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PATTERN RECOGNITION AND INNATE IMMUNE SIGNALING IN HUMAN DENDRITIC CELLS

by Attila Szabó

Supervisor: Prof. Dr. Éva Rajnavölgyi, Ph.D., D.Sc.

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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By **Attila Szabó**, M.Sc., M.A.

Supervisor: Prof. Dr. Rajnavölgyi Éva, Ph.D., D.Sc.

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the Examination Committee:	Prof. Dr. László Maródi, Ph.D., D.Sc.
Members of the Examination Committee:	Dr. Edit Buzás, Ph.D., D.Sc.
	Dr. Péter Bay, Ph.D.

The Examination takes place at the Department of Immunology, Medical and Health Science Center, University of Debrecen, 4th of February, 2013.

Head of the Defense Committee:	Prof. Dr. László Maródi, Ph.D., D.Sc.
Reviewers:	Prof. Dr. Sandra Gessani, Ph.D.
	Dr. József Kónya, Ph.D.
Members of the Defense Committee:	Dr. Edit Buzás, Ph.D., D.Sc.
	Dr. Péter Bay, Ph.D.

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1. Introduction

Dendritic cells (DCs) involve a heterogeneous population of hematopoietic cells that co-evolved with the adaptive immune system. They are efficient orchestrators of innate and adaptive immune responses mediated by their ability to sense environmental changes, internalize and process proteins, and present peptide fragments to T lymphocytes. Their activity has been shown to be critical in both the induction and maintenance of self tolerance and initiation of immune responses. The 2011 Nobel Prize in Medicine awarded to Bruce Beutler, Jules Hoffmann and Ralph Steinman further supported the admission of the field of innate immunity and its impact on modern medical sciences. The Prize was shared for the discoveries of the role of Toll-like receptors (TLRs) in fruit fly immunity, TLR activities in mammals and the identification and characterization of DCs as initiators of the adaptive immunity. Membrane-bound TLRs and the cytoplasmic RIG-I-like receptors (RLRs) are two important families of innate sensors. Upon activation both receptor types mediate pro-inflammatory and interferon responses, two crucial branches of early-phase host defenses. In the past decade novel vaccination strategies based on the specific activation or co-stimulation of these receptors have also been developed to increase the efficacy of protective immune responses to combat virus infections.

Our work was aimed to study the functional activity of TLRs and RLRs in different human DC subsets under various conditions, such as acute or chronic virus infection and inflammation. We also established an *in vitro* model to investigate the mechanism of action of a vaccine component with adjuvant effect in human DCs.

1.1 Dendritic cell subsets and their functions

The description of DCs in 1972 by Steinman and Cohn and the functional characterization of their antigen-presenting capability was a major advance in immunology. The finding that epidermal Langerhans cells (LCs) are functionally identical to splenic DCs led to a unified model of DC ontogeny. DCs belong tothe hematopoietic system and have a relatively short *in vivo* turnover time in both mice and humans. Based on their origin, tissue localization and functional properties, human DCs can be classified into two major types: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). As cDCs and pDCs found in peripheral tissues are non-dividing cells, they must be continuously replenished from bone

marrow-derived hematopoietic stem cells (HSCs). The majority of human DCs derive from myeloid precursors and give rise to various subsets, such as LCs, interstitial (dermal or tissue) DCs, and monocyte-derived DCs (moDCs).

During pathogenic invasion or inflammation, resting DCs become activated resulting in their transition to a mature cell type with altered chemokine receptor expression. This process ensures the rapid migration of activated DCs through the lymphatics to draining LNs. Here, activated DCs carrying their accumulated antigenic content act as highly potent APCs and instruct T lymphocytes to differentiate into effector and memory cells. Microbial compounds are strong activators of resting DCs and mediate stimulatory signals through conserved pattern recognition receptors (PRRs). In response to these stimuli, steady state tissue resident DCs exit inflamed tissues and transport peripheral antigens to secondary lymphoid organs where these cells can initiate the adaptive immune response by triggering naïve T cell activation. At the same time, monocytes enter the inflamed tissues and give rise to phagocytic APCs including DCs, thereby counterbalancing the rapid egress of DCs. The newly differentiated moDCs may function as resting tissue resident APCs or as sources of inflammatory cytokines. In addition, these cells may migrate to peripheral lymphoid organs and trigger the activation of naïve T lymphocytes.

Human CD1molecules belong to the family of surface glycoproteins expressed by thymocytes, epidermal LCs, and a subpopulation of B cells. The family consists of four homologous proteins: CD1a, CD1b, CD1c, and CD1d, and the CD1 gene locus on human chromosome 1 contains five potential CD1 genes. The major function of CD1membrane proteins is to present self- or pathogen-derived lipids to activate CD1-restricted T lymphocytes. Unlike MHC class II molecules, the membrane expression of CD1a does not depend on DC maturation, but little is known about the transcriptional and/or ligand-dependent regulation of this process. Previously, our group identified two human moDC subsets that differed in their phenotypic and functional characteristics. The $CD14^{low}$DC-$SIGN^{+}PPAR\gamma^{high}CD1a^{-}$ moDC subset, referred to as $CD1a^{-}$ DC, has been identified throughout lymph nodes and was characterized by efficient phagocytic activity to engulf bacteria and apoptotic cells. Depending on environmental cues, these cells develop into $CD14^{-}$DC-$SIGN^{+}PPAR\gamma^{low}CD1a^{+}$ inflammatory cells with high cytokine secretion and membrane expression of CD40 and E-cadherin. This $CD1a^{+}$ DC subset is detectable in the interfollicular areas of reactive lymph nodes. The ratio of $CD1a^{-}$ to $CD1a^{+}$ moDC varies among individuals and is negatively regulated by serum lipids and synthetic PPARγ ligands,

which have been shown to ameliorate immune pathology in mice infected by highly pathogenic influenza virus.

In vitro generated human moDC subsets provide a rich source of cells with remarkable plasticity upon responding to various signals and thus emerged as promising candidates for various immunotherapies and have been utilized for targeting vaccines against cancers and viruses.

1.2 Innate pattern recognition by DCs – the role of TLR and RLR families under various conditions

DCs express a wide array of PRRs that are distributed to various cellular compartments. The cell type specific expression and the intracellular compartmentalization of PRR determine the synergistic or inhibitory cross-talk of these receptors, the interplay of the coupled signaling pathways they trigger, and the effector molecules they produce. An important event in the history of immunology was the description of the Drosophila Toll protein, which is essential for immune responses against *Aspergillus fumigates*. The TLR family is an important class of PRRs through which the innate immune system detects invasive microorganisms. TLRs are also important in the non-immediate phase of the immune response, such as the recruitment of phagocytes to infected tissue areas. Recent studies revealed that TLRs recognize several organisms, such as bacteria, fungi, protozoa, and viruses. Once TLRs have been activated, they initiate various signal transduction cascades that initiate and regulate the immune response through the transcription factors NF-κB, IRFs and mitogen-activated protein kinases (MAPKs) such as p38, ERK1/2, and c-Jun N-terminal kinase (JNK). This process results in the expression of a common set of genes whose products, like cytokines, chemokines, and co-stimulatory molecules, are essential in the orchestration of both innate and adaptive immunity.

MyD88 was the first adapter found to be essential for TLR signaling. MyD88 had already been identified as a protein generally expressed in myeloid tissues and could be induced when myeloid precursors were differentiated in the presence of IL-6. Later, this protein was found to be crucial for the signaling processes of several TLRs and IL-1RI. In general, MyD88 is able to mediate the signaling of all TLRs except TLR3 and to some extent TLR4. MyD88-dependent signaling of TLRs is very similar to the downstream processes of IL-1RI in terms of the kinetics of activation and the induction of NF-κB and MAPKs. Thus the ligation of a TLR results in the association of MyD88 to Interleukin-1 receptor-associated

kinases (IRAKs). Initially, IRAK-1 seemed to play an important role in IL-1RI signaling as the overexpression of IRAK-1 led to the activation of downstream signaling and IRAK-1-deficient HEK293 cells did not respond to IL-1. After TLR activation, IRAK-1 becomes phosphorylated, dissociates from the receptor complex and binds to TRAF6, an essential cytosolic downstream element that links IL-1R/TLR complexes to the activation of NF-κB and MAPKs.

Besides NF-κB and MAPK triggering, the MyD88-dependent signaling pathway also leads to the activation of interferon-regulatory factors (IRFs). This process is of extreme importance because these factors control the production and regulation of interferons, a cytokine family with strong antiviral and anticancer activity. These IRFs act as transcription factors, and after activation-induced phosphorylation they form homo- and heterodimers, whichtranslocate into the nucleus where they regulate type I interferon gene expression.

During their differentiation, moDCs downregulate CD14, upregulate CD1a and DC-SIGN, and acquire the ability to express CCR7, which is required for migration to lymphoid tissues. Such differentiation of immature moDCs is unlikely to occur in inflamed tissues where the developing cells constantly receive stimulatory signals from microbial compounds, inflammatory mediators, and tissue damage. It has been extensively documented that long term activation leads to functional exhaustion of macrophages and DCs. Therefore, DC inactivation by constant stimulatory signals counteracts the development of potent monocyte-derived APCs in inflamed tissues. There are several molecular mechanisms implicated in macrophage and DC exhaustion. These include increased or decreased expression of signaling components, the release of soluble mediators that interfere with DC functions, and the altered expression of molecules that regulate gene expression. Although several pathways have been implicated in the functional exhaustion of long-term activated macrophages and DCs, the relative contribution of these pathways is not fully understood. It is yet to be clarified whether these pathways cooperate or operate within different conditions and/or time frames, or multiple inhibitory mechanisms act in a redundant manner.

RLRs include retinoic acid-induced gene 1 (RIG-I), melanoma-differentiation associated gene-5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), all of which possess a DExD/H box helicase domain. RIG-I and MDA5 are structurally homologous proteins with RNA helicase activity, being able to unwind double stranded RNA in an ATP-dependent manner. However, this event does not lead to downstream signaling *per se*. The two CARD domains of RIG-I and MDA5 are located at the N-terminal end of the proteins. RIG-I- and MDA5-mediated signaling is coordinated by another CARD domain containing

protein, the CARD adaptor inducing interferon-β (CARDIF) alternatively referred to as IPS1, MAVS, or VISA. This adaptor is localized to the mitochondrial membrane and thus links innate immunity to an organelle that evolutionarily originated from aerobic bacteria. RLRs are essential for the production of type I IFNs and pro-inflammatory cytokines in response to viral infections. However they exhibit different specificity and "select" among different virus taxa. Studies demonstrated that RIG-I is highly specific for RNA viruses, such as influenza A (*Orthomyxoviridae)*, whereas MDA5-deficient cells fail to produce interferon in response to *e.g.* picornaviruses. Besides viruses as natural ligands for RLRs, several other molecules are able to induce RIG-I and MDA5 activation under experimental conditions. Poly-riboinosinic:poly-ribocytidylic acid (polyI:C or pI:C) is a synthetic dsRNA that acts as a potent synthetic inducer of type I IFNs that selectively activates TLR3 and RLRs. This specificity is due to the length of the nucleic acid chain rather than its base composition, as commercial or "long" pI:C is not detected by RIG-I. However, partial digestion of pI:C by the specific endonuclease RNaseIII results in the generation of trimmed "short" pI:C of about 300 bp in length, which is able to activate RIG-I but not MDA5. The mechanism behind this size discrimination is not yet known.

Upon activation by dsRNA, RIG-I undergoes a conformational change that exposes its CARD domain. This is followed by an oligomerization step that involves the interaction of the CARDs of RIG-I and IPS1. MDA5 also signals through IPS1 in the same manner. This leads to the downstream activation of NF-κB and IRFs. In the last decade, cumulative studies revealed the details of virus sensing and signaling machinery of RLRs. It is now clear that RIG-I and MDA5 are of critical importance in innate antiviral host defense. However, discoveries of many signaling regulators as well as other PRRs coupled to interacting signaling processes make the picture more complicated. Indeed, many aspects of RLR pathway regulation need to be clarified, especially because RLRs were shown to be potential disease modifiers in humans.

Recent studies showed that TLRs and RLRs are involved in the regulation of systemic immune homeostasis to a much greater extent than initially expected. The efficacy of future PRR-based therapies is highly dependent on understanding the details of TLR and RLH signaling and their interaction.

1.3 Dendritic cell subtypes as targets of vaccination

Novel vaccination strategies that target DCs are emerging and involve delivering antigens to the appropriate DC subset along with stimulatory signals to result in prompt activation of innate immunity. DCs continuously monitor their environment with the help of their internalizing receptors that mediate the uptake of soluble and particulate material. Immature DCs are ideal targets of vaccine design due to their high phagocytic capacity. They possess unique mechanisms to promote antigen processing and presentation. Particulate antigens, such as intact microbes or their products, apoptotic cells, artificial beads, or other particulate formulates used for vaccination can be delivered to distinct intracellular compartments of DCs based on their expression pattern of PRRs. Microbes or artificial beads carrying both antigenic structures and TLR ligands induce phagosome maturation and the presentation of relevant peptide segments via MHC class II proteins on the cell surface. However, in the absence of coupled TLR signals peptides from phagocytosed structures (*e.g.* non-infected apoptotic cells) even if internalized together with microbes are not immunogenic because of incomplete antigen processing and phagosome maturation. The means how DCs get activated has an impact on their cytokine profile and physical interactions with other cells, and thus the mode of DC activation influences the functional activity of other immune cells. For example, proper DC modulation allows DCs to regulate the effector functions of T cells, the magnitude and characteristics of antibody responses and long-term immunologic memory.

IC31® is a two-component adjuvant consisting of the artificial antimicrobial cationic peptide KLK as a vehicle and the TLR9 stimulatory oligodeoxynucleotide ODN1a. Several *in vivo* studies in the murine system revealed the Th1 and/or Th17 polarizing effect of IC31® when used as an adjuvant in combination with mycobacterial antigens, and the efficacy of IC31® in anti-mycobacterial vaccination of healthy volunteers was also shown. Besides its beneficial immunological effects, the unique functional characteristics of IC31® and its components have also been described. KLK was shown to facilitate the uptake and delivery of ODN1a into TLR9-positive intracellular vesicular compartments of human moDCs and based on these properties IC31® was implicated to have a profound effect on immune responses triggered by TLR9 agonists. TLR9-mediated stimulation has been intimately linked to type I interferon production and previous studies in a mouse model revealed the capability of IC31® to induce peptide-specific cytotoxic T cell (CTL) activation in a Stat1 phosphorylation dependent manner, indicating the role of interferons as adjuvants. However, the exact mechanisms involved in IC31®'s actions have not been identified.

2. Aim of the studies

- To analyze the effects of different activation-induced inhibitory factors on the cytokine production of moDCs that received TLR4 stimulation early during their differentiation;
- To study the molecular background of endotoxin tolerance in moDCs by examining the detailed signaling events of TLR4 after chronic LPS stimulation;
- Investigate the expression of RLH sensors and the main components of the RLH-pathway in moDCs during steady-state conditions and after activation by various stimuli;
- To examine the contribution of RLHs to the inflammatory- and IFN response of moDCs upon pI:C and influenza A virus activation;
- To assess the activity of RLHs in the $CD1a^+$ and $CD1a^-$ DC subsets;
- Functional characterization of RLHs in $CD1a^+$ and $CD1a^-$ DCs by means of RNA-interference and ELISPOT assays;
- To identify the mechanisms of actions of IC31® adjuvant in human DCs at the molecular and functional levels by comparing the distribution and accumulation of the adjuvant in human PBMCs;
- To monitor the effects of IC31® on DC differentiation, subset distribution, activation of key transcription factors and signaling cascades, and cytokine secretion;

3. Materials and Methods

Isolation of monocytes, differentiation, activation and characterization of cDCs by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation of heparinized leukocyte-enriched buffy-coats of healthy donors drawn at the Regional Blood Center of Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance 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 Centre (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. PBMCs were separated by a standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation with anti-CD14 microbeads according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). After separation on a VarioMACS magnet, 96–99% of the cells were $CD14^+$ monocytes as measured by flow cytometry. Monocytes were cultured in 12-well tissue culture plates at a density of 2×10^6 cells/ml in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium) and 100 ng/ml IL-4 (Peprotech EC, London, U.K.). On day 2, the same amounts of GM-CSF and IL-4 were added to the cell cultures.

Phenotyping of resting and activated DCs was performed by flow cytometry using anti-CD83 and anti-CD1a antibodies (Abs) and isotype-matched control Ab (BD Pharmingen, San Diego, CA). Fluorescence intensities were measured by FACS Calibur cytometer (BD Biosciences, Franklin Lakes, NJ), data were analyzed by the FlowJo software (Tree Star, Ashland, OR).

Fluorescent dye conjugated KLK(-FITC) and ODN1a(-Cy5) uptake was performed in cell culture medium at 37°C and 0°C as control. The cell population of interest was gated according the forward and side light scatter properties.

Activation of dendritic cells

Bacterial lipopolysaccharide (LPS) (Sigma, Schnelldorf, Germany), human recombinant IFNγ (Peprotech, Rocky Hill, NJ), high molecular weight polyinosinic:polycytidylic acid (pI:C), CL075 and CpG2216 (InvivoGen, San Diego, CA) were used at concentrations indicated in the Figure legends. Purified live and inactivated A/Brisbane/59/7 (H1N1) influenza virus (kindly provided by the National Influenza Laboratory, Hungary) of 6×10^6 PFU/mL was used for *in vitro* treatment of 1×10^6 per mL sorted DC in serum-free AIMV medium for 24h.

To prepare cell lysates for Western blotting, DC were activated for 24h, to collect supernatants for ELISA and prepare cell lysates for Q-PCR for 18 – 24h. KLK (10 nmol/ml), ODN1a (0.4 nmol/ml) and IC31 (mixture of KLK+ODN1a) were used at similar working concentrations throughout the study.

RNA isolation, cDNA synthesis and QPCR

For real-time quantitative polymerase chain reaction (QPCR) total RNA was isolated by TRIzol reagent (Invitrogen; Carlsbad, CA). 1.5-2 μg of total RNA were reverse transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen) and Oligo(dT)15 primers (Promega, Madison, WI). Gene-specific TaqMan assays (Applied Biosystems) were used to perform QPCR in a final volume of 25 μl in triplicates using AmpliTaq DNA polymerase and ABI Prism 7900HT real-time PCR instrument (Applied Biosystems, Foster City, CA). Amplification of 36B4 was used as normalizing control. Cycle threshold values (Ct) were determined using the SDS 2.1 software. Constant threshold values were set for each gene throughout the study.

siRNA experiments

Gene-specific siRNA knockdown was performed by SilencerSelect siRNA (Applied Biosystems) transfection at day 3 of *in vitro* DC differentiation using GenePulser Xcell instrument (Bio-Rad, Hercules, CA). Silencing of helicase genes was performed by RIG-I and MDA5 siRNA mix, Silencer Negative Control non-targeting siRNA (Applied Biosystems)

was used as a negative control. The efficacy of siRNA treatments was tested before and after DC activation on days 5 and 6, respectively by Q-PCR and Western blotting.

Western blotting

Cells were lysed in Laemmli buffer and the protein extracts were tested by Ab specific for TLR3 (Abcam, Cambridge, UK), MDA5 (Lifespan, Seattle, WA), RIG-I, IκB-α, phospho-IκB-α, IRF3, phospho-IRF3 (Cell Signaling, Danvers, MA, US) and β-actin (Sigma) diluted to 1:1000; secondary Ab were used at 1:5000. Anti-rabbit or anti-mouse (pIκB-α) Ab conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont Buckinghamshire, UK) were used as secondary Ab. The SuperSignal enhanced chemiluminescence system was used for probing target proteins (Thermo Scientific, Rockford, IL). After the membranes had been probed for the target protein, they were stripped and re-probed for β-actin.

Cytokine measurements

Culture supernatants of DCs were harvested 24 hours after activation and the concentrations of IL-1β, IL-6, IL-8, TNF-α, CXCL10/IP-10 and IL-12p70 were measured using OptEIA kits (BD Biosciences). The level of secreted IFNβ was measured by a Human Interferon beta ELISA Kits (Cell Sciences, Canton, MA) according to the manufacturer's protocol. Absorbance measurements were performed with a Synergy HT reader (Bio-Tek Instruments, Winooski, VT, USA) at 450 nm.

IFNγ ELISPOT assay

Activated DC (2×10^5 cells/well) were co-cultured with naïve autologous T cells (10^6 cells/well) in serum-free AIMV medium for 5 days at 37°C in humidified atmosphere containing 5% CO_2. Phytohaemagglutinin (PHA) and Concanavalin A (ConA) activated T cells were used as positive controls, non-treated DC+T cell co-cultures and T cells without DC served as negative controls. Detection of cytokine secreting T cells was performed by the avidin-horseradish peroxidase system (NatuTec). Plates were analyzed on ImmunoScan plate reader (CTL Ltd., Shaker Heights, OH).

Immunohistochemistry (IHC) and immunofluorescence (IF) staining

Immunostaining was performed on human tissues obtained from formalin-fixed and/or paraffin-embedded surgical specimens. RIG-I and MDA5 Ab were used at a dilution of 1:50

(Cell Signaling and Lifespan, respectively). As a reference antibody, affinity purified rabbit Ab to S100 protein 1:1000; Novocastra) was used. Biotin-free EnVision^{+}-HRP system (DAKO) and VIP chromogen (Vector Labs, UK) were used for detection according to the manufacturer's instructions. Single and double immunofluorescence (IF) staining were performed as described (Gogolak et al. 2007). A tyramide-coupled red fluorescent amplification kit using tetramethylrhodamine (TMR of TSA-Fluorescent System; PerkinElmer Life Sciences) with DAPI (blue fluorescence; Vector Labs) nuclear counterstaining was used for visualization. For double IF, the first Ab labeling was followed by the second Ab treatment using preformed Ab-biotinylated-$(Fab')_2$ complex and streptavidin-FITC fluorochrome (green). To ensure the specificity of immunostainings isotype-matched control IgG (DakoCytomation) was used. The topographic identity of immunostained S100 positive DCs was determined on serial sections of reactive LN in parallel with intracellular RIG-I and MDA5 expression, and the double immunolabeled samples served as positive specificity controls. Microphotographs were taken by Olympus BX51 microscope equipped with excitation filters for green (FITC), red (rhodamine), and blue (DAPI) fluorescence and a DP70 digital camera (Olympus Europe, Hamburg, Germany).

Statististical analysis

Data are presented as mean ± standard deviation (SD). p values were calculated using Student's *t* test. $p < 0.05$ was considered as statistically significant; $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

4. Results

4.1 The role of IRAK-1 in TLR4 signaling of moDCs during chronic LPS stimulation

4.1.1 LPS-induced IRAK-1 downregulation is sufficient to inhibit further moDC activation

Several groups reported that TLR4 and IRAK-1 proteins are degraded in response to long-term LPS triggering in DCs and in macrophages whereas the inhibitory protein IRAK-M can be upregulated upon chronic DC activation. In this study, we compared TLR4 expression in moDCs developing with or without LPS for 2 days using flow cytometry or Western blot and found no sign of decreased TLR4 expression in the presence of LPS. Thereafter we studied IRAK-1 and IRAK-M protein levels in moDCs developing in the presence or absence of LPS using western blot and we detected the downregulation of IRAK1 by day 2 in the presence of LPS. IRAK-M levels slightly decreased as well, indicating that an upregulation of IRAK-M might not stand as the mechanism underlying moDC endotoxin tolerance. Our results indicate that the activation-induced IRAK1 downregulation might play an important role in the functional exhaustion of moDCs as this event alone can lead to decreased cytokine production by activated DCs.

4.1.2 LPS-tolerized moDCs shift from the dominance of the MyD88 to the TRIF-dependent signaling pathway

Previous studies have indicated a developmental blockade in moDC differentiation in response to persistent TLR activation or an impaired TLR signaling as the underlying mechanism for LPS-induced tolerance. To better understand how early activation may influence moDC functionality we studied the survival, differentiation and signaling abilities of moDCs developing in the presence of LPS. In order to identify which TLR-induced signaling pathways are impaired in moDCs that received an early LPS stimulation we studied MAPK, NF-κB and IRF-3 activations in these cells. Activation of MAPKs is attributed to signals transmitted by the MyD88-dependent arm of the TLR pathways that might be particularly affected by the downmodulation of IRAK-1. Accordingly, LPS-induced phosphorylation of the Erk1/2 and p38 kinases as well as phosphorylation of the CREB/ATF-1 transcription factors often occurring via p38 activation, were abrogated by LPS pre-treatment of developing moDCs. On the contrary, DCs differentiating in the absence of LPS

responded readily with Erk1/2, p38 and CREB/ATF-1 phosphorylation to LPS stimulation. We also found a partial activation of NF-κB despite decreased MyD88-dependent signal transduction that might indicate functional MyD88-independent, TRIF-dependent signal routes. Indeed, we found strong IRF-3 phosphorylation in response to TLR3 or TLR4 ligation by pI:C and LPS respectively, in both LPS pretreated and control moDCs. IRF-3 phosphorylation was rather elevated in LPS pretreated cells and increased IRF-3 activation was accompanied by higher IFNβ expression in LPS pretreated moDCs as compared to control cells. Similar to the observed effect on IFNβ, pI:C induced higher expression of other genes sensitive for TRIF-dependent regulation (IFNα1, IFNα2 and CCL5) when the cells received LPS pretreatment, whereas we did not observed a similar consistent effect on CXCL10 expression. Overall, our results indicated the downregulation of MyD88-dependent TLR signals in response to LPS pretreatment of developing moDCs. The TRIF-dependent TLR pathways, on the other hand might remain functional following persistent LPS stimulation.

4.1.3 moDCs activated at an early developmental state have limited ability to modulate chemokine receptor expression

We compared gene expression levels of chemokines (CXCLs and CCLs) in moDCs cultured with or without LPS for 2 days and observed a significant increase in the expression of CCL5, CCL18, CCL19, CCL23, CCL24, CCL26, CXCL1, CXCL2 and CXCL5 in the presence of LPS that suggests an increased ability of the LPS-treated moDCs to attract both resting and activated T cells, as well as granulocytes. In addition to such possible contribution to the cellular influx associated with tissue inflammation, LPS-treated moDCs might increase their motility by cleaving extracellular matrix constituents as suggested by the elevated MMP7, MMP9 and MMP12 mRNA levels in these cells. We also studied CCR5 and CCR7 expression on moDCs that received activation signals during the first day of their culture and compared these cells to moDCs that received the same activation signals at a more differentiated stage, at day 5 of the culture. Interestingly CCR5, a chemokine receptor that primes migration to inflamed peripheral tissues, was not downregulated and CCR7 was not induced when moDCs received activation signals early in their development. On the contrary, moDCs that developed for 5 days without activation downregulated CCR5 in response to LPS, HKSA, zymosan or CD40L and several of the tested activation signals induced the expression of CCR7 on these cells. These results showed that the inability of moDCs to modulate their chemokine receptors early during their differentiation might limit egress from

peripheral tissues and predispose these cells to short-term inflammatory functions in the periphery. Longer differentiation free of activation signals might be required for the acquisition of a migratory phenotype in response to late activation; however such differentiation pattern may not occur in inflamed tissues.

4.2 RLH-mediated production of IFNβ by the human CD1a$^+$ dendritic cell subset and its role in anti-viral immunity

4.2.1 Baseline expression and induction of RIG-I and MDA5 in human moDCs

The cytosolic RLR sensors RIG-I and MDA5 share dsRNA specificity with membrane TLR3 but their role in human DC biology is poorly understood. We first mapped the expression of RIG-I and MDA5 in resting moDC and found that they express both sensors albeit at lower levels than monocytes, but inducible by ATRA, LPS, and pI:C. The results obtained with three independent donors characterized by increasing ratios of CD1a$^+$ cells showed that mRNA expressions of the helicases correlate to the presence of CD1a$^+$ cells. These results altogether demonstrate that besides membrane TLR3, resting moDC also express cytosolic helicases that can be induced by various stimuli.

4.2.2 Subset dependent expression and activity of RLR-related genes in moDCs

To assess the expression of RIG-I and MDA5 and the components of the related signaling cascade in moDC subsets we sorted the cells to CD1a$^+$ and CD1a$^-$ fractions and subjected them to mRNA and protein analysis. Both resting moDC subsets were shown to express RIG-I and MDA5, the downstream transcription factor IRF3 and the effector cytokine IFNβ1. Interestingly, CD1a$^+$ cells exhibited significantly higher baseline expression of RIG-I, MDA5, IRF3 and IFNβ1 genes than CD1a$^-$ cells. Furthermore, activation by increasing doses of pI:C resulted in the coordinated upregulation of RLR, IRF3 and IFNβ1 mRNA preferentially in CD1a$^+$ cells. Activated CD1a$^+$ moDCs also displayed higher levels of RIG-I and MDA5 proteins as compared to the CD1a$^-$ subset.

4.2.3 Activation of NF-κB and IRF3 mediated signaling pathways in CD1a$^+$ and CD1a$^-$ moDCs

To determine the contribution of NF-κB and the IRF3 – IFNβ signal transduction pathways in CD1a$^+$ and CD1a$^-$ moDCs we compared the levels of phosphorylated IκBα and IRF3. As a result of pI:C stimulation we detected rapid activation of both pathways and found

obvious differences in IκBα and IRF3 phosphorylation levels detected in the two DC subsets. Concordant to the differential expression of RIG-I and MDA5 receptors, $CD1a^+$ and $CD1a^-$ moDCs not only differed in the expression levels of the key molecules, but also in the functional activity of the downstream signaling machinery. Comparison of the produced cytokine levels also revealed statistically significant differences (n=9) for IL-6, TNFα, IL-12p70, CXCL10, and IFNβ (p=0.006) showing that the main producer of these cytokines is the $CD1a^+$ subset. These results indicate that due to their distinct RLR expression and signaling activity the $CD1a^+$ and $CD1a^-$ moDC subsets participate in the inflammatory and interferon responses to different extent.

4.2.4 Type I IFNβ but not inflammatory cytokine production is controlled by RLR in human $CD1a^+$ moDCs

We showed previously that upon pI:C activation, the production of inflammatory cytokines and type I IFNβ is primarily attributed to $CD1a^+$ moDCs. As the dsRNA analogue pI:C is a shared ligand of both RLRs and TLR3, to dissect the roles of RLH- and TLR3-mediated signaling cascades we used the siRNA technology and silenced the RLR or TLR3 genes in $CD1a^+$ cells. Silencing of TLR3 gene resulted in dramatic down regulation of the secretion of pro-inflammatory cytokines IL-6, TNFα and CXCL10 known to be under the control of NF-κB. Interestingly, the production of these cytokines was not affected significantly, when the RIG-I/MDA5 genes were downregulated, whereas the production of IFNβ decreased significantly. These results show that upon pI:C stimulation the production of IFNβ is controlled preferentially by RLR expression and function, whereas TLR3 acts as a regulator of pro-inflammatory cytokine secretion in human $CD1a^+$ moDCs.

4.2.5 Contribution of $CD1a^-$ and $CD1a^+$ moDC subtypes to the anti-viral immune response

The role of type I IFNs in priming adaptive T-cell responses is well established in both mice and human. Type I IFNs are able to enhance T-cell activity via APCs, which exert a direct effect on the autocrine and/or paracrine secretion of these cytokines and influenza virus is known to activate RIG-I specifically. We hypothesized that $CD1a^+$ DC demonstrated to produce large amounts of IFNβ should be more efficient to prime T-cells in an *in vitro* human virus-infection model than $CD1a^-$ cells. To determine the functional relevance of DC subtype-specific regulation of IFNβ secretion we sought to analyse the involvement of $CD1a^+$ and $CD1a^-$ moDC in priming influenza virus-specific T lymphocyte responses. When the

autologous naïve $CD8^+$ T cells were activated by sorted $CD1a^+$ or $CD1a^-$ DC infected by live virus, the number of IFNγ producing T cells was significantly higher in the co-cultures containing $CD1a^+$ cells as compared to cultures with $CD1a^-$ cells underlying the preferential contribution of the $CD1a^+$ DC subset in virus-specific CTL stimulation. Silencing RIG-I/MDA5 expression in moDCs by specific siRNA resulted in a marked reduction in the number of IFNγ secreting T lymphocytes in both subsets demonstrating the RIG-I/MDA5 dependence of the $CD8^+$ T cell response. These results confirm the functional role of RLRs and the related signaling pathway in regulating influenza virus-specific $CD8^+$ T cell activation triggered preferentially by $CD1a^+$ moDC.

4.2.6 Ex vivo examination of RIG-I and MDA5 expression in human tonsil and lymph node dendritic cells

Monocyte-derived DC has been classified as migratory and inflammatory cells that could be indentified in both peripheral tissues and lymph nodes. To analyze the tissue distribution of RIG-I/MDA5 expressing DC we performed immune histochemistry (IHC) and immune fluorescence (IF) staining of tonsil and resting or reactive lymph node tissues. Immunoperoxidase (IP) staining revealed the appearance of MDA5 and RIG-I expressing cells in perifollicular regions of non-reactive lymph nodes. Double staining with S100, a typical DC marker demonstrated that a subset of DCs was positive for RIG-I. MDA5 and RIG-I positive DCs with typical morphology have also been detected in the interfollicular areas of reactive lymph node. These results show that $CD1a^+$ DCs expressing RIG-I and/or MDA5 are detectable under physiological and pathological conditions and can be identified in lymphoid tissues.

4.3 The two-component adjuvant IC31® boosts type I interferon production of human monocyte-derived dendritic cells via ligation of endosomal TLRs

4.3.1 Accumulation of IC31® and its components in human peripheral blood mononuclear cell populations

Cell type-specificity and cellular compartmentalization of PRRs has an important impact on the functional activity of various adjuvants. Thus, we first tried to identify the cell types of human PBMC preferentially involved in the accumulation of IC31® by using fluorescence-labeled KLK and ODN1a components of the adjuvant. Our results suggested that moDCs are the primary targets of IC31®. We also compared the fluorescence intensities

in $CD1a^+$ and $CD1a^-$ moDCs both at 0^oC and 37^oC, and found only marginal differences suggesting the contribution of both cell types in interacting with IC31® and its components in a temperature-independent manner.

4.3.2 Effect of IC31® on monocyte-derived dendritic cell differentiation and activation

To check the long and short term effects of IC31® on DC differentiation we set up various *in vitro* experimental systems. In the first system (*protocol A*) moDCs were differentiated in the presence of IC31® or its components added to monocytes on day 0 and to differentiating moDCs on day 2. In the second system (*protocol B*) the procedure was extended by an additional treatment on day 5 together with the activation of moDCs by LPS+IFNγ or by various TLR ligands to mimic inflammatory conditions. In the third system (*protocol C*), IC31® and its components were added on day 5 only, combined with the activation stimuli. When monocytes were differentiated according to *protocol A*, IC31® dramatically inhibited the generation of $CD1a^+$ cells and this effect could be attributed to KLK, as ODN1a had no effect. IC31® also inhibited the LPS+IFNγ-induced activation of DCs, while ODN1a had no effect indicating that IC31®interferes with moDC differentiation and activation, and KLK had an essential role in these effects.

4.3.3 IC31® modulates the cytokine profile of human monocyte-derived dendritic cells

Consistent with its inhibitory effect on moDC differentiation, we found that IC31® in combination with LPS+IFNγ according to *protocol B* inhibited the secretion of TNF-α and IL-6, but not IL-1β. We also observed a statistically significant increase of IFNβ secretion induced by IC31® and its KLK component, whereas ODN1a had no effect. Furthermore, our results revealed that IC31® and KLK abolished IκB-α phosphorylation, while slightly increased and extended the phosphorylated state of IRF3. These results suggest that IC31® is not able to activate the NF-κB pathway but rather supports the type I interferon response and this modulatory effect is attributed to KLK, the peptide component of IC31®.

4.3.4 Effect of IC31® on the type I interferon response of moDCs

Considering the moderate effect of the adjuvant on interferon secretion, we tested the effects of IC31® and its components present in the course of the entire *in vitro* moDC differentiation process (according to *protocols B* and *C*). The expression profiles of IRF and type I IFN family genes in DCs showed that repeated administration of IC31® during DC differentiation increased the expression of all IRF and type I IFN genes as compared to a

single adjuvant treatment at day 5. These findings suggest that if circulating monocytes as precursors of moDCs encounter IC31®, they stimulate type I interferon production during moDCs development, but it has a less prominent effect on already differentiated moDCs.

4.3.5 Mechanism of IC31® action at the level of pattern recognition receptors responsible for type I interferon responses

Type I IFN responses can be mediated by various PRRs localized either to the cytosol (RLRs) or to intracellular vesicular compartments (TLRs). To test the involvement of RLRs in IC31®-modulated production of IFNβ induced by pI:C we silenced the RIG-I and MDA5 genes by specific siRNA. Down-regulation of RLRs had no effect on pI:C-induced IC31®-enhanced expression of IFNβ measured at both gene and protein levels. Conversely, silencing of TLR3 resulted in dramatic abrogation of IFNβ mRNA and cytokine levels underlying the type I IFN-enhancing activity of IC31® acting through the vesicular TLR3-mediated signaling pathway. This observation led us to hypothesize that IC31® primarily interacts with PRRs localized to the endo/lysosomal membrane of cDCs. To check the possible booster effect of IC31® on IFNβ production we used CL075 and CpG2216 for triggering TLR7/8 and TLR9, respectively. Similar to TLR3-targeted stimulation, activation of TLR7/8 resulted in significantly increased IFNβ production in the presence of IC31® treated according to protocol B. Interestingly, CpG2216 stimulation of DCs did not induce IFNβ production *per se*, however, repeated pre-treatments by IC31® or ODN1a (*protocol B*) resulted in moderate secretion of IFNβ. Thus IC31® was able to render TLR9 functional in moDCs and this effect could be attributed to the ODN1a component. As TLR9 gene expression was not affected by the adjuvant we propose that targeting IC31® or ODN1a to endosomal vesicles ensures sufficient concentration of the CpG2216 ligand to trigger TLR9 in human moDCs.

5. Discussion

Persistent macrophage and DC activation by TLR ligands leads to powerful inhibitory mechanisms blocking further activation by the same or heterologous stimuli. Several inhibitory factors induced in response to TLR stimulation have been identified but it is still unclear, how these factors contribute to tolerance for further activation. Since DCs developing from monocyte precursors in inflamed tissues might be particularly affected by the constant presence of microbial compounds and inflammatory mediators, we decided to study which inhibitory pathways are activated in moDCs in the presence of early and persistent TLR4 stimulation. Although not tested here, it is possible that certain inhibitory factors could modulate the expression of particular genes of DCs thereby inducing a qualitative tuning of cellular functions.

We found, that IRAK-1 downregulation occurring in moDCs receiving early activation signals through TLR4 during differentiation might by itself be sufficient to inhibit further activation through TLRs, as demonstrated by the strong inhibitory effect of siRNA induced IRAK-1 downregulation on IL-12 secretion. We showed IRAK-1 downregulation and decreased MyD88-dependent signaling activity in response to early LPS activation in moDC development in the absence of any detectable change in the survival rate. Some activation stimuli, including zymosan, HKSA or CL075 inhibited the upregulation of CD1a and the downregulation of CD14 in a subset of the developing moDCs by day 2. These findings suggest that downmodulation of TLR signal intensity during early moDC activation might induce tolerance to further activation irrespective of the differentiation stage of the cells.

Recent reports showed that migratory DC differentiation in the peripheral tissues might be impaired if the activation signals reach the monocyte precursors before their commitment to the DC differentiation pathway. Our data support this hypothesis by showing that activation of early moDC precursors leads to inflammatory cytokine and chemokine production but the cells, at early stage of DC differentiation, have a limited ability to modulate their chemokine receptor expression required for lymph node homing. The cytokine producing ability of the developing inflammatory moDCs can be terminated by the functional exhaustion before the cells differentiate to mature DCs capable of reprogramming their chemokine receptor profile. Early activation of developing moDCs may thus set the threshold of DC migration to LNs, thereby limiting the continuous transfer of inflammatory signals to T lymphocytes.

Identification of DC subsets with specific functions has recently emerged as a new challenge of DC biology. DCs developing under inflammatory conditions support protection against pathogens, while DC subsets differentiating under steady state play a role in the control and resolution of inflammation and tissue destruction. This functional divergence is determined by the destination site of DC precursors and the subtype-specific and compartmentalized expression of PRRs. In contrast to the restricted expression of nucleotide recognizing TLRs, RLRs specialized for the recognition of shorter or longer dsRNA present in multiple cell types.

In our work we compared the expression and functional activity of RLR family members in two previously characterized human moDC subsets distinguished by the expression and activity of PPARγ that controls the expression of type I CD1a molecules. To assess the functional importance of $CD1a^+$ and $CD1a^-$ moDC subsets in the RLR-mediated inflammatory cytokine and type I IFN responses, and their role in priming influenza virus-specific T cell responses we performed *in vitro* and human *ex vivo* experiments. Our results show that the baseline expression of RIG-I/MDA5, IRF3 and IFNβ, key elements of the downstream signaling cascade, are significantly higher in $CD1a^+$ cells than in their $CD1a^-$ counterparts. Activation of these DC subsets by specific ligands, *i.e.* pI:C or influenza virus revealed that the coupled signaling machinery is more active in the $CD1a^+$ moDC subset than in $CD1a^-$ cells. These results altogether show that in human moDC the RLR cascade acts in a subtype specific manner. Furthermore, the results of our siRNA experiments revealed that in the inflammatory $CD1a^+$ moDC subset, the TLR3 – NF-κB and RLH – IFNβ pathways control the production of different sets of cytokines and due to their subset-specific activity work independently. The putative *in vivo* role of the $CD1a^+$ RLR expressing DC subtype is suggested by the appearance of these cells in tonsils and reactive lymph nodes.

We also showed that the increased activity of the RLH – IRF3 – IFNβ signaling pathway results in efficient priming of naïve autologous $CD8^+$ T lymphocytes by the $CD1a^+$ subset, and silencing of RIG-I/MDA5 abrogates this effect. Furthermore, the presence of RLR positive DCs in human lymphoid tissues suggests the possible importance of these cells during infections. Thus our findings not only describe the underlying mechanism of IFNβ production by moDC subsets but also identify the $CD1a^+$ DC subtype as a potential target for improving the efficacy of prophylactic and/or therapeutic vaccines against intracellular pathogens. As human moDCs are widely used in various clinical settings and considered as gold standards of DC biology, we propose that identification of human moDC subsets with

specialized functions may have an important impact on designing vaccines against viruses and tumor cells.

Provoking potent cellular immune responses against intracellular pathogens, which often cause persistent infections, requires the priming and/or boosting of inflammatory T-lymphocytes by properly activated DCs. Analyzing the response of human monocytes and differentiating moDC to IC31® we found that i) IC31® was efficiently accumulated in human blood-circulating monocytes and moDCs; ii) in the presence of IC31® the generation of inflammatory $CD1a^+$ DCs was inhibited, and failed to induce phenotypic changes of DC activation while decreased the secretion of TNF-α and IL-6 cytokines with a concomitant increase of IFNβ secretion; iii) long term presence of IC31® prevented IκB-α phosphorylation but extended the phosphorylated state of IRF3 indicating their independent activation; iv) the IC31® adjuvant exhibited a booster effect on ligand-induced vesicular TLR-mediated induction of IFNβ secretion.

The adjuvant effect of IC31® has already been characterized and its intracellular localization and role in TLR9-dependent moDC activation has also been shown. However, complete understanding of its mode of action and its effects on PRR-coupled signaling cascades in human moDCs has not been analyzed so far. To assess the long and short term *in vitro* effects of IC31 on moDC differentiation we set up different treatment protocols. The experimental data revealed that IC31® and its KLK component inhibited the transition of $CD1a^-$ cells to $CD1a^+$ moDCs and interfered with moDC activation. As a functional consequence these cells were unable to exhibit their typical phenotypic and functional changes upon moDC activation. This semi-mature differentiation and activation state of moDCs was associated with inactive IκB-α with the unusual capability to secrete high amounts of IFNβ. Thus IC31® could be identified as a moDC-modulatory adjuvant that has a profound effect on the balance of NF-κB and IRF3-mediated signaling pathways and thus fails to induce pro-inflammatory cytokine secretion. Further analysis of the type I interferon and NF-κB-mediated signaling pathways demonstrated that both IC31® and KLK increased type I interferon responses mediated by vesicular TLRs. This modulatory effect could be attributed in part to KLK, which facilitates the transport and accumulation of TLR ligands to the endosomal compartment to stimulate resident TLRs. Although these TLRs may interfere with each other's functional activities, our results point to the synergistic action of IC31® and TLR-induced interferon signaling and secretion. Besides increasing the magnitude of adaptive immune responses, adjuvants also play an instructive role in driving immune responses to the most appropriate directions to confer protection against pathogens. TLR activation inducing

lysosome maturation is a crucial requirement of antigen presentation by DCs, efficient $CD4^+$ T cell activation and Th1 polarization. As DCs produce increased levels of IFNβ when IC31® is co-administered with endosomal TLR-ligands we propose that augmented type I IFN responses known to facilitate cross-presentation may potentiate both $CD4^+$ and $CD8^+$ T cell responses. We also suggest that IC31® is an effective adjuvant of microbial proteins acting through the endosomal TLR system of moDCs and thus offers a potent tool for future vaccination strategies against intracellular pathogens and cancer.

6. Summary

Innate immunity represents the phylogenetically earliest host defense mechanism against invading microbes and endogenous harmful materials. Its function involves the *recognition* of **self- and non-self**, **innocuous and harmful**, the initiation of defensive *signaling processes*, and the *coordinated mobilization of effector cells*. Dendritic cells (DCs) are major participants in innate immune responses, acting as "sentinels" and ensuring the well-regulated immune surveillance and properly controlled response processes in multicellular organisms. Professional antigen-persenting cells (APCs), such as monocytes, macrophages, and DCs, play important roles in the regulation and coordination of defense mechanisms. In our work we investigated the role of Toll-like receptors and RIG-I-like receptors (TLRs and RLRs) in the regulation of inflammation and type I interferon (IFN) responses of human DC populations. We also studied the role of the two-component adjuvant IC31® in human
leukocytes and monocyte-derived DCs (moDCs).

We found, that:

- the TLR4-mediated, LPS induced IRAK-1 downregulation by itself can induce persistent moDC inactivation;
- studying cellular functions in line with activation induced negative feedback mechanisms we showed that early activation of developing moDCs via TLR4 receptor allows transient cytokine production only, followed by the downregulation of effector functions and the preservation of a tissue resident non-migratory phenotype;
- our results also revealed that the expression of RLR genes and proteins, as well as the activity of the coupled signaling pathways are significantly higher in the $CD1a^+$ moDC subset than in its phenotypically and functionally distinct counterpart;
- the requirement of RLR-mediated signaling in $CD1a^+$ moDCs for priming naïve $CD8^+$ T lymphocytes was confirmed by RIG-I/MDA5 silencing that abrogated these functions;
- our results demonstrate the DC subset-specific activation of RLRs, the underlying mechanisms behind the cytokine secretion profiles of the

responding cells, and identify $CD1a^+$ moDCs as an inflammatory and migratory DC subset with specialized functional activities;

- we also provide evidence that migratory $CD1a^+$ DCs are found in human tonsil and reactive lymph nodes;
- we showed that the vaccine compound IC31® is accumulated in blood-circulating monocytes, MHC class II-positive cells, and moDCs;
- in the presence of IC31®, the generation of inflammatory $CD1a^+$ moDCs and the secretion of inflammatory cytokines is inhibited, but the production of IFNβ increases;
- IC31® was identified as a DC-modulatory adjuvant that sets the balance of NF-κB- and IRF3-mediated signaling pathways to the production of IFNβ;
- the adjuvant activity of IC31® on the IFN response was shown to be exerted through TLRs residing in the vesicular compartment of moDCs.

Our findings draw the attention to the importance of the functional collaboration of cytoplasmic and membrane-bound pattern recognition receptors. The practical significance of this lies in the targeted modification possibilities of the functions of DC subtypes, and the controlled regulation of cellular immune responses representing crucial options in the practical use of DCs for prophylactic and therapeutic purposes.

7. Publications

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H-4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu

List of other publications

3. **Szabó, A.**, Osman, R.M., Bacskai, I., Kumar, B.V., Agod, Z., Lányi, Á., Gogolák, P., Rajnavölgyi, É.: Temporally designed treatment of melanoma cells by ATRA and polyI.
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Oral presentations:

Gogolak P., Benko Sz., **Szabo A.**, Rajnavolgyi E.: Dendritic cell subsets as primary targets of vaccines. *3rd Semmering Conference 2007 on Challenges for vaccine development: Medical needs and social implication, Vienna, Austria, 2007.*

Szabo A.: Type I interferon production by dendritic cells: The role of intracellular helicases. *2nd Molecular Cell and Immune Biology (MCIB) Winter School, Krompachy, Slovakia, 6.-9. January, 2009.*

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Szabo A., Bene K., Gogolak P., Dezso B., Rajnavolgyi E.: Dendritic cell subsets as adjuvants of immunomodulatory vaccines. *25th Annual Meeting of the European Macrophage and Dendritic Cell Society (EMDS), Brussels, Belgium, 22.-24. September 2011.*

Szabo A., Bene K., Varga R.E., Lanyi A., Rethi B., Gogolak P., Rajnavolgyi E.: Human $CD1a^+$ dendritic cells mediate efficient anti-viral immune responses via RIG-I and MDA5 signaling. *75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award, Szeged, Hungary, 22.-25. March 2012.*

Szabo A., Osman R.M., Bacskai I., Rajnavolgyi E.: Consecutive treatment of human melanoma cells by ATRA and polyI:C results in distinct inflammatory cytokine and chemokine responses via TLR3 and MDA5. *75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award, Szeged, Hungary, 22.-25. March 2012.*

Szabo A., Osman R.M., Bacskai I., Kumar B.V., Agod Z., Lanyi A., Gogolak P., Rajnavolgyi E.: Temporally designed treatment of melanoma cells by ATRA and polyI:C results in enhanced chemokine and IFNß secretion controlled differently by TLR3 and MDA5. *3rd European Congress of Immunology (ECI), Glasgow, Scotland, 5.-8. September 2012.*

8. Keywords

Dendritic cell, Toll-like receptor, RIG-I-like receptor, NF-κB, IRF3, signaling, innate immunity

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