LIPA	A_23_P97860	3988	2.9619	1.43E-03
MTHFD1L	A_24_P235049	25902	2.9547	1.75E-05
CTTNBP2NL	A_23_P318420	55917	2.9322	1.23E-04
BCAR3	A_23_P97394	8412	2.9302	6.21E-04
TRAF4	A_23_P15654	9618	2.9292	4.94E-05
GBP1	A_23_P62890	2633	2.9272	7.44E-03
SORBS3	A_33_P3409625	10174	2.9231	6.51E-08
SQLE	A_23_P146284	6713	2.9221	1.96E-04
CD209	A_33_P3272493	30835	2.9130	8.45E-05
SMYD5	A_23_P91015	10322	2.9069	8.55E-05
MORC4	A_23_P171117	79710	2.9069	1.11E-03
SLC1A4	A_24_P191312	6509	2.9039	8.17E-03
SMC2	A_33_P3357322	10592	2.8939	1.48E-03
OLFML2B	A_33_P3401243	25903	2.8889	6.81E-04
KHDRBS3	A_33_P3332081	10656	2.8889	3.42E-03
CKS1B	A_32_P206698	1163	2.8819	2.01E-05
IQCK	A_23_P324523	124152	2.8719	4.01E-03
CMAH	A_33_P3281572	8418	2.8659	1.27E-03

IL4I1	A_33_P3405424	259307	2.8659	1.04E-04
FABP5	A_32_P204676	2171	2.8620	9.28E-05
SH3RF1	A_24_P100551	57630	2.8461	8.57E-03
MCM2	A_32_P103633	4171	2.8392	1.03E-06
FABP5	A_23_P59877	2171	2.8284	1.15E-04
SPATS2L	A_33_P3400389	26010	2.8167	3.74E-08
RHEBL1	A_33_P3264895	121268	2.7876	3.30E-06
TRIM36	A_23_P110569	55521	2.7827	3.83E-04
A_19_P00802810	A_19_P00802810		2.7779	2.68E-05
XYLB	A_23_P212423	9942	2.7769	9.69E-04
LAG3	A_23_P116942	3902	2.7359	5.79E-03
HLA-DPA1	A_33_P3234277	3113	2.7245	2.20E-06
GIPC1	A_33_P3390102	10755	2.7245	2.30E-05
SDSL	A_33_P3227472	113675	2.7085	2.48E-03
MOBKL2B	A_24_P32085	79817	2.7048	8.76E-04
DLL1	A_23_P167920	28514	2.7038	1.11E-05
ZNF823	A_23_P39050	55552	2.6973	5.72E-06
ACOT7	A_24_P205589	11332	2.6926	4.36E-05
TUBA1B	A_23_P128147	10376	2.6870	8.21E-05
METTL1	A_23_P47790	4234	2.6805	8.46E-05
MAP4K1	A_23_P5002	11184	2.6749	1.07E-04
CENPP	A_33_P3245321	401541	2.6537	1.31E-03
C3orf26	A_23_P132874	84319	2.6500	9.65E-05
TUBA1C	A_24_P58529	84790	2.6372	4.04E-07
CC2D2A	A_23_P348383	57545	2.6308	6.08E-03
GPATCH4	A_23_P97265	54865	2.6308	8.06E-04
PPM1J	A_23_P201939	333926	2.6217	5.18E-06
A_33_P3346533	A_33_P3346533		2.6190	9.59E-03
UACA	A_33_P3323718	55075	2.6153	3.51E-03
CMAH	A_23_P351467	8418	2.6045	4.53E-04
GINS1	A_33_P3340025	9837	2.5982	1.23E-03
HLA-DPB1	A_24_P166443	3115	2.5955	4.49E-06
FOXRED2	A_24_P147765	80020	2.5937	9.02E-05
CHEK2	A_23_P109452	11200	2.5883	1.06E-05
CDC25B	A_23_P210726	994	2.5847	1.91E-03
CD209	A_24_P305345	30835	2.5838	1.80E-05
GBP4	A_24_P45446	115361	2.5767	4.37E-03
METTL1	A_33_P3700794	4234	2.5660	1.73E-04
CMAH	A_33_P3281567	8418	2.5633	8.10E-05
HLA-DQB1	A_33_P3424222	3119	2.5571	6.28E-03
C7orf58	A_24_P943781	79974	2.5535	6.04E-03
AK097103	A_33_P3374398		2.5535	8.67E-03
LOC100130401	A_33_P3347035		2.5509	1.61E-03
ALKBH8	A_33_P3318357	91801	2.5500	8.64E-03
SLC2A1	A_23_P571	6513	2.5438	1.14E-03
MTHFD1L	A_32_P135243	25902	2.5385	2.08E-05
HSPH1	A_33_P3348752	10808	2.5385	1.40E-09

FABP5	A_24_P673063	2171	2.5315	4.62E-04
PFAS	A_23_P402610	5198	2.5193	7.52E-04
XYLT1	A_24_P787897	64131	2.5140	5.87E-04
PLAU	A_23_P24104	5328	2.4889	3.24E-03
MAD2L1	A_23_P92441	4085	2.4751	7.14E-04
PAPSS2	A_24_P940166	9060	2.4734	2.46E-03
HLA-DOA	A_33_P3278968	3111	2.4572	1.08E-04
A_19_P00801394	A_19_P00801394		2.4504	1.16E-03
C5orf4	A_23_P501831	10826	2.4470	1.74E-03
CKS1B	A_23_P45917	1163	2.4444	2.97E-07
BC038245	A_23_P166508		2.4394	2.29E-03
C10orf2	A_23_P138461	56652	2.4385	4.59E-03
SDC3	A_33_P3241884	9672	2.4284	3.76E-03
CCDC58	A_32_P215113	131076	2.4275	3.20E-04
TRAF4	A_33_P3402035	9618	2.4259	2.45E-06
HLA-DRA	A_32_P87697	3122	2.4233	6.22E-04
ISOC2	A_24_P99795	79763	2.4200	1.97E-04
HSPH1	A_23_P88119	10808	2.4175	2.32E-07
RAB39B	A_24_P408603	116442	2.4099	1.18E-03
TRAF5	A_23_P201731	7188	2.4058	1.44E-03
USP13	A_23_P40989	8975	2.3991	1.12E-03
ATF7IP2	A_23_P129466	80063	2.3958	1.74E-04
MLLT4	A_33_P3278407	4301	2.3958	6.58E-04
PEA15	A_33_P3220827	8682	2.3875	4.62E-06
SEPT11	A_33_P3310475	55752	2.3867	1.23E-03
MAP1D	A_23_P90790	254042	2.3759	2.12E-03
THC2680929	A_33_P3397910		2.3644	4.19E-06
UTRN	A_33_P3234855	7402	2.3620	2.80E-03
CEP78	A_33_P3219641	84131	2.3563	7.31E-06
HABP4	A_33_P3592015	22927	2.3530	5.13E-05
GPAM	A_24_P227069	57678	2.3457	2.05E-03
THC2556327	A_33_P3271634	3115	2.3440	2.49E-05
HLA-DPA1	A_23_P30913	3113	2.3440	9.71E-07
UGCG	A_23_P313389	7357	2.3416	1.16E-03
FAM86B2	A_33_P3269740	653333	2.3206	7.28E-04
C6orf108	A_33_P3372451	10591	2.3198	2.77E-03
ARL4C	A_33_P3323722	10123	2.3070	8.72E-04
OBSL1	A_23_P300484	23363	2.3054	1.78E-04
PEA15	A_24_P410952	8682	2.2974	5.71E-06
CREBL2	A_24_P56194	1389	2.2942	2.44E-04
C1orf54	A_23_P74778	79630	2.2934	1.59E-04
GAS2L3	A_32_P189204	283431	2.2934	1.09E-03
BCL2	A_23_P352266	596	2.2871	8.17E-03
WDHD1	A_23_P25873	11169	2.2784	3.23E-03
ALDH18A1	A_23_P1361	5832	2.2728	4.00E-03
ADAT2	A_33_P3268798	134637	2.2650	8.27E-05
GPATCH4	A_33_P3211263	54865	2.2618	9.05E-03

HDGFRP3	A_23_P344451	50810	2.2603	4.69E-03
C6orf66	A_24_P171983	29078	2.2595	4.15E-06
BCKDHB	A_24_P914513	594	2.2408	9.96E-03
RICS	A_23_P161686	9743	2.2384	3.01E-03
HSPA8	A_33_P3390758	3312	2.2353	4.84E-04
PTTG2	A_23_P18579	10744	2.2245	9.69E-04
CD74	A_23_P70095	972	2.2245	1.45E-04
ZNF329	A_23_P413634	79673	2.2046	6.34E-03
NRP2	A_33_P3297415	8828	2.2046	9.88E-03
PTGER4	A_23_P148047	5734	2.2030	4.55E-05
SGPP1	A_23_P347048	81537	2.2015	7.97E-04
PPARGC1B	A_23_P213959	133522	2.1969	4.56E-04
PID1	A_23_P21485	55022	2.1962	1.46E-03
MCM6	A_23_P90612	4175	2.1931	4.32E-04
ULK2	A_23_P55107	9706	2.1901	1.68E-05
CIITA	A_32_P209960	4261	2.1833	5.54E-05
GOT1	A_23_P63825	2805	2.1765	1.07E-04
GINS4	A_33_P3340040	84296	2.1750	1.90E-04
CBLN3	A_33_P3412900	643866	2.1675	2.60E-03
FMNL2	A_32_P193080	114793	2.1644	6.53E-04
CCL17	A_23_P26325	6361	2.1622	3.58E-04
VEGFB	A_23_P1594	7423	2.1592	1.65E-04
UTRN	A_32_P524014	7402	2.1570	8.83E-04
ACSF2	A_23_P4190	80221	2.1517	3.41E-08
ABI2	A_24_P933418	10152	2.1495	8.11E-05
UTRN	A_33_P3234864	7402	2.1428	8.03E-03
PAOX	A_33_P3336387	196743	2.1421	1.25E-05
SNORA55	A_33_P3648417	677834	2.1287	1.19E-03
HLA-DRB1	A_24_P343233	3123	2.1287	2.21E-03
ZFP82	A_23_P416813	284406	2.1258	8.75E-03
HAUS5	A_33_P3269338	23354	2.1251	5.67E-04
SLIT1	A_33_P3411035	6585	2.1184	1.08E-04
LOC100128994	A_33_P3265872		2.1177	9.92E-04
SACS	A_33_P3405500	26278	2.1177	4.77E-04
DST	A_23_P303718	667	2.1148	5.74E-03
NPM3	A_23_P52298	10360	2.1126	2.51E-05
BAG2	A_23_P356554	9532	2.1104	2.06E-03
PAOX	A_23_P385771	196743	2.1104	2.52E-05
NME1	A_23_P152804	4830	2.1038	1.45E-03
HTR7	A_23_P500381	3363	2.0980	1.81E-04
HSD17B8	A_23_P81973	7923	2.0951	1.60E-03
FARP2	A_33_P3350728	9855	2.0936	7.65E-03
GAMT	A_24_P19228	2593	2.0929	2.73E-03
AI669688	A_33_P3367077		2.0929	2.14E-03
LOC653390	A_24_P475864	653390	2.0770	9.65E-03
PMVK	A_23_P114774	10654	2.0763	7.85E-06
BC035371	A_24_P376422		2.0712	5.20E-03

CR604707	A_32_P2634		2.0698	4.13E-03
PPARGC1B	A_33_P3816688	133522	2.0691	2.79E-03
OSBPL3	A_24_P377499	26031	2.0662	1.09E-04
PAICS	A_24_P200427	10606	2.0648	2.07E-05
EARS2	A_23_P152353	124454	2.0584	3.06E-04
RPL39L	A_23_P29594	116832	2.0569	9.46E-04
MOV10	A_23_P161125	4343	2.0548	3.06E-05
KCNK13	A_23_P3177	56659	2.0484	2.48E-04
C2orf7	A_23_P131596	84279	2.0477	1.97E-03
MCM4	A_23_P370989	4173	2.0477	2.15E-03
MTHFD1L	A_23_P214907	25902	2.0470	1.41E-03
HIG2	A_23_P20022	29923	2.0463	7.59E-03
FDX1L	A_24_P416660	112812	2.0420	1.10E-05
TTC27	A_23_P131227	55622	2.0413	2.98E-04
PCCA	A_23_P48358	5095	2.0371	9.79E-04
LRRC34	A_23_P41194	151827	2.0335	3.10E-03
ASAP3	A_23_P114689	55616	2.0314	1.27E-03
RFC4	A_23_P18196	5984	2.0314	1.09E-07
S1PR2	A_32_P827528	9294	2.0314	2.66E-03
CTNND1	A_23_P251316	1500	2.0279	1.88E-04
C2orf43	A_23_P420256	60526	2.0258	3.29E-03
A_19_P00327972	A_19_P00327972		2.0237	1.43E-04
ATF5	A_23_P119337	22809	2.0230	1.73E-03
HLA-DMA	A_24_P50245	3108	2.0223	1.21E-04
IMPDH2	A_33_P3214298	3615	2.0118	4.38E-04
FMNL3	A_33_P3415923	91010	2.0062	3.89E-04

Table 3 Agilent reporters with an at least two-fold upregulation in BDCA1^{+}CD14^{+} pro-iDCs relative to CD14^{+} CD16^{-} monocytes (Tukey test p-value ≤ 0.05).

Reporter Name	Reporter ID	Entrez Gene ID	Fold-change	Tukey test p-value
S100P	A_23_P58266	6286	52.201	5.73E-05
BPI	A_23_P131785	671	43.850	2.95E-06
ANXA8L2	A_32_P105549	244	32.447	6.80E-05
BPI	A_23_P131789	671	27.974	2.39E-04
HP	A_23_P206760	3240	25.510	6.85E-09
VNN3	A_23_P398449	55350	23.802	5.15E-10
DAPK2	A_24_P10233	23604	23.377	2.80E-06
TPST1	A_23_P145965	8460	23.103	3.03E-06
PADI4	A_23_P138262	23569	22.093	9.15E-10
SLCO4C1	A_32_P154342	353189	21.496	2.28E-05
LOC392382	A_24_P255384		19.400	4.47E-08
F5	A_32_P41604	2153	18.501	9.22E-13
TNFAIP6	A_23_P165624	7130	17.741	7.08E-08
VNN2	A_23_P122724	8875	17.539	2.70E-06
LOC572558	A_32_P157208	572558	17.220	4.46E-06
CXCL1	A_23_P7144	2919	16.359	1.20E-04
S1PR3	A_33_P3281283	1903	15.450	2.08E-04
MCTP2	A_24_P244944	55784	13.642	1.48E-07
HPR	A_33_P3289236	3250	13.595	2.63E-06
FAM82A1	A_23_P209625	1545	12.197	1.23E-03
LOC732146	A_33_P3277367		12.021	4.67E-03
SERPINB2	A_24_P245379	5055	11.975	9.60E-09
APCDD1	A_23_P337262	147495	11.696	1.14E-03
CYP1B1	A_33_P3290343	1545	11.692	1.11E-03
MCTP2	A_23_P65789	55784	11.416	6.00E-06
AQP9	A_23_P106362	366	11.236	4.74E-04
SERPINB10	A_23_P313981	5273	11.216	2.60E-09
PDE6H	A_23_P64913	5149	10.422	3.31E-05
C9orf47	A_33_P3379061	286223	10.407	2.80E-06
TDRD9	A_32_P208350	122402	10.378	1.27E-03
RPH3A	A_23_P76234	22895	10.307	3.81E-07
CMTM2	A_23_P152234	146225	10.289	2.98E-05
STEAP4	A_33_P3555937	79689	9.866	4.55E-08
MGAM	A_23_P42897	8972	9.687	9.71E-08
ALOX5AP	A_24_P347378	241	9.616	1.63E-04
MOSC1	A_33_P3247205	64757	9.590	2.79E-06
KCNJ15	A_33_P3376140	3772	9.517	4.01E-07
PRRT4	A_33_P3407529	401399	9.445	4.95E-06
C9orf47	A_33_P3248638	286223	9.177	4.23E-09
DYSF	A_23_P39931	8291	9.091	1.39E-03
A_19_P00322955	A_19_P00322955		8.843	2.67E-06
MOSC1	A_33_P3293362	64757	8.806	9.59E-07
TSPAN2	A_24_P62659	10100	8.601	3.25E-06
ALDH1A1	A_23_P83098	216	8.553	1.65E-05
CES1	A_33_P3241269	1066	8.477	6.37E-03

CRISPLD2	A_23_P106602	83716	8.444	3.65E-04
PANX2	A_33_P3588134	56666	8.401	2.03E-08
LGALS12	A_23_P139198	85329	8.188	1.79E-03
ADAMTS5	A_33_P3372426	11096	8.114	6.09E-06
AKR1C3	A_23_P138541	8644	8.109	4.68E-04
A_19_P00809432	A_19_P00809432		8.086	4.01E-03
PRL	A_32_P65616	5617	7.887	3.56E-06
SYNE1	A_33_P3335910	23345	7.563	6.54E-05
ADAM19	A_23_P81369	8728	7.519	4.56E-03
ZBTB16	A_23_P104804	7704	7.480	5.34E-07
C2orf39	A_23_P308722	92749	7.298	6.39E-04
SELL	A_33_P3400273	6402	7.288	7.67E-06
S100A12	A_33_P3385785	6283	7.280	1.54E-05
ENST00000442512	A_33_P3283964	390732	7.160	2.00E-04
AFF2	A_33_P3293888	2334	6.921	2.74E-05
CES1	A_33_P3389704	1066	6.842	6.30E-03
LRRC4	A_33_P3263217	64101	6.840	7.86E-04
KRT23	A_33_P3299761	25984	6.795	1.22E-04
SPRY1	A_23_P144476	10252	6.641	4.17E-04
ALDH1A1	A_33_P3379644	216	6.632	1.73E-06
CES1	A_23_P206733	1066	6.573	1.88E-04
LOC100128276	A_33_P3326447	100508384	6.536	1.13E-04
HAL	A_23_P61637	3034	6.338	5.16E-05
STEAP4	A_33_P3318322	79689	6.296	1.71E-04
A_19_P00316135	A_19_P00316135		6.251	9.57E-05
S100A12	A_23_P74001	6283	6.114	3.57E-03
STX1A	A_23_P168556	6804	6.078	9.50E-05
A_19_P00804329	A_19_P00804329		5.973	2.91E-03
ANKRD18A	A_33_P3238941	253650	5.965	8.57E-05
CR1	A_23_P256821	1378	5.918	5.32E-03
CYP4F3	A_33_P3294277	4051	5.864	8.35E-04
HTRA1	A_23_P97990	5654	5.820	6.79E-04
SELP	A_23_P137697	6403	5.788	1.21E-05
A_19_P00332072	A_19_P00332072		5.768	1.07E-03
TNFSF14	A_24_P237036	8740	5.706	1.99E-03
CXCL1	A_33_P3330264	2919	5.589	2.83E-04
KRT23	A_23_P78248	25984	5.523	2.23E-04
SPINK8	A_33_P3258542	646424	5.476	1.86E-04
C9orf47	A_24_P316601	286223	5.428	2.31E-03
MAPK13	A_24_P406132	5603	5.421	1.42E-04
PRLR	A_33_P3416757	5618	5.411	5.63E-04
DGAT2	A_23_P53198	84649	5.389	1.86E-04
GPR177	A_24_P256380	79971	5.354	2.86E-04
PNPLA1	A_32_P526255	285848	5.322	7.28E-05
KCNJ15	A_33_P3267532	3772	5.289	6.68E-04
KIAA1257	A_32_P163739	57501	5.287	4.47E-05
TMEM38A	A_23_P101392	79041	5.263	8.67E-07

CDH26	A_23_P502957	60437	5.231	1.55E-04
CLTCL1	A_23_P143559	8218	5.202	1.24E-05
CETP	A_23_P49376	1071	5.175	7.52E-03
CLEC4D	A_33_P3258977	338339	5.135	1.52E-05
PADI2	A_23_P201747	11240	5.042	6.82E-04
ADAM19	A_23_P374082	8728	4.966	4.42E-03
A_19_P00317277	A_19_P00317277		4.938	2.75E-03
CLEC4D	A_33_P3352578	338339	4.902	3.41E-06
ST6GALNAC3	A_33_P3275055	256435	4.885	1.68E-03
A_19_P00321148	A_19_P00321148		4.754	2.47E-03
TREML3	A_24_P170439	340206	4.727	6.43E-03
RGL4	A_23_P306941	266747	4.713	1.85E-05
LRRC6	A_23_P112004	23639	4.640	1.60E-03
SPOCK1	A_24_P354689	6695	4.640	9.00E-04
NFE2	A_23_P13753	4778	4.633	3.10E-03
RPH3A	A_33_P3395384	22895	4.633	2.06E-05
ABCC6	A_23_P100539	368	4.580	9.20E-06
RILPL1	A_23_P169934	353116	4.568	4.53E-03
LOC100131892	A_33_P3384427		4.555	2.03E-04
PRLR	A_23_P167468	5618	4.535	1.65E-05
A_19_P00316185	A_19_P00316185		4.491	3.04E-06
SERINC2	A_24_P145629	347735	4.488	3.04E-03
CLEC4E	A_33_P3318509	26253	4.458	1.05E-03
A_19_P00322394	A_19_P00322394		4.409	1.13E-03
FRY	A_33_P3223495	10129	4.376	1.12E-03
A_19_P00801649	A_19_P00801649		4.364	5.42E-03
A_19_P00801354	A_19_P00801354		4.342	1.66E-05
TOX2	A_23_P154566	84969	4.333	6.66E-03
TREM1	A_33_P3319905	54210	4.305	1.56E-05
LOC389634	A_32_P221305	389634	4.292	9.20E-03
ZFYVE28	A_33_P3277579	57732	4.280	2.05E-03
A_19_P00807643	A_19_P00807643		4.218	4.48E-03
LOC646626	A_32_P703	646626	4.211	4.26E-04
ZFYVE28	A_32_P104746	57732	4.206	4.89E-05
F12	A_33_P3233871	2161	4.102	2.98E-03
CTH	A_23_P126103	1491	4.101	2.03E-03
SEMA3C	A_23_P256473	10512	4.061	5.63E-04
PADI2	A_24_P187970	11240	4.054	1.83E-03
A_19_P00318057	A_19_P00318057		4.040	3.18E-04
LOC151438	A_33_P3479449	100506328	4.025	1.50E-03
AX721082	A_33_P3251198	23236	4.017	2.44E-04
A_19_P00331762	A_19_P00331762		4.007	7.91E-05
LOC100128668	A_33_P3415653	100128668	3.916	9.82E-03
ELOVL3	A_23_P149858	83401	3.908	4.84E-04
C1orf226	A_23_P103511	400793	3.889	5.03E-03
C8orf46	A_23_P353614	254778	3.883	5.78E-05
A_19_P00321593	A_19_P00321593		3.858	1.28E-05

VCAN	A_23_P144959	1462	3.849	1.55E-03
CPAMD8	A_23_P67198	27151	3.834	2.57E-05
LAMC1	A_23_P201628	3915	3.818	8.64E-03
A_19_P00318551	A_19_P00318551		3.804	2.08E-04
A_19_P00321594	A_19_P00321594		3.780	3.10E-03
A_19_P00316518	A_19_P00316518		3.774	4.79E-04
LIN7A	A_23_P99253	8825	3.770	7.58E-07
IRS2	A_24_P154037	8660	3.749	9.48E-06
WLS	A_33_P3311551	79971	3.731	8.32E-05
ZNF697	A_32_P19716	90874	3.724	3.03E-03
BASP1	A_23_P213385	10409	3.690	3.98E-04
SLC22A1	A_23_P145569	6580	3.658	6.36E-04
SLC2A14	A_32_P47754	144195	3.640	3.60E-03
SLC2A3	A_23_P139669	6515	3.600	3.02E-03
A_33_P3304748	A_33_P3304748		3.578	5.65E-06
ENST00000229824	A_33_P3424328	401258	3.566	1.48E-03
ADM	A_23_P127948	133	3.566	1.53E-06
DKFZp313P036	A_24_P221968	643802	3.555	2.48E-03
LOC497257	A_32_P219148	497257	3.516	1.52E-03
RNU4ATAC	A_33_P3839897	100151683	3.510	3.09E-03
ENST00000340798	A_33_P3383762		3.510	7.47E-06
ATP2A3	A_23_P207632	489	3.463	1.72E-05
A_19_P00317616	A_19_P00317616		3.427	6.75E-04
C17orf76	A_23_P368484	388341	3.396	1.59E-03
MPP7	A_33_P3229477	143098	3.389	2.24E-03
NOL3	A_23_P206371	8996	3.383	8.27E-06
ENST00000401931	A_33_P3243230	3576	3.350	5.34E-03
RNU105B	A_33_P3399534	26767	3.344	1.02E-03
ENST00000400513	A_33_P3223487	10129	3.330	1.16E-04
MAP2K6	A_23_P207445	5608	3.277	3.01E-08
TMEM144	A_33_P3304372	55314	3.275	7.29E-03
LOC402778	A_33_P3358601	402778	3.273	4.60E-03
PLCB1	A_23_P6099	23236	3.257	9.10E-04
OR7E140P	A_33_P3211793		3.252	1.58E-03
MT1F	A_23_P15174	4494	3.252	1.73E-03
SYCP2	A_23_P210675	10388	3.233	9.70E-03
NEK8	A_24_P99639	284086	3.228	1.04E-03
ARHGAP24	A_33_P3236030	83478	3.220	7.36E-03
LOC389634	A_33_P3243500		3.211	4.29E-03
A_33_P3279311	A_33_P3279311		3.202	4.68E-04
A_19_P00326336	A_19_P00326336		3.194	6.04E-05
A_33_P3333327	A_33_P3333327		3.172	3.80E-03
A_33_P3400023	A_33_P3400023		3.148	5.76E-03
A_33_P3262074	A_33_P3262074		3.134	6.11E-03
PRR7	A_33_P3319780	80758	3.106	4.89E-06
UNQ9368	A_24_P927716	643036	3.088	2.33E-03
MREG	A_23_P108948	55686	3.088	3.10E-03

PGM5	A_24_P254949	5239	3.085	2.61E-03
DYSFIP1	A_33_P3287472	116729	3.082	1.25E-05
TMEM170B	A_32_P178966	100113407	3.080	8.41E-05
A_33_P3615955	A_33_P3615955		3.069	5.10E-03
ORM2	A_23_P9485	5005	3.054	8.90E-04
HSPA1L	A_23_P70547	3305	3.020	1.24E-04
ENST00000338061	A_33_P3251193	23236	3.015	1.59E-05
LOC257358	A_32_P23308	257358	3.006	1.05E-03
SSPO	A_33_P3277173		2.997	5.68E-03
THC2715267	A_33_P3308219		2.992	3.45E-04
LOC647121	A_24_P684186	133418	2.969	2.08E-04
FRY	A_33_P3223497	10129	2.951	1.89E-05
JAG1	A_23_P210763	182	2.935	2.04E-03
LOC389634	A_33_P3243502	389634	2.919	2.06E-03
LOC389634	A_33_P3364651		2.910	1.76E-03
LOC389634	A_33_P3365553	389634	2.896	9.50E-04
LOC399744	A_33_P3369213	399744	2.883	2.80E-05
SLC11A1	A_33_P3317009	6556	2.882	5.59E-04
DDEF1IT1	A_23_P146325	29065	2.876	6.80E-03
RNASE2	A_23_P151637	6036	2.867	1.89E-04
RNASE3	A_23_P163025	6037	2.852	4.79E-03
TXNDC3	A_23_P259611	51314	2.851	3.88E-03
LOC100129186	A_33_P3342967	100129186	2.828	2.52E-04
AIM2	A_32_P44394	9447	2.826	3.92E-04
RAB27A	A_24_P373174	5873	2.813	2.01E-07
TMEM170B	A_33_P3352278	100113407	2.792	5.47E-05
LRP3	A_33_P3212188	4037	2.739	1.50E-04
PLAUR	A_23_P16469	5329	2.733	6.98E-03
HLX	A_23_P126266	3142	2.729	1.44E-04
DKFZp547G183	A_33_P3732466	55525	2.728	1.43E-04
FLJ22662	A_23_P87709	79887	2.713	7.26E-04
MGST1	A_23_P36658	4257	2.693	2.33E-05
TSHZ3	A_23_P361014	57616	2.672	2.08E-03
FMO5	A_33_P3376214	2330	2.671	8.10E-04
FXYD6	A_23_P150394	53826	2.664	6.51E-04
A_33_P3229037	A_33_P3229037		2.664	3.43E-03
DKFZp761E198	A_24_P9883	91056	2.660	6.06E-03
PECR	A_23_P91140	55825	2.658	2.20E-03
EGR1	A_23_P214080	1958	2.656	7.07E-03
ABHD5	A_23_P250294	51099	2.655	1.93E-05
A_33_P3304081	A_33_P3304081		2.652	1.23E-04
OMG	A_23_P55286	4974	2.641	3.26E-03
ACPL2	A_23_P57760	92370	2.627	1.19E-03
LOC644727	A_33_P3289251	644727	2.609	5.08E-05
FRY	A_33_P3223488	10129	2.604	4.56E-05
MGC13005	A_32_P227317	84771	2.595	1.69E-04
A_19_P00807435	A_19_P00807435		2.588	9.30E-04

SAG	A_23_P5853	6295	2.585	9.19E-03
LOC399744	A_33_P3248252	399744	2.581	1.54E-04
tcag7.907	A_33_P3362397	402483	2.579	8.91E-03
tcag7.907	A_32_P75425	401149	2.536	2.27E-03
RNF24	A_24_P333019	11237	2.529	8.07E-05
ABCC6	A_23_P15272	368	2.506	1.95E-03
A_19_P00802450	A_19_P00802450		2.502	2.84E-03
KIF13A	A_33_P3242659	63971	2.501	3.32E-05
AMPH	A_23_P31273	273	2.498	1.36E-03
CSF3R	A_33_P3881262	1441	2.498	5.29E-03
SNX21	A_24_P318897	90203	2.488	2.11E-03
A_19_P00803229	A_19_P00803229		2.486	2.47E-04
AK097080	A_24_P818010	100133161	2.474	5.89E-04
ACSL1	A_23_P110212	2180	2.461	4.83E-04
ATP2A3	A_24_P202319	489	2.450	6.67E-06
ABCC6	A_33_P3298043	368	2.445	1.27E-03
AK126778	A_33_P3373203		2.436	3.80E-03
FBXL16	A_23_P406385	146330	2.421	1.39E-03
TREML2	A_24_P214858	79865	2.417	6.75E-05
A_19_P00322101	A_19_P00322101		2.407	1.13E-03
PTPRN2	A_32_P79434	5799	2.404	3.01E-03
SPATA13	A_33_P3298105	221178	2.400	3.37E-03
IGF2R	A_23_P334021	3482	2.399	1.19E-03
AK125099	A_32_P190461		2.398	2.31E-04
LOC441268	A_33_P3415648		2.398	6.34E-05
PCSK5	A_23_P257003	5125	2.384	2.58E-04
LOC401357	A_24_P659122	401357	2.383	6.74E-04
A_33_P3221055	A_33_P3221055		2.376	4.76E-05
ADAM8	A_24_P300777	101	2.376	4.85E-05
SNX21	A_23_P154585	90203	2.373	2.60E-03
FLJ45445	A_33_P3238777	399844	2.363	1.35E-04
A_19_P00328825	A_19_P00328825		2.357	6.24E-04
BAG4	A_24_P930926	9530	2.354	7.32E-03
tcag7.907	A_32_P11230	399744	2.347	2.12E-05
FAM151B	A_33_P3728979	167555	2.347	2.87E-03
A_19_P00322727	A_19_P00322727		2.345	7.22E-06
LOC100291714	A_33_P3423565		2.342	5.87E-04
MTMR3	A_33_P3235335	8897	2.338	7.43E-09
PTPLA	A_33_P3235410	9200	2.334	4.47E-03
EPHX1	A_23_P34537	2052	2.321	3.11E-04
FLJ45445	A_33_P3225873	399844	2.320	8.35E-03
A_33_P3390673	A_33_P3390673		2.320	8.92E-04
TRIM7	A_24_P277615	81786	2.316	9.01E-04
DKFZp434F142	A_23_P111797	84214	2.313	1.01E-05
LOC100133331	A_33_P3287883	100133331	2.310	8.84E-06
A_19_P00322493	A_19_P00322493		2.309	5.33E-05
A_19_P00323307	A_19_P00323307		2.309	4.90E-03

IL31RA	A_33_P3691168	133396	2.309	1.65E-03
ALOX15B	A_23_P60627	247	2.307	3.58E-03
PRRG4	A_23_P127663	79056	2.302	4.35E-04
IVNS1ABP	A_33_P3280875	10625	2.297	1.51E-04
TRIM7	A_23_P30315	81786	2.293	2.64E-03
AOC3	A_23_P426305	8639	2.290	3.75E-03
ROPN1L	A_23_P121885	83853	2.289	1.80E-05
LOC731275	A_33_P3226380	731275	2.284	1.73E-04
FRY	A_23_P105862	10129	2.282	1.75E-04
ULK1	A_24_P73370	8408	2.281	1.12E-06
MXD1	A_23_P408094	4084	2.276	5.20E-03
ICAM3	A_23_P164691	3385	2.274	4.54E-03
FLJ45445	A_33_P3248900	399844	2.270	2.88E-05
LOC100132217	A_33_P3362249	100288292	2.268	5.29E-05
A_33_P3292126	A_33_P3292126		2.265	1.58E-05
LOC100132217	A_33_P3308101	401357	2.259	8.36E-07
SCCPDH	A_23_P62807	51097	2.254	2.42E-03
A_19_P00318861	A_19_P00318861		2.249	3.56E-05
A_19_P00329574	A_19_P00329574		2.244	3.06E-03
LOC731275	A_24_P691775	401149	2.236	1.31E-04
LOC731275	A_33_P3229380	731275	2.234	4.59E-04
A_19_P00316995	A_19_P00316995		2.233	6.54E-03
A_19_P00316010	A_19_P00316010		2.232	4.63E-03
A_19_P00320602	A_19_P00320602		2.215	9.38E-03
LOC349114	A_33_P3268629	349114	2.215	6.06E-06
MTMR3	A_33_P3235330	8897	2.214	6.05E-04
METTL9	A_24_P202139	51108	2.210	3.97E-05
LOC729218	A_33_P3256088	401149	2.209	4.58E-05
tcag7.907	A_24_P586390	100133331	2.205	4.75E-06
PGD	A_23_P126623	5226	2.205	2.11E-04
SLC22A4	A_23_P156180	6583	2.202	7.80E-04
LOC349114	A_33_P3365747	349114	2.199	6.02E-05
SPATA13	A_23_P390116	221178	2.192	3.06E-03
THC2677526	A_33_P3380236		2.188	4.71E-03
THC2656683	A_33_P3268910	10252	2.185	3.80E-03
LMTK2	A_33_P3266923	22853	2.176	4.25E-03
A_19_P00811179	A_19_P00811179		2.173	1.44E-03
ENST00000366583	A_33_P3349883	3964	2.173	3.49E-03
RAB37	A_33_P3314594	326624	2.172	5.63E-03
MSL1	A_33_P3356341	339287	2.170	1.01E-06
EMB	A_33_P3359900	133418	2.167	2.06E-03
AK097701	A_33_P3220425		2.166	3.10E-03
THC2764236	A_33_P3360867		2.164	1.82E-05
LOC100133060	A_33_P3249731		2.163	4.14E-04
A_19_P00319069	A_19_P00319069		2.162	6.61E-03
LOC729603	A_33_P3410859	729603	2.159	9.99E-03
A_19_P00802874	A_19_P00802874		2.156	3.19E-04

IGF2BP3	A_23_P19987	10643	2.155	2.19E-03
LOC158863	A_33_P3576797	158863	2.154	3.48E-03
LIPN	A_33_P3323939	643418	2.154	6.42E-03
LOC653056	A_33_P3256095		2.153	5.46E-04
LOC100132217	A_33_P3343206	100288292	2.144	1.41E-05
A_19_P00322866	A_19_P00322866		2.142	6.89E-04
KBTBD7	A_23_P25605	84078	2.141	1.14E-03
A_19_P00315808	A_19_P00315808		2.139	7.10E-08
UPB1	A_23_P120822	51733	2.133	6.69E-03
A_19_P00320417	A_19_P00320417		2.132	9.64E-03
MEGF9	A_32_P129894	1955	2.132	2.42E-03
LOC253981	A_32_P116206	768211	2.128	4.59E-04
PRO2852	A_33_P3538279	114224	2.125	6.96E-04
MAP4K4	A_23_P90804	9448	2.124	2.24E-03
MST150	A_23_P414273	85027	2.123	8.24E-04
APP	A_33_P3296479	351	2.123	1.74E-05
LOC100130360	A_32_P62211		2.121	3.11E-03
AF130850	A_33_P3499046		2.121	1.84E-03
THC2539584	A_33_P3392677		2.118	1.45E-04
A_19_P00813409	A_19_P00813409		2.115	6.93E-05
RAB3IP	A_23_P128174	117177	2.110	1.25E-03
ENST00000402522	A_33_P3370812	23216	2.110	2.23E-03
BQ945477	A_33_P3213526		2.107	3.04E-03
SEPX1	A_23_P129486	51734	2.099	5.71E-04
ANXA1	A_23_P94501	301	2.096	9.90E-03
LOC100132217	A_33_P3300843	401357	2.094	4.16E-05
A_19_P00324174	A_19_P00324174		2.080	8.12E-03
EVI2B	A_23_P66694	2124	2.076	1.77E-05
TACC3	A_23_P212844	10460	2.074	1.71E-04
ZNF516	A_33_P3225690	9658	2.074	1.15E-03
RTN3	A_23_P76684	10313	2.056	9.69E-08
DEXI	A_24_P144377	28955	2.053	3.36E-03
CMTM1	A_23_P106661	113540	2.051	7.82E-03
BC045716	A_32_P191895	100129960	2.043	3.05E-03
TALDO1	A_32_P18258	6888	2.037	7.87E-05
A_19_P00322932	A_19_P00322932		2.033	6.65E-04
COLQ	A_23_P212126	8292	2.028	9.96E-03
A_19_P00316992	A_19_P00316992		2.027	5.05E-04
DGKD	A_23_P210253	8527	2.027	1.11E-04
SOD2	A_23_P134176	6648	2.025	2.04E-04
LOC100131190	A_33_P3278200	4600	2.024	1.41E-07
DAB2IP	A_23_P123848	153090	2.016	2.06E-04
A_19_P00813109	A_19_P00813109		2.010	1.35E-03
A_19_P00321324	A_19_P00321324		2.009	2.23E-05
A_19_P00802257	A_19_P00802257		2.008	5.52E-03
LOC731275	A_33_P3232624	100288069	2.008	1.03E-03
LOC401357	A_33_P3365985	401357	2.001	3.76E-05

EPM2AIP1	A_24_P627984	9852	2.000	4.89E-03
DGCR2	A_24_P125881	9993	2.000	1.92E-05

Table 4 Agilent reporters with an at least two-fold downregulation in BDCA1^{+}CD14^{+} pro-iDCs relative to CD14^{+}CD16^{-} monocytes (Tukey test p-value ≤ 0.05).

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10 Selbstständigkeitserklärung

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung erlaubter und angegebener Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichter Literatur entnommen wurden, sind als solche kenntlich gemacht.

Ich versichere, dass diese Arbeit in dieser oder ähnlicher Form nicht an einer anderen Hochschule eingereicht wurde und ich noch keinen entsprechenden Doktorgrad besitze. Die Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin ist mir bekannt und ist akzeptiert.

_____

Robert Toschka Bergisch Gladbach, 28.02.2014

11 List of publications

Preliminary title: **Human peripheral blood contains a subset of pro-inflammatory DC with the capacity to induce T_H17 responses**
Robert Toschka, Jutta Kollet, Alexander Scheffold, Stefanie Gamradt, Christian Ostalecki, Andrzej Dzionek
[Manuscript in preparation]

_____

Robert Toschka Bergisch Gladbach, 28.02.2014

12 Curriculum Vitae

The official version of this PhD thesis does not contain personal data.

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