

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

*Human plasmacytoid dendritic cells: from identification
to specific antiviral function*

by

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DEBRECEN, 2015

ABBREVIATIONS

APCs	-	Antigen-presenting cells
BDCA	-	Blood dendritic cell antigen
BM	-	Bone marrow
CARD	-	Caspase activation and recruitment domain
CARDIF	-	CARD adaptor-inducing interferon- β
CDP	-	Common dendritic cell progenitors
CLP	-	Common lymphoid progenitors
CMP	-	Common myeloid progenitors
DC	-	Dendritic cell
FACS	-	Fluorescence-activated cell sorting
Flt3	-	Fms-like tyrosine kinase-3
G-CSF	-	Granulocyte colony-stimulating factor
GM-CSF	-	Granulocyte-macrophage colony-stimulating factor
IDO	-	Indoleamine (2,3)-dioxygenase
IFN	-	Interferon
IFNAR	-	Interferon- α/β receptor
IKK	-	I κ B kinase
IL	-	Interleukin
IPC	-	Interferon-producing cells
IPS-1	-	IFNB-promoter stimulator-1
IRAK	-	Interleukin-1 receptor-associated kinase
IRF	-	Interferon regulatory factor
ITIM	-	Immunoreceptor tyrosine-based inhibition motif
LGP2	-	Laboratory of Genetics and Physiology 2
LN	-	Lymph node
MAVS	-	Mitochondrial antiviral signaling protein
MDA5	-	Melanoma differentiation-associated gene 5
MHC	-	Major histocompatibility complex
PAMP	-	Pathogen-associated molecular patterns
pDC	-	Plasmacytoid dendritic cells
pDC-L	-	Plasmacytoid dendritic cell leukemia/lymphoma
PRR	-	Pattern recognition receptor

RAG-1	-	Recombination activating gene 1
RIG	-	Retinoic acid induced gene
RLH	-	RIG-like helicases
SLE	-	Systemic lupus erythematosus
TANK	-	TRAF family member-associated NF-kappa-B activator
TBK-1	-	TANK-binding kinase 1
TRAF	-	TNF receptor-associated factor
TRAIL	-	Tumor necrosis factor-related apoptosis-inducing ligand
VISA	-	Virus-induced signaling adaptor

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SUPPLEMENTARY 1

Zoltan Magyarics, Aniko Csillag, Kitti Pazmandi, Eva Rajnavolgyi and Attila Bacsi:
Identification of plasmacytoid pre-dendritic cells by one-color flow cytometry for
phenotype screening.

Cytometry A. 2008 73(3):254-8.

SUPPLEMENTARY 2

Attila Szabo*, **Zoltan Magyarics***, Kitti Pazmandi, Laszlo Gopcsa, Eva Rajnavolgyi
and Attila Bacsi: TLR ligands upregulate RIG-I expression in human plasmacytoid
dendritic cells in a type I IFN-independent manner.

Immunol Cell Biol. 2014 92(8):671-8.

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1 Introduction

Dendritic cells (DCs) are professional antigen presenting cells effectively linking the innate and the adaptive arms of the immune system. Since their discovery by the late Ralph Steinman and his co-workers, multiple subsets of DCs have been identified, including the identification of the previously described type I interferon producing cells (IPC) as plasmacytoid dendritic cells (pDCs). The rarity of this cell type in human peripheral blood necessitated the use of various model systems in DC research. In this work, first we aimed to optimize the flow cytometric identification of pDCs in peripheral blood of healthy donors using exclusively Blood Dendritic Cell Antigen (BDCA) 4 positivity.

Similarly to conventional DCs, pDCs effectively connect the innate and adaptive immune responses by secreting cytokines and triggering T-cell responses by antigen presentation upon detection of pathogens. DCs express various innate immune receptors, the so-called pattern recognition receptors (PRRs), on their cell surface and within intracellular compartments to detect pathogens. These ancient receptors are able to recognize evolutionally conserved motifs associated to microbes, and subsequently trigger cellular immune responses leading to inflammation and the production of antimicrobial molecules.

Plasmacytoid DCs express a unique set of intracellular receptors geared towards the detection of viral nucleic acids. Membrane-bound Toll-like receptors (TLRs) and the cytoplasmic RIG-I-like helicases (RLHs) are two important families of such innate sensors. Previous evidence supported the notion that pDCs exclusively use TLRs and do not depend on the cytoplasmic recognition of viral replication intermediates by RLHs. To investigate the role of RLHs in viral recognition by pDCs, we assessed the *in vitro* function of leukemic pDCs and used these cells as models for further studies. The second aim of this work was to elucidate the potential impact of TLR-activation on the expression and function of RLHs and to identify potential co-operation between the TLR and RLH mediated virus recognition pathways in pDCs.

2 Background

2.1 Dendritic cells: linking innate and adaptive immunity

Dendritic cells, identified by Steinman and Cohn in 1973 [1], are a heterogeneous population of hematopoietic cells that serve as an important bridge between innate and adaptive immune responses as professional antigen presenting cells (APCs). Their functional characterization, including the discovery of their role in antigen presentation, was a key milestone in immunology [2,3]. Based on their origin, tissue localization and functional properties, human DCs can be classified into two major types: conventional DCs (cDCs) and the later identified pDCs. As part of the innate immune network, cDCs are present in all peripheral tissues, and detect environmental changes, including the presence of pathogens (reviewed in [4]). Resting DCs are activated by pathogenic invasion or inflammation, which results in their maturation and rapid migration through the lymphatics to draining lymph nodes. During this course, DCs internalize and process proteins and other antigens, and after arrival to the lymph nodes, they present peptide fragments of the antigens through major histocompatibility complex (MHC) molecules, together with co-stimulatory molecules to T-cells. The coordinated activity of DCs has been shown to be critical for keeping the balance between maintenance of self-tolerance and induction of effective immune responses. Most of our knowledge about the role of DCs is stemming from experimental evidence based on the extensively studied conventional DCs; however, pDCs significantly differ from cDCs both in terms of tissue localization and in their functional role. The following sections summarize the key properties of pDCs that are different from cDCs.

2.2 The discovery and origin of plasmacytoid dendritic cells

Plasmacytoid pre-dendritic cells represent a rare but multipotent cell population of innate immunity. Human pDCs represent only 0.2%–0.8% of peripheral blood mononuclear cells (PBMC). They are smaller (8–10 μm in diameter) than CD14^+ monocytes or cDCs, but bigger than resting lymphocytes and display plasma cell morphology with eccentric kidney-shaped nucleus. In particular, whereas the CD11c^+ blood immature myeloid dendritic cells display dendrites, immature pDCs have no long outgrowths, but they acquire dendritic morphology upon *in vitro*

culturing in presence of IL-3 (Figure 1). The phenotype of human immature pDCs is $CD4^+CD45RA^+HLA^-DR^+CD123^{high}ILT3^+ILT1^-CD11c^-lineage^-$. They do not express the lineage-specific markers for all the known cell types within the immune system, including surface and cytoplasmic immunoglobulin and CD19 (B cells), TCR and CD3 complexes (T-cells), CD14 (monocytes), CD16 and CD56 (NK cells), and CD11c (myeloid DCs). Although pDCs express MHC class II molecule and myeloid antigen such as CD68, they do not express most of the antigens expressed on myeloid cells, such as CD11b, CD13, CD14, or CD33. More recently, *Matsui et al.* identified CD2, a cell adhesion molecule, as a surface antigen that distinguishes two human pDC subsets [5]. The $CD2^{high}$ subset of pDCs expresses lysozyme and displays cytolytic capacity, which is lacking in the $CD2^{low}$ subset.

Murine pDCs, identified soon after the discovery of their human counterparts, are characterized by their $B220^+Ly49Q^+CCR9^+$ phenotype [6-9].

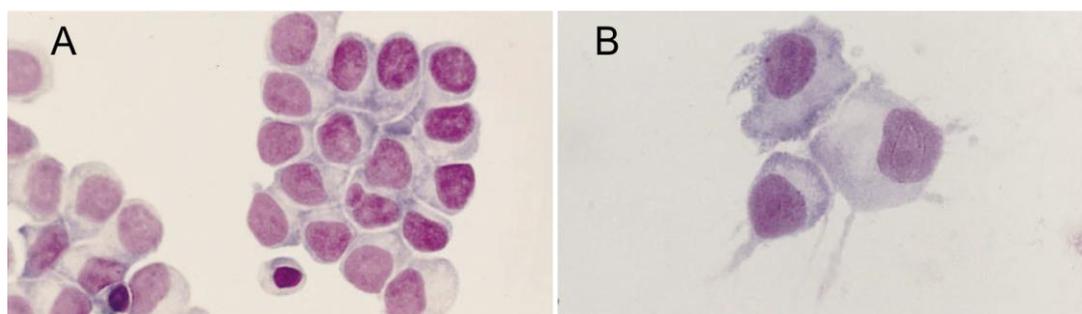


Figure 1. Freshly isolated (A) and *in vitro* matured (B) pDCs. The presence of interleukin 3 for 6 days in the *in vitro* culture of pDCs results in morphological changes resembling monocyte-derived DCs. From *Grouard et al., The Journal of experimental medicine 1997;185:1101-1111.*

Plasmacytoid dendritic cells were initially named as T-associated plasma cells, reflecting their plasma cell morphology and their localization to the T-cell area of reactive lymph node [10] and the ‘plasmacytoid T-cells’ designation was proposed in 1983 based on their CD4-expression [11]; however, this was soon questioned based on the lack of CD3, a key lineage marker of T-cells [12], and the name ‘plasmacytoid monocytes’ was proposed. The identity and the proper name of this special cell type has remained controversial for one more decade due to their unclear link to the later identified professional IPCs [13]. The characterisation of IPC was hindered by their rarity, rapid apoptosis after isolation, and lack of lineage markers. This enigmatic cell type was once more discovered as $CD4^+CD11^-$ immature DCs [14]. The same authors

also reported the *in vitro* differentiation of pDC into a morphological state resembling myeloid DC; however, the lack of key myeloid lineage markers (CD11b, CD13 and CD33) clearly distinguished them from classical DC, and they assumed that these cells are Langerhans cell (LC) precursors migrating to the skin.

In the late 1990ies, a new unifying concept emerged: the plasmacytoid T-cells (also called plasmacytoid monocytes by this time), the more recently identified IPC and the CD11⁻CD4⁺ pre-DCs described by *O'Doherty et al.* are likely to represent the same cell type, and these cells are able to develop into DCs upon IL-3 and CD40-ligand treatment *in vitro* [15]. This concept was proposed by *Yong-Jun Liu*, summarizing the key facts: 1) CD4⁺CD3⁻CD11c⁻lineage⁻ plasmacytoid cells do not express B- or T-cell receptors; 2) they represent DC precursors, but likely originate from lymphoid rather than myeloid lineage; 3) the key survival and differentiation factor of this cell type is IL-3 [16]. As a supportive observation, IPC purified from human peripheral blood express CD4 but no CD11c on their surface, they resemble pDC by morphology, and are able to secrete substantial amount of type I IFNs upon stimulation with ultraviolet (UV)-irradiated herpes simplex virus [17]. Besides the consensus on the identity of human pDCs, another key finding at that time was the identification of the murine analogue of human pDCs as Ly6C⁺/B220⁺/CD11c^{low}/CD4⁺ cell type [6,18-20]. This discovery opened up a new area of research: the quest for the origin of pDCs.

Initial reports suggested a lymphoid origin of pDCs, mainly based on the lack of certain myeloid markers (e.g. CD11c and CD13) and the expression of key lymphoid surface antigens (e.g. CD45RA), as well as the presence of rearranged immunoglobulin heavy chain (IgH) D-J genes [21], but their relationship to either of these lineages remained unclear. The cytokine fms-like tyrosine kinase-3 (Flt3) ligand (Flt3-L) was identified as a key soluble factor for development of DCs, both conventional and plasmacytoid ones. *Björck et al.* showed that murine pDCs could be mobilized from bone marrow by treating the animals with Flt3-L and GM-CSF [22].

In vitro cultures of murine bone marrow cells and transfer of bone marrow precursors into irradiated host shed more light on the development of pDCs. Using passive transfer of precursors, it was demonstrated that pDCs might originate both from common myeloid precursors (CMPs) or common lymphoid precursors (CLPs) [23,24], given that the progenitor cells express Flt3 receptor [25,26]. Further mouse studies showed that a special lymphoid-like transcription program, including the

expression of Recombination activating gene 1 (RAG-1) and pre-T-alpha receptor, is activated during development pDCs regardless of their CLP or CMP origin [27]. Experiments using human progenitors confirmed the potential of both CMPs and CLPs to give rise to pDCs *in vitro* [28]. *Comeau et al.* identified pDCs of myeloid-like intermediate phenotype in Flt3-L treated individuals, further supporting the concept of dual lymphoid-myeloid origin [29]. However, it remained unclear whether pDCs and cDCs share a common progenitor cell beyond the CMP/CLP stage. In 2007, two independent groups identified common DC progenitors (CDP) in murine bone marrow as lineage-negative cells expressing Flt3, moderate amounts of c-Kit and M-CSF receptor [30,31] and according to most recent results, mouse pDCs differentiate from either common DC progenitors or lymphoid-primed multipotent progenitors [32].

A common element of the CLP and CMP pathways is the expression of the helix-loop-helix transcription factor E2-2 during pDC development, both in mice and humans [33,34]. Furthermore, mice lacking type I IFN receptor show impaired pDC-development, and this has been attributed to the reduced expression of Flt3 on CLPs, suggesting that pDC production is dependent on a type I IFN/Flt3 signaling interaction [35].

Collectively, observations suggest a flexible development pathway for DCs, where pDCs might originate from a variety of precursors (Figure 2); Shortman and co-workers summarized this model in two recent publications [36,37]. Early-stage data obtained by ‘molecular barcoding’ of murine lymphocyte-primed multipotent precursor (LMPP) cells showed a great extent of early commitment of these cells to DC-lineage, and this technique might answers the still open questions of pDC-development. It is important to note that most of the data about origin of pDCs are derived from studies involving murine progenitors and transfer of early progenitor cell into irradiated host; therefore, these might have limited relevance to the development of pDCs under steady state conditions *in vivo*.

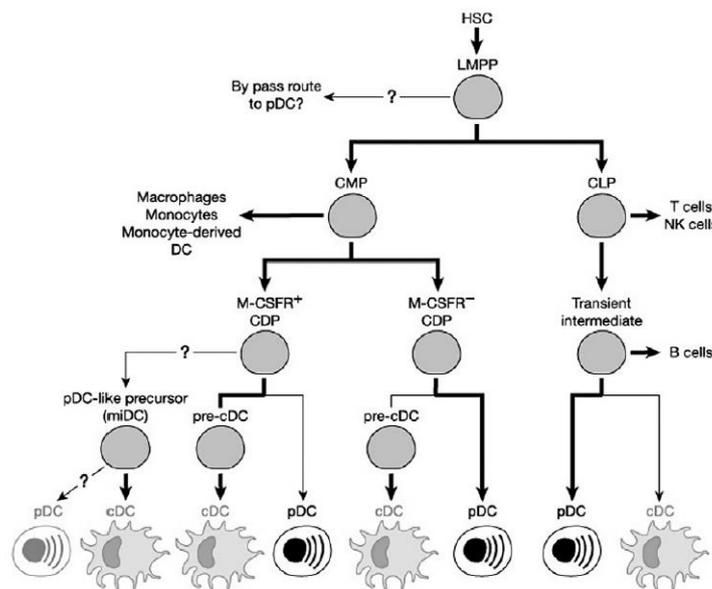


Figure 2. Developmental pathways to pDCs

Heavy arrows mark major, while thin arrows mark minor pathways leading to pDCs or cDCs, respectively. The relative importance of these pathways is dependent on the environmental conditions. The key diversity of pDCs is the presence or lack of 'lymphoid' markers (e.g. IgH gene DJ rearrangements); pDCs emerging from M-CSFR negative CDPs might carry these, while those originating from M-CSFR positive CDPs do not. *From Shortman et al., Advances in immunology 2013;120:105-126.*

2.3 Innate immune receptors of plasmacytoid dendritic cells

A prominent role of the innate immune system is the recognition of microbial components that are essential for the given microorganism, but serve as a 'danger signal' for the host promoting an efficient adaptive immune response against the recognized organism or structure. The pathogen-associated molecular patterns (PAMPs) are recognized by germ line-encoded PRRs that are usually expressed constitutively in the host [38]. Importantly, similar microbial structures might be expressed on non-pathogenic microbes; this underlines the importance of differentiation between harmful microbial patterns and those that should be tolerated by the immune system. This process is controlled by 'danger signals', e.g. tissue damage induced by invasive bacteria or viruses. An important aspect of this process is that certain PRRs also sense host-derived structures that become available during tissue damage or non-controlled cell death as opposed to apoptosis. Unlike other body cells expressing a limited number of PRRs, professional APCs, such as DCs, B-cells and macrophages, display a broad repertoire of PRRs to detect pathogen-derived molecules in their environment. Thus, DCs are at the crossroads of immune responses and need to tightly control and coordinate innate and adaptive immune responses.

2.3.1 Toll-like receptors of plasmacytoid dendritic cells

An important group of PRRs is the family of Toll-like receptors (TLRs), expressed on the cell surface or on the membrane of intracellular compartments. Thirteen members of the TLR family have been identified so far, and ten of them are found in humans [39]. TLRs are type I trans-membrane glycoproteins consisting of a cytoplasmic signaling domain called the Toll/IL-1 receptor (TIR) domain and different extracellular domains. These extracellular “leucine-rich repeats” (LRRs) consist of 19-25 tandem LRR motifs (24-29 amino acid-long each), containing the motif XLXXLXLXX (reviewed by *Akira* [40]). The activation of TLRs results in the expression of cytokines, chemokines and co-stimulatory molecules essential for the coordination of innate immune responses and shape the adaptive responses as well (reviewed by *Kawai and Akira* [39]).

The two major DC lineages, cDCs and pDCs, express a characteristic combination of TLRs ensuring the recognition of a dedicated array of pathogenic or damaged host structures (summarized on Figure 3). Plasmacytoid DCs are tailored to recognize RNA and DNA viruses as they express a special combination of TLRs, namely TLR7 and TLR9 [41,42]. TLR7 recognizes viral single stranded RNA (ssRNA), but several synthetic compounds such as loxoribine, resiquimod (R848) and imiquimod (R837) also bind to this receptor [43]. TLR9 recognizes viral double-stranded (ds) DNA genomes rich in unmethylated CpG sequences; these hypo- or unmethylated DNA-sequences are normally absent from eukaryotic genomic DNA. Synthetic CpG oligodeoxyribonucleotides (CpG ODN) are also recognized by this receptor [44]. Both receptors are synthesized in the endoplasmic reticulum and transported into endosomes; once in the endosomal membrane, TLR9 is converted into an active form by proteolytic cleavage [45,46]. Certain viruses enter pDCs by fusion, when a fraction of the cytosol is encircled by a membrane to generate a vacuole that can fuse to TLR7/9 endosomes [47]. Examples include Vesicular stomatitis virus and Sendai virus, and these viruses generate replicative intermediates that are redirected from the cytosol into TLR7/9 endosomes by autophagy. Other viruses, like Herpes simplex virus and Coxsackievirus B, are internalized through receptor-mediated endocytosis and delivered to the endosomes [48-50].

Although pDCs are rare, they produce over 1000-fold more of type I interferon (IFN) (most prominently, IFN- α and IFN- β) upon TLR-stimulation than any other cell types including cDCs. The resulting type I IFN response of pDC is

dependent on MyD88, IRAK, TNF receptor-associated factor 6 (TRAF6) and the IKK complex, and the signaling events initiated by TLR7 and TLR9 engagement are similar [51]; the common endpoint is induction of interferon regulatory factor (IRF) 7 activation. The functional consequences of TLR-activation and the resulting cytokine release are described in section 2.4.

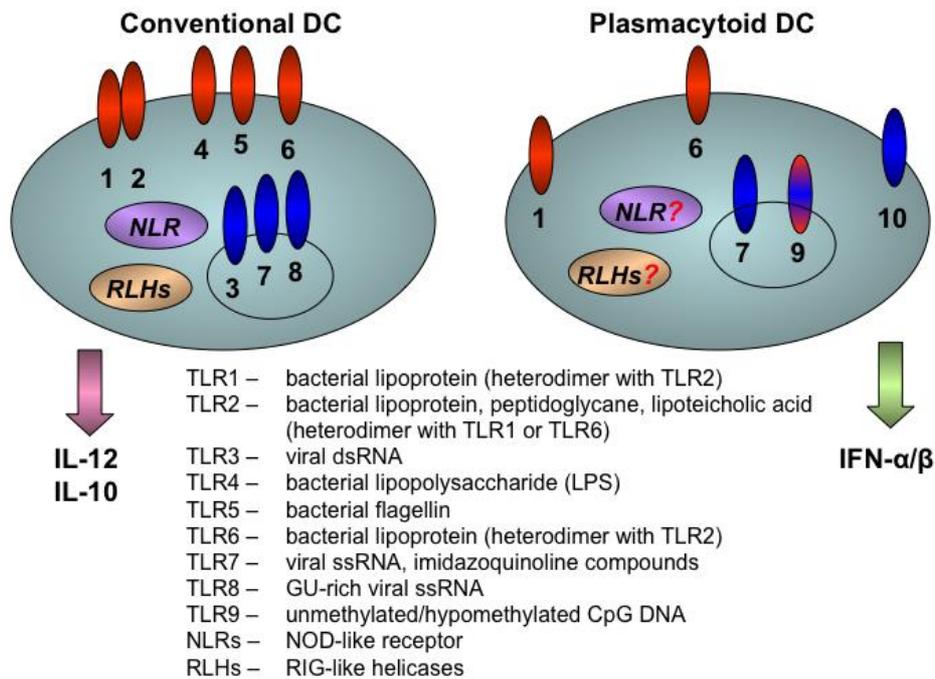


Figure 3. Expression of Toll-like receptors by conventional (myeloid) and plasmacytoid dendritic cells. Although plasmacytoid DCs express fewer TLR-types, they express a unique combination of nucleic acid sensing TLRs, TLR7 and -9 in their endosomal compartment. *From Benko et al., Biological chemistry 2008;389:469-485.*

2.3.2 RLHs in plasmacytoid dendritic cells

In addition to TLRs that are expressed in cellular or vesicular membranes, PRRs also involve conserved intracellular sensors, such as NACHT-LRRs [52], Nod-like receptors (NLRs) [53], retinoic acid induced gene (RIG)-like helicases (RLH), and intracellular DNA-binding sensors [54,55] that also recognize PAMPs. RIG-like helicases belong to the family of DExD/H-box helicases and comprise RIG-I, melanoma differentiation-associated gene 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2). Unlike TLRs, these receptors are located in the cytoplasm and are therefore able to detect replication intermediates of RNA viruses. They contain CARD signaling domains that allow interaction with the mitochondrial adaptor

molecule CARD adaptor inducing interferon- β (CARDIF), alternatively referred to as IFN β -promoter stimulator-1 (IPS-1), mitochondrial antiviral signaling protein (MAVS), or virus-induced signaling adaptor (VISA) [56,57]. Conformational changes induced by specific helicase ligands expose the CARD domain of RIG-I or MDA5, and this is followed by the interaction of the CARDS of RIG-I/MDA-5 and IPS-1. IPS-1 activates the I κ B kinase (IKK)-related kinases, IKKi and TANK-binding kinase 1 (TBK-1) via TRAF3 [58,59]. The activation of these kinases results in subsequent phosphorylation of transcription factors IRF3 and IRF7, leading to the production of type I IFNs. IPS-1 also induces the expression of pro-inflammatory cytokine and chemokine genes by activating the nuclear factor- κ B (NF- κ B) transcription factor through the FADD and caspase-8/10 pathway [60-62].

The specific recognition of viral RNAs by RLH is mediated by their C-terminal regulatory domain (CTD) [63,64]. Extensive research identified blunt-ended RNAs containing a free 5' triphosphate end (5' ppp), followed by a short double stranded region of at least 19 base pairs as the ligand of RIG-I [65]. Later studies described that the helicase core region binds to the double-stranded part of the RNA ligand, while specificity of 5' triphosphorylated RNA binding is determined by the CTD [66]; the binding does not have sequence specificity as contacts are made exclusively with the phosphoribose backbone of the RNA and determined primarily by the conformation of RNA [67]. In the absence of RNA ligand, RIG-I exists in an 'auto-inhibited' conformation, where the CARD domains are sequestered by a helical domain inserted between the two helicase moieties [68,69]. Others described the activation of RIG-I by longer dsRNA ligands (~100 - 400 bp) lacking a 5' triphosphorylated end; in this case, RIG-I molecules cooperatively assemble side by side on the dsRNA strand [70].

Similarly to RIG-I, the primary ligand of MDA5 is also dsRNA; however, the fine specificity of the two structurally similar helicases are different. Unlike RIG-I, MDA5 preferentially binds to long dsRNA [71], and the virus recognition pattern of the two receptors is also slightly different. RIG-I is highly specific for RNA viruses including Human influenza A, Newcastle disease virus and Sendai virus belonging to Paramyxoviridae, Vesicular stomatitis virus and Japanese encephalitis virus (Flaviviridae) [71,72]. In contrast, MDA5 seems to be important in the recognition of picornaviruses (including Encephalomyocarditis virus, Mengo virus and Theiler's

virus), caliciviruses and coronaviruses, e.g. Murine norovirus-1 and Murine hepatitis virus [73-75]. Certain viruses are recognized both by RIG-I and MDA5; examples include Hepatitis C (HCV), Dengue and West Nile viruses [76,77].

Besides natural viral ligands, several synthetic molecules are able to induce RIG-I and MDA5 activation. Poly-riboinosinic:poly-ribocytidylic acid (poly I:C or pI:C) is a synthetic dsRNA that activates TLR3 and RLHs and induces type I IFN responses [78,79]. Commercially available 'long' pI:C is recognized by MDA5; on the contrary, partially digested (by the specific endonuclease RNaseIII) pI:C of about 300 base pairs in length is able to activate RIG-I but not MDA5. Besides pI:C, another RNA species, 5'-triphosphate containing RNA (5'pppRNA) is also a highly selective ligand for RIG-I [80,81]. This RNA species is either a genome constituent or the product of *in vitro* transcripts of most RNA viruses.

The third member of the RLH family, LGP2, has no CARD-domain and therefore incapable of activating cytokine responses upon viral stimulus. Based on this inability, it was initially considered to be a negative regulator of RIG-I/MDA5 pathway [57,82]. A more recent study using LGP-deficient mice demonstrated decreased IFN-production after infection with Vesicular stomatitis virus or Encephalomyocarditis virus, suggesting that the ATPase of LGP2 facilitates viral recognition by RIG-I and MDA5 [83].

The signaling events leading to type I IFN production as a result of activation of RLHs and TLRs in pDCs are summarized on Figure 4.

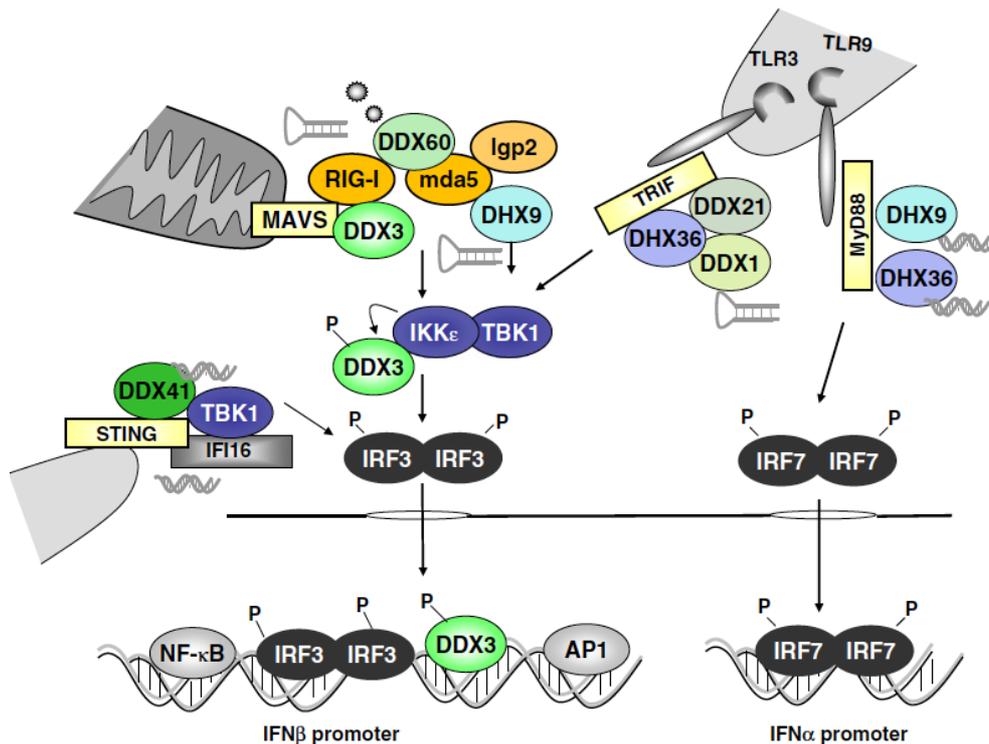


Figure 4. Signaling of DExD/H-box helicases leading to expression of type I interferons. While activation of endosomal TLR9 leads to phosphorylation of IRF-7 transcriptions through MyD88-dependent signaling pathway, cytoplasmic nucleic acid sensors signal through IKK/TBK-1 dependent pathway, leading to the phosphorylation of IRF-3. These two distinct pathways lead to differential activation of IFN- α/β promoters. From Fullam and Schroder, *Biochimica et biophysica acta* 2013;1829:854-865.

The expression and functional role of RLHs in pDCs remained poorly understood. The first key observation emerged from a mouse study by *Kato et al.* demonstrating that pDCs primarily employ the TLR-system to recognize viruses [72]. In this study, conventional and plasmacytoid DCs isolated from RIG-I^{-/-} mice were stimulated with Newcastle disease virus; while cDC lacking RIG-I produced significantly reduced amount of type I IFNs, the IFN-response of pDCs was not impaired. The second key notion against the role of RLHs in pDCs during the normal course of viral infection and recognition also came from a mouse study. *Kumagai et al.* showed that recognition of non-replicating, heat-inactivated NDV is dependent on TLR-7 and MyD88, but independent of IPS-1, the key adaptor molecule for RIG-like helicases [84]. This supported the concept of TLR-dominant viral recognition of pDCs under normal conditions over RLH-mediated recognition of cytoplasmic replication intermediates. Importantly, none of the studies verified this hypothesis in human pDCs. *Ablasser et al.* examined the expression and function of RIG-I in human pDCs. In this study, marginal levels of RIG-I and very low amount of MDA5

were detected in primary human pDCs compared to monocytes, under steady state conditions. Further supporting the marginal role of RLHs in viral recognition of pDCs, no type I IFN response could be detected from pDCs after treatment with RIG-I ligand 5' triphosphate RNA; the same result was obtained when pDCs were pre-stimulated with IFN- β [85]. More recently, *Goutagny et al.* studied the role of RIG-I and TLR7 in the recognition of Human metapneumoviruses in murine and human pDCs [86]. In this study, pDCs isolated from MAVS-deficient mice responded normally to both viruses and experiments with isolated primary human pDCs confirmed the TLR7-dependent, but RIG-I independent recognition of HMPVs upon infection. TLR-mediated signaling pathways are relatively well characterized in human pDCs [87], but none of the previously published studies investigated the potential interaction between TLR- and RLH-mediated signaling. Therefore, we aimed to study the effects of stimulation by endosomal TLRs on RIG-I expression in human pDCs.

2.4 Tissue distribution and functions of human plasmacytoid dendritic cells

The pattern of pDC trafficking is substantially different from that of cDCs. The precursors of cDCs leave the bone marrow and migrate to lymphoid organs and peripheral tissues, where they differentiate into resident or migratory DCs. In contrast to the cDCs, pDCs leave the bone marrow as fully developed effector cells and migrate to the peripheral tissues and, under steady-state condition, further to the lymph nodes via the afferent lymphatics. Flt3-L was identified as a key factor for pDC-development, but more recent evidence from murine studies shows that a further soluble mediator, CXCL-12 (also known as stromal cell-derived factor-1 [SDF-1]), recognized by CXCR-4, also contributes to pDC-differentiation in the bone marrow [88]; the role of the CXCL-12/CXCR-4 axis in humans is unknown.

The differentiated pDCs are mobilized from the bone marrow by Flt3-L and G-CSF [89,90]. Once leaving the bone marrow, pDCs appear to migrate into the T cell-rich areas of the secondary lymphoid tissues through high endothelial venule (HEV) in lymph nodes and mucosa-associated lymphoid tissues in a CXCL9 and E-selectin dependent manner [91]. The constant migration of pDCs to the splenic white

pulp is dependent on CCR7 and CXCR4 chemokine receptors expressed on pDCs, and their ligands, CCL21 and CXCL12, respectively [92]. Under inflammatory conditions, pDCs leave the bloodstream and accumulate at the site of infection and the draining lymph nodes; the underlying pathways are discussed in section 2.4.1. In addition to chemokines guiding migration to the site of infection/inflammation, pDCs can respond to two agonists released by damaged tissues; one of them is adenosine, which can engage the Adenosine Receptor A1 [93] and the other is F2L, which can trigger the formyl peptide receptor FPR3 [94]. Early studies indicated that murine pDCs have an average life span of about 2 weeks [19], while life span of pDCs in humans is not known.

2.4.1 The virus experts of the immune system

The key function of pDCs is the recognition of viral infections and subsequent type I IFN production as well as the initiation of adaptive immune responses. Their sentinel role is supported by their special set of pathogen recognition receptors targeted for efficient recognition of viral nucleic acid products, as summarized in section 2.3. Upon encountering specific stimuli, TLR9 and TLR7 deliver intracellular signals through the cytosolic adapter MyD88, which acts as a docking site for IRAK1/4, TRAF3, IKK α , and IRF7 [95,96]. IRF7 is then phosphorylated and translocated into the nucleus where it induces the transcriptional activation of the type I IFN genes [96].

Although most of the human blood cell types were reported to secrete type I IFNs upon viral infection, pDCs are responsible for the majority of type I IFN production in viral infections [17], secreting up to 1000-fold more IFN than other cells. During this process, pDCs dedicate approximately 60% of their new transcriptional activity to make type I IFNs [97]. Initially, the underlying difference in pDCs was suspected to be continuous expression of IRF-7 [98-100], but this could not explain the robust IFN-production in contrast to cDCs. Later, *Honda et al.* revealed that a special spatiotemporal regulation of MyD88–IRF-7 signaling is critical for the potent type I IFN responses in pDCs [101]. In this model, the TLR9-MyD88 complex is retained for approximately 30 minutes in transferrin receptor (TfR)-positive early endosomes after recognition of the TLR-ligand type A CpG, which maintains a prolonged induction of IRF-7 and in turn induces massive amount of type I IFN.

Although the special endosomal trafficking of TLR9 agonists causes strong activation of the IFN- α pathway, the activation of NF- κ B pathway and therefore, maturation of pDCs is less efficient [102]. Later studies using murine cells further supported this concept, also demonstrating the critical role of the inhibitory receptor Ly49Q [103]. Type A CpG, preferentially forming multimeric complexes, primarily induce type I IFN responses, while activation with monomeric type B CpG leads to maturation of pDCs. However, *Guiducci et al.* demonstrated that the endosomal localization, rather than oligomer formation, is the key factor determining the fate of pDC-responses; oligomerized CpB could induce sustained type I IFN production, while monomeric type A CpG induced pDC-maturation similarly to monomeric CpG. As an intermediate phenotype, type C CpG localizing both to TfR-positive early, and lysosome-associated membrane protein (LAMP)-1-positive late endosomes induces type I IFN release and maturation as well [104].

Initial studies suggested that the production of type I IFNs by pDCs is largely independent of IFN- α/β receptor (IFNAR) feedback signaling [105]. However, recent evidence suggests that an autocrine signaling circuit amplifies type I IFN secretion through IFNAR [84,106].

The type I IFNs produced by pDCs are central mediators of antiviral immunity [107], and they act on the cells of both innate and adaptive immunity. IFN- α has a strong adjuvant effect on antibody production [108], activates $\gamma\delta$ T-lymphocytes, and increases the cytotoxic activity of natural killer (NK) cells [109]. An important feature of the viral recognition by pDCs is that type I IFN responses can be initiated even by inactivated or non-replicating viruses [110].

Plasmacytoid DCs produce further cytokines; besides IRF-7, MyD88 also recruits IRF5, which in turn activates NF- κ B and induces the transcription of genes encoding a multitude of pro-inflammatory cytokines and chemokines [111]. This process also initiates the maturation of pDCs into antigen presenting cells, as discussed in the next chapter.

2.4.2 Plasmacytoid dendritic cells as antigen-presenting cells linking innate and adaptive immunity

After completing their important mission as the primary type I IFN secreting cell type of the immune system, pDCs differentiate into APCs and participate in the

initiation of secondary immune responses. This process is governed by the IRF-5 dependent induction of NF- κ B transcription factor. During maturation of pDCs, the expression of MHC class II and co-stimulatory molecules (CD80, CD86 and CD40) is increasing, resulting in the capacity to present antigens and provide co-stimulation to CD4⁺ T-cells. During differentiation, they acquire a „dendritic” morphology, slightly similar to cDC. In addition to TLR7 and TLR9, this process can be activated via CD40, a member of the TNF receptor superfamily [15,112]. By preferentially stimulating the NF- κ B pathway, CD40 is a weaker inducer of type I IFN as compared to TLR7 and TLR9, but it can effectively promote the maturation of pDCs and their antigen presenting capacity.

The induction of the NF- κ B pathway also results in secretion of IL-6, TNF- α , and pro-inflammatory chemokines by pDCs, which influences both innate and adaptive immune responses. Upon treatment with Sendai virus, the type I IFN producing pDCs can drive the development of IFN- γ producing helper T-cells [113]. Type I IFNs also increase NK cell-mediated cytotoxicity [114] and promote the differentiation and maturation of cDCs [115-117]. Plasmacytoid DCs secrete multiple chemokines, including CXCL9 (MIG), CXCL10 (IP-10), CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CCL5 (RANTES), and these are important signals for the migration of activated CD4⁺ and CD8⁺ T cells to the sites of infection [118-120].

The production of λ IFNs (IL-28A, IL-28B and IL-29, also called type III IFNs) was initially demonstrated in mice [121,122]; more recently, *in vitro* cultured human pDCs were also reported to secrete type III IFNs in response to TLR9-stimulation [123].

The extent to which pDCs are able to present antigens and induce secondary immune responses has been a topic of debate. Freshly isolated pDC do not express high levels of MHC class II and CD80/CD86 co-stimulatory molecules and they are inefficient inducers of antigen-specific T-cell activation and proliferation, but still able to drive naive CD4⁺ T-lymphocytes into IL-10 producing regulatory T-cells (Tr1) [124] suggesting a role in maintaining peripheral tolerance. More recently, the importance of pDCs in central tolerance was also demonstrated [125,126]. Lung pDCs are important in preventing asthmatic reaction to inhaled antigens, independent on their activation state [127]. They maintain their tolerogenic potential even after activation by IL-3 and CD40-ligand and polarize naïve CD8⁺ T-cells into an IL-10

producing phenotype with poor secondary proliferative and cytolytic responses [128]. However, they also contribute to the sustainment of CD8⁺ T-cell responses against influenza virus [129] and multiple studies demonstrated their efficient presentation of endogenous antigens via MHC I and II molecules (reviewed in [130]). In terms of external antigens, pDCs can uptake soluble proteins by pinocytosis or receptor-mediated endocytosis [127,131] and they effectively present such exogenous peptides through MHC class molecules [132]. On the other hand, the antigen presentation by pDCs might result in down-regulation anti-tumor immune responses [133].

Cross-presentation is also a key element of antiviral immune responses since it allows presentation of antigens acquired from autologous cells, making DCs capable to stimulate naive CD8⁺ T-cells [134]. Early studies suggested that pDCs do not cross-present exogenous antigens [135] although murine studies raised the possibility of cross-priming by pDCs *in vivo* [136]. More recently, cross-presentation of vaccine formulates consisting of lipopeptides and HIV-1 antigens by human pDCs was reported [137,138]. However, these *in vitro* observations have not been confirmed yet *in vivo*.

2.5 Plasmacytoid dendritic cells in human diseases

2.5.1 Plasmacytoid dendritic cells in HIV and other viral infections

As the virus experts of the immune system, the potential response of pDCs against human immunodeficiency virus (HIV) soon became a major area of research. Plasmacytoid DCs express CD4, CXCR4 and CCR5 [139], potential surface targets for entry of HIV, and multiple studies concluded that the loss of pDCs in HIV-patients correlates with viral load and the onset of opportunistic infections [140-143]. Interestingly, highly active retroviral therapy seems to be unable to prevent the loss of pDCs [144-146]. HIV activates pDCs to produce high levels of IFN- α and lower levels of TNF- α , but minimally up-regulates co-stimulatory molecules, regardless of the HIV strain used; *Beignon et al.* showed HIV RNA is the most potent activator of pDCs [147]. Depletion of pDCs from PBMC reduced the type I IFN response induced by HIV-infected cells 10-fold, clearly demonstrating the importance of pDCs in anti-HIV responses [148].

In contrast to the initial encounter of HIV, the impact of pDC activation during chronic HIV infection seems to be detrimental rather than beneficial. A murine *in vivo* study demonstrated that chronic TLR9-activation leads to destruction of lymph node organization and subsequent immunosuppression [149]. Furthermore, expression of indoleamine (2,3)-dioxygenase (IDO) by pDCs favours regulatory (Treg) T-cell development and has a negative impact on effector T-cells responses [150]. Activated pDCs also express TRAIL, and can induce the apoptosis of CD4⁺ T-cell during chronic HIV-1 infection; notably, the level of TRAIL-expression on pDCs seems to correlate with the viral load [151,152]. HIV-activated pDCs seem to maintain a type I IFN producing profile and do not effectively mature into antigen-presenting DCs [153]. Studies involving patients efficiently controlling HIV-1 support the hypothesis that the potent initial type I IFN responses are important, but in the chronic phase, the limited viral load results in moderate type I IFN levels and TRAIL expression is critical for HIV-control [154]. More recent evidence from studies of pDC-depleted humanized mice indicates that the decrease in IFN- α production leads to reduced cell death and higher number of CD4⁺ T-cells [155]; these results show the dual role of pDCs during HIV-infection.

The potential role of pDCs in other viral infections is somewhat less characterized as their contribution in HIV infection; among the more studied viruses are the members of the alpha herpes virus family. Plasmacytoid DCs infiltrate lesions of Herpes simplex virus and Varicella zoster virus in humans [156-159] and several cell surface molecules on pDCs are able to mediate the uptake of herpes viruses including CD111/HVE-C, CD112/HVE-B, and CD270/HVE-A [156]. The current model for pDC function in alpha herpes virus infections suggests a dual role of pDCs – first as professional IPCs mediating local antiviral effect, then as professional APCs that re-enter the blood from the site of infection, and home to lymph nodes by HEV-CD62L interaction for efficient antigen presentation [160].

The contribution of pDCs to immune responses against respiratory viruses remains a matter of debate. Initial *in vivo* studies suggested a direct antiviral and T-cell modulatory role for the type I IFN production secreted by pDCs [161-163]. However, the *in vitro* observation that pDCs can induce influenza-specific CD8⁺ T-cell responses [112,164,165] could not be verified by *in vivo* studies using pDC-deficient or pDC-depleted mice [166,167] and contradicting data were published regarding the ability of pDCs to induce virus-specific NK-cell and CD8⁺ T-cell

responses [168,169]. The interpretation of mouse studies and their relevance to human disease conditions is further complicated by the existence of different pDC-subpopulations in mice. Among the three subpopulations, CD8 $\alpha^+\beta^-$ and CD8 $\alpha^+\beta^+$ pDCs can induce tolerance, likely through induction of Tregs, while CD8 $\alpha^-\beta^-$ pDCs have pro-inflammatory potential.

2.5.2 The role of plasmacytoid dendritic cells in autoimmune diseases

Besides their protective role in viral infections and the tolerogenic capacity in the immune system of the gastrointestinal tract, the involvement of pDCs in autoimmune diseases also became a well-studied aspect of the biology of pDCs. Multiple studies indicate that chronic activation of pDCs and the maintained secretion of type I IFNs in the absence of infection could lead to autoimmunity. Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by antibody response against self-antigens, prominently DNA and ribonucleoproteins. Well before the identification of pDCs, many publications reported the increased type I IFN levels in the peripheral blood of SLE patients [170-172] and the prominent IFN- α/β signature in the transcriptome of peripheral blood mononuclear cells of SLE patients [173-176]. Furthermore, a clinical study seeking potential genetic factors associated with an increased risk of SLE-development identified higher IFN levels as an inherited risk factor [177]. Together with the emerging picture of pDCs as professional APCs specialized in the recognition of nucleic acids, these data soon turned the interest of researchers towards the newly identified cell type and identified pDCs to be of significant importance for the development of SLE (reviewed by *Gilliet et al.* [178]).

Initial *in vitro* evidence showed that DNA from apoptotic cells and IgG from SLE patients, assumed to contain anti-DNA antibodies, are able to induce type I IFN production from PBMC and pDCs are responsible for this IFN-response [179,180]. In the skin lesions of patients with SLE, DNA or RNA containing immune complexes are internalized by pDCs and stimulate TLR7 and TLR9 [181], while sera of SLE patients contain immunogenic complexes composed of neutrophil-derived antimicrobial peptides and self-DNA, also able to trigger TLR-9 responses from pDCs [182]. Activation of the IFN- α/β system in SLE correlates with disease activity and severity [183] and IFN- α ultimately contributes to SLE disease progression by

many ways [184]; it promotes the differentiation of activated B-cells into plasmablasts, triggers B-cell expansion upon co-stimulation by TLR-ligands and lowers the activation threshold for auto-reactive B cells [185-187]. Recent studies showed that hydroxychloroquine and C-reactive protein exert their observed positive effect on SLE patients by inhibiting type I IFN secretion by pDCs [188,189].

An important aspect of the contribution of pDCs to the progression of SLE is their potential involvement in tissue lesions. Similarly to the skin lesions mentioned above, pDCs also migrate to other organs involved in SLE, e.g. to the kidneys. At the same time, their number in the peripheral blood is decreased [190,191]. As a next step, two studies addressed the important aspect of potential accumulation of DCs in the kidney of SLE patients, simultaneously describing the accumulation of myeloid DC and pDCs in kidneys of SLE patients during lupus nephritis [192,193]. One of these studies also demonstrated a correlation between the amount of BDCA1⁺ mDCs and BDCA2⁺ pDCs in the peripheral blood and the activity of the disease; in patients with active SLE, the number of both cell types was reduced [192].

Besides SLE, the role of pDCs in the development of psoriasis vulgaris was also demonstrated. After initial studies indicating the potential role of pDCs in psoriasis development [194], the root cause initiating this chronic autoimmune skin disease was identified to be the antimicrobial peptide LL-37 which is able to convert self-DNA into structures that are sequestered in endosomal compartments of pDCs, triggering type I IFN production through TLR9 [195]. Vitamin D analogs are used in the treatment of psoriatic skin lesions; these compounds might exert their effect through inhibition of pDC function resulting in decreased T cell proliferation and secretion of the Th1 cytokine IFN- γ [196].

Multiple sclerosis (MS) is considered to be an immune-mediated disorder of the central nervous system and viral pathogens have been implicated in the etiology and pathogenesis of the disease (reviewed by *Haahr and Hollsberg* [197]). Patients with MS have an increased risk of disease exacerbation at the time or shortly after clinical viral infections [198]. This potential link between viral infections and MS turned the interest of researchers towards pDCs as professional antiviral antigen presenting cells. A clinical study showed decreased cell surface expression of CD86 and 4-1BBL co-stimulatory molecules on pDCs from MS patients *ex vivo*, compared to pDCs from healthy individuals and pDCs from MS patients were also impaired in terms of type I IFN production [199]. Animal studies of experimental autoimmune

encephalomyelitis, the most widely used animal model of MS, support the notion of pDC involvement in MS [200-202]. The most recent *in vitro* studies using human pDCs found a link between pDC activation leading to MS exacerbation and the beneficial effect of IFN- β treatment of MS patients; IFN- β in MS patients is able to inhibit the secretion of chemokines (CCL3, CCL4, CCL5) and IFN- α [203].

2.6 Plasmacytoid dendritic cell leukemia/lymphoma and pDC-derived cell lines

Similarly to the discovery of pDC, described in section 2.2, the identification and naming of their malignant counterparts also followed a tortuous path. Plasmacytoid dendritic cell leukemia/lymphoma (pDC-L) is an extremely rare hematopoietic malignancy characterized by a rapid and aggressive clinical course and poor prognosis. The clinical symptoms typically begin with isolated cutaneous lesions followed by systemic dissemination, including involvement of bone marrow, peripheral blood, lymph nodes and other tissues [204]. This entity was initially described as histiocytic lymphoma / histiocytic associated hematologic malignancy [205] or as agranular CD4⁺CD56⁺ cutaneous lymphoma/hematodermia [206]. Based on their expression of CD56, they were also related to NK-cells and referred to as blastic/blastoid NK-cell leukemia [207] or NK-lymphoma/leukemia [208,209] and this classification was accepted by the WHO committee in 2000, although a CD4⁺CD56⁺, ‘monocytic plasmacytoid’ phenotype was also listed in this document [210]. When *Lucio et al.* reported a case of a CD123-expressing lymphoma for the first time, CD56-expression was not investigated, thus a link to the CD4/CD56 positive ‘NK-lymphoma’ was not established [211]. In 2001, *Rakozy et al.* suggested for the first time that CD4/CD56 positive malignancies might represent a diverse group of diseases [212] and others also supported this conclusion [207]. Finally, *Chaperot et al.* reporting on CD4/CD56 positive leukemia cells from 7 patients could verify the pDC-like phenotype of this entity by functional characterization [213]; this hypothesis was confirmed by others [214,215]. Based on an extensive review of case studies and of the data collected by the French leukemia workgroup called ‘Groupe d’Etude Immunologique des Leucémies’ (GEIL), *Jacob et al.* proposed the new name ‘early pDC leukemia/lymphoma’ for the pDC-derived lymphoma/leukemia cases

[216]. Other authors also supported this conclusion suggesting a re-classification of NK-cell related lymphomas [217,218]. The WHO/European Organisation for Research and Treatment of Cancer (EORTC) classification for cutaneous lymphomas designated this entity as CD4/CD56⁺ hematodermic neoplasm or ‘early’ plasmacytoid dendritic cell leukemia/lymphoma (pDC-L) [219,220]. The incidence and reported characteristics of pDC-derived malignancies was estimated to be <1% of acute lymphoma cases as reviewed by *Bueno et al.* [221].

In the clinical practice, the diagnosis of pDC-L is often delayed due to the rarity and atypical presentation of the disease. In principle, the diagnosis of pDC-L is based 1) clinical symptoms; 2) morphologic findings; 3) immunophenotypic profile determined by flow cytometry; and 4) cytogenetic and molecular data. Due to the significant overlap of the immunophenotypic profile with other hematopoietic neoplasms (e.g. T-cell lymphoma or acute T-cell leukemia), extensive immunophenotypic analysis is needed for a definitive diagnosis (reviewed in detail by *Shi and Wang* [222]). A ‘typical’ pDC-L profile has previously been reported as follows: CD4⁺CD56⁺ lineage⁻ CD45RA⁺/RO⁻ CD11c⁻ CD116^{-/+low} CD123⁺ CD34⁻ CD36⁺ HLA-DR⁺ [215]. A novel scoring system for the diagnosis of pDC-L and atypical pDC-L has recently been developed that includes a limited number of lineage markers (CD4⁺, CD56^{+/-}, CD11c⁻, MPO⁻, cCD79a⁻ and cCD3⁻) and requires investigation of CD123, BDCA-2 and BDCA-4 expression; as CD56 was reported to be absent in multiple pDC-L cases, this should not be considered as a mandatory marker for the diagnosis of pDC-L [223]. Cytogenetic and FISH analysis revealed that two-thirds of pDC-L cells showed cytogenetic anomalies at the time of diagnosis, but no single anomaly could be considered as specific for this disease as gross genomic imbalances predominate over gene-specific alterations. Cytogenetic aberrations were concentrated only on 6 major chromosomal targets: anomalies of 5q (particularly 5q21 or 5q34 regions), 12p13, and 13q; loss of 6q or deletion of 6q23-pter; monosomy 15p and chromosome 9 [224]. Other authors reported recurrent deletions of chromosomes 4 (4q34), 9 (9p13-p11 and 9q12-q34) and 13 (13q12-q31) [225].

As recently suggested, the ultimate way to diagnose pDC-L is to perform functional characterization of the malignant cells [226]. However, this process is time-consuming and requires special methods that are often unavailable in diagnostic laboratories; thus the differential diagnosis tends to be phenotype-based. Functional

studies on pDC-L are few, but earlier experiments showed that pDC-L cells are able to produce IFN- α and subsequently differentiate into mature antigen presenting cells to present viral antigens to allogeneic naïve T-cells [213,227]. However, a study showed that certain pDC-L samples are unable to secrete IFN- α , while the producers tend to secrete lower amounts than normal pDC [214]. It is therefore important to conduct further studies to allow reliable diagnosis of pDC-L supported by functional properties.

The mean age of pDC-L patients is between 60 to 70 years, but it might occur at any age – pediatric cases have also been reported. The male to female ratio is 3:1, and the underlying factors of this difference are currently unknown. The clinical manifestation of pDC-L typically involves cutaneous lesions – in most cases, these are asymptomatic, solitary or multiple nodules, plaques, or bruise-like lesions ranging from a few millimeters to up to 10 centimeters in diameter (Figure 5). Although a minority of pDC-L patients lacks cutaneous lesions at diagnosis, skin lesions develop quickly during the course of disease. The presence of extra-cutaneous symptoms upon diagnosis is frequent, and these symptoms involve the bone marrow, peripheral blood and the regional lymph nodes. During the progression of pDC-L, patients typically develop fulminant leukemia, particularly in the terminal stage of the disease. The clinical course of pDC-L is aggressive, with a median survival of 12 to 14 months, practically independent of the initial presentation of the disease. A likely reason behind the poor prognosis is the lack of consensus treatment due to the rarity of the disease. Most patients receive CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or hyper-CVAD (alternating combination of course A: cyclophosphamide, vincristine, doxorubicin; and course B: methotrexate and cytarabine) chemotherapy. Despite the typically good response to initial chemotherapy, the disease often relapses and usually resistant to previous used chemotherapeutic agents. The only treatment shown to result in durable remission is high-dose chemotherapy followed by allogeneic stem cell transplant from matched related or unrelated donors.

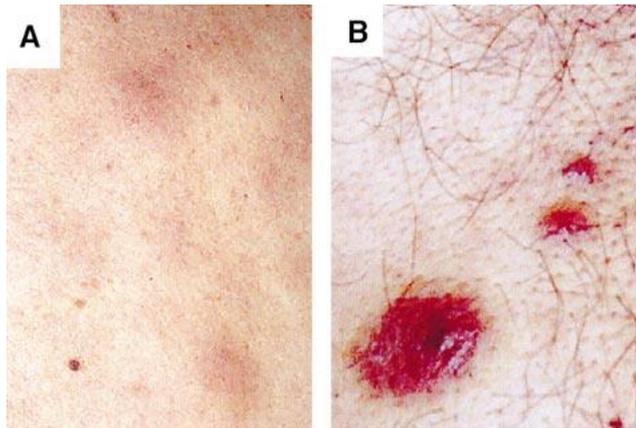


Figure 5. Clinical appearance of pDC-L skin lesions in patients

Skin lesions are present in 80% of patients at diagnosis, most commonly as purple plaques infiltrating the dermis. From Feuillard *et al.*, *Blood* 2002;99:1556-1563.

Cells isolated from bone marrow or peripheral blood of pDC-L patients might serve as research tools to understand pDC biology. In a previous study, our research group performed an extensive flow cytometric characterization of pDC-L cells from a 71-year-old male patient. In this case the CD4⁺CD56⁺lineage⁻ pDC-L cells were characteristically positive for CD36, CD38, CD40, CD45, CD45RA, CD68, CD123, CD184, HLA-DR, BDCA2 and granzyme-B [228]. The basic immunophenotype of malignant cells from skin lesions and lymph nodes together with the clinical feature of the disease allowed us the diagnosis of pDC-L. Genotyping by FISH detected del 13q and monosomy 9. The cryopreserved bone marrow samples of this patient served as source of malignant pDCs used in functional studies.

Besides studies involving pDC-L cells obtained from patient' samples, multiple research groups attempted to generate cell lines from malignant pDCs. Currently, two cell lines and their derivatives are available: CAL-1 [229] and GEN2.2 [230,231]. After short term *in vitro* culturing in presence of GM-CSF and IL-3, CAL-1 cells change morphologically into the mature DC appearance with many long dendrites and able to secrete TNF- α ; however, they are unable to secrete IFN- α , which makes this cell type less appealing as a model system replacing normal pDCs. GEN2.2 cells are considered to represent a model system superior compared to CAL-1, as these cells are able to produce type I IFNs upon encountering viral stimulus. The long-term culture and maintenance of this cell line requires the presence of MS-5 feeder cells. However, the impact of the pro-inflammatory cytokines secreted by MS5 cells on GEN2.2 cells is unknown; furthermore, GEN2.2 are considered to have weak antigen presentation capabilities (Kitti Pazmandi, unpublished data). To address this weak antigen presentation by GEN2.2 cells, a new derivative, GEN3 was established

and shown to effectively cross-present viral antigens from influenza-virus expressed cells [232].

2.7 Methods to study plasmacytoid dendritic cells in mixed cell populations

The scarcity of pDCs has traditionally been a barrier for researchers, further complicated by the lack of pDC-specific cell surface markers. These two factors forced combined isolation approaches, like the depletion of lineage-positive cell types by magnetic bead separation and subsequent cell sorting by FACS, described in the earliest studies [15]. Although the IL-3 receptor α chain (CD123) was suggested as a potential marker of pDC [233], this antigen does not have the specificity needed to isolate pDCs from certain mixed lymphocyte populations, especially not from lymph nodes or tonsils. The need for two-step isolation and the lack of specific markers led to unreliable confirmation of cell purity, and this was proposed to be one of the reasons behind the long-lasting debate about the IL-12 production of human pDC [234]. In 2000, two potentially DC-specific cell surface antigens were described and named as BDCA-2 and -4, later classified as CD303 and CD304 (also known as neuropilin-1), and monoclonal antibodies as well as cell separation kits soon became available [235]. However, the rarity of this cell type still remains a significant limitation, as one blood bag (400 mL peripheral blood) or the derived buffy coat contains approx. 1×10^6 pDCs. Furthermore, pDCs rapidly die upon *in vitro* culturing, and the IL-3 used in the medium to promote survival also induces limited phenotypic maturation of the cells.

To overcome the limited availability and poor *ex vivo* survival of pDCs, multiple authors suggested studies involving pDCs maintained in mixed populations. *Olshalsky et al.* proposed for the first time the characterization of pDCs with flow cytometry in PBMC [236] and *Ida et al.* extended this approach for evaluating functional properties of pDCs in peripheral blood leukocytes [237]. More recently, *Della Bella et al.* described a 6-color panel for analysis of *ex vivo* activated dendritic cell subsets in whole blood [238]. In these studies, three fluorescence channels were used for pDCs identification using lineage-cocktail, anti-HLA-DR, and anti-CD123 antibodies. This method was optimized for the most reliable identification of pDCs,

but offers only one or no open channel for further phenotypic analysis of these cells on a typical instrument used in the clinical diagnostic environment. Despite the availability of BDCA-2 and BDCA-4 antibodies, the lineage/HLA-DR/CD123 marker combination was used in more recent studies [239,240]. Implementation of BDCA antibodies for DC identification potentially allows capturing DCs by two channels and the screening of large number of samples with a simpler staining and analyzing method [241,242].

3 Aims of the study

- To optimize the flow cytometric identification of pDCs in peripheral blood of healthy donors using exclusively Blood Dendritic Cell Antigen 4 positivity.
- To assess whether leukemic pDCs resemble the functional activity of their normal counterparts.
- To investigate whether leukemic pDCs are suitable as model system to study the role of RLHs in viral recognition by pDCs.
- To elucidate the potential impact of TLR-activation on the expression and function of RLHs and to identify potential co-operation between the TLR- and RLH-mediated virus recognition pathways in pDCs.

4 Materials and methods

4.1 Flow cytometric identification of pDC

To evaluate the single-marker identification of pDCs in peripheral blood and to assess their phenotype, we used fresh peripheral blood samples of healthy adult donors. After obtaining informed consent from each donor, blood samples (~10 mL) were collected to BD Vacutainer™ tubes containing K-EDTA as anticoagulant, by trained personnel at the Department of Internal Medicine. The protocol was approved by the Institutional Review Board of the University of Debrecen under permit No. RKEB/IKEB 2741-2008. All samples were processed within 60 minutes after collection. Briefly, 250 µL of blood sample was dispensed to each of the 5 mL plastic tubes (BD Biosciences, San Jose, CA, USA) and the antibodies were added to the tubes at pre-optimized concentration as described below. Following 30 minutes of incubation on ice, the lysis of red blood cells (RBC) was performed using 4 mL of 1X FACS Lysing Solution (BD Biosciences) containing paraformaldehyde as fixative. Following lysis of RBCs, the samples were centrifuged at 300 g, washed with 4 mL of PBS containing 0.5% bovine serum albumin and 0.05% sodium azide, and re-suspended in 300 µL of the same buffer. Samples were analysed on a FACSCalibur flow cytometer equipped with FL4 option (BD Biosciences). The following antibodies were used in these studies:

- 5 µL anti-HLA-DR (MHC Class II antigen presenting molecule)-FITC (clone G46-6) and 3 µL anti-CD123 (IL3-receptor)-PECy5 (clone 9F5) mAbs (both from BD Biosciences) for two-color identification of pDCs.
- 3 µL (1 µg/mL) of anti-BDCA-4 (neuropilin-1)-APC (clone AD5-17F6, Miltenyi Biotec, Bergisch Gladbach, Germany) monoclonal antibody for single color identification of pDCs.
- 3 µL CD4-FITC (clone RPA-T4) and 3 µL CD123-PE (Clone 9F5) mAbs (both from BD Biosciences) for further confirmation of single-color identification of pDCs.
- Single-color labelled cells for compensation setup were stained with 5 µL anti-CD3-FITC (clone UCHT1, BD Biosciences), 5 µL anti-CD3-PE (clone UCHT1, BeckmanCoulter, Hialeah, FL) or 3 µL anti-CD123-PECy5 (BD Biosciences) antibodies, respectively.

Hardware compensation was performed by single-color labelled cells from the same sample, while software compensation for FL1, FL2, and FL3 fluorescent channels was calculated and applied using FlowJo software (TreeStar, Ashland, OR, USA). For FL1 and FL2 channels the compensation was also evaluated with fluorescently labelled CaliBrite beads (BD Biosciences). Throughout data acquisition, $5-8 \times 10^5$ total events were collected from every sample corresponding to $0.5-1.3 \times 10^4$ pDCs per sample; data acquisition was terminated on time, $t=300$ seconds. List mode data files were analysed using FlowJo software (TreeStar).

4.2 Analysis of phenotypic changes of pDCs in whole blood following TLR-ligand treatment

To activate pDCs, the fresh blood samples were treated with TLR-7 ligand imiquimod (R837; Invivogen, San Diego, CA) at a final concentration of $9.06 \mu\text{M}$ ($2.5 \mu\text{g/mL}$). Stock solution of the TLR7 ligand was prepared at a 10-fold higher concentration than the final concentration and stored at -20°C . For pDC activation, $25 \mu\text{L}$ of imiquimod stock solution was added to $225 \mu\text{L}$ blood and samples were incubated at 37°C in 5% CO_2 humidified atmosphere for 24 hours. Surface staining was performed as described above for non-stimulated samples. The following antibodies were used in these studies:

- Phenotyping of pDCs after activation with imiquimod was performed using $5 \mu\text{L}$ of anti-HLA-DQ (MHC Class II antigen presenting molecule)-PE (clone HLADQ1) and $5 \mu\text{L}$ of anti-CD62L (L-selectin adhesion molecule)-PE (clone Dreg56) mAbs (both from BD Biosciences) in two-color identification method.
- For phenotyping together with one-color identification, we used $5 \mu\text{L}$ of anti-HLA-DQ-FITC (clone Tü169) together with $5 \mu\text{L}$ of anti-CD62L-PE mAbs (both from BD Biosciences).

Data collection, compensation setup and analysis of list mode files were performed as described in section 4.1. For phenotyping, pDC gate was set to achieve $>93\%$ pure pDC-population. To assess average fluorescent intensities for pDC population, median values were calculated and used.

4.3 Isolation and culture of primary and leukemic pDC

Leukocyte-enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center, Debrecen, Hungary. Written, informed consent was obtained from the donors prior to blood donation, and their data were processed and stored according to the directives of the European Union. Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare, Little Chalfont, UK). Primary pDCs were separated from peripheral blood mononuclear cells by negative selection using magnetic cell enrichment kit on a QuadroMACS magnet (both from Miltenyi Biotec). This process is based on the retention of non-pDC cell types labeled with a cocktail of magnetic bead conjugated specific antibodies (containing mAbs against CD3, CD14, CD16, CD19, CD20, CD56 antigens) during elution through a cell separation magnet. The homogeneity of the pDC fraction was 91 – 96%, as confirmed by flow cytometry. The purified cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 110 mg/L Na-pyruvate (both from Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/mL), streptomycin (100 µg/mL), 10% fetal calf serum (all from Invitrogen) and 10 ng/mL recombinant IL-3 cytokine (PeproTech, Rocky Hill, NJ, USA), which are essential for *in vitro* survival of primary pDCs.

Leukemic pDCs were isolated from the bone marrow samples of a 71-year-old patient diagnosed with pDC leukemia [228]. The Ethical Committee of the National Medical Center, Institute of Hematology and Immunology, Budapest, Hungary approved the study. For cell separation, $2.5 - 5 \times 10^7$ bone marrow cells were incubated with 10 µL anti-CD123-PECy5 antibody (clone 9F5, BD Biosciences) for 30 minutes at room temperature and subsequently washed two times in PBS (PAA Laboratories, Pasching, Austria). Cell sorting of pDC-L cells was performed on a FACSDiVa cell sorter (BD Biosciences), based on their CD123 positivity and light scatter properties. The purity and viability of the sorted cells was evaluated on a FACSCalibur cytometer using 7-amino-actinomycin D (Sigma-Aldrich) staining (5 µg/mL) for viability assessment. Before cell sorting, the frequency of pDC-L cells

was on average $61.5 \pm 3\%$ ($n=3$) in the bone marrow samples. After cell sorting, more than 95% of the cells displayed the pDC phenotype and the viability ranged from 87% to 93%. The separated cells were cultured at a final density of 10^6 cells/mL in flat-bottom Nunclon 48-well cell culture plates (Thermo Fisher Scientific, Rochester, NY, USA) in RPMI-1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 110 mg/L Na-pyruvate (both from Sigma-Aldrich), penicillin (100 U/mL), streptomycin (100 μ g/mL) and 10% fetal calf serum (all from Invitrogen).

4.4 Functional characterization of leukemic pDCs

To analyze phenotypic changes induced by TLR-activation, leukemic plasmacytoid dendritic cells cultured in RPMI-1640 medium were treated with 9.06 μ M (2.5 μ g/mL) imiquimod (InvivoGen), 5 μ M type A (CpG 2216), type B (CpG 2006) CpG (both from Hycult Biotechnology, Uden, The Netherlands), or a combination of imiquimod and type B CpG. Phenotyping of TLR-activated pDC-L cells was performed by staining the cells with 5 μ L of anti-HLA-DQ-PE (Clone No. HLADQ1) or 5 μ L of anti-CD86/B7-2-PE (Clone No. IT2.2) mAbs (both from BD Biosciences) in a final volume of 100 μ L. (Antibody concentration was not specified by the manufacturer, the amount used was pre-determined in setup experiments.) Following 30 minutes incubation on ice, the samples were washed with 1 mL PBS containing 0.5% bovine serum albumin and 0.05% Na-azide (both from Sigma), resuspended in 300 μ L of the same buffer, and analyzed on a FACSCalibur flow cytometer. Compensation setup and offline data analysis was performed as described in section 4.1.

Following 24 or 48 hours of activation, cell culture supernatants were harvested and stored at -80°C until further analysis. Concentrations of secreted cytokines in the supernatants were measured by ELISA or cytometric bead array. The amount of IFN- α was measured using the ELISA kit of PBL Biomedical (Piscataway, NJ, USA). To measure the concentration of IL-6 and TNF- α , the FlowCytomix Flex Set (Bender MedSystems / eBioscience, Vienna, Austria) was used and samples were analyzed on a FACSArray bioanalyser (BD Biosciences). The detection limit of the Flow Cytomix assay was 27.44 pg/ml for both IL-6 and TNF- α , and the maximum bias of the calculated standard values was 12% for all the cytokines measured.

To assess the T-cell stimulatory capacity of leukemic pDCs, we performed co-culture experiments with heterologous CD3⁺ T-cells and TLR-activated pDC-L cells. T-cell activation was monitored by means of ELISPOT using 96-well polyvinylidene difluoride (PVDF)-backed plates (Millipore, Billerica, MA, USA), pre-coated overnight at 4°C with 100 µL/well of anti-IFN-γ capture antibody (NatuTec, Frankfurt, Germany). Plates were washed 5 times after coating with sterile ELISPOT coating buffer (NatuTec), and blocked at room temperature for 1 hour with complete RPMI-1640 medium. T-cells used in this assay were selected from PBMC of healthy donors by using the magnetic anti-CD3 selection kit (Miltenyi Biotec). Activated pDC-L cells (10⁴-10⁵ cells/well) were incubated with T-cells (10⁶ cells/well) in RPMI-1640 for 4 days at 37°C in a humidified atmosphere containing 5% CO₂. T-cells activated by phytohemagglutinin (10 µg/ml) and concanavalin A (10 µg/ml, both from Sigma-Aldrich) were used as positive controls, whereas untreated T-cells and T-cells co-cultured with IL-3-treated pDC-L cells served as negative controls. The transferred co-cultures (2 × 10⁵ cells/well) were incubated in complete medium in a final volume of 200 µL/well in ELISPOT plates under the same conditions for 24 hours. Plates were washed three times using coating buffer and 100 µL/well of biotinylated anti-IFN-γ detection antibody (BioLegend) was added and incubated for 2 hours at room temperature. For signal detection, we used avidin-conjugated horseradish peroxidase (NatuTec; 100 µL/well, incubation for 45 minutes at room temperature) and freshly prepared AEC Substrate Solution (NatuTec; 100 µL/well, incubation for 30 to 60 minutes at room temperature). The substrate reaction was stopped by three washing steps with 200 µL / well distilled water. Plates were dried and analyzed by an ImmunoScan plate reader (CTL Ltd., Shaker Heights, OH, USA).

4.5 Analysis of cytoplasmic nucleic acid sensor RIG-I

To analyze the expression of RIG-I, freshly isolated primary pDCs and pDC-L cells were treated with imiquimod (InvivoGen) at a final concentration of 9.06 µM (2.5 µg/mL) and type A (CpG 2216) or type B (CpG 2006) CpG (both from Hycult Biotechnology) at a final concentration of 5 µM for 8 hours in Q-PCR or for 24 hours in Western blot and ELISA experiments. In separate experiments, cells were incubated with imiquimod or type CpG A for 24 hours at concentrations indicated in

Figure 15, and then washed two times with fresh medium. Thereafter, 5'ppp-dsRNA treatment of the cells was performed in freshly added medium. The introduction of 5'ppp-dsRNA was performed with the LyoVec transfection system (InvivoGen) according to the manufacturer's recommendations. The LyoVec+5'ppp-dsRNA complex containing 1 µg/mL working concentration of the RIG-I ligand was added to the cells, and the supernatants of the cultures were collected for ELISA after 16 h (IL-6) or 24 h (IFN- α) of incubation. Control experiments were performed with "LyoVec-only" and LyoVec+control-oligo complexes (provided by InvivoGen).

To analyze the relative changes in gene expression, Q-PCR was performed as described previously [243]. Total RNA was isolated by TRIzol reagent (Invitrogen) and 1.5-2 µg of the total RNA were reverse transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen) and Oligo(dT)15 primers (Promega, Madison, WI, USA). Gene-specific TaqMan assays (Applied Biosystems, Foster City, CA, USA) were used to perform Q-PCR. Reactions were carried out in triplicates in a final volume of 25 µL using AmpliTaq DNA polymerase and ABI Prism 7900HT real-time PCR instrument (Applied Biosystems). Amplification of 36B4 was used as normalizing control. Cycle threshold values were determined using the SDS 2.1 software (Applied Biosystems). Constant threshold values were set for each gene throughout the study. The sequence of the primers and probes are available upon request.

Concentration of secreted cytokines in the cell culture supernatants were measured by ELISA. The amount of IFN- α was measured using an ELISA kit from PBL InterferonSource. Level of IL-6 secreted by primary pDCs was measured using OptEIA™ ELISA kit (BD Biosciences) after 24 hours of stimulation.

To measure the expression of RIG-I on protein level, protein extraction was performed by lysing the cells in lysis/loading buffer (0.1% SDS, 100 mM Tris pH 6.8, bromophenol blue, 10% glycerol, 5 v/v% β -mercaptoethanol). Proteins were denatured by boiling for 5 minutes. Samples were separated by SDS-PAGE (10% gels), and transferred to nitrocellulose membranes. Nonspecific binding was blocked by TBS-Tween-5% non-fat dry milk for 1 hour at room temperature. Anti-RIG-I, anti-STAT1, anti-phospho-STAT1 (Ser727), anti-phospho-STAT1(Tyr701) (Cell Signaling, Danvers, MA, USA) and anti- β -actin antibodies (Sigma-Aldrich) were used at a dilution of 1:1000. Membranes were washed three times in TBS-Tween, and then incubated with anti-rabbit secondary antibody conjugated to horseradish

peroxidase (GE Healthcare) at a 1:5000 dilution at room temperature for 30 minutes. After three washes with TBS-Tween, protein samples were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific). After the membranes had been probed for RIG-I or phospho-STAT1, they were stripped and re-probed for β -actin or native STAT1. For densitometry analysis, we calibrated the imaging system using a gray optical wedge (Kodak, Rochester, NY, USA). Consistent optical and light conditions were maintained during the whole CCD camera capturing session, performed with a Kodak Image Station 2000mm device (Kodak).

To block IFNAR1 receptors, cells were treated with 50 μ g/mL anti-IFNAR1 monoclonal antibody (Abcam, Cambridge, UK) for 1 hour prior to activation with 10 ng/mL recombinant human IFN- α (R&D Systems, Minneapolis, MN, USA) or TLR-7/9 ligands. To analyze RIG-I expression by Q-PCR, the total RNA from activated cells was extracted at 3 hours after IFN- α or TLR-7/9-specific stimulation.

4.6 Statistics

One-way ANOVA followed by Bonferroni *post hoc* test was used for multiple comparisons. All analyses were performed by using GraphPad PRISM software, version 5.04. Differences were considered to be statistically significant at $p < 0.05$. Significance is indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when compared to control sample and by # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ for comparison among treatment groups.

5 Results

5.1 Identification of pDCs in whole blood by one-color flow cytometry

To set up our standard methods to identify pDCs, we stained peripheral blood samples with anti-HLA-DR-FITC and anti-CD123-PECy5 antibodies and analyzed them by two-color flow cytometry. The first analysis gate was set up to exclude remaining erythrocytes and cell debris and to define the population of mononuclear cells. Within this gate, pDCs were first identified as being HLA-DR-FITC and CD123-PECy5 positive ones. The distribution of the cells within the “pDC gate” was analyzed by back gating on the light scatter parameters and confirmed a homogenous cell population (Figure 6).

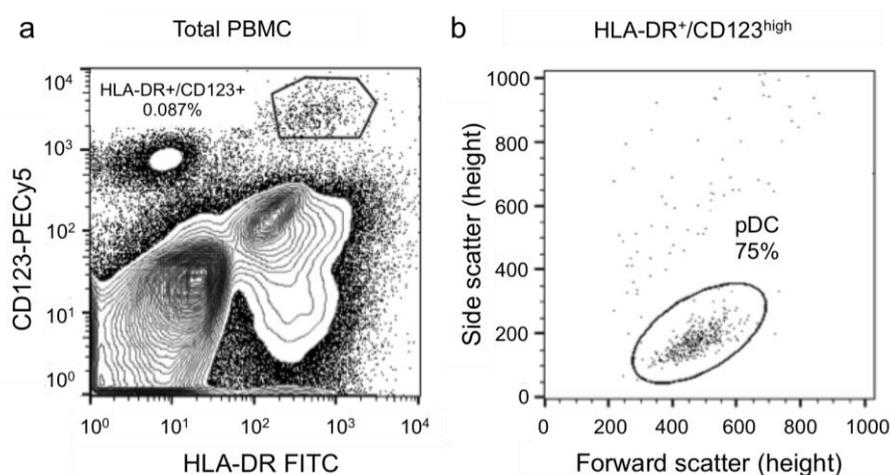


Figure 6. Two-marker identification of pDCs in peripheral blood samples.

pDCs are identified by their double positive staining with anti-HLA-DR-FITC and anti-CD123-PECy5 mAbs as the first step (a). The homogeneity of the cell population is then confirmed using the light scatter parameters (b). Gating on CD123^{high}/HLA-DR⁺ cells results in a >80% pure pDC population as assessed by forward and side scatter properties. Shown values are percentages of gated cells within all events shown on the cytogram.

In the exploratory single-channel identification method, blood samples were stained with BDCA-4-APC, CD4-FITC, and CD123-PE antibodies. In this sample, pDCs were first gated on BDCA-4-APC positivity and side scatter properties. The homogeneity of the resulting cell population was confirmed by back gating on forward and side scatter parameters (Figure 7). In a separate analysis, the identity of

the resulting cell population was confirmed by its positivity for both CD4-FITC and CD123-PE (Figure 8). This phenotype is consistent with the phenotype reported for pDCs as the simultaneous expression of CD4 and CD123 is unique among cells of the peripheral blood of healthy adults [244]. These results suggest that BDCA-4 antigen might be suitable as a standalone surface marker for the identification pDCs in peripheral blood, if used in combination with light scatter parameters.

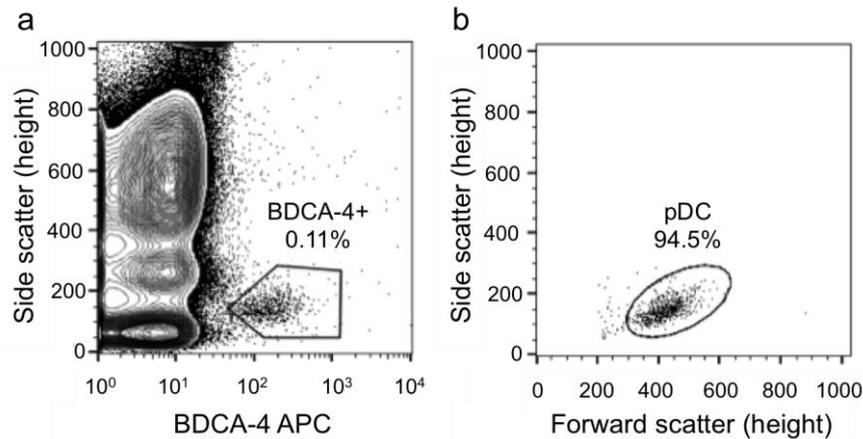


Figure 7. Single-marker identification of pDCs in peripheral blood samples. One-color identification of pDCs was done based on BDCA-4-positivity as the only surface marker (a). The homogeneity of the resulting cell population was confirmed by light scatter properties (b). Shown values are percentages of gated cells within all events shown on the cytogram.

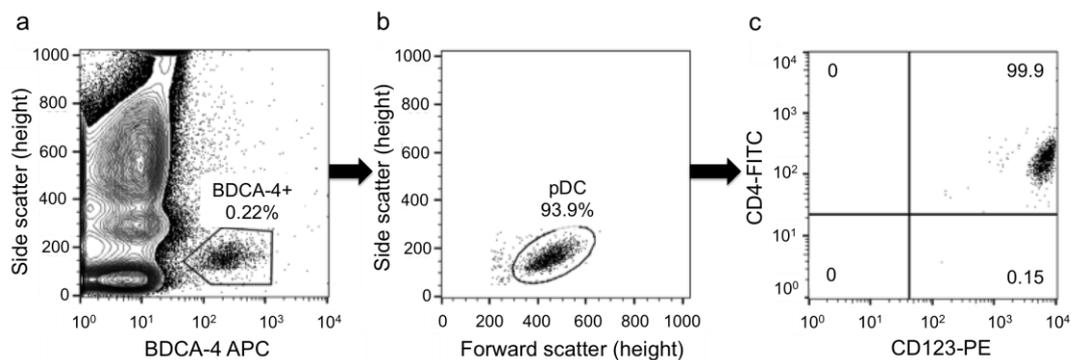


Figure 8. Confirmation of the one-color identification of pDCs in peripheral blood samples. Cells gated on BDCA-4-APC positivity and side scatter properties (a) and back-gated on forward and side scatter parameters (b). Confirming the identity of this cell population, the gated cells are positive for both CD4-FITC and CD123-PE (c). Shown values are percentages of gated cells within all events shown on the cytogram.

Our results indicated that in freshly analyzed peripheral blood, BDCA-4 identifies the pDC-population and provides a useful tool in a diagnostic setting, where

the complexity of surface staining antibody panels should be kept as low as possible. However, the expression of cell surface antigens might be altered during isolation of the cells, or more importantly, even during short-term *in vitro* culturing necessary to study rare cell types in mixed population. As methods employing mixed cultures (mainly PBMC) are widely used to study pDCs (reviewed in section 2.7), we aimed to assess the suitability of the single-color, BDCA-4 based identification method for such studies. To this end, we analyzed the phenotypic changes of pDCs in blood samples from five healthy young adults after treatment with 9.06 μM (2.5 $\mu\text{g/mL}$) TLR7 ligand (imiquimod). The number of pDCs and the changes in the expression levels of CD62L and HLA-DQ on pDCs were assessed 24 hours post stimulation by using both the one- and two-color identification methods; these results are summarized in Table 1. The difference between the numbers of pDCs determined by one- or two-color detection was the highest in the sample from Donor 4 (10.1%, 966 vs. 877 events in pDC-gate) and the lowest in the sample from Donor 3 (1.9%, 794 vs. 779). Upon treatment with TLR7 ligands, the expression of MHC class II molecules (HLA-DR and DQ) is increasing, while the expression of the cell surface adhesion CD62L is decreasing. In whole blood samples treated with imiquimod, both one- and two-color methods were able to measure the changes in the expression of HLA-DQ and CD62L on pDCs. By using the two-color identification method, we measured lower ratios of median fluorescent intensities (untreated versus treated cells) related to CD62L expression, and higher ratios of median fluorescent intensities related to HLA-DQ levels. The one-color identification method detected lower bio-variability in both CD62L and HLA-DQ expression (coefficient of variation, 14 and 7% versus 36 and 14%). Importantly, the expression of BDCA-4 remained constant following TLR7-ligand treatment and the *in vitro* culturing for 24 hours does not compromise the reliability of the single-color identification method.

2-color identification							
	pDC count*	CD62L			HLA-DQ		
		Control	Imiquimod	Ratio**	Control	Imiquimod	Ratio**
Donor #1	1061	869	636	0.73	10	20	2.00
Donor #2	722	593	327	0.55	332	623	1.88
Donor #3	794	397	113	0.28	461	1102	2.39
Donor #4	966	2054	718	0.35	18	49	2.72
Donor #5	842	832	487	0.59	6	16	2.67
Mean/S.D.***				0.50/0.18			2.33/0.38

1-color identification							
	pDC count*	CD62L			HLA-DQ		
		Control	Imiquimod	Ratio**	Control	Imiquimod	Ratio**
Donor #1	1097	729	564	0.77	13	19	1.46
Donor #2	775	585	358	0.61	77	94	1.22
Donor #3	779	419	250	0.60	77	104	1.35
Donor #4	877	1561	894	0.57	20	28	1.40
Donor #5	806	862	641	0.74	19	28	1.47
Mean/S.D.***				0.66/0.09			1.38/0.10

*pDC count determined in 250 μ l peripheral blood sample, **Ratio of median fluorescent intensities of imiquimod-treated vs. control cells, ***S.D., standard deviation.

Table 1. Cell surface expression of CD62L and HLA-DQ on pDCs of fresh blood samples after 24 hours of imiquimod (2,5 μ g/mL) treatment.

To test the reproducibility of the one-color identification method, we obtained fresh blood samples from the same individual on days 0, 4, and 8, respectively. Cells were treated with imiquimod at a final concentration of 9.06 μ M (2.5 μ g/mL) within 60 minutes after venipuncture. After 24 hours of incubation, the changes in the expression levels of HLA-DQ and CD62L activation markers on pDCs were investigated by using both identification methods. Our data indicate that the measurements performed by either one-color or two-color identification methods are highly reproducible. The mean of ratios of median fluorescent intensities (untreated vs. treated cells) corresponding to CD62L expression was 0.79 (range, 0.74–0.84; standard deviation/S.D./, 0.05) when analyzed by the two-color method, and 0.77 (range, 0.67–0.83; S.D., 0.09) when detected by the one-color assay. The two-color method measured higher increase in HLA-DQ expression; the mean of ratios of median fluorescent intensities was 2.21 (range, 2.16–2.26; S.D., 0.05), while this parameter was 1.56 (range, 1.52–1.59; S.D., 0.04) measured by the one-color assay. The coefficient of variation was 11 and 2% for CD62L and HLA-DQ expression measured by the one-color identification method, whereas 6 and 2% derived from the two-color assay.

5.2 Phenotypic and functional characterization of pDC-L cells activated by TLR ligands

Phenotypic characterization of pDC-L cells activated by TLR ligands

To investigate the responsiveness of pDC-L cells to TLR ligands, we sorted the CD123 positive cells from cryopreserved bone marrow samples of a patient diagnosed with pDC-L. The percentage of pDC-L cells defined by CD123 positivity and scatter parameters was $61.5 \pm 3\%$, as determined in three independent experiments. After sorting, more than 95% of the cells displayed the pDC phenotype (Figure 9) and the viability measured by 7-amino-actinomycin D staining ranged from 87% to 93%.

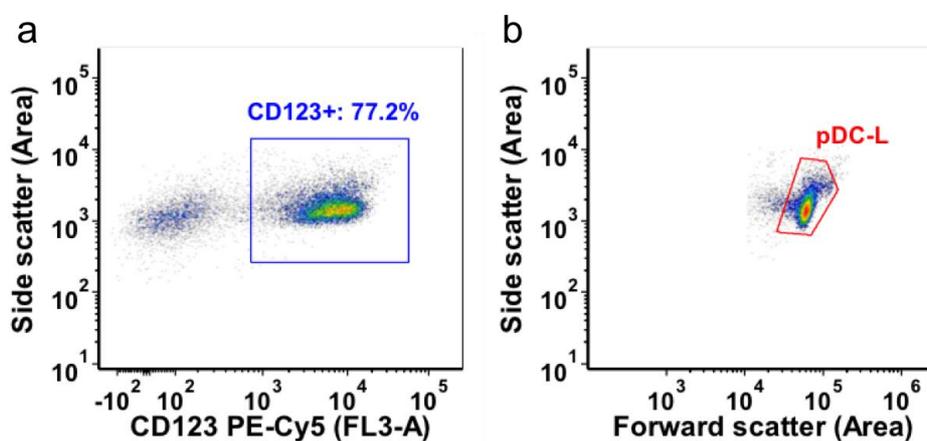


Figure 9. Isolation of resting pDC-L cells.

pDC-L cells stained with PE-Cy5 conjugated anti-CD123 mAbs were sorted by gating on CD123 positivity and side scatter profile (a). The purity of the resulting cells population was analyzed by back-gating (b).

To assess pDC-L cell activation, we measured the surface expression of CD86, and HLA-DQ molecules 24 hours and 48 hours after treatment with the TLR7 ligand imiquimod and the TLR9 ligand type A or type B CpG. Imiquimod was the most potent enhancer of CD86 expression and type B CpG had lower but still pronounced effect, whereas the treatment with type A CpG resulted only in slight increase in CD86 expression (Figure 10, upper panel). Combined treatment with imiquimod and type B CpG resulted in CD86 expression comparable to that induced by imiquimod alone. The phenotypic changes were more obvious at 48 hours than at 24 hours (Figure 10, upper panel). In contrast to the co-stimulatory molecule CD86,

the expression of HLA-DQ was most efficiently induced by the combined treatment with imiquimod and type B CpG (Figure 10, lower panel), suggesting that concomitant stimulation of pDC-L cells through TLR7 and TLR9 has a synergistic effect on the membrane expression of HLA-DQ. This observation indicates an independent regulation of the expression of these functionally important cell surface molecules. These results also revealed that pDC-L cells exhibit similar phenotypic changes after activation by TLR ligands to their normal counterparts [100,124].

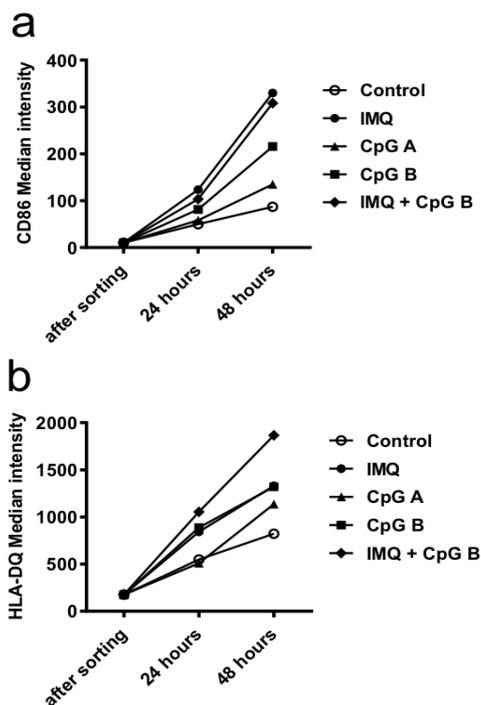


Figure 10. Phenotypic characterization of activated pDC-L cells.

Expressions of CD86 (a) and HLA-DQ (a) were measured on sorted cells 24 hours and 48 hours after activation with 9.06 μ M (2.5 μ g/mL) imiquimod (●), 5 μ g/mL type A CpG (▲), 5 μ g/mL type B CpG (■) or imiquimod plus type B CpG (◆), as well as on non-treated control cells (○). Expression levels in a representative measurement (n=3) are plotted as fold increase in median fluorescence intensity compared to isotype matched control antibodies.

Cytokine production of pDC-L cells treated with TLR ligands

To assess the cytokine secretion of pDC-L cells exposed to TLR ligands, we measured the concentrations of pro-inflammatory cytokines TNF- α , IL-6 and IFN- α in the culture supernatants of pDC-L cells 24 and 48 hours after activation by TLR7 and 9 ligands using a bead-based flow cytometric assay or ELISA. Increased levels of TNF- α were detected in the supernatants of pDC-L cells treated with TLR7 ligand imiquimod or TLR9 ligand type B CpG, but not with type A CpG (Figure 11, left panel). Co-stimulation with imiquimod and type B CpG resulted in lower levels of secreted TNF- α as compared to exposure to either TLR ligand individually (Figure 11, left panel). A similar pattern of cytokine secretion was observed for IL-6;

however, IL-6 concentrations were similar or higher at 48 hours than at 24 hours after treatment, reflecting the different kinetics of TNF- α and IL-6 cytokine responses (Figure 11, right panel). Although IFN- α secretion is a hallmark of pDC activation, we were unable to detect substantial amounts of this cytokine in the culture supernatants of leukemic cells. In control experiments, normal pDCs secreted high levels of IFN- α after imiquimod or type A CpG stimulation, indicating that the applied TLR ligands are able to induce IFN- α cytokine secretion (Figure 17, a).

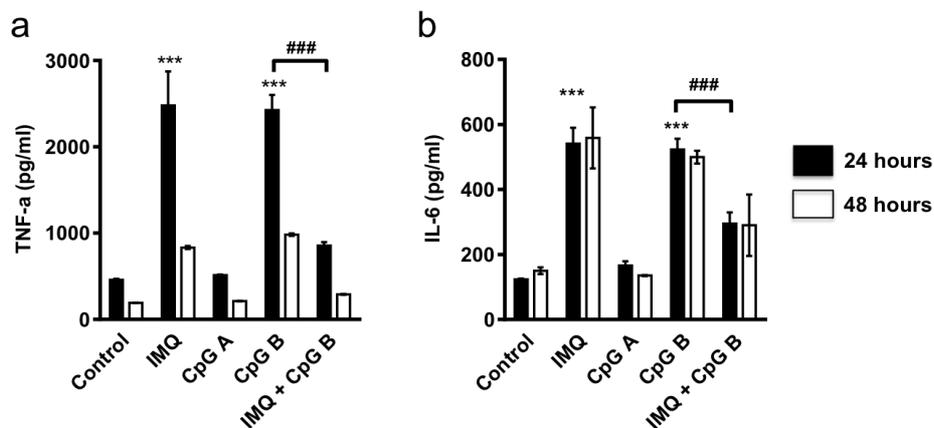


Figure 11. Cytokine production of activated pDC-L cells.

Secreted TNF- α (a) and IL-6 (b) in culture supernatants of pDC-L cells were measured 24 hours (black bars) and 48 hours (white bars) after activation by ELISA. Data are presented as means \pm SD of triplicate measurement within one of two individual experiments. *** $p < 0.001$ versus non-treated control; ### $p < 0.001$ for imiquimod (IMQ) plus CpG B treatment vs. CpG B treatment alone.

T-cell stimulatory potential of activated pDC-L cells

As leukemic pDCs exhibited phenotypic changes resembling those of their normal counterparts, we aimed to examine the T-cell priming capacity of pDC-L cells. To this end, TLR-activated pDC-L cells were co-cultured with naïve allogeneic T-lymphocytes. The number of IFN- γ -producing T-cells was detected by the ELISPOT assay. High allostimulatory capacity of pDC-L cells activated by both type A and type B CpG was observed, whereas exposure to imiquimod had a lower but still significant effect on the T-cell activating potential of pDC-L cells. Interestingly, but in line with our previous findings, the concurrent stimulation of pDC-L cells with the TLR7 ligand imiquimod and TLR9 ligands (both type A and B CpG) abrogated their allostimulatory activity, resulting in significantly lower number of IFN- γ -

producing T-cells (Figure 12). These results demonstrate that the allostimulatory potential of pDC-L cells can be dramatically enhanced by TLR9- and to a lesser extent by TLR7-mediated signals that are translated to potent Th1 type T cell responses. Based on our results this is promoted by increased pro-inflammatory cytokine secretion (Figure 11) and expression of co-stimulatory and antigen presenting MHC class II molecules (Figure 10) induced by TLR stimulation. However, these results also indicate that TLR7- and TLR9-mediated activation of pDCs is not collaborative but rather interfere with each other.

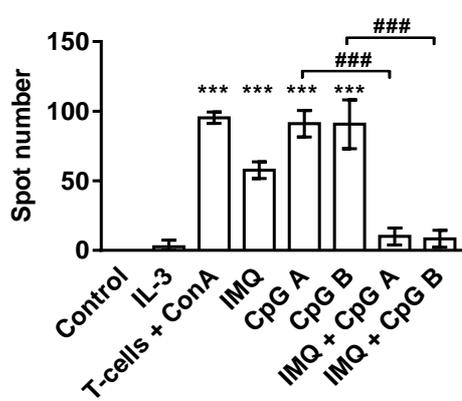


Figure 12. T-cell activating potential of pDC-L cells detected by IFN- γ ELISPOT.

TLR ligand-treated pDC-L cells were co-cultured with allogeneic CD3⁺ T-cells for 4 days. T-cells stimulated with Con A and PHA for 24 - 48 hours were used as positive controls. T-cells incubated alone or co-cultured with IL-3-treated pDC-L cells were used as negative controls. Spot numbers of 2×10^5 cells were detected in the co-cultures of TLR7 and/or TLR9 ligand-activated pDC-L cells and T-cells. Data are presented as means \pm SD of triplicates within one of two individual experiments. *** $P < 0.001$ vs. non-treated control, ### $P < 0.001$ for imiquimod (IMQ) plus CpG A treatment vs. CpG A treatment alone and IMQ plus CpG B treatment vs. CpG B treatment alone.

5.3 TLR ligands up-regulate the expression of RIG-I in pDCs in a type I interferon independent manner

Previous studies showed very low expression levels of RIG-I in murine and human pDCs under steady state conditions and could not demonstrate the activation of RIG-I in response to viral stimulation [72,85]. However, none of them investigated the expression and function of RIG-I following TLR-stimulation of the cells. In order to investigate the effects of exposure to TLR ligands on the expression of RIG-I in pDCs, we isolated primary human pDCs from peripheral blood and treated them with increasing concentrations of the TLR7 ligand imiquimod (from 0.25 to 2.5 $\mu\text{g}/\text{mL}$) and the TLR9 ligands type A or type B CpG (from 0.5 to 5 $\mu\text{g}/\text{mL}$). In accordance with previous reports, the expression of RIG-I was undetectable in untreated primary pDCs at both mRNA and protein levels (Figure 13, a-d). However, both type A CpG

(Figure 13, a and c) and imiquimod (Figure 13, b and d) treatments increased the expression of RIG-I in a dose-dependent manner, while stimulation of the cells with type B CpG did not induce the expression of RIG-I (data not shown). Kinetic measurements performed using 2.5 $\mu\text{g}/\text{mL}$ imiquimod or 5 $\mu\text{g}/\text{mL}$ type A CpG revealed that the expression of RIG-I mRNA could be detected as early as 2 hours after activation by either ligands and peak expression is measured at 6 hours after TLR-stimulation (Figure 13, e and f).

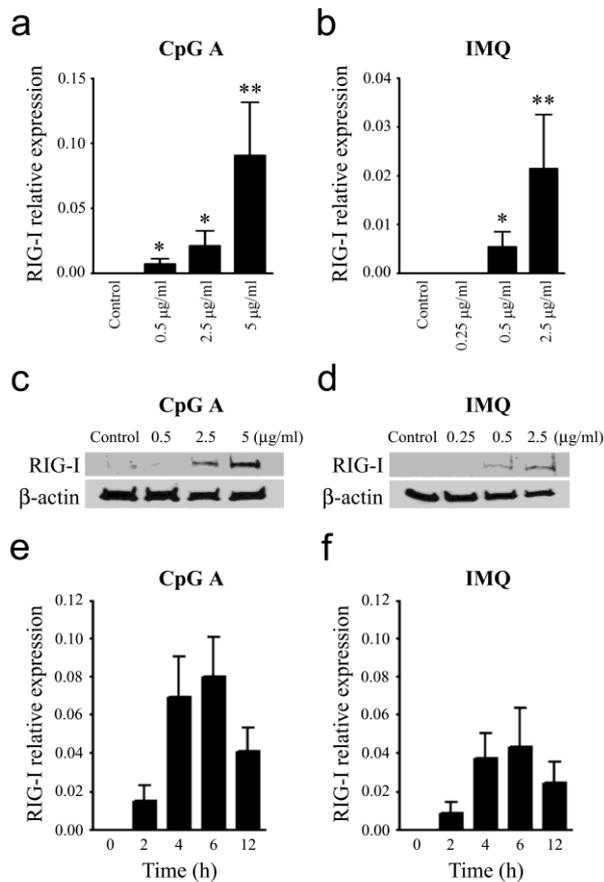


Figure 13. Expression of RIG-I in primary pDCs after stimulation by CpG A and imiquimod (IMQ).

Expression level of RIG-I was measured by Q-PCR (8 hours) (a, b) and western blotting (24 hours) (c, d) after activation of pDCs at the indicated concentrations of type A CpG or IMQ. * $P < 0.05$ and ** $P < 0.01$ versus untreated control. Kinetics of RIG-I expression was analyzed by Q-PCR after treatment of pDCs with 5 $\mu\text{g}/\text{mL}$ type A CpG and 2.5 $\mu\text{g}/\text{mL}$ IMQ (e, f). Gene expression data are presented as means \pm s.d. of triplicates within one representative experiment out of the two independent ones. Relative expression is calculated compared to the expression of 36b4 housekeeping gene.

As our previous results and literature data [245,246] indicated that TLR7 ligands are able to interfere with concurrent TLR9-stimulation, we aimed to analyze the effect of TLR7 and TLR9 co-stimulation on the expression of RIG-I. As observed for the induction of cytokine expression, co-stimulation of primary pDCs with type A CpG and imiquimod resulted in a lower level of RIG-I expression than the activation of cells with either one of the TLR ligands applied individually (Figure 14, a and b). Similarly to primary pDCs, cultured pDC-L cells also showed reduced RIG-I

induction upon co-stimulation with imiquimod and type A CpG as compared to either one of the ligands alone (Figure 14, c).

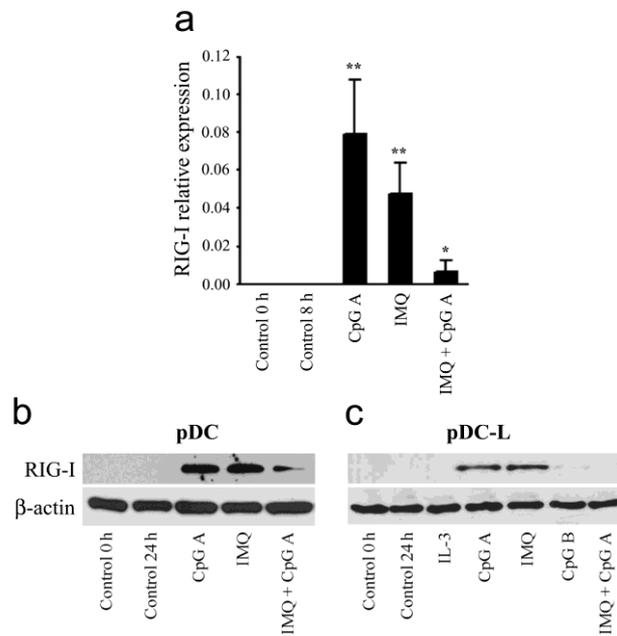


Figure 14. Expression of RIG-I in primary pDCs and in malignant pDC-L cells after co-stimulation with type A CpG and imiquimod (IMQ).

Expression of RIG-I followed by stimulation of primary pDCs with 5 $\mu\text{g}/\text{mL}$ type A CpG, 2.5 $\mu\text{g}/\text{mL}$ of IMQ or the combination of both TLR ligands was determined by Q-PCR (8 hours) (a) or western blotting (24 hours) (b). RIG-I protein expression was also determined in pDC-L cells 24 hours after stimulation (c). As primary pDCs were cultured in the presence of 10 ng/mL recombinant human IL-3 cytokine, some experiments with pDC-L cells were also performed in this medium.

Based on these results, we aimed to verify the potential functional consequence of up-regulation of RIG-I following endosomal TLR-stimulation. To test this hypothesis, blood-derived primary pDCs were treated with 5 $\mu\text{g}/\text{mL}$ type A CpG or 2.5 $\mu\text{g}/\text{mL}$ imiquimod for 24 hours. After removal of the culture supernatants the cells were re-stimulated in fresh medium containing 1 $\mu\text{g}/\text{mL}$ 5'ppp-dsRNA, a highly specific synthetic ligand of RIG-I [80]. As expected due to the low steady-state expression of RIG-I in freshly isolated primary pDCs and in un-stimulated cultured cells (Figure 14, a and b), exposure to 5'ppp-dsRNA without previous TLR-mediated activation did not result in IFN- α or IL-6 secretion (Figure 15, a-d). However, if primary resting pDCs were pre-treated with type A CpG (Figure 15, a and c) or imiquimod (Figure 15, b and d), treatment with 5'ppp-dsRNA was able to induce the production of IFN- α (Figure 15, a and b) and IL-6 (Figure 15, c and d) in a dose-dependent manner. Importantly, only pDCs that had previously been activated with either TLR7 or TLR9 ligands were able to respond to 5'ppp-dsRNA. These results suggest that ligation of endosomal TLRs brings about the ability of pDCs to sense of and respond to cytosolic viral RNA through the RIG-I receptor.

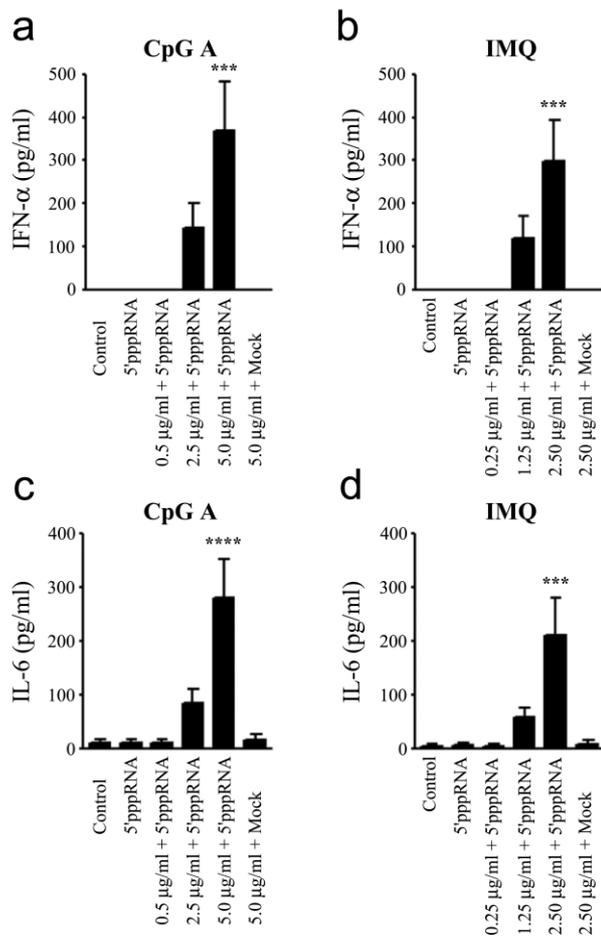


Figure 15. Cytokine secretion of primary pDCs in response to 50-ppp-dsRNA after induction of RIG-I expression with type A CpG or imiquimod (IMQ).

Primary pDCs were treated with the indicated concentrations of type A CpG (panels a, c) or imiquimod (IMQ; panels b, d) for 24 hours and washed twice with fresh medium. The cells were then treated by 5'-ppp-dsRNA (1 µg/mL) using the LyoVec transfection system in fresh medium. Mock treatments were performed with 'LyoVec-only' and LyoVec + control-oligo complexes. The concentration of the secreted IFN-α (a, b) and IL-6 (c, d) cytokines in the culture supernatants, collected after 16 hours (IL-6) or 24 hours (TNF-α) of incubation, was measured by ELISA. Data are presented as means±s.d. of triplicates within one representative experiment out of three independent ones. ***P<0.001 and ****P<0.0001 versus untreated control.

Previous studies reported that TLR7 stimulation results in the expression of early IFN-inducible genes even in the absence of type I IFNs [247]. Based on this observation we next investigated whether the up-regulation of RIG-I in primary pDCs following activation of TLR7 or TLR9 receptors is independent on type I IFN-mediated signals. In this set of experiments, primary pDCs were treated with an antibody recognizing and functionally blocking the IFN-α/β receptor 1 (IFNAR1), shared by IFN-α, -β and -ω, prior to stimulation by type A CpG or imiquimod (in 2.5 µg/mL and 5 µg/mL concentration, respectively). The efficacy of receptor blockage was controlled by measuring the expression of Mx1 and OAS1 genes recognized as early, type I IFN-induced factors [248]. Blocking of IFNAR1 receptors by the addition of 50 µg/mL antibody almost completely prevented Mx1 and OAS1 up-regulation in type I IFN-stimulated primary pDCs (Figure 16, a and b). Importantly, the same treatment did not modify the ability of type A CpG or imiquimod to elevate RIG-I expression levels significantly (Figure 16, c and d). Furthermore, treatment of primary pDCs for 6 hours with various doses (ranging from

10 to 100 ng/mL) of recombinant human IFN- α in control experiments did not lead to RIG-I up-regulation tested at both mRNA and protein levels (data not shown). These data collectively suggest that the up-regulation of RIG-I expression following TLR-ligand treatment is independent of autocrine type I IFN signaling.

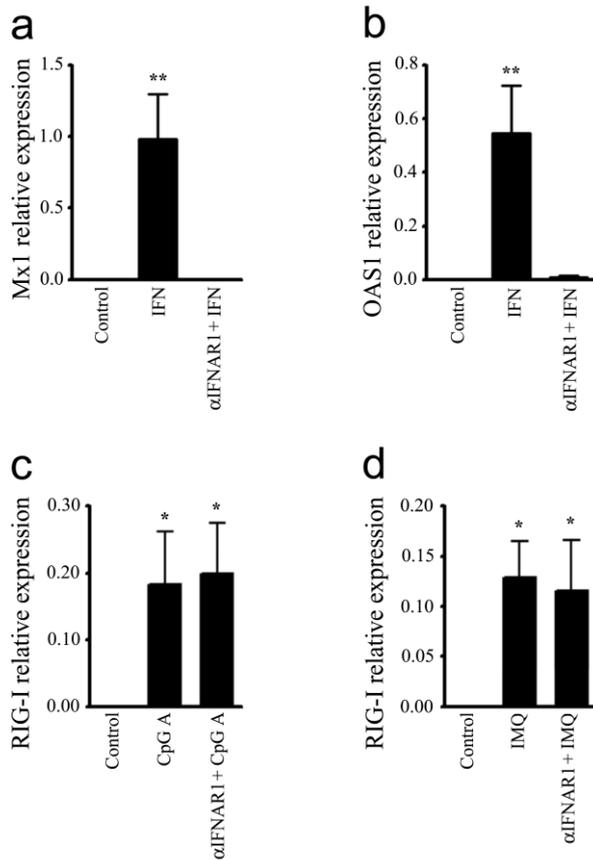


Figure 16. Induction of RIG-I expression in the primary pDCs after type A CpG and imiquimod (IMQ) treatment in the presence or absence of anti-IFNAR1 blocking antibodies.

To confirm the blocking potential of anti-IFNAR1 antibodies, primary pDCs were pre-incubated with or without 50 μ g/mL anti-IFNAR1 monoclonal antibodies for 1 h, washed and stimulated with 10 ng/mL recombinant human IFN- α for 3 hours. Relative expression levels of the early type I IFN-induced factors Mx1 (a) and OAS1 (b) were measured by Q-PCR. To control the dependence of RIG-I up-regulation in primary pDCs induced by type I IFN-mediated signals, the cells were treated with or without 50 μ g/mL anti-IFNAR1 for 1 h before activation by 5 μ g/mL type A CpG (c) or 2.5 μ g/mL imiquimod (d) for 3 hours. Gene expression data are presented as means \pm s.d. of triplicates within one representative experiment out of the two independent ones. Relative expression is calculated compared to the expression of 36b4 housekeeping gene. * P <0.05 and ** P <0.01 versus untreated control.

To further confirm the involvement of a type I IFN-independent mechanism of TLR ligand-induced up-regulation of RIG-I expression in pDCs, we harnessed the defective type I IFN secreting capacity of previously characterized malignant pDC cells isolated from cryopreserved bone marrow samples of a patient diagnosed with pDC leukemia [228]. Our previous control experiments revealed that non-malignant primary pDCs secrete high levels of IFN- α as a result of imiquimod or type A CpG stimulation, but pDC-L cells failed to do so (Figure 17, c). However, these cells displayed similar phenotypic and functional characteristics following TLR-stimulation (Figure 10) as described for non-malignant pDCs indicating that pDC-L cells might have a functional signaling pathway leading to these changes. To confirm that TLR-mediated signaling is unimpaired in pDC-L cells on a molecular level as

well, we measured the expression of IFNA genes after TLR stimulation at the mRNA level. Data from these experiments showed that the expression of IFNA gene transcripts was significantly increased as a result of imiquimod or type A CpG treatment for 6 hours; of note, treatment with type B CpG did not induce remarkable changes in either IFNA-1 or IFNA-2 expression levels (Figure 17, b and c). Furthermore, co-stimulation with imiquimod and type A CpG induced lower expression of IFNA genes than either of these TLR ligands individually.

To further verify the functionality of the IFN- α signaling pathway, we also assessed the expression of the IFN regulatory factor 7 (IRF-7), a master regulator of type I IFN production upon TLR-ligand stimulation at the mRNA level. Our results showed that the expression pattern of IRF-7 was similar to that of IFNA-1 and IFNA-2 genes (Figure 17, d). While administration of imiquimod or type A CpG triggered a remarkable increase in IRF-7 levels, treatment with type B CpG rather had an inhibitory effect on IRF-7 expression. Combined treatment with imiquimod and type A CpG induced lower expression of IRF-7 than either of the ligands individually (Figure 17, d). These results are in line with previous observations on IFN- α production by non-malignant primary pDCs tested after exposure to different TLR ligands [42] and suggest that the TLR induced signaling pathway is functional in pDC-L cells even though the secretion of type I IFN proteins is impaired.

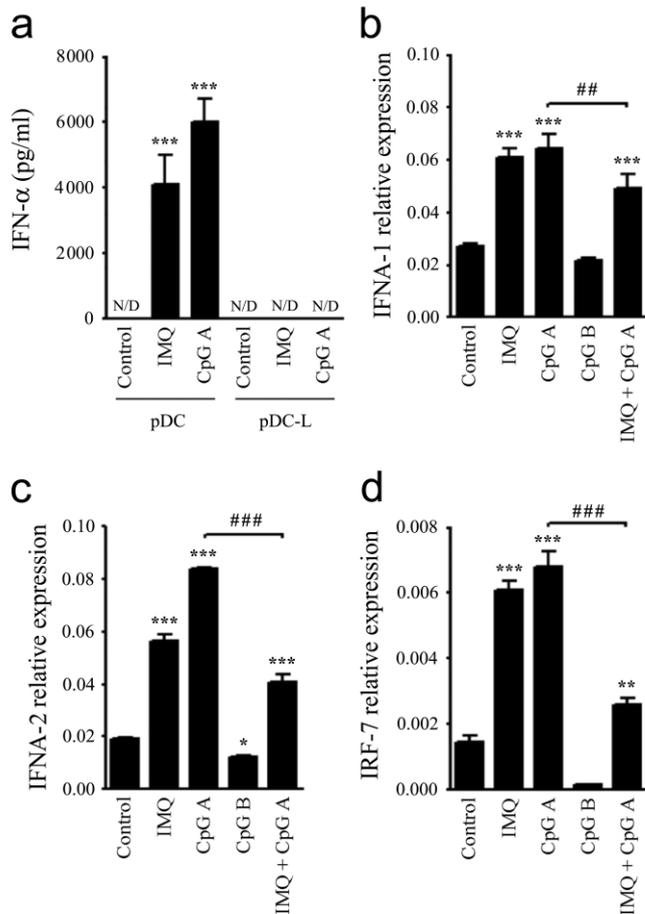


Figure 17. Type I interferon production and RIG-I expression of the activated primary pDCs and malignant pDC-L cells.

The amount of secreted IFN-a (a) in the culture supernatants of primary pDCs and malignant pDC-L cells was measured 24 hours after activation with 2.5 µg/mL imiquimod (IMQ) by cytometric bead array. Relative expressions of IFNA-1 (b), IFNA-2 (c) and IRF-7 (d) genes in pDC-L cells were measured 6 hours after stimulation with the indicated TLR agonists by Q-PCR. TLR agonists were applied at the following concentrations: type A CpG and type B CpG, 5 µg/mL; IMQ, 2.5 µg/mL. Data are presented as means±s.d. of triplicates within one representative experiment out of the two independent ones. *P<0.05, **P<0.01 and ***P<0.001 versus non-treated control, ##P<0.01 and ###P<0.001 for imiquimod plus type A CpG treatment versus type A CpG treatment alone (b–d).

It has been previously demonstrated that in TLR7-activated GEN2.2 cells (a human pDC-derived cell line; see section 2.6) the expression of several “IFN-inducible” genes is independent on the presence of type I IFN, but they remained dependent on p38 mitogen-activated protein kinase (MAPK)-mediated STAT1 phosphorylation on Tyr701 [247]. Beside this phosphorylation site STAT1 can also be phosphorylated at Ser727, and type I IFNs are known to be able to induce STAT1 phosphorylation both on serine and tyrosine residues [249]. To define the possible role of STAT1 in early signaling events leading to RIG-I expression in primary pDCs upon stimulation with endosomal TLR ligands, we examined the phosphorylation of STAT1 at both residues within a 90-minutes time period. Stimulation of primary pDCs for 90 minutes by TLR7 or TLR9 ligands induced STAT1 phosphorylation on Tyr701 but not on Ser727 (Figure 18). This finding raised the possibility that a MAPK-dependent, but IFNAR1-independent STAT1 activation is involved in endosomal TLR-induced up-regulation of RIG-I in human pDCs.

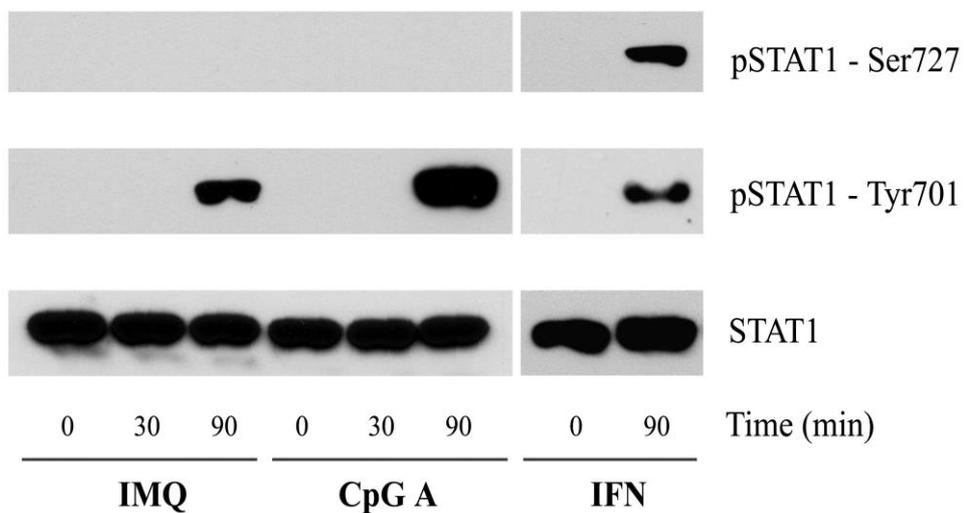


Figure 18. Induction of STAT1 phosphorylation in primary pDCs by CpG A or imiquimod (IMQ) treatment. Time course of STAT1 phosphorylation was detected in primary pDCs after treatment with type A CpG (5 $\mu\text{g}/\text{mL}$), IMQ (2.5 $\mu\text{g}/\text{mL}$) or recombinant human IFN- α (10 ng/mL). Whole-cell lysates were prepared and 20 mg of the cell extract per assay was used to determine STAT1 phosphorylation at the Tyr701 and Ser727 residues and the total STAT1 levels by western blotting. Results from a representative experiment out of the three independent ones are shown.

6 Discussion

Plasmacytoid DCs represent key players of the innate immune system and provide a functional link between adaptive and innate immunity. Because of their special and versatile properties pDCs become a major focus of interest in DC biology. Alterations in pDC counts in peripheral blood, in their migration and function were described in various diseases [42,250,251]. Among them, SLE represents a severe autoimmune disease with relapses associated with the infiltration of pDCs in the kidney lesions [192,193]. Importantly, *Fiore* and her co-workers demonstrated the correlation between migration of pDCs into kidney, decreased pDC counts in the peripheral blood and activity of the disease [192]. Monitoring of pDC counts in peripheral blood of SLE patients might provide a minimally invasive assessment of tissue involvement during various stages of the disease. However, single-marker identification strategies for such analysis were missing so far; previous studies employed complex, multi-marker strategies to identify pDCs in peripheral blood, mostly depending on their positivity for HLA-DR, CD123 and CD4 surface molecules [238,252-256]. Other studies demonstrated that BDCA-2 and BDCA-4 antigens are expressed only by pDCs among peripheral blood cell types [235], raising the possibility of using these antigens as unique markers of pDCs. *Robak* and her co-workers reported a flow cytometric evaluation of dendritic cell subtypes in SLE patients, using BDCA-2 to identify pDCs [241,257]. Although BDCA-2 is the most specific marker of pDCs, it was shown that its expression depends on the activation state of the cells [246]. In SLE, where continuous activation of pDCs occurs, BDCA-2 might not be a reliable independent marker for pDCs in whole blood or PBMC [258].

Based on these considerations, we assessed the potential use of BDCA-4 as sole surface marker to identify pDCs in peripheral blood of human donors. Our results indicate that BDCA-4-positivity confirmed by light scatter properties is suitable for the identification of pDCs in peripheral blood. To confirm the applicability of the one-color method for functional analyses, we treated the blood samples with TLR7 ligand (imiquimod), identified the pDCs with the help of anti-BDCA-4 mAbs and used two other channels to detect the activation state of these cells. Expression levels of HLA-DQ and CD62L molecules on pDCs were investigated in response to stimulation with imiquimod. HLA-DQ membrane proteins are expressed primarily on

professional antigen-presenting cells and their expression is up-regulated after activation of pDCs [259]. CD62L (also referred to as L-selectin) allows pDCs to migrate from the blood to lymph nodes through high endothelial venules. CD62L has been shown to be down-regulated on pDCs as they are activated [112]. To compare the feasibility of the one- and two-color measurements, we also investigated the effects of imiquimod treatment with the two-color method. The two different methods detected higher differences in the expression levels of HLA-DQ than in those of CD62L. This result may be explained by the utilization of the same CD62L-PE antibody in both the one- and two-color identification methods and by usage of different, FITC- and PE-conjugated, HLA-DQ-specific antibodies in the two assays. Results of repeated analysis of blood samples from the same individual indicated that the one-color identification method is highly reproducible and its sensitivity is equal to the two-color method when using the same conjugated antibodies. Conclusively, our data show that BDCA-4-positivity remains a valuable marker for pDCs even after TLR-activation of these cells, suggesting that BDCA-4 is a suitable independent marker of pDCs for screening large numbers of blood samples. Multicolor flow cytometric methods are valuable tools for monitoring disease states where specific changes involve alterations in the number of pDCs or in their phenotype and/or function. These studies need reliable identification of pDCs using the minimal possible number of fluorescent channels. Although flow cytometers used in research laboratories often have more than 4 fluorescent channels, the instruments validated for *in vitro* diagnostic use typically have not more than 3-5 channels. In this respect it is useful that pDCs can be identified in whole blood using only one fluorescent channel while leaving the other channels open for identification of other cell types, phenotyping of pDCs or functional studies. The use of one-color identification of pDCs by anti-BDCA-4 mAbs and light scatter parameters offers a simple method for further phenotyping and functional analyses that are of great importance, because further studies are needed to identify the specific patterns of pDCs in different disease states.

The main difficulty of the pDC research is the rarity of these cells, as they represent a minor population of 0.1–0.6% in peripheral blood mononuclear cells. Another technical limitation of pDC studies is their short-term (24–96 hours) viability *in vitro*. The novel one-color flow cytometric method described here might facilitate

the phenotyping and functional analysis of these cells in fresh blood samples, as the investigators can use all but one fluorescent channels for phenotyping.

Besides *ex vivo* activation of pDCs in mixed cell populations like PBMC or peripheral blood, malignant pDCs might be useful models to study certain aspects of dendritic cell biology. We have isolated leukemic pDCs from bone marrow samples of a patient whose pDC leukemia was previously characterized by *Gopcsa* and his co-workers [228] and performed functional characterization of these cells. Signaling through TLR7 or TLR9 activates pDCs, induces the secretion of pro-inflammatory cytokines such as IL-6 and TNF- α [260] and triggers the production of type I IFNs through a MyD88-dependent pathway that involves the phosphorylation of IRF7 by IRAK-1 and IKK α [261]. To investigate whether pDC-L cells share functional properties with normal pDCs, we analyzed their response to treatment with type A or B CpG, which acts through TLR9, and imiquimod, which targets TLR7. It has been shown that type A CpG induces type I IFN responses, whereas type B CpG predominantly activates pDCs in a manner that results in phenotypic changes and pro-inflammatory cytokine production [262]. The functional activity of type B CpG is attributed to its single stranded monomeric form present in lysosome-associated membrane protein 1-positive endosomes, while type A CpG forms aggregates with longer retention time in early transferrin receptor-positive endosomes [104] thus favoring the prolonged activation of the MyD88-TRAF6-IRAK1-IRF7 complex and the robust production of IFN- α [95]. The TLR7 ligand imiquimod is able to trigger both the production of type I IFN and the activation/maturation program of pDCs [263]. We observed that similarly to circulating normal pDCs, TLR7- and TLR9-mediated stimulations by imiquimod and type B CpG, respectively, but not by type A CpG were able to induce the activation of pDC-L cells as demonstrated by the increased expression of T-cell co-stimulatory CD86 and antigen-presenting HLA-DQ molecules on the cell surface and by the secretion of pro-inflammatory cytokines TNF- α and IL-6. Retention of the TLR signaling complex within early endosomes of pDCs has been shown to correlate with IRF-7 recruitment and the induction of type I IFN production [101]. In line with these results, we demonstrated these events in pDC-L cells, as treatment with imiquimod and type A CpG induced significant increases in the expression of IRF-7, IFNA-1 and IFNA-2 mRNA indicating the functionality of the coupled signaling pathways (Figure 17). However, we failed to detect the secretion of IFN- α in the culture supernatants of TLR-ligand activated

pDC-L cells. Our observation is in agreement with previous data showing the lack of IFN- α secretion by malignant cells isolated from the bone marrow [213]. The finding that bone marrow-derived leukemic pDCs are not competent for type I interferon production even though their signaling machinery is intact suggests that pDC-L cells may acquire this potential in the periphery through additional signals that may contribute to the symptoms of leukemia patients.

We also aimed to assess the impact of dual TLR7 and TLR9 stimulation on pDC-L cells. Concurrent treatment with imiquimod and type B CpG had a moderate effect on CD86 expression and IL-6 production, but remarkably increased the cell surface expression of HLA-DQ as compared to imiquimod treatment alone (Figure 10). Interestingly, in all other experiments where combined treatments were used, TLR7-mediated signals inhibited the TLR9-mediated ones. Recently, similar inhibitory effects of simultaneous TLR7- and TLR9-mediated activation have been observed. *Marshall* and his co-workers found that the capacity of TLR9 ligands to induce potent IFN- α responses is markedly reduced by concurrent TLR7 stimulation; however, the expression of CD80/CD86 and the secretion of IL-6 are not altered by the presence of TLR7 ligands [246]. *Berghofer* and her co-workers demonstrated that while TLR7 signaling dominates the outcome of TLR7/TLR9 co-stimulation and can strongly suppress IFN- α production, it can also promote activation of pDCs via enhanced IL-8 and CD40 expression [245]. The mechanism by which TLR7 ligands achieve their inhibitory effect is still unclear. It seems that they neither mediate their effect through blocking CpG from binding to TLR9, since pre-treated cells retain their responsiveness to CpG even 4 hours after exposure to TLR7 ligands, nor do TLR7 ligands alter the endosomal localization of CpG within the pDC [246]. One possible explanation for the molecular background of the observed inhibitory effect could be the competition of TLR7 and TLR9 receptors for the N-terminal domain of the membrane spanning protein Unc93B1 [264,265] that interacts with the transmembrane domain of these structurally related TLR receptors in the acidified endo-lysosomal compartments [266]. It also cannot be excluded that imiquimod suppresses TLR9-mediated responses in a TLR7-independent molecular mechanism [267,268]. Upon encounter with TLR ligands or viruses, pDCs participate in adaptive immune responses by directing activation of naïve T-cells [113]. We found that similar to normal pDCs, pDC-L cells stimulated with TLR ligands individually

possess potent T-cell priming ability (Figure 12). However, co-stimulation of pDC-L cells with TLR7 and TLR9 ligands significantly reduced their T-cell stimulatory potential. This observation is in line with previous findings showing that simultaneous stimulation with TLR7/8 and TLR9 agonists results in a suppression of B-cell proliferation and IgM responses [269]. *Bagchi* and co-workers found that simultaneous and sequential activation of both the MyD88-dependent and MyD88-independent pathways leads to synergy and priming, respectively, while TLR agonists that act through the same pathway induce tolerance [270]. Based on these data we propose that the co-stimulation of professional antigen presenting cells by closely related TLRs down-regulates responses to prevent overstimulation of the adaptive immune system as demonstrated by the almost complete abrogation of the T cell response.

In conclusion, we demonstrate that despite chromosomal aberrations (del 13q and monosomy 9), the phenotypic and functional characteristics of pDC-L cells activated by TLR7 and TLR9 ligands are identical to those of normal pDC. We also show that despite the lack of their ability to release type I IFNs into the culture medium, pDC-L cells offer a useful tool for analyzing the activity and the inhibitory rather than synergistic collaboration of the vesicular TLR7 and TLR9 receptors, which are both capable of inducing type I interferon responses and activating allogeneic T-cell responses.

Previous studies have shown that primary pDCs, conventional DCs and alveolar macrophages secrete large amounts of type I IFNs and thus are considered as the primary source of type I IFNs during viral infections. Plasmacytoid DCs detect RNA and DNA viruses by two endosomal receptors, TLR7 and TLR9 [41]. Due to the vesicular localization of these TLRs, the recognition of viral nucleic acids occurs when viruses and/or their components are ingested by pDCs and transported into the lysosomes in a TLR transmembrane domain dependent manner or via the process of autophagy [47,271]. In contrast, conventional DCs and alveolar macrophages were shown to detect replicating viral RNA intermediates by cytosolic RLRs [272]. However, the potential involvement of RLRs in viral recognition by pDCs remained poorly understood. Previous studies supported the notion that pDCs primarily employ the TLR-system to recognize viruses; this was supported by the observation that RLRs are expressed to a very low extent in both murine and human pDCs under steady-state conditions [72,84-86]. As the potential interaction between TLR- and

RLR-mediated viral recognition was not evaluated, we aimed to assess the impact of TLR-activation on the expression of RLRs. We demonstrated for the first time that the expression of the RIG-I receptor is dramatically up-regulated in a type I IFN-independent manner in pDCs upon stimulation via TLR7 or TLR9, challenging the current paradigm that RIG-I has no significant function in these cells. We also demonstrate an inhibitory rather than synergistic collaboration of the vesicular TLR7 and TLR9 receptors in the induction of this phenomenon suggesting that co-activation of TLR7 and TLR9 in pDCs does not support uncontrolled RIG-I expression but rather keeps the response under the control of TLR specificity.

Vesicular TLRs sense viruses in a replication-independent manner; by this pathway, pDCs can recognize viruses for which they are not serving as host cells. Furthermore, as material delivery from the endosomal compartment to the cytosol occurs in pDCs to facilitate cross-presentation [273], it could be presumed that viral replication intermediates can come into contact with cytosolic RLRs upon phagocytosis of the debris of infected cells. Our data support the existence of such mechanisms but also delineate a novel mechanism for TLR-RLR collaboration in pDCs. We have found that following activation by TLR7/9 ligands, pDCs are able to sense 5'ppp-dsRNA. These results suggested that TLR7/9-mediated signals are able to prepare pDCs for sensing cytosolic viral nucleic acids by up-regulating RIG-I expression and consequently for boosting antiviral responses. A previous study using respiratory syncytial virus demonstrated that pDCs are in fact capable of detecting ssRNA viruses that enter the cytosol directly and the recognition of replicating viruses leads to IFN- α production independently on endosomal TLRs [274]. In these experiments pDCs responded to cytosolic viral replication without previous TLR-mediated activation, which could be explained by the fact that pDCs produce only marginal level of RIG-I under steady-state conditions, but they are able to express considerable amount of MDA5 at the mRNA level [275]. Another study has demonstrated that MDA5 is indispensable for sustained expression of type I IFN in response to paramyxovirus infection in mice [276]; however, the involvement of MDA5 in sensing respiratory syncytial virus infection by human pDCs remains to be determined.

As type I interferons secreted by pDCs act through autocrine feedback loop, we aimed to test whether TLR7/9-triggered up-regulation of RIG-I expression in pDCs depends on type I IFN-mediated signals. To this end, we blocked IFNAR1 by

specific antibody and, in separate experiments, we used pDC-L cells isolated from bone marrow samples of a former patient with pDC leukemia defective in type I IFN secretion [228]. We have shown for the first time that TLR7- and TLR9-mediated signals are able to up-regulate RIG-I expression in both primary pDCs and pDC-L cells via a type I IFN-independent mechanism. This finding was unexpected, because RIG-I was considered as one of the IFN-inducible genes in several human cell types [277]. Interestingly, MxA, CXCL10 and TRAIL, all involved in the inhibition of virus replication and described to be tightly regulated by IFNs, were shown to be rapidly expressed in TLR7-stimulated pDCs in the absence of type I IFNs. The existence of a novel pathway downstream of TLR7 ligation and involving MAPK-mediated early STAT1 phosphorylation on Tyr701 has been identified behind this phenomenon [247]. In an earlier study, 2-hour treatment of human pDCs with CpG DNA induced MAPK-dependent phosphorylation of STAT1 on both Tyr701 and Ser727 in a type I IFN independent manner. In control experiments, stimulation of cells with type I IFN also led to STAT1 phosphorylation on both residues; however, it was not influenced by the MAPK pathway [249]. In our experiments STAT1 phosphorylation could be detected exclusively on the tyrosine residue when tested 90 minutes after TLR7/9 ligation. This observation is in good agreement with a recent report suggesting that the phosphorylation of this tyrosine by an intermediate MAPK-dependent tyrosine kinase precedes the direct serine phosphorylation in STAT1 by MAPK [247]. However, the tyrosine kinase responsible for partial phosphorylation of STAT1 remains to be identified.

In all experiments where combined treatments were used, we observed antagonistic effects of TLR7- and TLR9-mediated signals. Similar inhibitory effects of simultaneous TLR7- and TLR9-mediated activation have been observed by *Marshall* and co-workers, who have found that the capacity of TLR9 ligands to induce potent IFN- α responses is markedly reduced by concurrent TLR7 stimulation [246]. *Berghofer* and her co-workers also demonstrated a strong inhibitory effect of TLR7 stimulation on IFN- α production following CpG-A- and CpG-C-oligodeoxynucleotide treatment of pDCs [245]. The molecular background of these antagonistic effects might be explained by the competition of TLR7 and TLR9 receptors for the N-terminal domain of the membrane spanning protein Unc93B1 [264,265] that is known to interact with the transmembrane domain of these

structurally related TLR receptors in the acidified endo-lysosomal compartments [266]. It is also possible that imiquimod antagonizes TLR9-mediated responses in a TLR7-independent molecular mechanism [267]. Our results show that upon recognition of viral replication intermediates, early type I IFN production depends on TLR-mediated signals, whereas the second wave of type I IFN responses is guided by RLR signaling. These results however, raise the question of which biological situations would need pre-stimulation by strong type I IFN inducers (endosomal TLR ligand) to establish subsequent responsiveness to a late and weak IFN inducer (cytosolic dsRNA). In this context, the TLR-driven acute but transient activation of pDCs resulting in direct release of huge amount of IFNs into the lymph and to blood circulation [278] should be considered in contrast to pathological conditions, including viral infections going on in non-lymphoid tissues [279]. We suggest that at the site of infection moderate RIG-I-mediated production of IFNs by recruited pDCs may be sufficient for supporting potent antiviral responses, while unraveling the significance of this spatiotemporal regulation requires further studies.

7 Summary

Innate immunity is a conserved host defense mechanism and its key functions of are 1) to differentiate between harmful and non-harmful materials; 2) to initiate the first line of immune response; and 3) to recruit and prime the effector cells of the adaptive immune system. Dendritic cells (DCs) are key participants of these processes as part of the network of professional antigen presenting cells. More than a decade after the discovery of myeloid DCs, researchers identified the plasmacytoid DCs (pDCs), a small subset of DCs. Due to the rarity and poor *ex vivo* culturability of pDCs, various model systems had been considered, including study of pDCs in mixed populations (e.g. PBMC or whole blood) and the use of malignant pDCs or pDC-derived cell lines. Similarly, the lack of specific and suitable cell surface antigens complicated the identification of pDCs in whole blood.

In our work, we tested single-color flow cytometric identification of pDCs in whole peripheral blood based on positivity with Blood Dendritic Cell Antigen-4 (BDCA-4) as compared to two-color identification methods. We found that BDCA-4 is a suitable and specific antigen for single-color identification of pDCs in freshly drawn peripheral blood and its expression is not affected by treatment of pDCs with TLR7 agonist imiquimod, suggesting that BDCA4 alone is suitable to monitor changes in pDC counts in patients. It was described that organ involvement, specifically, the inflammatory infiltration of the kidneys correlate well with the decreased pDC counts in human systemic lupus erythematosus (SLE) as these cell migrate to lupus lesions and disappear from the circulation. Therefore, the analysis of pDC counts in peripheral blood offers a minimally invasive yet pathogenically relevant marker of kidney involvement and disease status is SLE.

Plasmacytoid DCs have a prominent role in antiviral immunity as professional type I interferon-producing cells, recognizing viruses in their endosomal compartment by Toll-like receptors. In contrast to recognition of viral replication intermediates in the cytoplasm of other cells by RIG-I-like helicase molecules (RLHs), this mechanism does not depend on viral replication and effectively detects non-replicating viruses. Multiple studies suggest that pDCs exclusively employ TLR-mediated viral recognition under steady-state conditions; however, the potential collaboration of TLRs and RLHs was not investigated. Therefore, we aimed to assess

the potential expression and function of RIG-I in pDCs following TLR-activation. We could demonstrate that:

- 1) PDCs up-regulate RIG-I upon treatment with TLR7 and 9 ligands;
- 2) The up-regulation of RIG-I is independent of type I IFN autocrine feedback regulation;
- 3) Co-stimulation with a TLR7 and TLR9 ligand showed inhibitory rather than synergistic effect on the up-regulation of RIG-I in primary pDCs.

Our results suggest that Toll-like receptors and cytoplasmic nucleic acid sensors might act in co-operation during viral infection. The detection of non-replicating viral particles by endosomal TLRs might sensitize and prepare pDCs for the appearance of viral replication intermediates in the cytoplasm. This concept represents a novel synergy between various innate immune recognition pathways.

Összefoglalás

A veleszületett immunitás egy ősi és konzervált védelmi mechanizmus, melynek fő funkciói 1) a veszélyes és veszélytelen anyagok megkülönböztetése; 2) az immunvédekezés első vonalának elindítása; és 3) az adaptív immunrendszer aktiválása. Ezen folyamatok kulcsfontosságú résztvevői a hivatásos antigénprezentáló sejtek közé tartozó dendritikus sejtek (DS-ek). Több mint egy évtizeddel a myeloid eredetű DS-ek felfedezését követően azonosították a kutatók a DS-ek alpopulációját képező plazmacitoid DS-eket (pDS-ek). A pDS-ek ritkasága és korlátozott *ex vivo* életképessége miatt különféle modellrendszereket alkalmaztak tanulmányozásukra; mint például vegyes sejtpopulációkon (például PBMC vagy teljes vér) vagy pDS eredetű tumorokon és sejtvonalakon történő vizsgálatok. A megfelelő pDS-specifikus sejt felszíni antigének hiánya megnehezítette a pDS-ek azonosítását a perifériás vérben.

Munkánk során a pDS-ek egyszínű, Blood Dendritic Cell Antigen-4 (BDCA-4) pozitivitáson alapuló azonosítását hasonlítottuk össze a korábban használt, két antigén jelölésen alapuló áramlási citometriás módszerekkel. Eredményeink szerint a BDCA-4 megfelelően specifikus antigén a pDS-ek perifériás vérben történő azonosításához, és kifejeződése Toll-szerű receptor (TLR) 7 agonista (imiquimod) kezelést követően sem változik, így önmagában is alkalmas a keringő pDS-ek számának meghatározására betegektől származó vérmintákban. Irodalmi eredmények szerint a szisztémás lupus erythematosus-ban (SLE) szenvedő betegekben a vesék gyulladásos sejtes beszűrődésével szorosan együtt jár a pDS-ek számának csökkenése a perifériás vérben. A pDS-ek számának követése egyszerű áramlási citometriás módszerekkel így minimálisan invazív módszert jelenthet a veseérintettség és a betegség státuszának megítéléséhez SLE-ben.

A plazmacitoid DS-ek hivatásos I-es típusú interferont termelő sejtekként kitüntetett szereppel bírnak az vírusellenes immunitásban. Más sejttípusokkal szemben a pDS-ek a vírusokat az endoplazmás retikulumban, TLR-ek segítségével ismerik fel. A vírusreplikáció közti termékeinek a citoplazmában történő RIG-I-szerű helikázok általi felismeréssel szemben, ez a mechanizmus nem replikálódó vírusok azonosítására is képes. Korábbi tanulmányok szerint a pDS-ek csak a TLR-eket fejezik ki nyugalmi állapotban, és ezen munkák egyike sem vizsgálta a RIG-I-szerű helikázok és a TLR-ek potenciális együttműködését. Vizsgálataink második részében

ezért arra kerestük a választ, hogy a TLR-ek aktivációja milyen hatással bír a RIG-I kifejeződésére és funkciójára. Vizsgálataink során igazoltuk, hogy:

1) A pDS-ek RIG-I receptort fejeznek ki TLR 7 és 9 specifikus ligandumaival történő aktiválást követően;

2) A RIG-I kifejeződése nem függ az I-es típusú interferonok autokrin visszacsatolásától;

3) A TLR 7 és 9 ligandumainak egyidejű alkalmazása nem szinergisztikus, hanem gátló hatású a RIG-I kifejeződésére a pDS-ekben.

Eredményeink alapján valószínűsíthető, hogy a TLR-ek és a citoplazmatikus nukleinsav receptorok összehangoltan működnek a vírusfertőzések során. A nem replikálódó vírusok TLR-eken keresztül történő felismerése a citoplazmatikus nukleinsav receptorok kifejeződését indukálva érzékenyítheti a pDS-eket vírusok replikációs termékeinek várható megjelenésére. Ez a mechanizmus eddig ismeretlen együttműködést tár fel a veleszületett immunitás különböző mintázatfelismerő rendszerei között.

8 References

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8.2 Publication list prepared by the Kenézy Life Sciences Library



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PUBLICATIONS



Register number: DEENK/15/2015. PL
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Subject: Ph.D. List of Publications

Candidate: Zoltán Magyarics
Neptun ID: D742NU
Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

- * 1. Szabó, A., **Magyarics, Z.**, Pázmándi, K., Gopcsa, L., Rajnavölgyi, É., Bácsi, A.: TLR ligands upregulate RIG-I expression in human plasmacytoid dendritic cells in a type I IFN-independent manner.
Immunol. Cell Biol. 92 (8), 671-678, 2014.
DOI: <http://dx.doi.org/10.1038/icb.2014.38>
IF:4.205 (2013)
2. **Magyarics, Z.**, Csillag, A., Pázmándi, K., Rajnavölgyi, É., Bácsi, A.: Identification of plasmacytoid pre-dendritic cells by one-color flow cytometry for phenotype screening.
Cytometry A. 73 (3), 254-258, 2008.
DOI: <http://dx.doi.org/10.1002/cyto.a.20529>
IF:3.259



* Szabó, A. and Magyarics, Z. contributed equally to this work.

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List of other publications

3. Rouha, H., Badarau, A., Visram, Z.C., Battles, M.B., Prinz, B., **Magyarics, Z.**, Nagy, G., Mirkina, I., Stulik, L., Zerbs, M., Jägerhofer, M., Maierhofer, B., Teubenbacher, A., Dolezilova, I., Gross, K., Banerjee, S., Zauner, G., Malafa, S., Zmajkovic, J., Maier, S., Mabry, R., Krauland, E., Wittrup, K.D., Gerngross, T.U., Nagy, E.: Five birds, one stone: Neutralization of α -hemolysin and 4 bi-component leukocidins of *Staphylococcus aureus* with a single human monoclonal antibody.
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5. Pázmándi, K., **Magyarics, Z.**, Boldogh, I., Csillag, A., Rajnavölgyi, É., Bácsi, A.: Modulatory effects of low-dose hydrogen peroxide on the function of human plasmacytoid dendritic cells.
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IF:5.271
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9. Jiao, H., Tóth, B., Erdős, M., Fransson, I., Rákóczi, É., Balogh, I., **Magyarics, Z.**, Dérfalvy, B., Csorba, G., Szaflarska, A., Megarbane, A., Akatcherian, C., Dbaibo, G., Rajnavölgyi, É., Hammarström, L., Kere, J., Lefranc, G., Maródi, L.: Novel and recurrent STAT3 mutations in hyper-IgE syndrome patients from different ethnic groups.
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11. **Magyarics, Z.**, Rajnavölgyi, É.: Professional type I interferon-producing cells: A unique subpopulation of dendritic cells.
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DOI: <http://dx.doi.org/10.1556/AMicr.52.2005.3-4.14>

Total IF of journals (all publications): 35,932

Total IF of journals (publications related to the dissertation): 7,464

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

22 January, 2015

9 Keywords

Plasmacytoid dendritic cell, plasmacytoid dendritic cell leukemia, Toll-like receptor, RIG-I-like receptor, signaling, innate immunity, flow cytometry

Tárgyszavak

Plazmacitoid dendritikus sejt, plazmacitoid dendritikus sejt leukémia, Toll-szerű receptor, RIG-I-szerű receptor, jelátvitel, veleszületett immunitás, áramlási citometria

10 Acknowledgments

I would like to thank my supervisor Dr. Attila Bácsi for his continuous support, excellent mentorship. I am thankful for Attila for teaching me the scientific way on thinking and asking the right questions as well as planning the right experiment to answer those.

I am thankful for Prof. Éva Rajnavölgyi for her continuous support and inspiration during my entire Ph.D. period.

I am indebted to Dr. Árpád Lányi, Dr. Kata Kis-Tóth and Dr. Szilvia Benkő, for the scientific discussions and their valuable suggestions as well as for the technical help during my years at the Department of Immunology. Special thanks for Anikó Csillag, Attila Szabó and for my student, Kitti Pázmándi for the technical discussions and nice moments within the lab. Importantly, I am thankful for Dr. Péter “Gogy” Gogolák for being an excellent teacher in flow cytometry and guiding me through my learning curves with cell sorting.

I would like to thank the whole staff of the Department of Immunology (University of Debrecen, MHSC) for the supporting and friendly environment.

And last, but not least I am sincerely grateful to my family, especially my wife Kata and to my kids helping me get through the difficult times, and for their emotional support and encouragement.