Human plasmacytoid dendritic cells: from identification to specific antiviral function

by

Zoltán Magyarićs, MD

Supervisor: Attila Bácsi, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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Head of the **Examination Committee:** Prof. Gábor Szabó, MD, PhD, DSc
Members of the Examination Committee: Imre Kacskovics, DVM, PhD, DSc
József Kónya, MD, PhD

The examination takes place at Room 2.209, Life Science Building, Department of Immunology, Faculty of Medicine, University of Debrecen, at 11:00 a.m. on 1st July, 2015.

Head of the **Defense Committee:** Prof. Gábor Szabó, MD, PhD, DSc
Reviewers: Prof. Andrea Szegedi, MD, PhD, DSc
György Nagy, MD, PhD

Members of the Defense Committee: Imre Kacskovics, DVM, PhD, DSc
József Kónya, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1:00 p.m. on 1st July, 2015.
1. Introduction

1.1. Main properties of plasmacytoid dendritic cells

Dendritic cells 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. 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 plasmacytoid DCs (pDCs). 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. The phenotype of human immature pDCs is CD4+CD45RA+HLA-DR+CD123highILT3+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). Murine pDCs were identified soon after the discovery of their human counterparts, and characterized by their B220+Ly49Q+CCR9− phenotype.

Before reaching consensus on the name and origin of pDCs, multiple names were used for this enigmatic cell type, including the terms ‘T-associated plasma cells’, or ‘plasmacytoid T-cells’, and ‘plasmacytoid monocytes’ and pDCs were later identified to be identical to previously described professional type I interferon-producing cells (IPCs). Similary to their naming, the developmental origin of pDCs remained a debated topic for a decade. The current view proposes a flexible development pathway for dendritic cells, where pDCs might originate from a variety of precursors including common myeloid and common lymphoid precursors as well.
1.2. 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, constitutively expressed pattern-recognition receptors (PRRs). Similar microbial structures might be expressed on pathogenic and non-pathogenic microbes, and the differentiation between harmful microbial patterns and those that should be tolerated by the immune system is controlled by ‘danger signals’, e.g. tissue damage induced by invasive bacteria or viruses. Importantly, certain PRRs also sense host-derived structures that become available during tissue damage or non-controlled cell death as opposed to apoptosis.

Unlike other cell types, professional antigen presenting cells, such as DCs, B-cells and macrophages, display a broad repertoire of PRRs to detect pathogen-derived molecules in their environment. An important group of PRRs is the family of Toll-like receptors (TLRs), localized to the cell surface or the membrane of intracellular compartments. Thirteen members of the TLR family have been identified so far, and ten of them are found in humans. TLRs are type I trans-membrane glycoproteins consisting of a cytoplasmic signaling domain called the Toll/IL-1 receptor domain and different extracellular domains. The activation of TLRs results in the expression of cytokines, chemokines and co-stimulatory molecules essential for the coordination of innate immune responses and shapes the adaptive responses as well. Plasmacytoid DCs express a characteristic combination of TLRs, namely TLR7 and TLR9, tailored to recognize RNA and DNA viruses. TLR7 recognizes viral single stranded RNA (ssRNA), but several synthetic compounds such as loxoribine, resiquimod (R848) and imiquimod (R837) also bind to this receptor. TLR9 recognizes viral double-stranded (ds) DNA genomes rich in unmethylated CpG sequences; these hypo- or un-methylated DNA-sequences are normally absent from eukaryotic genomic DNA. Synthetic CpG oligodeoxyribonucleotides (CpG ODN) are also recognized by this receptor. Plasmacytoid DCs produce over 1000-fold more type I interferons (IFNs) (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, TRAF6 and the IKK complex, and the common endpoint of TLR7 and TLR9 activation is induction of interferon regulatory factor (IRF) 7 activation.

In addition to TLRs, NACHT-LRRs, Nod-like receptors (NLRs), retinoic acid induced gene (RIG)-like helicases (RLH) also belong to the group of intracellular PRRs. 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. Conformational changes induced by specific helicase ligands expose the CARD signaling domain of RIG-I or MDA5, the activation of signaling pathway leads to the phosphorylation of transcription factors IRF3 and IRF7, resulting in the production of type I IFNs. 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. 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) was also identified as a highly selective ligand for RIG-I. This RNA species is either a genome constituent or the product of transcription in most RNA viruses.

The expression and functional role of RLHs in pDCs remained poorly understood. Initial studies involving murine cells suggested that pDCs primarily employ the TLR-system to recognize viruses and do not depend on RLHs. In primary human pDCs, marginal levels of RIG-I and very low amount of MDA5 were detected under steady state conditions and, treatment with RIG-I ligand 5’ triphosphate RNA did not result in type I IFN response. Importantly, 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.
1.3. Plasmacytoid dendritic cell leukemia/lymphoma and pDC-derived cell lines

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. This entity was initially described as histiocytic lymphoma / histiocytic associated hematologic malignancy or as agranular CD4⁺CD56⁺ cutaneous lymphoma/hematodermia. Based on their CD56 expression, they were also related to NK-cells and referred to as blastic/blastoid NK-cell leukemia or NK-lymphoma/leukemia. Although multiple studies speculated on possible link between pDCs and the CD4⁺CD56⁺ malignancies, Chaperot et al. could first verify the pDC-Like phenotype of CD4⁺CD56⁺ positive leukemia cells. Based on the data collected by the French leukemia workgroup (GEIL), the new name ‘early pDC leukemia/lymphoma’ was proposed for the pDC-derived lymphoma/leukemia cases. The WHO/European Organization for Research and Treatment of Cancer (EORTC) classification for cutaneous lymphomas finally designated this entity as CD4⁺CD56⁺ hematodermic neoplasm or ‘early’ plasmacytoid dendritic cell leukemia/lymphoma. The incidence of pDC-derived malignancies is estimated to be <1% of acute lymphoma cases.

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), an extensive immunophenotypic analysis is needed for a definitive diagnosis. A ‘typical’ pDC-L profile has previously been reported as follows: CD4⁺CD56⁺ lineage-CD45RA⁺/RO⁻ CD11c⁻ CD116⁻/low CD123⁺ CD34⁻ CD36⁺ HLA-DR⁺. 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.

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. Although a minority of pDC-L patients lacks cutaneous lesions at diagnosis, skin lesions develop quickly during the course of disease. 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. 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.

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 $\text{CD}^+\text{CD}56^+$ lineage- pDC-L cells were characteristically positive for $\text{CD}36$, $\text{CD}38$, $\text{CD}40$, $\text{CD}45$, $\text{CD}45\text{RA}$, $\text{CD}68$, $\text{CD}123$, $\text{CD}184$, $\text{HLA-DR}$, $\text{BDCA2}$ and granzyme-B. 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 derivates are available: CAL-1 and GEN2.2. 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.
1.4. Methods to study plasmacytoid dendritic cells in mixed cell populations

The scarcity of pDCs has traditionally been a barrier for researchers, and this issue is 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. A potential cell surface marker for selection is IL-3 receptor α chain (CD123); however, this antigen does not have the specificity needed to isolate this cell type from certain mixed lymphocyte populations, especially not from lymph nodes or tonsils. To date, two potentially DC-specific cell surface antigens, blood dendritic cell antigen (BDCA) 2 and 4, later classified as CD303 and CD304 (also known as neuropilin-1) were described. 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. $1 \times 10^6$ 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, including characterization of pDCs with flow cytometry in PBMC or evaluating functional properties of pDCs in peripheral blood leukocytes. The development of a 6-color panel for flow cytometric analysis of ex vivo activated dendritic cell subsets in whole blood was also reported. This method was optimized for the most reliable identification of pDCs, but complexity of the staining panel is beyond the limits of a typical flow cytometer used in the clinical diagnostic environment. Implementation of BDCA antibodies for DC identification potentially allows capturing DCs by one or two channels and the screening of large number of samples with a simpler staining and analyzing method. Our studies were focused on the validation of BDCA4 as a potential antigen for single-color identification of pDCs in peripheral blood samples.
2. Aims of the studies

- 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.
3. Materials and methods

3.1. 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 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, blood sample was dispensed to each of the 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 1X FACS Lysing Solution (BD Biosciences) containing paraformaldehyde as fixative. Following lysis of RBCs, the samples were centrifuged, washed with PBS containing 0.5% bovine serum albumin and 0.05% sodium azide, and re-suspended in 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:

- Anti-HLA-DR (MHC Class II antigen presenting molecule)-FITC (clone G46-6) and anti-CD123 (IL3-receptor)-PECy5 (clone 9F5) mAbs (both from BD Biosciences) for two-color identification of pDCs.
- Anti-BDCA-4 (neuropilin-1)-APC (clone AD5-17F6, Miltenyi Biotec, Bergisch Gladbach, Germany) monoclonal antibody for single color identification of pDCs.
- CD4-FITC (clone RPA-T4) and CD123-PE (Clone 9F5) mAbs (both from BD Biosciences) for further confirmation of one-color identification of pDCs.
- Single-color labelled cells for compensation setup were stained with anti-CD3-FITC (clone UCHT1, BD Biosciences), anti-CD3-PE (clone UCHT1, BeckmanCoulter, Hialeah, FL) or 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, \(5–8 \times 10^5\) events were collected from every sample corresponding to 500–1,300 pDCs per sample; data acquisition was terminated on time, \(t=300\) seconds. List mode data files were analysed using FlowJo software (TreeStar).

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

To activate pDCs, aliquots of the fresh blood samples were treated with TLR-7 ligand imiquimod (R837; Invivogen, San Diego, CA). After addition of TLR7 ligand, the cells were incubated at 37\(^\circ\)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 anti-HLA-DQ (MHC Class II antigen presenting molecule)-PE (clone HLADQ1) and 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 anti-HLA-DQ-FITC (clone Tü169) together with anti-CD62L-PE mAbs (both from BD Biosciences).

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

3.3. 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 ethical principles defined in the Helsinki Declaration developed by the World Medical
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). 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 L-glutamine, Na-pyruvate (both from Sigma-Aldrich, St. Louis, MO, USA), penicillin/streptomycin, 10% fetal calf serum (all from Invitrogen) and 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. The Ethical Committee of the National Medical Center, Institute of Hematology and Immunology, Budapest, Hungary approved the study. For cell separation, bone marrow cells were incubated with anti-CD123-PE Cy5 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 for viability assessment. Before cell sorting, the frequency of pDC-L cells was on average 61.5±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, supplemented as described for primary pDCs except the addition of IL-3.

3.4. 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 imiquimod (InvivoGen), 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 anti-HLA-DQ-PE (Clone No. HLADQ1)
or anti-CD86/B7-2-PE (Clone No. IT2.2) mAbs (both from BD Biosciences). 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 the same buffer, and analyzed on a FACSCalibur flow cytometer. Compensation setup and offline data analysis was performed as described above.

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).

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 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 (10^4-10^5 cells/well) were incubated with T-cells (10^6 cells/well) in RPMI-1640 for 4 days at 37°C in a humidified atmosphere containing 5% CO₂. T-cells activated by phytohemagglutinin and concanavalin A (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 × 10^5 cells/well) were incubated in complete medium in ELISPOT plates under the same conditions for 24 hours. Plates were washed three times using coating buffer and 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; incubation for 45 minutes at room temperature) and freshly prepared AEC Substrate Solution (NatuTec; incubation for 30 to 60 minutes at room temperature). The substrate reaction was stopped by three washing steps with distilled water. Plates were dried and analyzed by an ImmunoScan plate reader (CTL Ltd., Shaker Heights, OH, USA).
3.5. 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) and type A (CpG 2216) or type B (CpG 2006) CpG (both from Hycult Biotechnology) 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 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 hours (IL-6) or 24 hours (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. Briefly, 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. The assay IDs were as follows: RIG-I: Hs00184937_m1, IRF-7: Hs00185375_m1, IFNA-1: Hs00855471_g1, IFNA-2: Hs00999940_s1. Q-PCR reactions were carried out in triplicates 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).
To measure the expression of RIG-I on protein level, protein extraction was performed by lysing the cells in lysis/loading buffer. 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 anti-IFNAR1 monoclonal antibody (Abcam, Cambridge, UK) for 1 hour prior to activation with 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.

3.6. 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.
4. Results

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

To set up our standard method 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 analysis gates were set up to exclude remaining erythrocytes and cell debris and to identify pDCs as being HLA-DR-FITC and CD123-PECy5 positive mononuclear cells. 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. In the exploratory single-channel identification method, blood samples were stained with BDCA-4-APC, CD4-FITC, and CD123-PE antibodies and pDCs were first gated on BDCA-4-APC positivity. In a separate analysis, the identity of the resulting cell population was confirmed by its positivity for both CD4-FITC and CD123-PE, which is a unique phenotype for pDCs among cells of the peripheral blood of healthy adults. 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.

Our results indicated that in freshly analyzed peripheral blood, BDCA-4-positivity 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, 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 μg/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. 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 \textit{in vitro} culturing for 24 hours does not compromise the reliability of the single-color identification method.

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 µM (2.5 µg/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.

4.2. 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±3%, as determined in three independent experiments. After sorting, more than 95% of the cells displayed the pDC phenotype and the viability measured by 7-amino-actinomycin D staining ranged from 87% to 93%.
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. 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. 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, 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.

4.3. 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. 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. 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. 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.
4.4. **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. 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 and expression of co-stimulatory and antigen presenting MHC class II molecules 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.

4.5. **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. 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 µg/mL) and the TLR9 ligands type A or type B CpG (from 0.5 to 5 µg/mL). In accordance with previous reports, the expression of RIG-I was undetectable in untreated primary pDCs at both mRNA and protein levels. However, both type A CpG and imiquimod 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. Kinetic measurements performed using 2.5 µg/mL imiquimod or 5 µg/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. As observed for the induction of cytokine expression, co-stimulation of either primary pDCs or pDC-L cells with type A CpG and imiquimod resulted in a lower level of RIG-I expression compared to treatment with either of the ligands alone.

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 µg/mL type A CpG or 2.5 µg/mL imiquimod for 24 hours. After removal of the culture supernatants the cells were re-stimulated in fresh medium containing 1 µg/mL 5’ppp-dsRNA, a highly specific synthetic ligand of RIG-I. As expected due to the low steady-state expression of RIG-I in freshly isolated primary pDCs and in un-stimulated cultured cells, exposure to 5’ppp-dsRNA without previous TLR-mediated activation did not result in IFN-α or IL-6 secretion. However, if primary resting pDCs were pre-treated with type A CpG or imiquimod, treatment with 5’ppp-dsRNA was able to induce the production of IFN and IL-6 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.

Next, we 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. Primary pDCs were treated with an antibody recognizing and functionally blocking the IFN-alpha/beta receptor 1 (IFNAR1), 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. Blocking of IFNAR1 receptors almost completely prevented Mx1 and OAS1 up-regulation in type I IFN-stimulated primary pDCs. Importantly, the same treatment did not modify the ability of type A CpG or imiquimod to elevate RIG-I expression levels significantly. These data collectively suggest that the up-regulation of RIG-I expression following TLR-ligand treatment is independent of autocrine type I IFN signaling.
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 pDC-L cells. 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. 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.

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. 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. These results are in line with previous observations on IFN-α production by non-malignant primary pDCs tested after exposure to different TLR ligands 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.

It has been previously demonstrated that in TLR7-activated GEN2.2 cells (a human pDC-derived cell line), 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. 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. 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. Stimulation of primary pDCs for 90 minutes by TLR7 or TLR9 ligands induced STAT1 phosphorylation on Tyr701 but not on Ser727. 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.
5. Discussion

Alterations in pDC counts in peripheral blood, in their migration and function were described in various diseases. As a prominent example, relapses in SLE are associated with the infiltration of pDCs in the kidney lesions and correlation with decreased pDC counts in the peripheral blood and activity of the disease was demonstrated. Therefore, 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. Previous studies employed complex, multi-marker (usually HLA-DR, CD123 and CD4) strategies to identify pDCs in peripheral blood. These techniques are non-ideal, as the flow cytometers validated for in vitro diagnostic use typically have not more than 3-5 channels, leaving no space for phenotyping besides identification of pDCs. BDCA-2 and BDCA-4 antigens are expressed only by pDCs among peripheral blood cell types, thus might serve as unique markers of pDCs. However, the expression of BDCA-2 depends on the activation state of the cells, making it less ideal in SLE, where continuous activation of pDCs occurs. We assessed the potential use of BDCA-4 as sole surface marker to identify pDCs in peripheral blood of human donors and showed that BDCA-4-positivity confirmed by light scatter properties is suitable for the identification of pDCs. To confirm the applicability of the method for functional analyses, expression levels of HLA-DQ and CD62L molecules on pDCs were monitored in response to stimulation with imiquimod. The expression of HLA-DQ is up-regulated, while that of CD62L is down-regulated on pDCs as they are activated. Similarly to a multi-antigen panel, BDCA-4 identification method was also able to detect these changes. Results of repeated analysis of blood samples from the same individual indicated that our method is highly reproducible. Conclusively, our data suggest that the expression of BDCA-4 is not decreased after TLR-activation of these cells, and this antigen is a suitable independent marker of pDCs for screening large numbers of blood samples in the in vitro diagnostic environment. The novel BDCA-4-based 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.

As a further model system, 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.
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 and triggers the production of type I IFNs through a MyD88-dependent pathway that involves the phosphorylation of IRF7 by IRAK-1 and IKKα. To investigate whether pDC-L cells share functional properties with normal pDCs, we analyzed their response to treatment with TLR9-ligands type A or B CpG, and TLR7-ligand imiquimod. We observed that similarly to circulating normal pDCs, TLR7- and TLR9- 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. 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. However, we failed to detect the secretion of IFN-α in the culture supernatants of TLR-ligand activated pDC-L cells. 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. 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. 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 that interacts with the transmembrane domain of these structurally related TLR receptors in the acidified endo-lysosomal compartments.
Upon encounter with TLR ligands or viruses, pDCs participate in adaptive immune responses by directing activation of naïve T-cells. We found that similar to normal pDCs, pDC-L cells stimulated with TLR ligands individually possess potent T-cell priming ability. 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 ligands results in a suppression of B-cell proliferation and IgM responses. Based on these data we propose that the co-stimulation of professional APCs 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 the phenotypic and functional characteristics of pDC-L cells activated by TLR7 and TLR9 ligands are identical to those of normal pDC.

Plasmacytoid DCs detect RNA and DNA viruses by two endosomal receptors, TLR7 and TLR9 and the recognition of viral nucleic acids occurs when viruses and/or their components are ingested by pDCs and transported into the lysosomes. In contrast, conventional DCs and alveolar macrophages were shown to detect replicating viral RNA intermediates by cytosolic RLRs. Previous studies suggested that pDCs primarily employ the TLR-system to recognize viruses and do not depend on RLRs. 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. Furthermore, we observed 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. To assess the functional state of RIG-I expressed following activation by TLR7/9 ligands, we demonstrated that pDCs are able to sense 5’ppp-dsRNA, a synthetic RIG-I ligands. 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.
As type I IFNs 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. In our experiments, TLR7- and TLR9-mediated signals were able to up-regulate RIG-I expression in both primary pDCs and pDC-L cells even in presence of blocking antibodies against IFNAR1, suggesting a type I IFN-independent mechanism. 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. 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 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 tyrosin by an intermediate MAPK-dependent tyrosine kinase precedes the direct serine phosphorylation in STAT1 by MAPK.

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 should be considered in contrast to pathological conditions, including viral infections going on in non-lymphoid tissues. 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.
6. Discussion

Plasmacytoid DCs represent key players of the innate immune system and provide a functional link between adaptive and innate immunity. Alterations in pDC counts in peripheral blood, in their migration and function were described in various diseases. As a prominent example, relapses in SLE are associated with the infiltration of pDCs in the kidney lesions and correlation with decreased pDC counts in the peripheral blood and activity of the disease was demonstrated. Therefore, 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. Previous studies employed complex, multi-marker (usually HLA-DR, CD123 and CD4) strategies to identify pDCs in peripheral blood. These techniques are non-ideal, as the flow cytometers validated for in vitro diagnostic use typically have not more than 3-5 channels, leaving no space for phenotyping besides identification of pDCs. BDCA-2 and BDCA-4 antigens are expressed only by pDCs among peripheral blood cell types, thus might serve as unique markers of pDCs. However, the expression of BDCA-2 depends on the activation state of the cells, making it less ideal in SLE, where continuous activation of pDCs occurs. We assessed the potential use of BDCA-4 as sole surface marker to identify pDCs in peripheral blood of human donors and showed that BDCA-4-positivity confirmed by light scatter properties is suitable for the identification of pDCs. To confirm the applicability of the method for functional analyses, expression levels of HLA-DQ (membrane protein involved in antigen presentation) and CD62L (also referred to as L-selectin, a key ligand for migration through high endothelial venules) molecules on pDCs were monitored in response to stimulation with imiquimod. The expression of HLA-DQ is up-regulated, while that of CD62L is down-regulated on pDCs as they are activated. Similarly to a multi-antigen panel, BDCA-4 identification method was also able to detect these changes. Results of repeated analysis of blood samples from the same individual indicated that our method is highly reproducible. Conclusively, our data suggest that the expression of BDCA-4 is not decreased after TLR-activation of these cells, and this antigen is a suitable independent marker of pDCs for screening large numbers of blood samples in the in vitro diagnostic environment. As the rarity of pDCs 0.1–0.6% in PBMC) means a ked difficulty for research the novel BDCA-4-based 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.

As a further model system, 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 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 and triggers the production of type I IFNs through a MyD88-dependent pathway that involves the phosphorylation of IRF7 by IRAK-1 and IKKα. To investigate whether pDC-L cells share functional properties with normal pDCs, we analyzed their response to treatment with TLR9-ligands type A or B CpG, and TLR7-ligand imiquimod. We observed that similarly to circulating normal pDCs, TLR7- and TLR9- 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. 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. 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. 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. 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. 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. 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 that interacts with the transmembrane domain of these structurally related TLR receptors in the acidified endo-lysosomal compartments.

Upon encounter with TLR ligands or viruses, pDCs participate in adaptive immune responses by directing activation of naïve T-cells. We found that similar to normal pDCs, pDC-L cells stimulated with TLR ligands individually possess potent T-cell priming ability. 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 ligands results in a suppression of B-cell proliferation and IgM responses. Based on these data we propose that the co-stimulation of professional APCs 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 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 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.

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 and the recognition of viral nucleic acids occurs when viruses and/or their components are ingested by pDCs and transported into the lysosomes. In contrast, conventional DCs and alveolar macrophages were shown to detect replicating viral
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To assess the functional state of RIG-I expressed following activation by TLR7/9 ligands, we demonstrated that pDCs are able to sense 5'ppp-dsRNA, a synthetic RIG-I ligand. 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.

As type I IFNs 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. In our experiments, TLR7- and TLR9-mediated signals were able to up-regulate RIG-I expression in both primary pDCs and pDC-L cells even in presence of blocking antibodies against IFNAR1, suggesting 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. 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. 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 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 tyrosin by an intermediate MAPK-dependent tyrosine kinase
precedes the direct serine phosphorylation in STAT1 by MAPK. 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 recently reported in the literature. 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 that is known to interact with the transmembrane domain of these structurally related TLR receptors in the acidified endo-lysosomal compartments. 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 should be considered in contrast to pathological conditions, including viral infections going on in non-lymphoid tissues. 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 are 1) to differentiate between harmful and non-harmful antigens; 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 (APCs). More than a decade after the discovery of myeloid dendritic cells, researchers identified the plasmacytoid dendritic cells (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 (BDCA4) as compared to two-color identification methods. We found that BDCA4 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 cells 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 tissue 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 cooperation 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.

8. **Keywords**

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

List of publications related to the dissertation

DOI: http://dx.doi.org/10.1038/icb.2014.38
IF:4.205 (2013)

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IF:3.259

* Szabó, A. and Magyarics, Z. contributed equally to this work.
List of other publications

   mAbs. 7 (1), 243-254, 2014.
   DOI: http://dx.doi.org/10.4161/19420862.2014.985132
   IF:4.726 (2013)

   DOI: http://dx.doi.org/10.1128/CVI.00685-13
   IF:2.37 (2013)

   DOI: http://dx.doi.org/10.1016/j.freeradbiomed.2011.11.022
   IF:5.271

   Vaccine. 29 (23), 3982-3989, 2011.
   DOI: http://dx.doi.org/10.1016/j.vaccine.2011.03.081
   IF:3.766
DOI: http://dx.doi.org/10.4049/jimmunol.0803938  
IF: 5.745

DOI: http://dx.doi.org/10.1515/BC.2008.054  
IF: 3.035

DOI: http://dx.doi.org/10.1016/j.molimm.2008.07.001  
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Oral presentations

Zoltán Magyarics, Éva Rajnavölgyi, Attila Bácsi: Investigation of phenotypic properties and cytokine secretion profiles of plasmacytoid dendritic cells activated by various CpG-oligonucleotides. DCcrest07 DC-THERA Graduate School, 25-30th March 2007., Celerina/St. Moritz, Switzerland

Zoltán Magyarics, Éva Rajnavölgyi, Attila Bácsi: Phenotypic and functional studies on pDC-derived acute leukemia (pDCL) cells. DCcrest08 DC-THERA Graduate School, 9-14th March 2008., Celerina/St. Moritz, Switzerland


Poster presentations


Zoltán Magyarics, Attila Szabó, Kitti Pázmándi, László Gopcsa, Attila Bácsi, Éva Rajnavölgyi: Cytokine production and helicase expression of leukemic plasmacytoid dendritic cells. *2nd European Congress of Immunology (ECI), 13-16th September 2009, Berlin, Germany*
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