SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY (PHD)

# Investigating the activity and regulatory mechanisms of cytosolic sensors in plasmacytoid dendritic cells

by

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## I. INTRODUCTION

Plasmacytoid dendritic cells (pDC) are a very rare but essential subtype of dendritic cells (DC), our body's sentinels, and are key components of the antiviral response as professional type I interferon (IFN) producing cells. However, under pathological conditions, overactivation of pDCs and overproduction of IFNs can lead to chronic tissue damage and autoimmune reactions, and thus pDCs may play a role in the pathogenesis of several diseases.

DCs perform their main immunological functions through the activation of their pattern recognition receptors (PRRs). The dominant role of endosomal Toll-like receptor (TLR) 7 and TLR9 in pDCs has long been known, but little is currently known about their cytoplasmic receptors. Recently, we have shown that cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) also function in pDCs, and thus hypothesized that in addition to RLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) may also function in pDCs, determining the outcome of pDC-mediated immune responses. Thus, in our work, we aimed to comprehensively characterize the functionality of regulatory and inflammasome-forming NLRs in human pDCs. Furthermore, we wanted to identify those factors that may lead to NLRP3 inflammasome formation and inflammasome-associated interleukin (IL)-1β production in human pDCs.

Since overactivity of pDCs and the inflammasome can also lead to the development of autoimmune diseases, regulation and fine-tuning of IFN and IL-1β production is essential to avoid uncontrolled inflammation. Inflammatory responses and the activation of DCs and their cytokine secretion are regulated by several mechanisms, including receptor interactions. Thus, we wanted to study the regulatory role of regulatory NLRs in the RLR-mediated antiviral and inflammatory responses of pDCs, which would emphasize the importance of interactions of cytosolic sensors in pDCs. Furthermore, we wanted to investigate whether pDCs also exhibit the regulatory, reciprocal negative interaction between antiviral type I IFN and antibacterial NLRP3-dependent IL-1β pathways, which has been previously observed in other innate immune cells.

Our results may provide new insights into the biology of pDCs and are likely to contribute to a more comprehensive understanding of the role of pDCs in antiviral response and autoimmune diseases, which may lead to the development of new therapeutic approaches and increase the efficacy of current therapies.

## II. THEORETICAL BACKGROUND

### II.1 The significance of pDCs

PDCs are a special subgroup of DCs. Despite their extremely low incidence in the blood – they represent only 0.2-0.8% of peripheral mononuclear cells (PBMC) - they play a crucial role in antiviral immunity through their outstanding type I IFN producing capacity. Their role in human diseases is also mainly related to their IFN producing capcacity. The lack of their type I IFN producing capacity can lead to the development of persistent viral infections and, in an immunosuppressed state induced by cancer cells, which is also associated with inhibited IFN secretion, they can promote tumour growth. Conversely, overactivated pDCs may lead to the development of autoimmune pathologies associated with high IFN signature.

The endosomal nucleic acid sensing TLR7 and TLR9 are highly expressed in pDCs and are essential for the high production of type I IFN in pDCs. TLR7 recognizes single-stranded RNA, whereas TLR9 recognizes DNA containing unmethylated CpG motifs. Furthermore, human pDCs also express the cell surface receptors TLR1/2, TLR6 and TLR10. Moreover, the C-type lectin receptors mannose and dectin-1, 2, 3 receptors and the CD32 scavenger receptor have also been described in them. In addition to membrane-bound receptors, several cytosolic nucleic acid sensors also function in pDCs. Our group has described that RIG-I, a member of the RLRs, and melanoma differentiation associated protein (MDA) 5 are also active in pDCs. Furthermore, the cGAS-STING pathway is also functional in these cells. However, the expression profile of NLRs in pDC is less known.

The activation of PRRs in human pDCs leads to the production of type I IFNs and pro-inflammatory cytokines (TNF-α, IL-6) and chemokines (IL-8, CCL3, CCL4, CXCL10) via the interferon regulatory factor (IRF) and nuclear factor-kappaB (NF-κB) pathways, and thus to their active participation in various immunological processes.

### II.2 Cytosolic sensors

DCs, including pDCs, are equipped with various PRRs. PRRs are expressed primarily by antigen-presenting cells, but are also found in other innate immune cells and non-immune cells. The different PRRs recognise pathogen-associated molecular patterns (PAMP) from extra- and intracellular pathogens and damage-associated molecular patterns (DAMP).

A large number of intracellular receptors are specialised in the recognition of intrinsic PAMPs of pathogens, mainly nucleic acids. These include cytosolic PRRs such as RLRs, AIM2-like receptors (ALRs) and STING-activating cGAS, DAI and DDX41. RLRs recognise cytosolic RNA, while ALRs and proteins signalling via STING recognise cytosolic DNA. Some

types of NLRs may also be able to recognise cytosolic nucleic acids, but this is not the main function of these receptors and the outcome of their activation is different.

Among the cytosolic nucleic acid sensors mentioned above, our resrearch group is mainly interested in studying the functions of RLRs. While cDCs express these receptors at basal levels, their expression in pDCs is activation-dependent. RLRs are RNA sensors that recognize viral replication intermediates and are essential for the induction of the antiviral immune response and type I IFNs. There are three members of the protein family: RIG-I, MDA5 and the laboratory of genetics and physiology 2 protein. RIG-I mainly recognises short double-stranded (ds) RNA sequences containing 5′triphosphate (5′ppp) groups, whereas MDA5 mainly recognises long dsRNAs. After RNA binding and oligomerisation, RIG-I and MDA5 interact with their adaptor, mitochondrial antiviral-signaling protein (MAVS). MAVS then activates TANK-binding kinase 1 (TBK1) and IκB kinase (IKK), leading to the activation of IRF3 and IRF7, which in turn, together with NF-κB, induce the transcription of type I IFNs and other antiviral genes. Overall, this results in apoptosis of infected cells, protection of surrounding cells and activation of antigen-specific antiviral immune response. Thus, RLRs play a prominent role in controlling infections caused by RNA viruses.

NLRs also belong to the large family of intracellular sensors. Based on their specific functions, NLRs can be classified into four subgroups. Several NLRs are capable of forming multi-protein complexes, inflammasomes, whose activation is required for the formation of mature, biologically active forms of pro-IL-1β and pro-IL-18 pro-inflammatory cytokines. In contrast, regulatory NLRs regulate intracellular signalling cascades - such as NF-κB, type I IFN or mitogen-activated protein kinase (MAPK) pathways - induced by other PRRs. A special subset of NLRs is composed of NLRC5 and CIITA proteins, which act as enhanosomes and regulate the transcription of MHC genes. In addition, upon bacterial stimulation, some NLRs, such as NOD1 and NOD2, are able to bind the autophagy modulating protein ATGL16L1 to the cell membrane, which promotes autophagosome formation. Since little is known about the NLR expression profile of pDCs, our group aimed to elucidate the regulatory and inflammasome-forming NLR expression of these cells.

### *II.2.1 The NLRC5 and the NLRX1 regulatory NLRs*

Regulatory NLRs can act synergistically or antagonistically on signalling pathways induced by other PRRs, their effect depends on the type of cell, receptor or ligand. NLRs can regulate NF-κB, MAPK and type I IFN pathways, among others. NLRC5 and NLRX1 proteins belong to the group of regulatory NLRs.

While the role of NLRC5 as a transactivator is relatively well documented in the regulation of MHC I gene expression, little is known about its cytosolic functions and the results to date are controversial. NLRC5 may regulate several signalling pathways, for example, it may promote tumour formation by interfering with β-catenin, TGF-β and Akt pathways, and its role in the regulation of NLRP3 inflammasome activation has also been suggested, and it has been described to affect NF-κB signalling and type I IFN production. The role of NLRC5 in regulating signalling pathways is discussed below.

Mitochondrial NLRX1 has diverse functions depending on its localization and interaction partners. NLRX1 regulates NF-κB and type I IFN signaling, influences the MAPK pathway, modulates the production of reactive oxygen species (ROS), and affects major metabolic pathways as well as autophagy and cell death. In addition to regulating host cell-pathogen interactions, NLRX1 may also act as a tumour suppressor or, on the contrary, may promote metastasis formation. It has also been shown to play a protective role and inhibit the development and progression of various autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS) and inflammatory bowel diseases (IBD). NLRX1 may also contribute to the pathogenesis of metabolic disorders. The diverse effects of NLRX1 may be explained by the fact that its regulatory role may be highly cell type specific and the characteristics of regulation may be determined by the specific functional activity or metabolic profile of a given cell type. The regulatory role of NLRX1 in signalling pathways is discussed below.

### *II.2.2 The NLRP3 inflammasome*

Of the inflammasomes that activate caspase-1, NLRP3 is the best characterized. NLRP3 is a cytosolic protein expressed by many cell types, including neutrophils, macrophages, microglia, lymphocytes, epithelial cells, osteoblasts, neurons and DCs.

Canonical NLRP3 inflammasome activation occurs in two steps in most cell types. First, during the so-called priming phase, stimulation of cytokine receptors or other PRRs activates the NF-κB and activator protein-1 pathways, which enhance the transcription of NLRP3, pro-IL-1β and pro-IL-18. However, recent studies suggest that priming not only affects transcription but also regulates post-translational modifications of NLRP3. Subsequently, NLRP3 is activated and oligomerized in response to a secondary stimulus. Unlike most PRRs, NLRP3 can be activated by a wide range of stimuli. These may be DAMPs of endogenous or exogenous origin, such as crystals (e.g. uric acid crystals, silica, asbestos), extracellular adenosine triphosphate (ATP), heme, $K^+$ ionophores, toxins, and PAMPs from viruses, bacteria, fungi and

protozoa. The active caspase-1 then cleaves pro-IL-1β and pro-IL-18 to create the biologically active, mature form of these proteins. Caspase-1 also cleaves and activates the membrane pore-forming protein gasdermin D, which triggers a form of programmed cell death called pyroptosis. Impairment of the cell's osmotic potential leads to the release of intracellular contents, including the inflammatory cytokines IL-1β and IL-18, through the membrane pores. However, IL-1β and IL-18 can also be secreted, for example, via microvesicles, exosomes or secretory lysosomes independently of pyroptosis, in response to less potent activation signals.

The inflammation-inducing effects of IL-1β and IL-18 cytokines released upon activation are central to antimicrobial immune responses. However, polymorphisms of NLRP3 can lead to abnormal activation of the NLRP3 inflammasome. Increased secretion of IL-1β and IL-18 by innate immune cells induces systemic inflammation, which ultimately results in chronic tissue damage and the development of autoinflammatory or autoimmune diseases. Several autoimmune diseases, such as SLE, psoriasis, MS, rheumatoid arthritis (RA) or IBD have been associated with NLRP3 inflammasome overactivity.

### II.3 Controlling the activity of cytosolic sensors

Effector functions mediated by the activation of cells' cytosolic receptors are of paramount importance as they are required for effective immune responses and clearance of pathogens. However, over-activation and prolonged activation of these receptors can lead to tissue damage and development of various pathological conditions, thus their activation has to be regulated.

Our research group has previously explored some possible mechanisms for fine-tuning RLR activation in human DCs.We have described that mitochondrial ROS significantly enhances RIG-I mediated type I IFN production in pDCs through the enhancement of MAVS expression and Akt and IRF3 phosphorylation. In addition, we have shown that oxidative phosphorylation is also essential for IFN production in RIG-I agonist-treated pDCs. We further confirmed that mammalian rapamycin target activity is also required for the RLR-induced antiviral and inflammatory response of monocyte-derived DCs (moDCs) and pDCs, as it is essential for the phosphorylation of TBK1. However, the role of regulatory NLRs in the RLR-mediated immune response of DCs remains to be elucidated.

Therefore, we investigated the regulatory mechanisms based on receptor-receptor interactions of cytosolic sensors on the one hand and on interactions of cytokine responses produced as a consequence of receptor activition on the other hand in human DCs.

### *II.3.1 Regulation based on receptor-receptor interactions*

#### *II.3.1.1 Role of NLRC5 in the regulation of RLR-mediated immune response*

NLRC5 may influnce both innate and adaptive immune responses, although the results to date appear to be contradictory. It has been described that NLRC5 may be a positive regulator of type I IFN response during viral infections, as silencing of NLRC5 has been shown to impair IFN-α expression following Cytomegalovirus infection in human fibroblasts. Similar results have been reported by another group, who found that the absence of NLRC5 attenuated the Sendai virus and polyinosin:polycytidylic acid (polyI:C)-induced type I IFN response in THP1 cells and human primary dermal fibroblasts. In addition, Ranjan and colleagues showed in human airway epithelial cells overexpressing NLRC5 that the binding of NLRC5 to RIG-I resulted in increased IFN-β secretion and reduced influenza virus replication.

In contrast, another group of researchers showed that NLRC5 directly interacts with IKK and RIG-I to negatively regulate NF-κB and type I IFN pathways in different cell lines and primary cells. Namely, following viral infection or stimulation by specific agonists, NLRC5 competes with MAVS for binding, leading to the inhibition of IRF3 activation. The same group has shown that in embryonic fibroblasts, peritoneal and bone marrow-derived macrophages from NLRC5-deficient mice, IL-6 and IFN-β production is increased following vesicular stomatitis virus (VSV) infection or LPS stimulation, whereas bone marrow-derived DCs are unaffected by the absence of NLRC5. Interestingly, Kumar et al. found that NLRC5 does not play a role in cytokine induction in either bone marrow-derived macrophages or DCs in response to viral and bacterial infections. Furthermore, NLRC5 was also shown to bind directly to TBK1 and inhibit TBK1-mediated IFN-β promoter activation in HEK293T cells.

Taken together, the *in vitro* data strongly suggest that NLRC5 may regulate type I IFN response in the cytosol through interactions with RIG-I, MDA5 and TBK1. The discrepancy between the positive and negative effects may be explained by the use of different cell types and mouse models, overexpression or gene ablation techniques.

#### *II.3.1.2 Role of NLRX1 in the regulation of RLR-mediated immune response*

Increasing evidence suggests that NLR family members play a key role in antiviral immune responses. Moore and colleagues have demonstrated in their *in vitro* system using HeLa and HEK293T cells that NLRX1 is a negative regulator of RIG-I and possibly MDA5-induced antiviral signalling by competing with these receptors for binding MAVS. Furthermore, under *in vivo* conditions, Allen et al. also demonstrated a negative regulatory role for NLRX1 in virus-induced inflammation in a mouse model of influenza infection. Their

results showed that although NLRX1-deficient mice eliminated the virus more rapidly, they were characterized by increased IL-6 and IFN-β levels, more severe lung injury and morbidity.

NLRX1 may also indirectly inhibit RIG-I-MAVS signalling. In a human hepatoma cell line, the nucleotide-binding domain of NLRX1 was shown to inhibit hepatitis C virus-induced RIG-I-MAVS signaling by recruiting PCBP2 protein to MAVS. PCBP2 induces K48-coupled polyubiquitination and subsequent proteasomal degradation of MAVS, thus limiting type I IFN production. In addition, NLRX1 can be used by many other viruses to modulate the antiviral response of the host and thus promote viral replication and survival.

In contrast, several research groups have reported that the absence of NLRX1 does not affect RLR signalling. The absence of NLRX1 has no effect on antiviral and inflammatory responses in bone marrow-derived macrophages and mouse embryonic fibroblasts from NLRX1-deficient mice following Sendai virus infection. Furthermore, NLRX1 silenced HEK293T cells were characterized by a normal type I IFN response following Sendai virus exposure.

These previous results clearly indicate that the mode of NLRX1-mediated regulation of antiviral responses is cell type specific and determined by virus characteristics that may directly or indirectly modulate NLRX1 function.

### *II.3.2 Regulation based on interactions of cytokine responses following receptor activation*

Since the IL-1 family of cytokines are extremely potent, regulatory mechanisms are "built into the system" to regulate their production and secretion at multiple levels. The activation of the NLRP3 inflammasome may be regulated by cytokines and protein interactions. Type I IFNs have immunosuppressive properties in chronic viral and bacterial infections. For this reason they are also used as "anti-inflammatory agents", for example in the treatment of MS. The main mechanism by which IFNs exert their immunosuppressive effects is through inhibition of the inflammasome-IL-1β axis.

Type I IFNs can directly inhibit IL-1 signalling by inducing IL-1 receptor antagonist expression and also downregulate IL-1β secretion in a signal transducer and activator of transcription (STAT) 1-dependent manner. In terms of mechanism of inhibition, IFN-β first induces IL-10 expression. IFN-β-induced IL-10 then binds to the IL-10 receptor, which activates STAT3 signaling and inhibits pro-IL-1α and pro-IL-1β mRNA expression. IFN-β also induces the expression of cholesterol 25-hydroxylase (CH25H). This gene encodes an enzyme that converts cholesterol to 25-hydroxycholesterol (25-HC), a lipid with antiviral properties. 25-HC is a ligand for sterol regulatory element binding protein (SREBP) and inhibits the

activity of SREBP, which is required for the induction of IL-1β transcription. Furthermore, IFNs also stimulate the expression of iNOS. In turn, the produced nitric oxide nitrosylates NLRP3, which prevents NLRP3 oligomerisation. IFN-β can also inhibit NLRP3 activation by inhibiting ROS production. In macrophages, IFN-β induces the expression of cytokine signaling suppressor 1 (SOCS1), which inhibits Rac1-mediated ROS generation that would be required for NLRP3 activation.

In addition to the proteins that make up the inflammasome complex, other proteins may also interact with NLRP3. Pyrin-only proteins (POPs) and CARD-only proteins (COPs) are small cytoplasmic decoy proteins that contain a single homotypic protein binding domain and their main function is to regulate inflammation. POP1 and POP2 bind to ASC and inhibit NLRP3 activation. In contrast, the five human COPs regulate caspase-1 activation.

Interestingly, IL-1 can also inhibit the IFN pathway. In hepatocytes, IL-1β is able to inhibit IFNα/β-induced STAT1 phosphorylation through a proteasome-dependent mechanism. Another study has also described IL-1-dependent inhibition of IFN-β production in human fibroblasts. Prostaglandin E2 may underlie this inhibition. Thus, it can be concluded that there may be a mutual negative interaction between type I IFN and NLRP3-dependent IL-1β pathways.

Thus, in our work, in addition to exploring the expression profile of cytosolic receptors in human pDCs and the regulatory mechanisms of these receptors, we were also curious to see whether they are expressed in professional type I IFN-producing cells, i.e. pDCs, whether the NLRP3 pathway could be functional at all and, if so, what conditions are required for its activation, and what interactions might exist between type I IFN and NLRP3-dependent IL-1β pathways, which have not yet been investigated in these cells.

## III. AIMS OF THE STUDY

During our studies we wanted to investigate the expression pattern of cytosolic, reglatory NLRs and their role in the regulation of the RLR signaling pathway in human pDCs compared to moDCs, in *in vitro* model systems. Furthermore, among the inflammasome forming-NLRs, we also aimed to elucidate the conditions for NLRP3 inflammasome activation and its regulation by type I IFNs in human pDCs. In our experiments, we sought to answer the following questions:

I/1 What are the baseline and activation-induced NLRX1 and NLRC5 expression levels in human pDCs and moDCs?
I/2 How does silencing of NLRX1 and NLRC5 affect the RLR-induced antiviral and inflammatory response of pDCs and moDCs?

II/1 What is the baseline expression of NLRP3 inflammasome components in human pDCs and how is this affected by different activation signals?
II/2 Which secondary signals are required for inflammasome activation in pDCs?
II/3 What interactions exist between the type I IFN and the NLRP3-dependent IL-1β secretory pathways in pDCs?

## IV. MATERIALS AND METHODS

### IV.1 Cell lines and culture conditions

The human pDC cell line (GEN2.2) used for our experiments was provided by Dr. Joel Plumas and Dr. Laurence Chaperot (Research and Development Laboratory, French Blood Bank Rhône-Alpes, Grenoble, France, CNCMI number 2938). The GEN2.2 cell line was grown on a layer of mitomycin C (Sigma-Aldrich, St. Louis, MO, USA)-treated murine MS5 feeder cells (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, cat. no. ACC 441) in complete RPMI 1640 medium (Sigma-Aldrich). For experiments, GEN2.2 cells were removed from the feeder layer and seeded in 24-well plates at a concentration of $5\times10^5$ cells/500 µl in complete RPMI 1640 medium. Cells were grown and incubated at 37°C in 5% CO2 humidified atmosphere. For GEN2.2 and MS5 cell lines, Mycoplasma contamination was excluded with MycoAlert® Mycoplasma Detection Kit (Lonza, Basel, Switzerland), the cell cultures were monitored once a month.

### IV.2 Collection and processing of human peripheral blood samples

The blood samples from psoriasis patients were provided by the Regenerative Medicine and Cellular Pharmacology Research Laboratory (Department of Dermatology and Allergology, University of Szeged). The sample collection was approved by the National Public Health and Medical Officer Service (NPHMOS) and the National Medical Research Council (administrative number: 13740-5/2021/EÜIG and 4969; 90/2021-SZTE IKEB, protocol code: PSO-CELL-01).

Men or women aged 25 to 65 years suffering from plaque psoriasis with a psoriasis area sensitivity index (PASI) greater than 15 were included in the study. Patients who received systemic treatment (biological or traditional) or total body phototherapy were excluded. Peripheral blood of healthy, gender and age-matched donors was used as a control in experiments with psoriasis samples. To characterize the NLRP3 pathway in healthy individuals, 23–55-year-old volunteers were selected for peripheral blood donation.

Then, 25 mL of peripheral blood was collected from psoriatic and healthy donors into 10 mL BD Vacutainer® Plastic whole blood tubes with K2EDTA (BD Biosciences, San Jose, CA, USA). The blood was diluted 1:1 with physiological saline (B. Braun, Melsungen, Germany) and then PBMCs were separated by Ficoll-Paque Plus (Cytiva Sweden AB, Uppsala, Sweden) gradient centrifugation. Freshly isolated PBMCs were seeded in FACS tubes at a density of $1 \times 10^6$ cells/ 500 mL complete RPMI 1640 medium (Sigma-Aldrich).

### IV.3 Isolation and differentiation of primary cells

Primary cells were isolated from human buffy coats derived from healthy blood donors, drawn at the Regional Blood Center of the National Blood 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, Faculty of Medicine (Debrecen, Hungary). The blood was diluted 1:1 with physiological saline and then Ficoll Paque Plus gradient centrifugation was used to separate the PBMC layer.

Primary human pDCs were isolated from PBMCs by positive selection using the human CD304 (BDCA-4/Neuropilin-1) MicroBead Kit (Miltenyi Biotech, Bergish Gladbach, Germany). After magnetic separation, the purity (>98%) of the isolated pDC population was confirmed by flow cytometry. Cells were cultured in 48-well cell culture plates at a density of $5 \times 10^5$ cells/500 μl in complete RPMI 1640 medium. The medium also contained 50 ng/ml recombinant human IL-3 (Peprotech EC, Brussels, Belgium). Cells were incubated at 37°C in 5% CO2 humidified atmosphere.

For differentiation of primary human macrophages, monocytes were obtained by positive selection from the PBMC layer using anti-CD14-conjugated microbeads (Miltenyi Biotech). After magnetic separation, the purity of the isolated monocyte population (>98%) was confirmed by flow cytometry. Monocytes were cultured in 24-well cell culture plates at a density of $5\times10^5$ cells/500 μl in complete RPMI 1640 medium. The medium was supplemented with 50 ng/ml recombinant human macrophage colony stimulating factor (M-CSF, Peprotech). Cells were differentiated for 5 days and then the macrophages were activated with 250 ng/ml Ultrapure LPS (*Escherichia coli* 0111:B4, InvivoGen, San Diego, CA, USA) for the indicated times. Cells were incubated at 37°C in 5% CO2 humidified atmosphere.

For differentiation of moDCs, freshly isolated monocytes were seeded in 24-well cell culture plates at a density of $10^6$ cells/ml in complete RPMI 1640 medium. The medium also contained 80 ng/ml GM-CSF (Gentaur Molecular Products, London, UK) and 50 ng/ml IL-4 (PeproTech). Cells were incubated at 37°C in 5% CO2 humidified atmosphere.

### IV.4 Gene silencing experiments

GEN2.2 cells or freshly isolated monocytes were left untreated (control), transfected with no siRNA (mock), NLRX1- or NLRC5-specific Silencer Select Validated siRNAs and Silencer Select Negative Control siRNA (scr, Life Technologies) in Opti-MEM medium (Life Technologies) in 4-mm cuvettes (Bio-Rad Laboratories GmbH, Munich, Germany) using

GenePulser Xcell instrument (Bio-Rad). Following transfection the cells were seeded in cell culture plates for activation or differentiation as previously described.

**IV.5 Cell stimulation**

To induce RIG-I expression GEN2.2 cells were incubated with 0.25 μM CpG-A (ODN 2216, Hycult Biotech, Uden, The Netherlands) for 16 h. Cells were then washed, re-seeded in 24-well plates in fresh complete RPMI 1640 medium, and stimulated with 5′ppp-dsRNA (InvivoGen), a specific agonist of RIG-I, or high molecular weight polyI:C (InvivoGen), a RIG-I/MDA5 agonist, both complexed with the transfection reagent LyoVecTM (InvivoGen) according to the manufacturer's recommendations. To induce RIG-I in primary pDCs, cells were treated with 2.5 μM CpG-A for 16 h prior to western blot analysis. For moDCs, half of the medium was removed, replaced by fresh medium then stimulation with 5′ppp-dsRNA/LyoVecTM or polyI:C-HMW/LyoVecTM complex was performed as described for GEN2.2 cells. For live virus infection untreated and CpG-A pre-treated GEN2.2 cells and moDCs were infected with VSV (MOI 1 and 10), respectively for 18 or 24 h.

To prime the NLRP3 inflammasome, GEN2.2 cells were incubated with 1 μM CpG-A, 1 μM CpG-B (ODN 2006, Hycult Biotech) or 5 μg/ml Imiquimod (InvivoGen) in fresh, complete RPMI 1640 medium for the indicated times. In order to activate the inflammasome secondary activation signals including 20 μM nigericin (InvivoGen), 5 mM ATP (Invivogen), 250 μg/mL Alum (Thermo Scientific,), 300 μg/mL MSU (Sigma-Aldrich) or 0.5 μg/mL antimycin A (AMA, Sigma-Aldrich) were added to the cells after priming without a washing step and then the cells were incubated for 1, 2, 4 or 6 h.

In separate experiments, GEN2.2 cells were primed with *E. coli* ATCC 11775, *Bacillus subtilis* ATCC6051, *Lactobacillus rhamnosus* ATCC53103, *Candida albicans* ATCC10231, VSV (Indiana serotype) or HSV 1 (KOS serotype, ATCC-VR-1493) microbes. A MOI of 10 was used for bacteria and viruses, while a MOI of 0.01 was used for *Candida albicans*. Bacteria and *Candida albicans* were provided by Dr. Valter Péter Pfliegler (Faculty of Science and Technology, Department of Molecular Biotechnology and Microbiology, University of Debrecen, Debrecen, Hungary). *E. coli* and *Bacillus subtilis* were cultured on LB agar, *Lactobacillus rhamnosus* on MRSA medium and *Candida albicans* on YPD agar, and cell suspensions were counted using a Thoma chamber. The viruses were obtained from Dr. Eszter Csoma (University of Debrecen, Department of Medical Microbiology, Debrecen, Hungary), African green monkey kidney epithelial Vero cell line (ATCC-CCL-81, Manassas, VA, USA) was used for virus propagation, and plaque assay was performed to determine the viral titer of

the suspension. After microbial priming, cells were incubated with 20 μM nigericin (InvivoGen) until the indicated times. In some experiments, cells were pre-treated with VSV or HSV (MOI 10) for 3 h before *E. coli* and nigericin treatments (InvivoGen).

In parallel experiments, 1 μM MCC950 (Invivogen) or 20 μM Z-YVAD-FMK (BioVision Incorporated, Milpitas, CA, USA) was added to the cells for the last 30 min of priming, then cells were exposed to the secondary signals without any washing step.

Experiments with CpG-B were also repeated in the presence of 50 μg/ml recombinant human IFN-α (Abcam, Cambridge, UK). IFN-α was added to the cells 30 min before CpG-B treatment, and then the cells were treated with nigericin without a washing step. Similarly, experiments with CpG-A were repeated in the presence of 10, 50 or 100 ng/ml recombinant human IL-1β (Peprotech). IL-1β was added to the cells 30 min before the CpG-A treatment.

**IV.6 Flow cytometric analysis of the cells**

Flow cytometry was used to analyse the phenotype of moDCs. The analysis was performed using antibodies specific to their cell surface markers and their isotype-matched control antibodies (all from BioLegend, San Diego, CA, USA). The viability of electroporated moDCs was also determined by flow cytometry using 7-aminoactinomycin-D (7-AAD, 10 μg/ml, Sigma-Aldrich) staining. Fluorescence intensity was measured using a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

In samples from healthy blood donors and psoriasis patients, intracellular staining and flow cytometry was used to assess the expression of inflammasome components in the primary monocyte and pDC populations. To this end, PBMCs were first stained with anti-CD14-FITC (BioLegend) and anti-BDCA4-APC (CD304, Neuropilin-1, BioLegend), and then fixed and permeabilized using the BD Cytofix/CytopermTM Plus Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's instructions. After washing, cells were incubated with antibodies specific for the inflammasome components for 30 min. After washing, the cells were incubated with PE-conjugated donkey anti-rabbit IgG1 secondary antibody (clone: Poly4064, BioLegend) for 30 min. Rabbit IgG (clone DA1E, Cell Signaling) was used as isotype control. As a final step, cells were taken up in 2% paraformaldehyde (Alfa Aesar, Haverhill, MA, USA). Fluorescence intensity was measured using a FACS Calibur flow cytometer and data were evaluated using FlowJo software. Monocytes were identified as $CD14^+$ cells and $BDCA4^+$ cells in the 400-600 FSC region were considered as pDCs.

**IV.7 Quantitative real-time polymerase chain reaction (PCR)**

RNA was isolated from $5\times10^5$ GEN2.2 cells or $1\times10^6$ PBMCs using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Total RNA was treated with DNase I (Thermo Fisher Scientific) to exclude amplification of genomic DNA then reverse transcribed into cDNA using the Applied Biosystems High Capacity cDNA RT Kit (Thermo Fisher Scientific). The concentration and purity of RNA samples were measured by the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Gene expression assays were obtained from Thermo Fisher Scientific and Integrated DNA Technologies (Coralville, IA, USA). Quantitative PCR was performed using the ABI StepOne Real-Time PCR system (Thermo Fisher Scientific) and cycle thresholds were determined using StepOne v2.1 software (Thermo Fisher Scientific). Normalized expression of the respective mRNA was obtained by normalization to the PPIA housekeeping gene in all experiments.

**IV.8 Western blot analysis**

For western blotting, $5\times10^5$ GEN2.2 cells or moDCs were lysed in Laemmli buffer and denatured at 100°C for 10 min. Lysates were then separated on 7.5, 10 or 15% SDS-PAGE gels and electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories GmbH). Nonspecific binding sites were blocked with TBS Tween buffer (50 mM Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.4) containing 5% non-fat dry milk. Membranes were incubated with antibodies specific for the proteins of interest at a dilution of 1:10000. β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) was used as loading control at 1:5000 dilution. For the detection of primary antibodies, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (Cat. No. 1721011, Bio-Rad) or anti-rabbit (Cat. No. NA934, GE Healthcare, Chicago, IL, USA) secondary antibodies at 1:5000 and 1:10000 dilutions, respectively. The protein bands were then visualized using an enhanced chemiluminescent (ECL) system with SuperSignal West Pico or Femto chemiluminescent substrate (Thermo Fisher Scientific) and X-ray film exposure. Densitometric analysis of immunoreactive bands was performed using Image Studio Lite software version 5.2 (LI-COR Biosciences, Lincoln, NE, USA).

**IV.9 Enzyme-linked immunosorbent assay (ELISA)**

Supernatants from cell cultures were collected at the indicated time points and the levels of IL-1β, TNF, IL-6 and IL-8 cytokines and chemokines were determined using BD OptEIA

human ELISA kits (all BD Biosciences). IFN-α and IFN-β levels were measured using VeriKineTM Human Interferon Alpha and Interferon Beta ELISA kits (PBL Interferon Sources, Piscataway, NJ, USA). Tests were performed according to the manufacturer's instructions. Absorbance measurements were performed with a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 450 nm.

**IV.10 Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance (ANOVA) or unpaired Student t-test followed by Bonferroni post hoc test or paired Student t-test. Data analysis was performed using GraphPad Prism v.6 software (GraphPad Software Inc., La Jolla, CA, USA). All experiments were repeated at least three times. Differences were considered statistically significant at $p < 0.05$.

## V.RESULTS

### V.1 Investigating the regulatory role of regulatory NLRs in the RLR-mediated antiviral and inflammatory response of human DCs

#### *V.1.1 Exploring the NLRC5 and the NLRX1 expression in unactivated and TLR9 ligand CpG-A activated human GEN2.2 pDC cell line and primary pDCs*

To explore the possible interactions between NLR and RLR receptors, we first examined the expression patterns of these receptors in unactivated and CpG-A activated cells. Since only a very low percentage of primary human pDCs are found in peripheral blood, we performed most of our experiments on the GEN2.2 human pDC cell line. Our results show that NLRC5 is not expressed in unactivated cells, but its level is significantly increased by CpG-A treatment. In contrast, NLRX1 is constitutively present in cells. Examining the RLR pathway, we observed increased expression of RIG-I and MDA5 upon CpG-A treatment, whereas the level of the MAVS adaptor protein was decreased. TBK1, a key regulatory molecule in the MAVS-IFN signaling pathway, was not affected by CpG-A stimulation. Similar results were obtained for freshly isolated primary human pDCs.

#### *V.1.2 Investigating the role of NLRC5 and NLRX1 in the RLR-induced type I IFN and pro-inflammatory response of GEN2.2 cells*

We wondered whether NLRs could regulate the RIG-I-mediated antiviral responses of pDCs, and therefore performed siRNA-mediated gene silencing. The siRNAs significantly reduced (>80%) the levels of NLRC5 and NLRX1 proteins in unactivated and activated cells, respectively. Our results also showed that siRNA transfection did not alter the expression pattern of RLR signaling proteins (RIG-I, MDA5, MAVS).

After transfection, cells unactivated or pretreated with CpG-A were activated with the RIG-I specific ligand 5′ppp-dsRNA. Interestingly, the absence of NLRC5 and NLRX1 enhanced type I IFN production following 5′ppp-dsRNA treatment. This suggests that both NLRC5 and NLRX1 play a negative regulatory role in the RIG-I-mediated antiviral response of human pDCs. In contrast to type I IFNs, the secretion of TNF, IL-6 and IL-8 was not affected by silencing of NLRC5 or NLRX1. RIG-I stimulation also did not induce IκBα degradation. This may suggest that in pDCs, RIG-I induces primarily the production of type I IFNs as opposed to pro-inflammatory cytokines. In similar experiments, following CpG-A pretreatment, cells were stimulated with high molecular weight polyI:C/LyoVec, which is mainly recognized by MDA5. Depletion of NLRC5 and NLRX1 also increased the polyI:C-

induced secretion of type I IFNs, but did not affect the production of pro-inflammatory cytokines.

To confirm the results of our studies with synthetic RLR agonists and to model the role of NLRs during viral infection, we also infected GEN2.2 cells with live RNA virus. VSV-induced type I IFN production was enhanced by silencing of both NLRC5 and NLRX1, whereas the production of pro-inflammatory cytokines was not affected by depletion of either NLR. These findings suggest that both NLRC5 and NLRX1 may be negative regulators of the RLR-induced type I IFN response of human pDCs, but play no role in the RLR activation-mediated pro-inflammatory cytokine and chemokine responses of pDCs.

### *V.1.3 Analysis of the NLRC5 and NLRX1 expression profile of moDCs*

To test whether our results are cell type specific, we repeated our experiments with moDCs. We differentiated moDCs *in vitro* from CD14+ monocytes isolated from human blood in the presence of GM-CSF and IL-4. Our results show a progressive increase in NLRC5 and NLRX1 levels during moDC differentiation. Likewise, the levels of RIG-I, MDA5, MAVS and TBK1 are also increased during monocyte-moDC differentiation.

When monocytes were transfected with NLRC5 or NLRX1 specific siRNAs, we observed a large decrease in expression of both NLRs, and silencing did not affect the expression of RIG-I, MDA5 and MAVS in immature 5-day-old moDCs. Our results suggest that the absence of NLRs does not affect the differentiation process of moDCs, and that 5-day-old moDCs were CD14 positive and CD209 negative. The expression of cell surface markers specific for DCs (CD1a, CD1c and CD11c) and the viability of moDCs were not affected by transfection, as confirmed by 7-AAD staining.

### *V.1.4 Elucidating the regulatory roles of NLRX1 and NLRC5 in the RLR-mediated antiviral and pro-inflammatory response of moDCs*

Following transfection, activated moDCs were stimulated with 5′ppp-dsRNA on day 5. The RIG-I-induced type I IFN response was unaffected by silencing of NLRC5, whereas silencing of NLRX1 enhanced it. Silencing of NLRC5 did not affect the secretion of pro-inflammatory cytokines by moDCs either. However, the pro-inflammatory response was enhanced in NLRX1-deficient cells, consistent with the increased NF-κB activity. The high molecular weight polyI:C-LyoVec complex also induced the production of type I IFNs and pro-inflammatory cytokines in moDCs.

In moDCs, VSV also induced type I IFN and pro-inflammatory cytokine production at low concentrations (MOI 1), which was enhanced by NLRX1 depletion and unaffected by NLRC5 deficiency. Taken together, our results suggest that NLRX1 also negatively regulates the RLR-mediated antiviral IFN and pro-inflammatory responses in moDCs, whereas NLRC5 has no regulatory role in these processes. Thus, both NLRX1 and NLRC5 may be able to regulate RLR-mediated type I IFN and pro-inflammatory responses in human DCs, while their regulatory roles may be cell-specific.

## V.2 Exploring the interactions between the type I IFN and the NLRP3-dependent IL-1β secretory pathways in human pDCs

### *V.2.1 Examining the expression of NLRP3 inflammasome components in unactivated and activated human GEN2.2 pDC cell lines and primary pDCs*

We first explored the kinetics of basal and activation-induced expression of NLRP3 inflammasome components in GEN2.2 cells. Our results show that the NLRP3 receptor, ASC and pro-caspase-1 can be detected in cells at basal levels, whereas pro-IL-1β is expressed only to a very low extent without an activation signal. When GEN2.2 cells were activated with synthetic TLR ligand receptors or with live *E. coli* bacteria, we found that distinct activators affected the expression levels of inflammasome components differently. The greatest difference was observed in the ability of the two TLR9 ligands, CpG-A and CpG-B, to induce pro-IL-1β production. CpG-B highly induced pro-IL-1β production in pDCs at early incubation time points, i.e. 3 and 6 h, whereas CpG-A treatment did not induce IL-1β expression at all. When cells were activated with the TLR7 ligand Imiquimod, pro-IL-1β expression showed prolonged kinetics. Similar to CpG-B, *E. coli* induced high levels of pro-IL-1β production. Thus, our results suggest that CpG-B and *E. coli* were the best priming signals for this cell line.

We also saw a similar baseline and activation profile for primary human pDCs. NLRP3, ASC and pro-caspase-1 were also expressed at basal levels in almost the entire pDC population. However, without an activation signal, cleaved caspase-1, pro-IL-1β and cleaved-IL-1β expression was negligible in cells similar to the cell line. Also in primary cells, the expression of pro-IL-1β was mainly detectable after CpG-B and Imiquimod pretreatment.

### *V.2.2 Identification of activation signals required for mature IL-1β secretion of the human GEN2.2 pDC cell line and primary pDCs*

Next, we were interested to determine whether pDCs could cleave and convert inactive pro-IL-1β to its mature form. In CpG-B pretreated cells, MSU, aluminium hydroxide, nigericin

and AMA, which enhances mitochondrial ROS production, were used as secondary activation signals. Activation with CpG-B alone was not sufficient to generate the cleaved form of IL-1β. For pDCs, only the potassium ionophore bacterial toxin nigericin proved to be a potent secondary signal. No NLRP3 inflammasome activation was observed in pDCs in the presence of ATP, as the cells do not express the P2X7 receptor. Since only nigericin significantly induced the expression of the cleaved form of IL-1β, we used this bacterial toxin as a secondary signal in our further experiments. Imiquimod pretreatment also induced mature IL-1β secretion in the presence of nigericin, however nigericin used in combination with CpG-A did not prove to be an activating signal for the NLRP3 pathway. Among the microbial stimuli used, only pathogenic *E. coli* induced mature IL-1β secretion in the presence of nigericin, whereas no significant changes were observed for *Bacillus subtilis, Lactobacillus rhamnosus* and *Candida albicans* activation. Since IL-1β secretion by pDCs was inhibited in the presence of the specific NLRP3 inhibitor, it can be concluded that mature IL-1β production by pDCs was mainly NLRP3-dependent in response to a combination of priming and secondary activation signals. A specific inhibitor of caspase-1 significantly reduced nigericin-induced IL-1β secretion by pDCs pretreated with CpG-B and Imiquimod. Furthermore, the levels of the p20 subunit generated during cleavage of caspase-1 were significantly reduced upon application of both inhibitors, suggesting that the activation of the NLRP3 inflammasome is indeed required for mature IL-1β secretion in the cell line, leading to the cleavage of pro-caspase-1. Also in the primary cells, CpG-B or Imiquimod pretreatment in combination with nigericin exposure resulted only in inflammasome activation, whereas CpG-A was shown to be a weak activation signal. Thus, only by combining the appropriate priming and secondary activation signal in human pDCs, NLRP3 inflammasome activation and the generation of the mature form of the IL-1β cytokine can be induced.

### *V.2.3 Analysing the NLRP3 inflammasome activity of pDCs in the presence of type I IFNs*

As the two ligands of the TLR9 receptor induced IL-1β production in pDCs to different extents, we hypothesized that this difference was due to the distinct ability of the two ligands to induce different levels of type I IFN production. CpG-A induced high levels of IFN-α secretion in cells, whereas CpG-B had a negligible effect. It has been described for several cell types that IFN-induced proteins can inhibit inflammasome activation. Therefore, pDC activation by CpG-B was also repeated in the presence of IFN-α. Pro- and mature IL-1β formation were inhibited in the presence of the cytokine IFN-α in GEN2.2 cells. Thus, it can be concluded that type I IFNs also inhibit the activity of the IL-1β pathway in pDCs. Interestingly,

in the presence of the IL-1β cytokine, CpG-A-induced type I IFN production significantly decreased. This suggests a mutual negative interaction between type I IFN and IL-1β pathways.

### *V.2.4 Investigating the effects of viruses inducing increased type I IFN production on the NLRP3 inflammasome activity of human pDCs*

Next, we examined the ability of viruses that induce high type I IFN production to induce pro-IL-1β production in pDCs compared to bacterial stimuli. GEN2.2 cells were treated with RNA and DNA viruses (VSV and HSV) and *E. coli*. Both RNA and DNA viruses induced high IFN-α secretion compared to *E. coli*, but viruses hardly induced pro-IL-1β production compared to *E. coli*. We then examined whether the presence of viruses affects *E. coli*-induced IL-1β production in pDCs. Our results showed that pre-treatment with viruses significantly reduced *E. coli*-induced pro-IL-1β expression and that mature IL-1β concentrations were lower. We also validated our results with primary pDCs using flow cytometry with intracellular protein staining. An inhibitory effect of viral pretreatment was also observed in primary pDCs. Our results suggest that bacteria-induced IL-1β production by pDCs is suppressed in the presence of RNA and DNA viruses, which is likely due to the inhibitory effects of virus-induced type I IFNs on NLRP3 activation.

### *V.2.5 Examining the NLRP3 inflammasome activity of pDCs in autoimmune diseases associated with high IFN signature*

Finally, we wished to explore how the IL-1β producing capacity of human pDCs is altered under pathological conditions in which cells are exposed to high levels of type I IFNs. Thus, we also investigated the activity of the IL-1β pathway in pDCs from patients with plaque-type psoriasis associated with high IFN levels, as both overactivation of pDCs and overproduction of type I IFNs are involved in the pathogenesis of the disease. Accordingly, higher levels of IFN-α were measured in serum samples from patients, and IL-1β, TNF and IL-6 concentrations were also elevated. In total PBMCs from patients, we observed increased expression of NLRP3, ASC, caspase-1 and IL1B mRNAs, whereas within the pDC population of patients, we observed reduced baseline NLRP3 pathway activity compared to pDCs from healthy individuals. In pDCs from psoriasis patients, CpG-B and Imiquimod treatment in the presence of nigericin resulted in significantly lower pro-IL-1β, cleaved-caspase-1 and cleaved IL-1β positivity compared to pDCs from healthy donors. Thus, it is likely that high levels of type I IFN pathway activity in patients' pDCs inhibit the IL-1β pathway.

We also examined the expression of NLRP3 inflammasome components in the $CD14^+$ monocyte population, as monocytes play an important role in the pathomechanism of psoriasis, but their IFN production capacity is more limited, and we wanted to test how this shapes their NLRP3 activity. The major difference between the two cell populations is that the patients' monocytes have high levels of cleaved caspase-1 even in the absence of an activation signal. In the presence of similar activation signals (CpG-A, CpG-B, Imiquimod and nigericin), we did not observe a significant decrease in the expression levels of cleaved caspase-1, pro-IL-1β and cleaved IL-1β proteins in the patients' monocytes compared to the pDC population. Thus, patients' monocytes were characterized by higher NLRP3 activity.

To investigate the mechanism underlying the inflammasome inhibitory activity of type I IFNs in pDCs, we examined the expression of IFN-α-induced NLRP3 inhibitory proteins in human pDCs. In pDCs, the expression of CH25H, SOCS1 and COP1 can be induced to a large extent when cells are treated with CpG-A, an activation signal that triggers increased type I IFN production. In contrast, CpG-B did not induce the expression of these proteins that can inhibit NLRP3 activation. PBMCs from psoriatic patients also expressed significantly higher levels of CH25H, SOCS1 and COP1 mRNA compared to healthy donors. Thus, it is likely that CH25H induced by high levels of type I IFNs may inhibit IL-1β transcription, SOCS1 may inhibit NLRP3 activation, and COP1 may limit caspase-1 activation in pDCs. In conclusion, the lower NLRP3-dependent IL-1β production capacity of pDCs is due to their activation-induced high type I IFN production capacity, which inhibits NLRP3 inflammasome activation.

## VI. DISCUSSION

During the course of immune responses the goal is the detection of inflammation provoking agents by various PRRs expressed in cells of the natural immune system, including DCs, which, upon recognition, trigger signalling pathways mediating the innate immune response. The RLRs we studied are cytosolic sensors that specialise in the recognition of viral nucleic acids and trigger the production of type I IFNs or other pro-inflammatory cytokines via the mitochondrial MAVS adaptor protein. Although RLR signalling is essential for the control of viral infections, this receptor pathway is also tightly regulated to prevent excessive immune responses. RLR-mediated signalling of innate immunity is regulated by a number of molecules. This regulation may also be based on receptor-receptor interactions, as other cytosolic receptors, including several members of the NLR family, have been described to serve as checkpoints for immune activation. For example, among the NLRs, NLRC5 and NLRX1 may also directly interact with proteins of the RIG-I-mediated antiviral signalling pathway and thus influence the outcome of the particular signalling pathway.

NLRX1 - localised on the mitochondrial outer membrane - initially became known as a negative regulator of antiviral responses by interacting with MAVS. Since then, several studies have examined the role of NLRX1 in antiviral signalling both *in vitro* and *in vivo,* but conflicting results have been obtained. The inconsistencies in these studies suggest that the role of NLRX1 may be highly cell-specific, and we therefore wished to investigate the effect of NLRX1 on the RLR pathway in human pDCs, which are the "conductors" of the antiviral response. The functional specificity of pDCs differs from their myeloid "counterparts", so we also used moDCs as controls in our studies. Our results suggest that NLRX1 is constitutively expressed in human pDCs in a similar manner to moDCs and affects RIG-I-mediated type I IFN production in these cell types, but that it had no effect on pro-inflammatory cytokine secretion in pDCs, whereas it enhanced it in moDCs. Thus, our results suggest that NLRX1 acts predominantly as a negative regulator of the RLR signaling pathway in human DCs, but its effect is cell-specific.

In addition to NLRX1, we also investigated the role of NLRC5 in the regulation of the RLR pathway. NLRC5 expression is progressively increased in differentiating moDCs and also in pDCs upon TLR9 activation. Recent studies suggest that NLRC5 is involved in the modulation of the antiviral immune response, although the results are rather ambiguous regarding its regulatory role, similar to NLRX1. The role of NLRC5 in pDCs has not been investigated so far, however, our own results suggest that it negatively regulates RLR-induced type I IFN production in pDCs. However, IFN production by moDCs was not affected by

NLRC5 silencing. Furthermore, the absence of NLRC5 had no effect on the RLR-mediated pro-inflammatory cytokine response in either DC subtype. Taken together, our results suggest that NLRC5 negatively regulates only RLR-mediated type I IFN responses in human pDCs.

Thus, our work has shown that interactions between RLRs and regulatory NLRs also function in human pDCs, mainly with respect to the regulation of type I IFN production. The regulatory effect of regulatory NLRs is likely to be most pronounced in those cellular functions that are prioritized by the cell. Thus, the main function of regulatory NLRs is presumably to provide negative feedback mechanisms in a cell-type specific manner, regulating mainly those cellular functions whose excessive activity may upset immune homeostasis.

In our work, we have studied not only the regulatory but also the inflammasome-forming NLRs responsible for IL-1β-mediated immune responses. Very little is known about the possible role of the NLRP3 receptor and the effector functions of IL-1β, an inflammatory cytokine associated with its activity, in pDC-mediated immune responses.

Several studies have suggested that pDCs have low antibacterial activity compared to type 2 conventional DCs (cDC2), as NLRP3 and caspase-1, which are involved in antibacterial immunity, are expressed at low levels in these cells. Another recent study also identified cDC2s as potent IL-1β producing cells. Thus, based on the very low expression of pDC caspase-1 relative to the cDC2 subtype, these studies did not suggest that pDCs have inflammasome activity. However, in these studies, the expression values of pDCs could be masked by the extremely high expression levels of cDC2s. The functions of pDCs are more diverse than previously thought and they may play an important role not only as "virus-specific" immune cells but also in immune responses against bacteria and fungi, in which the NLRP3-dependent IL-1β pathway also shows significant activity.

Thus, we first investigated whether NLRP3 activity could be detected in human pDCs. At protein level, we could detect the NLRP3 receptor, the ASC adaptor protein and pro-caspase-1 in both unactivated and activated cells. However, among the inflammatory cytokines associated with NLRP3 activity, only the pro-form of IL-1β was detectable at the protein level and only after activation. Thus, in human pDCs, IL-1β may be mainly involved in NLRP3-mediated antimicrobial immune responses.

Interestingly, of the two different structural ligands of the endosomal TLR9 receptor, only CpG-B was shown to be a potent pro-IL-1β inducer, in contrast to CpG-A. The distinct TLR activation signals also resulted in different pro-IL-1β production kinetics. While the TLR9 agonist CpG-B induced pro-IL-1β production between 3-6 h, the TLR7 agonist Imiquimod showed prolonged pro-IL-1β production kinetics. It is likely that the ROS-inducing effect of

Imiquimod may play a role in the prolonged pro-IL-1β production kinetics in pDCs. It should be noted that macrophage subtypes also show different IL-1β production kinetics, suggesting that the quality of activation signals has a major impact on the IL-1β kinetics profile of cells.

In general, two signals are required for canonical NLRP3 activation. The priming signal enhances NLRP3 and pro-IL-1β expression via the NF-κB pathway and primes inflammasome assembly through post-translational modifications of NLRP3, which is then induced by a secondary signal, molecules which can have pathogenic, intrinsic or foreign origin. Our results suggest that in pDCs, treatment with synthetic TLR ligands alone is only a priming step and does not induce actual NLRP3 activation. It is known from literature data that mainly the LPS endotoxin provides a strong enough signal in monocytes through TLR4 activation to activate the inflammasome without secondary signals. In human pDCs, the Gram negative *E. coli* bacterium proved to be a strong priming signal similar to CpG-B, but did not induce mature IL-1β release by itself, as human pDCs, in contrast to mouse pDCs, do not express TLR4.

Thus, we investigated which secondary signals could induce NLRP3 assembly. Only the bacterial toxin nigericin, which acts as a potassium ionophore, induced mature IL-1β release in thr cells, which was detectable after one hour of nigericin treatment following priming. This may suggest that canonical NLRP3 inflammasome activation in pDCs requires $K^+$ efflux. Our results suggest that, unlike macrophages, pDCs do not express the P2X7 receptor required for ATP recognition, and thus ATP does not act as an NLRP3 activator in them, unlike monocytes, macrophages or moDCs.

Thus, among the activators we tested in pDCs, CpG-B and Imiquimod were found to be the best priming signals for pro-IL-1β induction and, together with nigericin, for the formation of mature IL-1β, compared to CpG-A. However, in the absence of an activation signal, we could not detect bioactive IL-1β in primary pDCs using TLR priming or secondary signaling alone.

Our results also highlighted that CpG-A, which induces a high type I IFN response, is unable to induce IL-1β production in pDCs, in contrast to CpG-B. CpG-A has poly-G motifs that greatly facilitate its aggregation and tetramer formation. Thus it is retained in early endosomes and leads to high levels of type I IFN secretion. CpG-B is not able to form tetramers and is thus rapidly transported to late endosomes where it induces mainly NF-κB activation. Thus, we hypothesized that the strong type I IFN production capacity of pDCs may also contribute to the lower NLRP3 inflammasome activity in these cells compared to myeloid cells. When pDCs were pretreated with recombinant human IFN-α or viruses inducing type I IFNs, neither CpG-B nor *E. coli* were able to induce higher IL-1β production, which confirmed our hypothesis. This is consistent with the high incidence of bacterial superinfections after viral

infections, which may be partly due to the inhibitory effect of the virus-activated IFN pathway on the antibacterial IL-1β pathway. However, IL-1β can also inhibit the secretion of type I IFNs, for example by enhancing PGE2 production. Thus, activation of the IL-1β pathway may also inhibit the type I IFN pathway, which we observed in our experiments, as the presence of IL-1β inhibited CpG-A-induced IFN-α production in pDCs.

Interactions between the type I IFN and IL-1β pathways play an important role in the regulation of the pathomechanisms of various autoimmune diseases. Furthermore, the inhibitory effect of type I IFNs may also be an important therapeutic option, for example in patients with MS in whom increased NLRP3 activity can be detected. It is important to highlight that overactivation of both pDCs and NLRP3 receptors plays a role in the pathomechanism of various autoimmune diseases. SNPs in NLRP3, which are mainly gain-of-function mutations, have been described in e.g. SLE, IBD, MS, RA, as well as psoriasis. It is known from the literature that pDCs may also play a role in the pathogenesis of psoriasis, as self-nucleic acids released during cell death in complex with overproduced LL37 antimicrobial peptides induce excessive activity of pDCs and increased production of type I IFN, which promotes the development of autoimmune processes.

Our results show that pDCs from psoriasis patients have significantly lower activation-induced NLRP3 activity. This also supports our hypothesis that high type I IFN pathway activity in pDCs results in low NLRP3 activity, as type I IFNs inhibit the NLRP3 pathway.

However, separately examining the $CD14^+$ monocyte population of patients, we observed higher baseline inflammasome activity compared to pDCs. Using similar activation signals, we did not find reduced NLRP3 activity in monocytes from psoriasis patients compared to the pDCs. We hypothesise that this may be due to the fact that NLRP3 activity of monocytes are not inhibited by type I IFNs as much, as monocytes have orders of magnitude less type I IFN-producing capacity compared to pDCs, and for a more precise comparison, in the case of monocytes we also used activation signals tailored to pDCs, and activation through these receptors is not the strongest stimulation for monocytes.

Finally, we wondered what might underlie the inhibitory effect of IFNs in pDCs. In other cells, type I IFNs can enhance the expression of several negative NLRP3 regulatory proteins. Our results suggest that in human pDCs, the expression of CH25H, SOCS1 and COP1 is greatly enhanced by a high IFN response-inducing activation signal. Furthermore, we found that the expression of these transcripts is significantly higher in PBMCs of psoriatic patients associated with high IFN-α levels. Thus, due to the high type I IFN pathway activity in pDCs,

CH25H may inhibit IL-1β transcription, SOCS1 may inhibit NLRP3 activation, and COP1 may inhibit caspase-1 activation.

In conclusion, the NLRP3 pathway may also function in human pDCs. However, we have demonstrated that NLRP3 activity in pDCs is lower than in other myeloid cells, probably due to their low caspase-1 expression and high type I IFN pathway activity. Namely, stimuli that elicit a high type I IFN response also increase the expression of NLRP3 pathway inhibitory proteins, which may inhibit NLRP3 inflammasome activation in pDCs at several points. Thus, the IL-1β-mediated immune response of pDCs may predominantly prevail during inflammation that is not associated with increased type I IFN pathway activity.

Thus, our work has demonstrated that pDCs, one of the dominant innate immune cells of the antiviral immune response, also express regulatory and inflammasome-forming NLRs. Furthermore, we have uncovered negative interactions between regulatory NLR and RLR receptors and between type I IFN and NLRP3-dependent IL-1β pathways. Increasing evidence suggests that abnormal type I IFN production due pathological RLR activation may play a role in the pathogenesis of autoimmune diseases. In addition to IFNs, increased IL-1β production associated with overactivation of the NLRP3 inflammasome also contributes to the breakthrough of tolerance and the development of autoimmune diseases. Therefore, the detailed elucidation and understanding of the molecular mechanisms underlying the negative regulation of innate immunity may contribute to the development of therapies for inflammation-induced autoimmune diseases. On the other hand, regulatory NLRs, which serve as molecular "brakes" on antiviral signalling, may be potential therapeutic targets to enhance the immune response to infections. Interventions aimed at modulating the balance between IFN and IL-1β cytokine production may provide an opportunity to promote the development of an effective antiviral or antibacterial response and thus determine the outcome of a given infection.

## VII. SUMMARY

NOD-like receptors (NLR) are highly conserved cytosolic pattern recognition receptors (PRR) of the immune system, which due to their multifunctional characteristics are not only involved in the recognition of pathogens and danger signals of tissue injury, but also affect almost all cellular functions, from autophagy to transcription activation. Furthermore, they have the ability to regulate signalling pathways of other PRRs and to form multiprotein inflammasome complexes, thus determining the outcome of both antibacterial and antiviral immune responses. Plasmacytoid dendritic cells (pDC) are the professional type I interferon (IFN) producing cells of our body. Due to their high IFN producing capacity, pDCs possess unique antiviral activity, and also play a role in the pathogenesis of autoimmune diseases, therefore they are important therapeutic targets. However, the role of cytosolic PRRs and their regulatory mechanisms are less understood in pDCs. Thus, the aim of our study was to investigate the regulatory receptor interactions of different NLRs and their inflammasome-forming capacity in human pDCs. We have shown that NLRX1 and NLRC5 negatively regulate the RIG-I-like receptor (RLR) mediated type I IFN production in pDCs and also in monocyte-derived dendritic cells (moDC), whereas the pro-inflammatory cytokine response is inhibited only by NLRX1 and only in moDCs. These results indicate that in addition to cell-specific effects, regulatory NLRs may serve as a negative feedback mechanism mainly for cellular functions that significantly determine the profile of the cell. We have also demonstrated that besides regulatory NLRs, the NLRP3 inflammasome functions in pDCs as well. Our results suggest that both strong NF-κB inducers and specific secondary signals that activate the inflammasome are required in pDCs to induce biologically active, mature IL-1β secretion. However, the NLRP3-dependent IL-1β secretion pathway can only be active in pDCs under inflammatory conditions in which the activity of the type I IFN pathway is not dominant, as conditions with high type I IFN levels increase the expression of several NLRP3 inhibitory proteins. Thus, our work has revealed novel receptor interactions and inflammasome activity in a cell type with a known role in many pathological conditions. The dysfunction of pDCs and NLRs is considered to be an important pathogenic factor in the outcome of infections and the development of autoimmune diseases, therefore the more comprehensive understanding of the cytosolic receptors of pDCs and their regulatory mechanisms may provide new targets for more effective therapeutic approaches in a number of pathologies.

# VIII. PUBLICATIONS

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Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
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## List of publications related to the dissertation

1. **Bencze, D.**, Fekete, T., Pfliegler, V. P., Szöőr, Á., Csoma, E., Szántó, A., Tarr, T., Bácsi, A., Kemény, L. V., Veréb, Z., Pázmándi, K. L.: Interactions between the NLRP3-Dependent IL-1β and the Type I Interferon Pathways in Human Plasmacytoid Dendritic Cells.
   *Int. J. Mol. Sci. 23* (20), 1-34, 2022.
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   IF: 6.208 (2021)
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**Total IF of journals (all publications): 46,541**
**Total IF of journals (publications related to the dissertation): 10,924**

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## IX. KEYWORDS

innate immunity, dendritic cell, plasmacytoid dendritic cell, monocyte-derived dendritic cell, RLR, NLR, NLRX1, NLRC5, type I interferon, antiviral response, inflammatory response, NLRP3, IL-1β, inflammasome, psoriasis, interaction, regulation, autoimmunity

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