SHORT THESIS FOR THE DEGREE OF PHYLOSOPHY (PHD)

# Regulation of immunomodulatory functions of dendritic cells by endogenous and exogenous factors

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Supervisor: Kitti Linda Pázmándi, PhD


UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR CELLULAR AND IMMUNE BIOLOGY

DEBRECEN, 2025

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The PhD Defense takes place at Department of Internal Medicine, Building A, University of Debrecen, at 13.00 p.m. on 08th of July 2025.

## I. Introduction

Dendritic cells (DC), as members of the innate immune system, play a prominent role in the recognition of pathogens and danger signals, as well as in the initiation of inflammatory processes and the presentation of antigens to T cells. Due to their unique immunomodulatory capacity, they significantly influence the direction of T-cell polarization, making human dendritic cells versatile therapeutic targets. Their functional activity is monitored and controlled by a variety of endogenous regulatory mechanisms, and DC functions can also be greatly influenced by exogenous stimulation signals.

In our work, we aim to manipulate the immunomodulatory capacity of DCs through various endogenous and exogenous ways. First, we investigated the regulatory role of the mammalian target of rapamycin (mTOR) in DC functions mediated by retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) specialized for the recognition of cytosolic replicating viruses. MTOR is one of the main intrinsic regulatory systems in DCs which mediates the differentiation, maturation and functionality of DCs. Our work has revealed the important role of mTOR signaling in the RLR-mediated antiviral and inflammatory cytokine responses and T cell activating capacity of human monocyte-derived DCs (moDCs) and plasmacytoid DCs (pDCs) using mTOR inhibitors. In further experiments, we investigated how the immunomodulatory effects of DCs can be influenced by modified exogenous stimulation signals. Therefore, we compared the effects of radiation-detoxified lipopolysaccharide (RD-LPS) and native LPS (N-LPS) on moDCs. We also investigated the effect of RD-LPS in ragweed-induced allergic airway inflammation and found that renaturation of the indoor environment with detoxified LPS may prevent the development of Th2-mediated allergic inflammation, as RD-LPS enhances the Th1 polarizing ability of DCs, which suppresses Th2 responses.

Thus, our work has revealed new possibilities for immunomodulation through endogenous and exogenous signaling to influence the functional activity of DCs and thereby the outcome of various inflammatory processes.

## II. Theoretical background

### II.1 The significance, origin and subtypes of DCs

The innate immune system is the first line of defence of our body, and its components include DCs, which immediately recognise danger signals or foreign structures that have entered the body and trigger immediate immune response.

As professional antigen-presenting cells (APS), DCs are also of great importance in establishing the link between the innate and adaptive immune system as well, due to their crucial role in antigen sensing, uptake, processing and presentation to T cells, and thus activating the adaptive immunity. In addition to naïve T cell activation, they are involved in the generation of memory T cells, and are also capable of facilitating B cell activation, thereby inducing the adaptive immune response and playing a role in the formation of immunological memory. Furthermore, DCs also contribute to the maintenance of stable immune homeostasis and immunological tolerance against their own tissues under physiological conditions.

DCs, except for follicular DCs with mesenchymal origin, can derive from CD34+ haematopoietic bone marrow stem cells (HSCs) in both myeloid and lymphoid developmental pathways under the influence of differentiation-promoting mediators such as granulocyte-macrophage colony stimulating factor (GM-CSF) and cytokine Fms-like tyrosine kinase-3-Ligand (Flt3L). During the myeloid differentiation pathway, pre-conventional or classical dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), and transitional dendritic cells (tDCs) originate in the bone marrow from granulocyte-monocyte progenitors (GMDPs) that exhibit high expression of interferon regulatory factor 8 (IRF8). Transitional dendritic cells (tDCs) are likely to represent a subpopulation of pDCs; however, their precise origin remains to be elucidated. Pre-cDCs are heterogeneous at the transcriptomic level and are pre-committed to cDC1 or cDC2 development. GMDPs with low IRF8 expression differentiate into monocytes and the DC3 subset via two other pathways. Finally, monocytes can differentiate into macrophages or monocyte-derived DCs (moDC) through two different developmental pathways. Two types of DCs can differentiate from monocyte-DC precursors (MDPs); cDC3 and moDCs, the latter mostly in response to an inflammatory stimulus. During

lymphoid development, DCs can also develop from lymphoid progenitors (LPs), such as pre-pDCs and the pDCs derived from them, as well as cDC1s, which can also differentiate along the lymphoid pathway.

**II.2 Specialized functions of DC subtypes in the blood**

Circulating DCs represent only 0.1-1% of mononuclear cells and the different DC subpopulations have specific, specialized immunological functions. cDC1s play an important role in the immune response against intracellular pathogens and tumours. cDC2s can polarize immune responses in several directions. Depending on the tissue microenvironment and the type of antigen, they can induce the polarization of naive CD4+ T cells into Th1, Th2 or Th17 cells, but they have a prominent role in the initiation of Th17 responses due to their high secretion of IL-23. The DC3 subgroup is characterized by a mixed monocyte-cDC2 phenotype and transcriptional profile. Similar to cDC2s, they are able to produce IL-12 and IL-23, and like monocytes, this DC subtype is characterized by high levels of IL-1β secretion. They are potent stimulators of naive CD4+ T cells, in particular inducing Th1 and Th17 polarization. They stimulate the proliferation of naive CD8+ T cells and may also promote the formation of tissue resident memory T cells.

During inflammation, human monocytes differentiate into monocyte-derived dendritic cells (moDCs), which primarily secrete IL-1β, TNF-α, IL-12, and IL-23, similar to cDC2s. Additionally, moDCs effectively induce the proliferation of naïve CD4+ T cells and, depending on the context, promote the differentiation of Th1, Th17, CD8+ T cells, and Tfh cells. Under *in vitro* conditions, CD14+ monocytes can be differentiated into moDCs in the presence of various cytokines, such as GM-CSF and IL-4, providing an excellent model for investigating the phenotypic and functional characteristics of immature and mature DCs.

Among the DC subgroups, pDCs play a key role in antiviral immunity. As professional type I interferon (IFN) producing cells, they have the unique property of being able to produce large amounts of type I IFNs, mainly IFN-α, very rapidly in response to viral stimuli. Type I IFNs act in an autocrine and paracrine manner to

initiate processes that prevent the spread of viruses and promote the elimination of viruses and virus-infected cells. Following viral infection, 95% of type I IFNs produced by mononuclear cells are derived from pDCs, as they are able to produce 200-1000 times more type I IFNs than any other white blood cells following viral exposure.

Most of the DC subtypes found in the blood continue to migrate to peripheral tissues under physiological conditions, where they undergo further differentiation and may be detected in all tissues except the brain. They do not divide in peripheral tissues but are continuously replenished because they have a relatively short *in vivo* lifespan (approximately 3-5 days). DC precursors in peripheral tissues and lymphatic organs are therefore likely DC subpopulations found in the blood. Based on their localization in the tissue they can be distinguished into migratory and tissue resident DCs. About half of the DCs found in lymph nodes are resident DCs, the other half are DCs with a mature phenotype that arrive via the lymphatic vessels. The latter transport antigens taken up at the periphery into lymph nodes, where the antigens are presented to T cells.

**II.3 Pattern recognition receptors (PRRs) of DCs**

Cells belonging to the innate immune system are able to detect via PRRs pathogen-associated molecular patterns (PAMPs) from microorganisms or damage-associated molecular patterns (DAMPs) of endogenous origin, which may be foreign or self-selected and are mainly released following tissue injury.

Transmembrane Toll-like receptors (TLRs) play a prominent role in the recognition of microbial structures, with 13 of them identified in mammals and 10 identified in humans. Some of these TLRs are localised on the cell surface, in the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10) and mainly recognise microbial cell wall components. Other TLRs (TLR3, TLR7, TLR8, TLR9) are localised in the endosomes and are mainly specialised in recognising nucleic acids of microbial or endogenous origin. Upon recognition, signaling pathways via TLRs activate transcription factor NF-κB, which can lead to the production of pro-inflammatory cytokines IL-6, IL-12 and TNF-α. Through IRF3 and IRF7 activation, it induces the production of mainly type I IFNs (INFα, INFβ).

During many pathogenic infections, pathogens can escape into the cytoplasm of the host cell and replicate there. Moreover, DCs also express a wide variety of cytoplasmic receptors, which are mainly specialized for the recognition of nucleic acids of pathogens. These include the cytoplasmic retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), the melanoma absent protein 2 (AIM2)-like receptors (ALRs), as well as the interferon gene stimulator (STING) activating cyclic GMP-AMP synthase (cGAS) receptors, and the IFN-regulatory factor DNA-dependent activator (DAI) and DDX41. RLRs recognise cytosolic RNA, while ALRs and proteins signaling via STING recognise cytosolic DNA. Cytosolic PRRs using mitochondrial antiviral signaling protein (MAVS) and STING adaptor protein mainly activate IRF3 and IRF7, in addition to the transcription factor NF-κB, and trigger the production of type I IFNs and pro-inflammatory cytokines, respectively. Some types of nucleotide-binding oligomerisation domain (NOD)-like cytosolic receptors (NLRs) may also be able to recognise cytosolic nucleic acids, but these receptors also form inflammasomes, signalosomes, enhanosomes and autophagosomes to help eliminate intracellular pathogens.

Among cytosolic nucleic acid sensors, we are mainly interested in studying the functions of RLRs. Previously, our group has published that similarly to cDCs circulating in the blood, moDCs also constitutively express RLRs, while pDCs, in which two types of RLRs, RIG-I and melanoma differentiation-associated protein 5 (MDA5), are either not expressed, or are expressed at very low levels. However, stimulation via endosomal TLRs (TLR7, TLR9) constitutively expressed in pDCs, in addition to the result of high levels of type I IFN production, is also able to induce expression of RLRs to a large extent independently of autocrine type I IFN effects. These observations indicate that although RLRs are absent from quiescent pDCs, they can be induced upon viral infection and thus may contribute to late phases of type I IFN responses of pDCs.

## II.4 Endogenous regulation of DCs functions

### *II.4.1 The importance of the mTOR signaling pathway in DCs*

The role of mTOR in DC biology has been extensively studied previously and it has been found that mTOR plays an important role in the regulation of innate immune cell functions, including the coordination of DC development, differentiation and function.

In cells, mTOR is expressed in two different multiprotein complexes, in mTORC1 and mTORC2. mTOR was named after its inhibitor, rapamycin, originally an antifungal agent, later characterised as an immunosuppressive agent, isolated from Streptomyces hygroscopicus bacterium. As a potent allosteric inhibitor of mTOR, rapamycin inhibits mTORC1 downstream signaling, while not affecting mTORC2 activity. Novel ATP-competitive catalytic inhibitors such as AZD8055, ADZ2014, Torin-1 and PP242 target the ATP-binding site in the mTOR domain and are thus able to inhibit the activity of both mTOR complexes. mTORC1 and mTORC2 complexes differ in both structure and function.

In DCs, mTORC1 responds to signals from different extracellular spaces, such as growth factors, cytokines or signals detected by PPRs, but also detects intracellular signals, including DAMPs, nutrient levels and cellular energy charge. The two main target molecules of mTORC1 are S6K and 4E-BP1. mTORC1 phosphorylates S6K1, which reaches full activity after further phosphorylation events and phosphorylates its downstream targets, 40S and S6, promoting ribosomal biogenesis, which results in protein synthesis and enhances cell proliferation. mTORC1 also influences mTORC2 activity via S6K, as it directly phosphorylates Rictor and thus inhibits mTORC2 functions. Furthermore, mTORC1 phosphorylates 4E-BP1, it detaches from the translation initiation factor and the eukaryotic translation initiation factor 4E (eIF4E) released from inhibition initiates cap-dependent translation. The autophagy inhibitory effect of mTORC1 is also well known, provided the right conditions, such as nutrients and energy, are available. Upstream regulators of mTORC2 are growth factors similar to those of mTORC1. mTORC2 directly phosphorylates Akt and its activity is mainly

manifested in changes in cell shape, as it plays an important role in modifying the cytoskeleton structure of actin.

In several studies, mTOR has been shown to be involved in the regulation of the immunomodulatory functions of DCs and to be able to influence the effector T cell activating capacity of DCs. Furthermore, the role of mTOR in both endogenous and exogenous antigen presentation and in the regulation of TLR-mediated immunological functions has been explored. Various TLR ligands such as polyI:C and LPS are also effective in inducing phosphorylation of the mTORC1 target molecule, S6K in DCs, which is inhibited by rapamycin. It was first described in detail in pDCs that the PI3K-mTOR-S6K pathway is crutial for the induction of type I IFNs mediated by TLRs. Subsequent studies confirmed that mTOR is essential for polyI:C-induced endosomal TLR3-mediated secretion of type I and type III IFNs by human moDCs and circulating CD1c+ cDCs. Several studies have also addressed the role of the mTOR pathway in the regulation of inflammatory cytokine production in the immune response against extracellular pathogens. mTOR has been identified as an essentialnegative regulator of the NF-κB signaling pathway and a positive regulator of the transcription signal transducer and activator 3 (STAT3) signaling pathway. Furthermore, several data support that mTOR differentially regulates cytokine production in myeloid DCs and moDCs stimulated with TLR4 ligand. The data also indicate that NF-κB and STAT3 induction is under the regulation of the mTOR pathway in DCs. In different DC subtypes stimulated with LPS, mTOR also differentially regulates the expression of cell surface molecules, which affects the phenotypic maturation of cells, therefore also the interactions of DCs with T cells, meaning antigen presentation. For example, it is known from previously published data that mTOR negatively regulates the ability of human mieolid DCs to induce T cell proliferation, whereas it is essential for moDCs to induce efficient T cell proliferation.

Different DC subtypes undergo different metabolic reprogramming following activation. Therefore, it is possible that the different metabolic demands of different DC subtypes result in different regulatory functions of mTOR. mTOR inhibitors may affect the cytokine production and phenotypic maturation of DCs differently in each

DC subtype. The available data reflect the fact that the mTOR dependence of TLR activation has been extensively studied in different DC subtypes, while the regulatory role of mTOR in RLR-dependent DC functions is yet to be fully understood.

## II.5 Exogenous control of DCs functions

### *II.5.1 The role of environmental microbes in the development of immune status: hygiene hypothesis and farm effect*

It is now widely accepted that symbiotic contact with beneficial microbes in the environment, skin, gut or lungs, is responsible for healthy immune homeostasis, leading to proper immune regulation and resulting in, for example, a low incidence of allergic diseases.

Many studies have shown that infections can in some cases have a beneficial effect on allergic or autoimmune diseases. It was first suggested more than 30 years ago by Strachan that infections and unhygienic contact may protect against the development of allergic diseases. Since then, numerous publications have addressed the so-called hygiene hypothesis in epidemiology, immunology and clinical sciences. In addition to indoor and outdoor air pollution, the increase in allergic diseases is likely to be driven by the decline in exposure to infections as a result of the Western lifestyle and increasing affluence. The hygiene hypothesis is supported by previous and recent epidemiological observations that have reported a reduced incidence of atopic disorders in children growing up on a conventional farm, who are exposed to a wide range of environmental microbial agents. Although the exact mechanism of this observed effect is not fully understood, it is presumably due to continuous exposure to high environmental endotoxin in the farm environment, which affects the regulation of innate immunity and promotes the induction of Th1 and regulatory T cells. Among the environmental factors that may provide protection, endotoxin from Gram-negative bacteria is highlighted as the most important.

It is important to highlight that the protective effects of farm exposure are strongest in the prenatal period and early childhood. In most related studies, the postnatal window of time is the first 3 weeks of life for mice and the first year of life for humans.

For example, the protective effect of the farm environment in allergic rhinitis lasts until school age and has a lasting effect into adulthood, especially with continued exposure. These results are consistent with the hypothesis that the potential for allergy prevention lies in childhood and may raise the question of whether further exposure beyond childhood is necessary to maintain the protective effect.

In vivo studies using mouse models have shown that inhaled endotoxin is likely to exert its protective effect on the respiratory mucosa. Low-dose exposure to endotoxin or agricultural dust was shown to protect mice from the development of house dust-induced asthma by reducing epithelial cytokine production, resulting in reduced DC activation and type II allergic inflammation. Furthermore, in neonatal mice, LPS stimulation had an asthma-preventive effect, associated with reduced mucosal metaplasia and mucin production following allergization with ovalbumin. In this experiment, normal thymic stromal lymphopoietin (TSLP) and IL-4 levels were accompanied by increased IL-12 and TNF-α expression. Furthermore, animal experiments also demonstrate that interventions with endotoxin may be most effective and safest when initiated before the onset of allergen sensitisation and atopic inflammation. In addition to timing, endotoxin dose and other environmental factors, host-microbiome interactions are also crucial in shaping immune responses to exposures.

Taken together, these observations suggest the possibility that renaturalising the indoor urban environment of infants seems a likely solution to prevent the development of allergic diseases. However, mimicking the pharmacological effect through the restoration of endotoxin levels in the urban environment is only possible with a tolerable dose and the use of a non-toxic, safe form of endotoxins. Intravenous administration of LPS induces severe toxicity in mammals, with a maximum tolerated dose in humans of only 1-4 ng/kg body weight. However, the toxicity of LPS is quite low when administered orally. For example, in a rat study, it was shown that oral administration of high doses (2 mg/kg) of LPS was not toxic to animals. Therefore, oral administration of LPS appears to be the most appropriate route of administration for various diseases, including allergic and lifestyle-related diseases.

### *II.5.2 Modulation of LPS toxicity*

LPS or endotoxin, located in the outer membrane, a major immunostimulatory component of bacteria, is ubiquitous in the environment and can directly influence the outcome of immune responses, and is therefore important in the pathophysiology of many diseases. LPS consists of three distinct regions: the *O*-polysaccharide region at the outermost part of the LPS molecule, the R-core polysaccharides and the endotoxically active part of the molecule, lipid A. The *O*-polysaccharide region in the outermost part of the LPS molecule is the major antigen targeted by the host antibody response. However, the potent immunostimulatory ability of LPS is largely attributed to the lipid A portion of the endotoxin.

Therefore, several attempts have been made using physical, chemical or biosynthetic engineering approaches to reduce the endotoxic activity of LPS or to separate toxic and immunostimulatory activity. One such example is the potassium methylate detoxified formulation of the so-called monophosphoryl lipid A (MPLA), which was produced in the 1980s. Diphosphoryl lipid A (DPL) is a detoxified version of LPS from *Salmonella minnesota* strain R595, which is able to stimulate the immune system without causing direct toxic effects. The toxicity of LPS can also be influenced by biosynthetic methods. By inhibiting the genes involved in LPS biosynthesis or inducing modifying enzymes, the toxicity of LPS can be reduced while retaining its adjuvant activity, which facilitates the production of safer live attenuated vaccines or outer membrane vesicle (OMV) vaccines.

Another effective form of detoxification of endotoxins is treatment with ionizing 60Co γ-irradiation, which degrades the structure of LPS and leads to loss of its biological functions. With increasing doses of radiation, the trilaminar, ribbon-like structure of the endotoxin becomes nodular and, in parallel with the morphological changes, repeating units of the *O*-side chain and fragments of the R-core liposaccharide molecules are lost. Radiation breaks down the simple sugars within the structure, such as galactose, galactosamine, glucosamine, glucose and heptose, reducing the antigenicity of the polysaccharide part. The R-core component, Kdo, is the most resistant sugar derivative to ionising radiation. The hydrophobic lipid A part of the

endotoxin is less degraded than the sugar components, even under relatively high radiation. However, among the fatty acids of lipid A, either partial conversion of β-hydroxymyristic acid to myristic acid or its selective loss has been observed in response to radiation. Furthermore, the amount of glucosamine and phosphate in the irradiated lipid A was also reduced. Lóránd Bertók and colleagues succeeded in producing a 150 kGy $^{60}$Co detoxified LPS formulation (RD-LPS, TOLERIN®) that showed reduced toxicity while retaining its beneficial immunomodulatory effect. Extensive animal and human studies have shown that these detoxified endotoxin preparations can be used to enhance natural resistance, to prevent various shock conditions (radiation sickness, septic-surgical shock, etc.), to improve immunosuppression or immunodeficiency of various origins, and to improve the efficacy and immunogenicity of vaccines made from inactivated viruses due to their adjuvant effect. There have also been encouraging results with TOLERIN® in acquired immunodeficiency syndrome (AIDS) and in its preceding condition (pre-AIDS), as well as in the restoration of bone marrow function in patients treated with anticancer agents.

## III. Objectives

**1.** Our primary goal was to investigate how endogenous regulatory processes, such as mTOR-mediated regulation, influence the RLR-mediated immunological functions of DCs. Thus, we first investigated:

- whether RLR activation is mTOR dependent?
- how inhibition of mTOR affects the phenotypic and functional maturation of moDCs and pDCs induced by RLR activation?
- how does inhibition of mTOR affect the naive T cell activating capacity of RLR-activated moDCs and pDCs?

**2.** Since dendritic cell (DC) functions are also influenced by exogenous factors from the environment, our second goal was to examine how the modulation of TLR-mediated exogenous stimulation signals affects the immunomodulatory properties of human DCs. Our investigations sought to answer the following questions:

- how does the toxicity of radiation-detoxified LPS (RD-LPS) change compared to native LPS (N-LPS)?
- how does TLR-mediated RD-LPS exposure affect the functions of DCs?
- how does RD-LPS activation affect the T cell polarizing ability of moDCs?
- can pre-exposure to RD-LPS prevent the development of ragweed-induced Th2-mediated airway inflammation in an *in vivo* mouse model?

## IV. Materials and methods

### IV.1 Cell lines and culturing of primary human cells

Our experiments were carried out using human pDC cell line (GEN2.2) provided by Dr. Joel Plumas and Dr. Laurence Chaperot (Research and Development Laboratory, French Blood Bank Rhône-Alpes, Grenoble, France; CNCMI number 2938).

Buffy coat samples were obtained from healthy blood donors and peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Paque Plus gradient centrifugation. Primary human $CD14^+$ monocytes were obtained from PBMC by positive selection using magnetic cell separation with anti-CD14-conjugated microbeads. Freshly isolated cells were differentiated into DCs in the presence of GM-CSF and IL-4.

Primary human pDCs were also isolated from PBMCs by positive selection using the human CD304 (BDCA-4/Neuropilin-1) MicroBead Kit.

Autologus and allogenic primary naive $CD8^+$ T cells were isolated from PBMC using the Human Naïve $CD8^+$ T Cell Isolation Kit and were used for DC-T cell co-culture experiments.

The collection of human heparinized leukocyte-enriched buffy coat samples was carried out according to the guidelines of the Helsinki Declaration and was approved by National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Faculty of Medicine (OVSzK 3572-2/2015/5200, Hungary).

### IV.2 Bacterial endotoxin preparations

N-LPS used for *in vivo* and *in vitro* experiments was extracted from *Escherichia coli* serotype O101RG/W by the hot aqueous-phenol method. Next, the extracted N-LPS was detoxified by $^{60}Co$ irradiation with under 150 kGy (Lot. number: 92040605/K/01; National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary). Toxicity of N-LPS and RD-LPS was tested on BALB/c mice. Separation and mass spectrometric detection of β-hydroxymyristic acid content in the

LPS extracts were carried out with an Infinity 1290 UHPLC coupled to a 6530 Accurate Mass Q-TOF LC/MS system.

**IV.3 Flow cytometric analysis of the phenotype and viability of DCs**

For phenotypical analysis of moDCs and GEN2.2 cells, cells were labeled with monoclonal antibodies specific for surface proteins, conjugated to fluorescent dyes. Cell viability was determined by 7-aminoactinomycin D (7-AAD) staining. Fluorescence intensities were measured using FACS Calibur flow cytometer and data were analyzed by FlowJo software.

**IV.5 Western blott analysis**

For western blotting $5 \times 10^5$ cells were lysed in Laemmli buffer and heated at 100°C for 10 min. The samples were separated on 7.5 or 10% SDS-PAGE, followed by electrotransfer to nitrocellulose membranes. After blocking with 5% non-fat dry milk, the membranes were incubated with specific primary antibodies. Beta-actin was used as a control. Primary antibodies bound to the membrane were conjugated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:5000 and 1:10000 respectively. Specific protein bands were visualized by the ECL system using SuperSignal West Pico or Femto chemiluminescent substrate and X-ray film exposure. The immunoreactive bands have undergone densitometric analysis performed by Image Studio Lite Software version 5.2.

**IV.5 Quantitative Real Time PCR**

First Total RNA was isolated from $5 \times 10^5$ cells using Tri Reagent, according to the manufacturer's instructions. Total RNA was treated with 1 μg DNase I, to exclude amplification of genomic DNA, and reverse transcription into cDNA was carried out using High Capacity cDNA RT Kit. Gene expression probes specific to the genes of interest were used in the reaction. Quantitative PCR was performed using the ABI StepOne Real-Time PCR System and cycle threshold values were determined by the

StepOne v2.1 Software. The relative amount of mRNA was obtained by normalizing to the PPIA housekeeping gene in each experiment.

**IV.6 Enzyme-linked immunosorbent assay (ELISA)**

Following treatments, cell culture supernatants were collected at the indicated time points, and IFN-α levels were measured using the VeriKine™ Human Interferon Alpha ELISA kit (PBL Interferon Sources, Piscataway, NJ, United States), while IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α concentrations were determined using the BD OptEIA human ELISA kits (BD Biosciences) following the manufacturer's instructions. Cellular lactate production was detected by the Glycolysis Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, Michigan, USA) according to the manufacturer's instructions. Absorbance values of the samples were detected by Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA) for both ELISA and lactate assay. The ELISA absorbance measurements were performed at 450 nm, while the lactate assay measurements were performed at 490 nm.

**IV.7 Measurement of intracellular reactive oxygen species (ROS) production in human moDCs**

For experiments with endotoxins, 5-day-old human moDCs were loaded with 50 μM fluorescent 2′,7′-dihydrodichlorofluorescein diacetate (H2DCFDA), and after a 2-hour LPS activation, changes in DCF fluorescence intensity were evaluated using a FACS Calibur Flow Cytometer in the FL1 (530 ± 15 nm) channel and data were analyzed using FlowJo v. 5.7.2 software.

**IV.8 T cell proliferation and activity investigated by flow cytometry**

For DC-T cell co-cultures, pretreated moDCs and primary human pDCs were washed twice and co-cultured with allogeneic naïve CD8+ T cells with anti-human CD3 monoclonal antibody. For T cell proliferation assays, T cells were loaded with 0.5 μM fluorescent carboxyfluorescein succinimidyl ester (CFSE). After a 5-day co-

cultivation, the CFSE fluorescence intensities of T cells were detected using a BD FACS Calibur flow cytometer.

For intracellular cytokine staining, after co-cultivation, T cells were restimulated with phorbol myristate acetate and ionomycin in the presence of a protein transport inhibitor, monensin for 5 hours. Afterwards, the cells were incubated with anti-CD8-FITC and an appropriate isotype control antibody, and fixed and permeabilized with BD Cytofix/Cytoperm solution. Cells were then incubated with APC-conjugated anti-IFN-γ and anti-granzyme B antibodies and control antibodies of the appropriate isotype. Fluorescence intensities were detected using a FACSCalibur flow cytometer and data were evaluated using FlowJo software.

**IV.9 Analysis of T-cell cytokine production by enzyme-linked immunosorbent assay (ELISPOT)**

Pre-treated moDCs were co-cultured with autologous or allogeneic human CD3+ pan-T cells, and after four days of co-cultivation, the number of IFN-γ producing T cells was determined using a commercially available human IFN-γ-specific ELISPOT kit, following the manufacturer's instructions.

**IV.10 *In vivo* mice experiments**

Six-week-old female BALB/c mice (4 – 10 groups, 8 mice per group) were treated daily for 70 days with 5 µg N-LPS or RD-LPS dissolved in 5 ml endotoxin-free water (Cat. No.: TMS011) in the form of an aerosol spray. Endotoxin-free water was used as control. During the 70-day-long LPS treatment period, mice were sensitized twice intraperitoneally, on days 60 and 64, with endotoxin-free ragweed pollen extract. On day 71, when LPS treatments were completed, allergic inflammation in was induced in animals with RWE given intranasally . Then, 72 hours after allergen treatment, mice were terminated and inflammatory cells from bronchoalveolar lavage fluid (BALF) were analysed. After cyto-centrifugation of the cells onto slides, preparations were stained with Wright-Giemsa and cell counts were determined by light microscopy.

The care and handling of animals was carried out according to the guidelines of the Helsinki Declaration, the European Union and the guidelines of the IASP Research and Ethical Issues Committee. All animal experiments were approved by the Animal Care and Welfare Committee of the University of Debrecen (#7/2011/DE MAB). Animals were kept in the pathogen-free animal facility of the University of Debrecen, where food and water were available at the animals' convenience.

**IV.11 Statistical analysis**

The data are presented as mean ± standard deviation (SD) in the figures. Shapiro-Wilk test was used to determine the distributions. For statistical analysis, one-way analysis of variance (ANOVA) was used for normally distributed data, followed by Bonferroni post-hoc test. For non-normally distributed data, the Kruskal-Wallis test was applied, with Dunn’s post-hoc test. Statistical significance was determined using GraphPad Prism v.6 software. Differences were considered statistically significant at $p<0.05$.

## V. Results

### V.1 Endogenous immunomodulation: investigating the regulatory role of mTOR in RLR activation-induced immune responses in human DC

#### *V.1.1 Analysis of mTOR activity in RLR-activated moDCs*

The regulatory role of mTOR in the immune responses of DCs was first investigated in moDCs using specific mTOR inhibitors. For our experiments, we used the specific inhibitor of mTORC1, rapamycin, and the ATP-competitive catalytic inhibitor AZD8055, which targets the ATP binding site in the mTOR domain, thus being able to inhibit the activity of both mTOR complexes simultaneously. First, we demonstrated that the RIG-I and MDA5 receptors are expressed at basal levels in moDCs, and that inhibition of mTOR does not affect expression of these receptors. Next, we examined whether RLR-mediated signaling activates mTOR signaling in moDCs. We examined the phosphorylation of S6K (p70S6K, Thr389), a key target molecule of mTORC1, and Akt (Ser473), a downstream target of mTORC2, by specific RIG-I stimulation (3p-hpRNA). Our results showed that RIG-I stimulation significantly enhanced the phosphorylation of p70S6K (Thr389), which peaked after 1 hour of activation, while the phosphorylation of p70S6K was significantly inhibited by rapamycin and AZD8055 pretreatment. Phosphorylation of Akt (Ser473) also increased significantly at 1 hour, which was effectively inhibited by AZD8055. In parallel experiments, moDCs were treated with polyI:C/LyoVecTM complex. The polyI:C/LyoVecTM complex is favored by MDA5 over RIG-I and therefore mainly considered as an activator of MDA5. Activation with polyI:C/LyoVecTM significantly increased the phosphorylation of both p70S6K and Akt, with p70S6K reaching its highest phosphorylation level after 1 hour and Akt after 3 hours of activation. The specific inhibitor of mTORC1, rapamycin, inhibited the phosphorylation of the mTORC1 target molecule, p70S6K, whereas Akt was not inhibited. However, the dual mTORC1/2 inhibitor AZD8055 effectively inhibited the phosphorylation of both p70S6K and Akt, the target molecule of mTORC2, in moDCs activated with polyI:C/LyoVecTM. These results show that activation via RLRs induces the activation

of mTOR signaling pathways in moDCs, demonstrating mTOR dependence of RLR receptors.

### *V.1.2 Effect of mTOR inhibition on RLR-mediated metabolic changes in moDCs*

Previously, our research group has shown that the activation of moDCs and pDCs by RIG-I has different metabolic conditions. In human moDCs, RIG-I activation results in a metabolic switch from oxidative phosphorylation to glycolysis, which promotes the production of type I IFN by the cells and enhances their T-cell activation capacity. In contrast, the RIG-I-mediated antiviral response of pDCs does not require a switch to glycolysis. Furthermore, mTOR activation is known to promote glycolysis in DCs following TLR stimulation. However, no data have been reported on the role of mTOR in the metabolic reprogramming of RLR-stimulated moDCs. Therefore, we further investigated the expression of glycolysis-related genes in RLE-activated moDCs in the presence of mTOR inhibitors, as well as the lactate concentration in the cell supernatant. Our results showed that in RLR-stimulated moDC, the expression of glycolytic genes (LDHA, HK2 and HIF1A) and also lactate production were significantly increased, which were all inhibited by mTOR inhibitors. These results suggest that mTOR is also essential for RLR-mediated glycolytic reprogramming of moDCs.

### *V.1.3 Investigation of the regulatory role of mTOR in phenotypic and functional changes in RLR-stimulated human moDCs*

During the phenotypic analysis, we examined the expression of the activation marker CD83, costimulatory molecules CD40, CD80 and CD86, and MHC molecules, such as HLA-DQ and HLA-ABC. Our results showed that mTOR inhibitors alone did not affect the basal expression of these proteins, while RLR agonists significantly increased the expression of cell surface activation molecules of moDCs. However, neither rapamycin, nor AZD8055 was able to significantly inhibit RLR stimulation-induced phenotypic maturation of moDCs.

In a next step, the concentration of cytokines produced in the supernatant of treated moDCs was examined by ELISA after 24 hours of activation. The cytokines tested included the major antiviral mediator IFN-α belonging to type I IFNs, and the

pro-inflammatory cytokines IL-6 and TNF. We found that untreated moDCs do not secrete type I IFNs and produce only very low levels of pro-inflammatory cytokines, the baseline levels of which are not affected by mTOR inhibition. However, in the presence of 3p-hpRNA or polyI:C/LyoVecTM treatment, the production of IFN-α, IL-6 and TNF cytokines was highly induced in moDCs, which was significantly reduced by mTOR inhibition. To confirm our observations, we also performed these experiments with live virus. For the activation of moDCs, we used a live VSV virus, whose RNA genome mainly provides activation at RLRs. Similar to synthetic ligands, we found that VSV infection increased the production of IFN-α, IL-6 and TNF cytokines in cells, which was significantly reduced by rapamycin and AZD8055 through inhibition of mTOR. In conclusion, inhibition of mTOR does not affect the phenotypic maturation of moDCs, but the cytokine production of activated moDCs is mTOR dependent.

Since TBK1 plays a crutial role in RLR signaling, we examined the kinetics of TBK1 phosphorylation in the presence of mTOR inhibitors by western blotting and found that RLR activation-induced TBK1 phosphorylation was significantly reduced when cells were pretreated with mTOR inhibitors prior to activation. However, when examining the role of p38 MAPK pathway, we found that p38 phosphorylation was high in cells even at baseline level, and was not significantly altered by treatment with either RLR agonists or mTOR inhibitors. In conclusion, the mTOR complex in moDCs may influence cellular cytokine production mainly by regulating TBK1 activity.

### *V.1.4 Analysis of mTOR activity in RLR-activated human pDCs*

The number of pDCs circulating in human peripheral blood is limited, therefore, most of our experiments were performed with the human GEN2.2 pDC cell line, which exhibits phenotypic and functional properties similar to primary human pDCs. First, we found that, similar to the results obtained for moDCs, mTOR inhibition does not alter the expression of RLR receptors in cells.

Furthermore, the results of western blot analysis showed that, similarly to moDCs, treatment of pDCs with 3p-hpRNA or polyI:C/LyoVecTM significantly increased the phosphorylation of p70S6K and Akt, which reached its peak after 1 hour

of activation. These results indicate that activation of RLRs also enhances mTOR activity in pDCs. However, pretreatment with rapamycin inhibited the phosphorylation of p70S6K in both unactivated and activated cells. The dual mTORC1/mTORC2 inhibitor AZD8055 blocked the phosphorylation of both mTOR targets. We also found that rapamycin treatment alone leads to an increase in Akt phosphorylation, which can be explained by the loss of the negative feedback between mTORC1 and p70S6K. These results are consistent with the data obtained in moDCs, suggesting that mTOR may have an important regulatory role in the regulation of RLR-mediated immune responses in both moDCs and pDCs.

### *V.1.5 Investigation of the regulatory role of mTOR in phenotypic and functional changes in RLR-activated human pDCs*

In contrast to moDCs, RLR activation of GEN2.2 cells did not increase the expression levels of CD40, CD80 and CD86 costimulatory and HLA antigen-presenting molecules. Similar to moDCs, pretreatment with mTOR inhibitors did not result in differences in the expression levels of these proteins. In contrast, RLR activation resulted in significantly increased levels of the activation marker CD83, which interestingly was further enhanced in AZD8055 pretreated cells, which may indicate some selective regulatory mechanism of mTOR in pDCs.

Afterward we examined the secretion of pro-inflammatory cytokines IFN-α, IL-6 and TNF from mTOR inhibited and RLR activated GEN2.2 cells. Our results are consistent with the changes observed in moDCs, where the secretion of IFN-α, IL-6 and TNF cytokines was significantly increased by RLR stimulation. However, in RLR-stimulated GEN2.2 cells, mTOR inhibitors significantly decreased the production of IFN-α and pro-inflammatory cytokines. To further confirm our results, similar to the moDC investigation, we also examined cytokine production in live virus-infected GEN2.2 cells and found that inhibition of mTOR also greatly reduced VSV-induced cytokine secretion by these cells. Furthermore, RLR activation also induces phosphorylation of TBK1 in pDCs, which peaked at 3 hours. However, phosphorylation of TBK1 was significantly reduced by mTOR inhibition. The

phosphorylation level of p38 in GEN2.2 cells was not significantly altered by RLR stimulation, and mTOR inhibition did not affect the phosphorylation level.

To conclude, our results suggest that, similar to moDCs, mTOR plays an important regulatory role in RLR-mediated type I IFN and pro-inflammatory cytokine responses in pDCs, probably through regulation of TBK1 activity.

### *V.1.6 Investigation of the regulatory role of mTOR in T cell activating functions of human DCs*

Our results show that moDCs and primary pDCs treated with 3p-hpRNA or polyI:C/LyoVec™ significantly induce CD8+ T cell proliferation. However, T cell proliferation is inhibited when moDCs and primary pDCs are pretreated with rapamycin prior to RLR stimulation. The dual mTOR inhibitor, AZD8055, however, proved to be much weaker and could only inhibit the T cell proliferation-inducing effect of moDCs to a much lesser extent compared to rapamycin. In the case of primary pDCs, AZD8055 did not significantly affect the ability of naïve T-cell proliferation-inducing cells stimulated by RLR activation.

In parallel experiments, we found that moDCs activated with 3p-hpRNA or polyI:C/LyoVec™ induced the production of IFN-γ and granzyme B by CD8+ T cells. However, when moDCs were treated with rapamycin prior to RLR activation, the ability of moDCs to activate CD8+ T cells was significantly reduced, which was not observed with AZD8055 pretreatment. By repeating these experiments with primary pDCs, similar results to moDCs were obtained. Based on these observations, it can be concluded that the T cell activating ability of human DCs is mainly influenced by selective inhibition of mTORC1, whereas the combined inhibition of mTORC1 and mTORC2 may possibly trigger some compensatory mechanism that could help to ensure the proper antigen presentation and thus T cell activating ability of DCs, but further studies are required to support this hypothesis.

## V.2 Exogenous immunomodulation: investigating the immunomodulatory effect of radiation-detoxified LPS on human moDCs

### *V.2.1 Investigation of the effects of native and radiation-detoxified endotoxin on viability and phenotypic maturation of human moDCs*

The aim of our series of experiments detailed below was to investigate the differences in the immunomodulatory effects of structurally radiation-modified endotoxin on human moDCs compared to the native form. After confirming that neither N-LPS nor RD-LPS affected the viability of moDCs at the doses used, we investigated the intracellular ROS-inducing ability of N-LPS and RD-LPS using redox-sensitive fluorescent dye ($H_2$DCF-DA). The endogenously generated ROS is essential for the antigen-presenting function of DCs. Our results show that treatment of moDCs with N-LPS or RD-LPS resulted in a significant increase in DCF fluorescence, but no significant difference in DCF fluorescence intensity between cells treated with N-LPS or RD-LPS, indicating the same intracellular ROS-inducing capacity of the two different types of LPS.

We then investigated how gamma-irradiation affected the immunostimulatory effect of LPS, which may induce the phenotypic maturation of moDCs required for interaction with T cells and the activation of T cells. To investigate the phenotypic changes in moDCs, changes in expression levels of various cell surface proteins, including the maturation marker CD83, the antigen-presenting molecule HLA-DQ, and costimulatory molecules CD40, CD80 and CD86, were measured after 24 hours of endotoxin stimulation by flow cytometry. Our results show that both N-LPS and RD-LPS significantly increased the expression of all cell surface proteins assessed compared to untreated cells. However, no statistically significant difference was found between the moDC-activating potential of the two types of LPS. In conclusion, these results suggest that N-LPS and RD-LPS can induce maturation of human moDCs to a similar extent.

### *V.2.2 Investigation of the effects of N-LPS and RD-LPS on cytokine production by human moDCs*

Our results show that both forms of LPS were able to significantly increase the moDC secretion of all measured cytokines (IL-6, TNF-α, IL-12 and IL-1β, IL-10) and

chemokine (IL-8). Although lower cytokine and chemokine concentrations were detected in the supernatant of moDCs treated with RD-LPS than in the supernatant of cells treated with the native form of LPS, significant differences between the effects of irradiated and native LPS could only be detected in the levels of IL-1β and IL-10. We also found that RD-LPS-induced protein secretion was TLR4-dependent in moDCs, similar to N-LPS, as the concentration of the cytokines tested remained at the level of control samples after stimulation with both N-LPS and RD-LPS under specific TLR4 inhibition. These results suggest that the irradiated form of the endotoxin can also be recognized by moDCs via TLR4, thus RD-LPS retained its ability to bind to TLR4.

### *V.2.3 Investigation of the ability of N-LPS and RD-LPS to induce endotoxin tolerance in human moDCs*

Endotoxin tolerance or LPS tolerance is a defence mechanism in which cells exposed to low endotoxin concentrations enter a transient state that reduces their sensitivity to a second dose of endotoxin. On day 4 of DC differentiation, human moDCs were pretreated with low-dose LPS (5 ng/ml), and on day 6 re-exposed to high-dose LPS (250 ng/ml). Then, after 24 hours of treatment, the concentrations of IL-12, IL-1β and IL-10 were determined by ELISA from cell culture supernatants. In our experiments, restimulation of moDCs with N-LPS or RD-LPS also resulted in significantly lower concentrations of IL-12, IL-1β and IL-10 cytokines in cell supernatants compared to a single high dose of LPS treatment. However, there was no significant difference in the amount of cytokines released between N-LPS-reactivated moDCs and RD-LPS-reactivated ones, except for the cytokine IL-10. Both single high-dose and repeated N-LPS treatments induced significantly higher levels of IL-10 production than similar treatments with RD-LPS. These results show that both N-LPS and RD-LPS can induce endotoxin tolerance in cells, however, both moDCs activated with a single dose of RD-LPS and restimulated with RD-LPS have a lower capacity to produce the cytokine IL-10 than cells exposed to the native form of endotoxin.

### *V.2.4 Investigation of the T cell activating effects of N-LPS and RD-LPS in human moDCs*

Having shown that there is a difference in the effects of N-LPS and RD-LPS moDCs on cytokine response, we were curious to see whether there is any difference between the T cell polarization-inducing effects of N-LPS-activated and RD-LPS-activated human moDCs. Our results show that human moDCs exposed to RD-LPS induce Th1-directed polarization much more strongly than cells activated with the native form of LPS in both autologous and allogeneic systems.

### *V.2.5 Study of the immunomodulatory effects associated with the myristic acid content of RD-LPS*

Since our results showed that N-LPS and RD-LPS can induce phenotypic maturation of moDC to the same extent, the stronger Th1-directed polarization-inducing capacity of RD-LPS-activated moDCs cannot be explained by the differential effects of endotoxins on DCs maturation. However, the inducing ability of IL-10 was different between the two types of endotoxins, and we sought to explore the molecular background of this observation in our further experiments. We suggested that the different immunostimulatory ability of the two endotoxin preparations could be due to the fact that in the case of RD-LPS, ionizing radiation disrupts the structure of the endotoxin and releases various free fatty acids that may have immunomodulatory properties. Previously, it was found that $^{60}$Co γ-irradiation at 150 kGy degraded the simple sugars in the structure of LPS and thus reduced the antigenicity of the polysaccharide moiety. Among the lipid A fatty acids of endotoxin, β-hydroxymyristic acid was partially converted to myristic acid or selectively degraded. Thus, we investigated the concentration of free fatty acids, β-hydroxymyristic acid in our LPS preparations by high-performance liquid chromatography-mass spectrometry (HPLC-MS) and found that the concentration of β-hydroxymyristic acid was three times higher in RD-LPS (1.82 μg/ml in 1 mg/ml LPS) than in N-LPS (0.53 μg/ml in 1 mg/ml LPS).

Based on these results, we suggested that higher concentrations of the free myristic acid derivative in RD-LPS may affect the IL-10-inducing potential of moDCs

exposed to RD-LPS, leading to lower IL-10 production. To test this hypothesis, we examined the IL-10 inducing capacity of native endotoxin with lower myristic acid content in the presence of myristic acid. Our results showed that treatment with myristic acid alone did not alter basal IL-10 secretion levels in moDCs, but completely inhibited N-LPS-induced IL-10 secretion by the cells. Thus, the higher myristic acid level in RD-LPS formulation may be responsible for the decreased IL-10 production in RD-LPS-treated moDCs, and it is likely that polarization towards Th1 may be more pronounced with lower IL-10 levels.

### *V.2.6 Investigation of immunomodulatory effects of RD-LPS in a mouse model of pollen-induced airway allergic inflammation*

Previously published data has shown that myeloid DCs in the airways play a key role in sensitization to inhaled antigens and in the development of Th2-dependent airway eosinophilia. Since our results showed that human moDCs treated with RD-LPS can induce stronger Th1 polarization than human moDCs treated with N-LPS, we hypothesized that the T-cell polarization-altering effect of RD-LPS in Th2 inflammation-associated allergic reactions may be able to reduce the symptoms of Th2 inflammation by enhancing Th1 dominance. Thus, we have also investigated the immunomodulatory effect of RD-LPS in an *in vivo* pollen-induced airway allergic inflammation mouse model.

In our mouse experiments, we first examined the toxicity of endotoxin preparations and found that RD-LPS, in contrast to N-LPS, is not toxic to mice. Furthermore, the results of the previous limulus amoebocyte lysate (LAL) study also showed that the limulus reactivity of RD-LPS was negligible compared to N-LPS (limulus reactivity: RD-LPS/N-LPS=1/1000).

Afterwards, six-week-old female BALB/c mice were treated for 70 days with a daily dose of 5 μg N-LPS or RD-LPS and endotoxin-free water ($H_2O$) as an aerosol spray was used as a control. Endotoxin-free ragweed pollen extract (RWE) was used to induce allergic inflammation. In mice where the mice environment was treated with endotoxin-free $H_2O$ as a control and then RWE exposure was used to induce Th2 allergic

inflammation, both total cell count and eosinophil cell count showed significant increases in BALF samples of the animals. When the mice were exposed to N-LPS or RD-LPS for 70 days, then induced to develop allergic inflammation, a slight decrease in the cell count of BALF samples was detected. However, in contrast to N-LPS, in RD-LPS-rich environments, we detected a significant reduction in the eosinophil count of allergized mice, which are the major inflammatory components of allergic responses and whose numbers correlate best with lung function and the degree of Th2-type airway inflammation. Based on these results, we hypothesize that environmental enrichment with non-toxic RD-LPS may provide protection against ragweed pollen-induced airway allergy.

## VI. Discussion

### VI.1 Endogenous regulation of DC functions: the role of mTOR in RLR-mediated antiviral responses of human DC

In the past decade, several studies have identified the mTOR complex as a central endogenous regulator of TLR-mediated DC functions, but the role of mTOR in RLR-dependent antiviral responses in human DCs has not been investigated, although recognition of viral-derived nucleic acids in the cytoplasm of DCs and immune responses against actively replicating viruses are mainly mediated by RLRs.

Endogenous regulatory mechanisms mediated by PI3K/mTOR also affect DC activation, survival, maturation, cytokine production and T cell stimulatory capacity. Furthermore, several RLR-activating viruses also affect PI3K/Akt/mTOR signaling in infected cells to promote cell survival and inhibit apoptosis. These previous observations suggest that immune responses mediated by RLRs may also be under mTOR regulation, which we have directly verified with the results of our own experiments. We have shown that, similarly to the activation of TLR signaling, specific RLR stimulation of human DCs also triggers activation of the mTOR signaling pathway. We found that activation of moDCs and pDCs with RIG-I/MDA5-specific ligands significantly enhanced p70S6K and Akt phosphorylation, reflecting increased mTORC1 and mTORC2 activity. Previous studies have described that the mTOR pathway also plays a central role in coordinating metabolic changes in DCs following activation and facilitates glycolytic reprogramming. We showed that both rapamycin and AZD8055 significantly reduced the production of lactate, the end product of glycolysis, and the expression of glycolysis-related genes in RLR-stimulated moDCs, reflecting the reduced glycolytic activity that occurs upon mTOR inhibition.

There are several, often contradictory, results in the literature on whether mTOR plays a positive or negative regulatory role in TLR-mediated phenotypic maturation and cytokine production of different subtypes of DC. Our results our clearly demonstrate that mTOR may function as a key positive regulator of RLR-mediated cytokine responses in human DCs, as both rapamycin and AZD8055 significantly reduced RLR-mediated cytokine secretion of IFN-α, IL-6 and TNF in both moDCs and pDCs. Our results are

consistent with previous studies reporting that mTOR inhibition reduces VSV-induced IFN-α production in Flt3L-dependent DCs. Furthermore, since we detected reduced TBK1 phosphorylation in the presence of both mTOR inhibitors following RLR activation, we suggest that mTOR regulates RLR-induced cytokine production in human DCs by phosphorylating TBK1.

In contrast to the cytokine responses, we could not detect significant changes in the phenotype of RLR-stimulated DCs when mTOR was inhibited, except for CD83 expression in pDCs, which was further enhanced by AZD8055 pretreatment in RLR-stimulated cells. Previously, our group has shown that upon rapamycin pretreatment CD83 levels were increased in TLR-activated CD1c+ cDCs, but not in moDCs. All these data suggest that the phenotypic effects of mTOR on DCs are highly dependent on cell type, activation mode and the type of receptor stimulated.

There are several data suggesting that mTOR is also involved in the regulation of antigen presentation by DCs and thus has the ability to influence the effector T cell activating capacity of DCs.

With our further results, we confirmed that rapamycin, an inhibitor of mTORC1, effectively reduces the CD8+ T cell proliferation-inducing capacity of RLR-stimulated moDCs and pDCs. However, surprisingly, the dual mTORC1/mTORC2 kinase inhibitor AZD8055 was only slightly able to reduce the T cell proliferation-inducing ability of RLR-stimulated moDCs, while it did not affect that of pDCs. Our observation is constistent with another study showing that rapamycin is a more effective inhibitor of CD8+ T cell proliferation induced by murine bone marrow DCs than the dual mTOR inhibitor AZD2014, which needs to be used at a dose 20-fold higher than rapamycin to achieve a similar inhibitory effect. Although, it should be noted that mTOR inhibitors are toxic to cells at higher doses, and we have also observed that AZD8055 at doses greater than 100 nM significantly reduces moDC survival. Similarly, Torin-1, another ATP-competitive mTOR inhibitor, was less effective than rapamycin in reducing the T-cell stimulatory capacity of TLR4-stimulated murine bone marrow DCs.

We also found that, in addition to T-cell polyfermentation, AZD8055 did not affect the ability of RLR-activated DCs to induce cytokine production by CD8+ T cells,

in contrast to rapamycin, which significantly reduced it. This phenomenon may be explained by the loss of a negative feedback loop mediated by mTORC2, which is suppressed by dual mTOR inhibitors. The dual PI3K/mTOR inhibitor NPV-BEZ235 has recently been shown to overactivate the mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway in human pancreatic cancer cells. Stimulation of DCs via PRRs also leads to the activation of various MAPKs, of which the MEK/ERK signaling pathway acts as a positive regulator of antigen presentation in moDCs. Interestingly, it seems that blocking mTORC2 alone enhances rather than inhibits the T cell stimulatory capacity of DCs. For example, the absence of mTORC2 in skin DCs induced enhanced CD8+ T-cell effector responses and increased skin graft rejection. In addition, in a mouse model, mTORC2-deficient myeloid DCs were shown to have enhanced allostimulatory capacity and greater ability to induce activation of IFN-y and IL-17-producing T cells following both TLR-dependent and TLR-independent stimulation. The mechanism of the enhanced antigen-presenting ability of mTORC2-deficient DCs has not yet been elucidated, and further studies are needed to explain the differences between the effects of mTORC1 and dual mTORC1/C2 inhibitors. However, our results indicate that the differential effects of first and second generation mTOR inhibitors on different immune cell functionalities should also be considered when developing new generation mTOR inhibitors.

### VI.2 Exogenous regulation of DC function: investigating the immunomodulatory effects of radiation-detoxified LPS in human moDCs

Among the exogenous factors in our environment, endotoxins may influence the functions of human DCs through their immunomodulatory properties.We investigated how the detoxified form of endotoxin may affect the immunological processes mediated by human DCs. According to the hygiene hypothesis exposure to environmental microbes and their endotoxins at an early age may be an effective activator of the innate immune response and may protect against the development of atopic diseases. However, the use of endotoxins as immunomodulatory agents is severely limited by their highly toxic nature, capable of inducing clinical symptoms including shock, fever, chills,

bronchoconstriction and non-specific bronchial hyperreactivity. Although, studies in animals and humans have shown that detoxification of LPS with 150 kGy of $^{60}$Co gamma radiation preserves the immunological adjuvant and immunomodulatory properties of the endotoxin and the detoxified form provides protection against endotoxin shock and radiation-induced diseases and stimulates the natural defence system.

First, we investigated the effects of native and radiotoxic endotoxins on the functional activity of human moDCs. The results of our *in vitro* experiments showed that human moDCs exposed to RD-LPS have similar ROS-forming capacity as RD-LPS-treated DCs and similar phenotypic maturation. Endogenous, intracellular ROS are essential mediators of antigen presentation by DCs, and phenotypic maturation of moDCs is required for interactions with T cells. Our further results showed that both forms of LPS significantly increased cytokine and chemokine secretion in moDCs, while moDCs treated with RD-LPS were characterized by a reduced inflammatory potential compared to moDC treated with N-LPS. The concentration of all measured secreted cytokines showed a decreasing trend compared to N-LPS-stimulated moDCs, but RD-LPS resulted in significantly lower protein levels only for pro-inflammatory IL-1β and anti-inflammatory IL-10 compared to N-LPS. The reduced pro-inflammatory effect of RD-LPS is probably due to detoxification. Previous studies have reported that ionizing radiation alters the structure of LPS and results in degradation of polysaccharide partsand alteration of the lipid A component. Irradiation significantly reduces glucosamine, phosphate, Kdo and fatty acid components in RD-LPS formulations, indicating a change in lipid A structure compared to native LPS formulations. It has been previously published that the length of the polysaccharide chain may also explain the differences in the inflammatory activity of LPS in different species, as *E. coli* LPS with a long polysaccharide chain generates weaker pro-inflammatory (IL-6, TNF) and anti-inflammatory (IL-10) cytokine responses than LPS with a shorter polysaccharide chain. Our experiments also demonstrated that a form of endotoxin detoxified by radiation was able to react with TLR4 and retained its binding capacity and specificity for this receptor.

It is important to note that in our experimental system both RD-LPS and N-LPS were able to induce endotoxin tolerance, as both inhibited the production of the pro-

inflammatory cytokines IL-12 and IL-1β after repeated LPS treatments. The fact that the non-toxic RD-LPS retained the ability to induce endotoxin tolerance could be considered a beneficial property of detoxified LPS. It has previously been described that the development of endotoxin tolerance in the body may be a crucial factor in alleviating allergic airway inflammation. In addition to secreting less inflammatory mediators upon secondary stimulation, endotoxin-tolerant cells also result in higher levels of pro-inflammatory cytokines, such as higher IL-10 and transforming growth factor beta (TGF-β) levels. However, in our system, neither native nor radiation-detoxified LPS resulted in increased IL-10 levels in endotoxin-tolerant moDCs compared to cells treated with a single dose of LPS. The observed difference is probably due to the heterogeneity of LPS molecules, since the type of LPS and the bacterial species from which it is derived can greatly influence the quality of the immune response.

Data from our ELISPOT measurements showed that treatment of human moDCs with RD-LPS has a higher potential to induce polarization of IFN-γ-producing Th1 cells in co-cultures than N-LPS. As there was no difference in intracellular ROS production and phenotypic maturation between the effects of N-LPS and RD-LPS, we speculate that differences in the induction of cytokine production may underlie the different Th1 inducing effect. N-LPS treatment resulted in significantly higher IL-1β and IL-10 cytokine levels compared to RD-LPS. IL-1β has previously been observed to affect Th2 differentiation, and culture of CD4+ T cells under Th2 polarizing conditions and in the presence of IL-1β resulted in a significant increase in IL-13 production compared to cells cultivated without IL-1β. Thus, it is likely that IL-1β from DCs may favour Th2-directed polarization, which overshadows the Th1 direction. IL-10 was originally described as a cytokine produced by Th2 cells, which has the ability to suppress cytokine production by Th1 cells, later it was found that activated DCs also produce. Our results suggest that the IL-10-inducing effect of N-LPS was more pronounced in moDCs than in RD-LPS treatments, suggesting that the lower Th1 polarizing capacity of N-LPS-treated DCs may be due to higher IL-10 levels. Furthermore, IL-10, IFN-γ limiting and Th2 cell promoting cytokines have been reported in several Th2-type inflammation in different *in vivo* mouse models. We hypothesized that the production of IL-10 by RD-LPS-treated moDCs could

also be affected by free fatty acids generated by ionizing radiation of endotoxin, as our studies showed that the concentration of β-hydroxymyristic acid was three times higher in RD-LPS preparations than in N-LPS. It is widely accepted that free fatty acids have immunomodulatory properties and influence the outcome of immune responses in the body. Our experiments have demonstrated that IL-10 production by LPS-treated moDCs is inhibited in the presence of myristic acid. Thus, we conclude that the higher myristic acid content of RD-LPS preparations may have contributed to the lower IL-10 production by moDCs exposed to RD-LPS. Due to the lower IL-10 levels, moDCs exposed to RD-LPS were able to induce IFN-γ-producing Th1 cells to a greater extent in co-cultures compared to DCs treated with the native form of LPS.

In our *in vivo* experimental system, prolonged pretreatment with RD-LPS (70 days) was able to significantly reduce ragweed pollen-induced airway eosinophilia in six-week-old BALB/c mice. In our experiments, we used a novel method of applying LPS in aerosol form prior to allergen exposure, which allowed the endotoxin to be distributed in the environment of laboratory animals, including bedding, food and drink. This technique helped us to better facilitate environmental renaturation and to model the conditions that characterize the environment of animal farms, where LPS levels are significantly higher than in downtown homes. Thus, endotoxin exposure of mice occurs not only by inhalation but also by oral route. Both inhalation and oral endotoxin treatment have been found to contribute to the prevention of atopy.

Our results suggest that compared with native LPS, the greater Th1 cell polarizing ability of RD-LPS may promote a shift of Th2-type inflammation towards Th1, which inhibits the development of Th2-mediated allergic processes. Therefore, we suggest that regular aerosol application of RD-LPS could contribute to the renaturation of urban environments and thereby reduce the risk of allergic disease. However, it is important to note that RD-LPS treatment may only be effective during the first year of life, when susceptibility to external microbial stimuli is still present.

## VII. Summary

In our work, we first aimed to elucidate the endogenous regulatory role of the mammalian target of rapamycin (mTOR) in the retinoic acid inducible gene (RIG)-induced effector functions of human monocyte-derived DCs (moDCs) and plasmacytoid DCs (pDCs). Our results show that RLR stimulation increased the phosphorylation of the downstream targets of mTOR complexes (mTORC1, mTORC2), p70S6 kinase and Akt, respectively, which was inhibited by the mTORC1 inhibitor rapamycin and the dual mTORC1/C2 kinase inhibitor AZD8055 in both DC subtypes. Furthermore, inhibition of mTOR in moDCs also inhibited RLR stimulation-induced glycolytic transition. Blockade of mTOR reduced the ability of RLR-stimulated moDCs and pDCs to produce type I interferon (IFN) and pro-inflammatory cytokines, whereas the phenotype of DCs was not affected. In addition, rapamycin inhibited the ability of both DC subtypes to induce IFN-γ and granzyme B-producing CD8+ T-cell proliferation and differentiation, which AZD8055 was unable to do. Thus, we have found that mTOR positively regulates RLR-mediated antiviral activity of human DCs and highlighted the differences between mTORC1 selective and dual mTORC1/C2 inhibition. We then investigated the immunomodulatory effects of environmental endotoxins in DCs. We wondered whether renaturation of the indoor environment with aerosolized, radio-detoxified lipopolysaccharide (RD-LPS) could prevent the development of Th2-type airway inflammation caused by ragweed. We first showed in our in vitro experiments that moDS exposed to RD-LPS have a higher Th1-polarizing capacity than moDS exposed to N-LPS. *In vivo* experiments, cages of six-week-old BALB/c mice were treated daily with aerosolized N-LPS or RD-LPS. After a ten-week treatment period, mice were sensitized and provoked allergic inflammation with ragweed pollen extract. Mice previously exposed to RD-LPS-rich milieu developed significantly less allergic airway inflammation than mice exposed to N-LPS-rich or LPS-untreated milieu. Thus, it can be suggested that early life renaturation of urban indoor environments with aerosolized, non-toxic RD-LPS may be a potential means of preventing Th2-mediated allergies.

## VIII. Publications

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Subject: PhD Publication List

Candidate: Beatrix Ágics
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
MTMT ID: 10076937

### List of publications related to the dissertation

1. Bácsi, A., **Ágics, B.**, Pázmándi, K. L., Kocsis, B., Sándor, V., Bertók, L., Bruckner, G., Sipka, S.: Radiation-Detoxified Form of Endotoxin Effectively Activates Th1 Responses and Attenuates Ragweed-Induced Th2-Type Airway Inflammation in Mice.
*Int. J. Mol. Sci. 25* (3), 1-22, 2024.
DOI: http://dx.doi.org/10.3390/ijms25031581
* These authors contributed equally to this work.
IF: 5.6 (2022)

2. Fekete, T., **Ágics, B.**, Bencze, D., Bene, K., Szántó, A., Tarr, T., Veréb, Z., Bácsi, A., Pázmándi, K. L.: Regulation of RLR-Mediated Antiviral Responses of Human Dendritic Cells by mTOR.
*Front. Immunol. 11*, 1-20, 2020.
DOI: http://dx.doi.org/10.3389/fimmu.2020.572960
IF: 7.561

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## List of other publications

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**Total IF of journals (all publications): 7,561**
**Total IF of journals (publications related to the dissertation): 7,561**

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

20 June, 2024

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

## VIII. Keywords

Innate immunity, dendritic cell, plasmacytoid dendritic cell, monocyte-derived dendritic cell, TLR, RLR, mTOR, RIG-I, MDA5, type I interferon, antiviral response, inflammatory response, ragweed, allergy, lipopolisaccharide (LPS), radiation-detoxified LPS (RD-LPS), BALB/c mice.

## IX. Acknowledgements

I would like to thank my supervisor, Kitti Linda Pázmándi, PhD, whose outstanding professional guidance, help and support made this thesis possible. I would also like to thank Tünde Fekete, PhD, who provided great assistance in my professional preparation and in the design and implementation of the experiments. I thank Angela Berki-Pál for her support in my laboratory work. I am thankful to Dóra Bencze, PhD for setting an example and for her encouragement. I would also like to thank Attila Bácsi, PhD, DSc, who gave me the opportunity to do my PhD studies and the experiments for my thesis at the Institute of Immunology.

I would also like to express my gratitude to Prof. Dr. Sándor Sipka and Prof. Dr. Géza Bruckner, who extensively studied the effects of RD-LPS and provided the essential professional foundation for the creation of this publication.

Particular thanks are due to Prof. Dr. Béla Kocsis and Dr. Viktor Sándor, who were of great help during the experiments, particularly through the mass spectrometric analysis of LPS extracts.

I would like to thank my mother, my family and friends who have always supported me throughout the years. I would especially like to thank my grandmother for her immeasurable support throughout my studies.

This thesis is dedicated to the memory of Prof. Dr. Lóránd Bertók, the "father of RD-LPS.