SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Modulation of human dendritic cell functionality by endotoxin tolerance and by the mammalian target of rapamycin

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The Examination takes place at the Department of Immunology, Faculty of Medicine, University of Debrecen, at 11:00 a.m. on 17th of December, 2014

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1:00 p.m. on 17th of December, 2014

1. Introduction

Dendritic cells (DCs) have been identified as the most potent antigen presenting cells (APC) that via continuously sensing their actual microenvironment are able to shape the outcome of both innate and adaptive immune responses. DCs play an unique role in directing immune responses through the recognition of harmful self or foreign environmental structures in peripheral tissues that results in DC activation and migration to the draining lymph nodes to stimulate effector T-lymphocyte proliferation and activation. However, DCs also induce central and peripheral tolerance ensured by the continuous introduction of innocuous antigens to T-cells under steady state conditions.

The unique role of linking innate and adaptive immunity, the functional plasticity and heterogeneity makes DCs optimal vaccine candidates for immune-based therapies. *Ex vivo* loading of DCs with adequate antigens can generate functional DCs, which cells induce antigen-specific T-lymphocyte proliferation and activation upon injection back to the patient with cancer or chronic infections. Recently, it has also been raised that DCs could be used as a tool to induce transplantation tolerance or to treat autoimmune disorders. To improve the therapeutic potential of DCs it is essential to gain a better understanding of DC biology, in particular how their tolerogenic and immunostimulatory properties could be modulated under different conditions or disease states.

The aim of the current study was to investigate how the functional abilities of DCs can be affected by persistent microbial stimulation and by the targeted suppression of the mammalian target of rapamycin (mTOR) and to identify the molecular mechanisms behind them.

1.1. Classification of human DC subsets and their role in maintaining homeostasis

DCs act as sentinels of the immune system and are present throughout the body, including the circulation, filtering organs, lymphoid tissues, mucosal surfaces and the skin. The wide variety of different environmental conditions requires high plasticity and indeed, DCs are composed of heterogeneous populations exhibiting diverse morphological, phenotypic and functional properties.

In the steady state dendritic cells originated from DC precursors can be classified into two classes: classical/conventional/myeloid (cDC) and non-classical/plasmacytoid DCs (pDC). Under inflammatory conditions this classification is further extended by the appearance of monocyte-derived DCs, which arise from monocyte precursors and are usually referred to as inflammatory DCs. Conventional dendritic cells can be subdivided into two further subsets: the blood dendritic cell antigen positive ($BDCA1^+/CD1c^+$) DCs and the $BDCA3^+/CD141^+$ DCs, which differ in their phagocytic, cytokine producing and T-cell simulatory capacity. pDCs and $CD1c^+$ DCs comprise approximately 1% of mononuclear cells in the blood and $CD141^+$ DCs also represent a minor subset of blood leukocytes (0.1% of mononuclear cells). Nevertheless, tissue resident and migratory DCs are abundant in the steady state and can efficiently fight against invading pathogens due to the expression of a wide range of pattern recognition receptors and their potent T-cell priming capacity.

DC acts as a double-edged sword since on one hand immunogenic DCs initiate potent innate and adaptive immune responses against invading pathogens, and on the other hand tolerogenic DCs promote and sustain tolerance and inhibit the induction of autoimmune responses. Numerous reports have confirmed the importance of this division of labor and crosstalk between DC subsets in orchestrating the immune system; however, several aspects of this regulation still remained to be elucidated.

1.2. Phenotypical and functional properties of *in vitro* generated DCs

Since DCs comprise only a small fraction (~1%) of peripheral blood mononuclear cells (PBMC) a search for finding more easily accessible progenitors has started. The first protocols for *in vitro* DC generation used bone marrow-derived $CD34^+$ HSCs; however, the limited number of these cells in peripheral blood (0.1%) and in the bone-marrow (1%) prompted the researchers to find more abundant progenitors. It was demonstrated in 1994 that DCs derived from blood mononuclear cells possess the typical features of immature DCs developed *in vivo*. Since then, monocytes are commonly used as progenitors to yield a rich source of DCs upon culturing with GM-CSF combined with different cytokines such as IL-4, IL-15, IFNα or TNFα. Commonly, monocytes are cultured with GM-CSF and IL-4 for 5-7 days to generate immature DCs that can subsequently be activated with microbial ligands or inflammatory cytokines to

obtain mature DCs. Lately, several papers suggested that functional moDCs can also be obtained within a shorter differentiation period (fast DC), thus better reflecting the *in vivo* situation. After 2 days of culture monocyte-derived cells already bear the phenotypic and functional characteristics of immature DC and have an equal potential to induce antigen-specific T-cell proliferation and IFNγ production as cells generated by the standard 7-day procedure. Another publication demonstrates a more efficient antigen processing capacity of 3-day DCs as compared to 7-day DCs, thus assigning fast DCs as optimal candidates for DC-based vaccines.

During their *in vitro* differentiation moDCs downregulate the monocytic marker CD14 and upregulate the DC-specific ICAM-grabbing non-integrin (DC-SIGN) and CD1a. These immature moDCs have many features of primary blood DCs such as the high expression of receptors that drive antigen-capture and migration. The maturation of DCs is coupled to the loss of phagocytic capacity accompanied by the upregulation of MHC II, maturation markers such as CD83 and costimulatory molecules such as CD80 and CD86, which boost their ability to present processed antigens to T-cells. A wide range of stimuli have been shown to activate DCs that affect the functional properties of maturing DCs differently. The maturation factors such as microbial compounds or a mixture of IL-1β, IL-6 and TNFα inflammatory cytokines mimic the *in vivo* infectious or inflammatory conditions and have a strong capacity to induce T helper type 1 cells (Th1) and cytotoxic T-lymphocytes (Tc). On the other hand addition of transforming growth factor β (TGFβ), IL-10 or corticosteroids results in the induction of tolerogenic DCs that can induce both $CD4^+$ and $CD8^+$ Tregs.

1.3. Pattern recognition receptors

DCs possess a broad range of pathogen sensors, termed pattern recognition receptors (PRRs) that can recognize conserved pathogen-associated molecular patterns (PAMPs) expressed by pathogens but not by host cells, thus enable DCs to discriminate between self and non-self structures. Later on, the Danger Model suggested by Polly Matzinger indicated that PRRs can also detect endogeneous molecules derived from damaged cells, referred to as damage-associated molecular patterns (DAMPs). PAMPs and DAMPs can initiate similar signaling pathways by inducing the transcription of genes involved in innate inflammatory immune responses. In mammals, 4 major classes

of PRRs have been described: Toll-like receptors (TLRs), RIG-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs).

The TLR family is one of the first and the best-characterized family of PRRs. So far 10 members of TLRs have been identified in humans and 13 in mice, more or less their ligands and mode of signaling have also been revealed. TLRs are transmembrane proteins and consist of three structural domains: a leucin reach repeat (LRR) ectodomain that mediates the recognition of PAMPs, a helical transmembrane domain and an intracellular Toll/IL-1R (TIR) domain initiating the downstream signaling pathways. Based on their cellular localization and ligand specificity the human TLR family members can be divided into two subgroups. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface and recognize different microbial membrane components such as proteins, lipids and lipoproteins. TLR3, TLR7, TLR8 and TLR9 are localized to intracellular vesicular compartments and detect nucleic acids such as single-stranded or double-stranded RNA (ssRNA or dsRNA) and DNA. Recently, it has been suggested that TLR10 with the aid of TLR2 might sense a wide variety of microbial compounds shared by TLR1, although a response to triacylated bacterial lipopeptide occured only under artifical conditions. TLR10 has also been identified as an innate sensor of viral infections; however, its specific ligand is still under investigation Ligand binding of TLRs results in the recruitment of one or more adaptor molecules and the subsequent initiation of diverse biological responses. All TLRs associate with the adaptor molecule, the myeloid differentiation primary response gene 88 (MyD88), except TLR3 that recruits the TIR-domain-containing adapter-inducing interferon-β (TRIF) to signal transduction. Exceptionally TLR4 can use both MyD88 and TRIF as adaptors and thus mediate more intense immune response.

CLR family members are calcium-dependent lektin-like receptors that bind a wide range of ligands derived from various microbes such as bacteria, viruses and fungi through their carbohydrate recognition domain. Activation of these receptors leads to the induction of both pro- and anti-inflammatory immune responses, therefore CLRs are suggested to play a pivotal role in the maintenance of immune homeostasis.

NLRs are cytoplasmic pathogen sensors with multidomain structure composed of a central nucleotid-binding oligomerization domain (NBD) and a C-terminal LRR sensor domain. The N-terminal portion of some NLRs has a caspase recruitment domain

(CARD) that is associated with the downstream signaling molecules. This NLR group includes members such as NOD1 and NOD2 recognizing peptidoglycan fragments of Gram-positive and Gram-negative bacteria. Recently, it has been found that beyond detection of peptidoglycans NOD2 is also involved in ssRNA-induced type I IFN production through a RIP2-dependent mechanism, thus mediating host defense upon viral infection.

The RLR family has three members: retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), all sensing viral or processed self RNA in the cytoplasm to induce innate signaling pathways and elicit inflammatory reactions. Intriguingly, beside detecting viral RNA genomes RLRs also act as sensors for cytoplasmic DNA and collaborate with TLRs and other signaling networks to modulate innate and adaptive immune responses.

All immune responses, including TLR-mediated signaling have the potential to damage the host leading to extensive tissue damage accompanied by pathological conditions such as autoimmune diseases, cancer, systemic inflammatory response syndromes (SIRS) including endotoxin shock or sepsis. Thus, a tight control and regulation of these responses is needed to avoid excessive inflammation and maintain homeostasis. Importantly, repeated exposure to endotoxin/lipopolysaccharide (LPS) renders innate immune cells such as DCs and macrophages hyporesponsive to subsequent challenges with LPS. This phenomenon, referred to as endotoxin tolerance (ET), is characterized by attenuated production of pro-inflammatory and increased production of anti-inflammatory mediators, thereby preventing overstimulation by the same danger signal allows the survival of a lethal secondary challenge with LPS. Several studies attempted to reveal the underlying molecular mechanism of endotoxin tolerance but it remained poorly defined so far. LPS stimulation is known to induce various numbers of negative regulators, that might provide inhibitory feedback mechanisms to terminate TLR4 downstream signaling. ET is usually linked with the upregulation of negative regulators such as IL-1 receptor-associated kinase (IRAK-M) and suppressor of cytokine signaling 1 (SOCS1) and with the over-expression of anti-inflammatory cytokines such as IL-10 and TGFβ. These findings indicate that LPS pre-

treatment more possibly induces reprogramming rather than an overall downregulation of gene expression.

1.4. The role of mTOR in regulating immune responses

The mammalian/mechanistic target of rapamyin (mTOR) is a serine/threonin kinase belonging to the PI3K related kinase (PIKK) family, which plays a central role in several cellular processes such as cell proliferation, differentiation, metabolism, motility and survival. mTOR exerts its modulatory effects through two multiprotein complexes: mTOR complex 1 (mTORc1) and mTOR complex 2 (mTORc2), that are connected to distinct upstream and downstream molecules, thus possess diverse functional attributes.

Recent studies revealed that mTOR plays a crucial role in the regulation of both innate and adaptive immune responses. Suppression of mTORc1 activity uncovered the essential role of mTOR signaling in the differentiation and maturation of DCs of different origin. Recently mTOR was identified as a critical regulator of inflammatory cytokine responses, since short-term treatment with rapamycin during TLR stimulation modulated the production of type I IFNs, pro-inflammatory and anti-inflammatory cytokines in various DC subtypes differently.

Most importantly, rapamycin treatment of pDCs impaired the TLR9-induced production of IFNα/β via suppressing the activity of IRF7 significantly. It has been demonstrated that the production of type I IFNs and pro-inflammatory cytokines is also suppressed in TLR7-activated human pDCs. Rapamycin also interfered with IFNβ production and phosphorylation of IRF3 suggesting the involvement of mTOR in the regulation of type I IFNs in keratinocytes. It was also demonstrated, that the dsRNA mimetic polyinosinic-polycystidylic acid (polyI:C)-induced c-Jun amino-terminal kinase (JNK) phosphorylation could be inhibited in the presence of rapamycin. Inhibiting JNK by SP600125 prevented the upregulation of pro-inflammatory cytokines and IFNβ following stimulation with the TLR3 ligand polyI:C. These results indicate that via regulating the activity of JNK mTOR collaborates with the mitogen-activated protein kinase (MAPK) pathway to upregulate IL-1β, TNFα and IFNβ upon stimulation with polyI:C. It is important to note that it has not been investigated before whether mTOR is involved in the regulation of TLR3-mediated production of type I IFNs in conventional DCs.

2. Aim of the studies

Aim 1. Study the development and control of endotoxin tolerance in moDCs

The phenomenon of ET has been studied and described in several pathologies such as sepsis, acure choronary syndrome and even cancer. A wide variety of innate immune cells are affected by ET rendering them in a transient state in which they are not able to respond to subsequent challenges with LPS. ET is mainly characterized by a decreased production of pro-inflammatory cytokines upon stimulation with endotoxin; however, little is known about the molecular mechanism behind it. To gain a deeper understanding in the regulation of ET we aimed at:

- exploring how long-term activation with low-dose LPS affects the phenotypic and functional properties of moDCs in response to further challenges with LPS.
- studying how various microbial compounds induce tolerance in moDCs.
- investigating the molecular mechanism of LPS-induced inhibition of DC functions during the early phase of moDC differentiation.
- screening the effects of a wide range of LPS-inducible inhibitory factors on moDC activation.

Aim 2. Study the regulation of IFN responses by mTOR in cDCs

mTOR controls many aspects of innate and adaptive immunity. Recent data have suggested an important role for mTOR in the regulation of type I IFN production by pDCs. However, to a lesser extent than pDCs, conventional DCs are also able to produce cytokines with potent antiviral activity such as type I/ III IFNs and IL-27 the potential regulatory role of mTOR has not been addressed in this issue. Thus, our goul was:

- to investigate the effects of mTOR inhibition on the phenotypic properties of moDCs and circulating CD1c+ DCs upon TLR3 ligation.
- to reveal how the mTOR-inhibitor rapamycin modulates the antiviral capacity of moDCs and CD1c+ DCs.
- to explore the molecular mechanism of inhibition exerted by rapamycin on polyI:C-induced production of type I and type III IFNs in moDCs.

3. Materials and Methods

Reagents

The TLR ligands LPS, CL075, HKSA, Zymosan, Pam3Cys and polyI:C were purchased from InvivoGen (San Diego, CA, USA). Soluble CD40L, IFNγ, TNF, IL-1, IL-6 and IL-4 were ordered from PeproTech EC (London, UK) and GM-CSF from Gentaur Molecular Products (Brussels, Belgium). Polyclonal IL-10 neutralizing antibody and the goat isotype control antibody were purchased from R&D Systems (Minneapolis, MN, USA). Rapamycin was obtained from Merck Millipore (Darmstadt, Germany). The PI3K inhibitor LY-294002 hydrochloride and DMSO were from SIGMA-Aldrich (Schnelldorf, Germany). Rapamycin and LY-294002 were used at a concentration of 100 nM and 10 μM, respectively.

Cell culture techniques

PBMCs were separated from buffy coats of healthy donors drawn at the Regional Blood Center of Hungarian National Blood Transfusion Service (Debrecen, Hungary) with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen. PBMCs were separated from buffy coats by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). Monocytes were isolated by positive selection using magnetic cell separation using anti-CD14-conjugated microbeads (Miltenyi Biotech, Bergish Gladbach, Germany). The purified cells were cultured at a density of 10^6- $2x10^6$ cells/ml in RPMI-1640 medium supplemented with 10% FBS (both from Life Technologies Corporation, Carlsbad CA, USA), 75-80 ng/ml GM-CSF (Gentaur) and 50-100 ng/ml IL-4 from Peprotech. cDCs were separated from PBMCs using the CD1c isolation kit (Miltenyi Biotech) and cultured in RPMI-1640 medium consisting 10% FBS plus 20 ng/ml GM-CSF. The purity of $CD1c^+$ DCs was 97.7±1.2% as measured by flow cytometry. Autologous naive $CD8^+$ T-cells were isolated from PBMC by using the human $CD8^+$ T-cell isolation kit (Miltenyi Biotech). Purity of naive T-cells was checked by cell staining with CD45RA-FITC and CD8-PE (both from BD Pharmingen) antibodies and the percentage of double positive cells was 87.3±5.6%.

T-cell stimulation with autologous moDCs

Activated moDCs were co-cultured with naive autologous $CD8^+$ T-lymphocytes in the presence of 1μg/ml anti-human CD3 mAb (BD Pharmingen) in RPMI medium at a ratio of 1:5. After 5 days of co-culture, the T-cells were restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (both from SIGMA-Aldrich) in the presence of GolgiStop (BD Biosciences, Franklin Lakes, NJ, USA) used according to the manufacturer's protocol for 4 hours. At the end of incubation the cells were labelled with CD8-PE, CD25-FITC and IgG1-FITC antibodies (both from BD Pharmingen), fixed and permeabilized by the BD cytofix/cytoperm solution and labeled with IFNγ-APC (BD Pharmingen) and foxp3-APC antibodies (R&D Systems).

Flow cytometry

Phenotyping of activated DCs was performed by flow cytometry using anti-PD-L1-PE antibody purchased from BD PharMingen (San Diego, CA, USA), anti-CD80-FITC, anti-CD86-PE from R&D Systems, anti-CD40-FITC, anti-CD83-PE, anti-HLA-DQ-FITC and isotype-matched control antibodes from BioLegend (San Diego, CA, USA). Fluorescence intensities were measured with FACSCalibur (BD Biosciences). Data analysis was performed with the FlowJo software (Tree Star, Ashland, OR, USA).

Cytokine measurements

Culture supernatants were harvested after 24 hours of cell activation. TNF and IL-12p70 cytokine secretion was analyzed in culture supernatants using the human BD OPTEIA™ TNF and IL-12p70 ELISA kits (BD Pharmingen). The concentration of IFNα and IFNβ was measured by the VeriKine™ Human Interferon Alpha and Beta ELISA kits, respectively (PBL Interferon Sources, Piscataway, NJ, USA). The level of secreted IFNλ1 and IL-27 was measured by the human IL-29 and IL-27 ELISA Ready-Set Go kit, respectively (eBioscience, San Diego, CA, USA).

Real-time quantitative PCR (Q-PCR)

Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and was reverse-transcribed using the High Capacity cDNA RT Kit of Applied Biosystems (Carlsbad CA). All gene expression assays were purchased

from Applied Biosystems. Results were normalized to the housekeeping gene cyclophilin or RNU48 in case of the miR assays (Integrated DNA Technologies, Coralville, IA, USA). Q-PCR was performed using the ABI StepOne Real Time PCR System (Applied Biosystems) and cycle threshold values were determined using the StepOne v2.1 Software (Applied Biosystems).

Western blotting

Protein extraction was performed by lysing the cells in Laemmli buffer. Proteins were denaturated by boiling for 10 minutes then samples were separated by SDS-PAGE using 7.5% or 10% polyacrylamide gels and electro transferred to nitrocellulose membranes (BIO-RAD Laboratories Inc, Germany). Nonspecific binding was blocked by TBS-Tween-5% non-fat dry milk for 1h at room temperature, except pIRF3, where TBS-Tween-5% BSA (BSA was purchased from PAA Laboratories GmBH, Pasching, Austria) was applied. Anti-IRF3, anti-pIRF3 (Ser396), anti-TBK1 (all from Cell Signaling, Danvers, MA, US), anti-β-actin (Sigma-Aldrich) and anti-TLR3 (Abcam, Cambridge, UK) antibodies were used at a dilution of 1:500 or 1:1000; secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used at 1:5000. Protein samples were visualized by ECL system (SuperSignal West Pico/ Femto Chemiluminescent Substrate; Thermo Scientific, Rockford, IL, US). The protein bands were scanned and band density was determined using Kodak 1D Image Analysis Software version 3.6 (Kodak Digital Science Imaging, Eastman Kodak Company, New Haven, CT, USA). Relative density was calculated by the ratio of pIRF3 versus IRF3 band intensities.

RNA interference

All gene-specific and negative control siRNAs were purchased from Life Technologies except of IRAK-M that was ordered from Thermo Scientific Dharmacon (Lafayette, CO, US) with its appropriate non-targeting control siRNA. TBK1-specific Silencer Select Validated siRNAs and Silencer Select Negative Control siRNA were purchased from Life Technologies. The microRNA LNA-inhibitors for miR-146a and miR-155 and microRNA controls were purchased from Exiqon. Precursors for miR-146a, miR-155 and non-targeting microRNA controls were purchased from Applied Biosystems.

Cells were transfected in Opti-MEM medium (Life Technologies) in 4-mm cuvettes (Bio-Rad) using GenePulser Xcell instrument (Bio-Rad).

Phospho-protein array

Human Phospho MAPK array kit was purchased from R&D Systems (Minneapolis, MN, USA). Monocytes were seeded in 6-well plates at a density of 10^6 cells per ml. After 3 days of culture DCs were activated by polyI:C and after 20 minutes of induction cells were rinsed with PBS and lysed with the provided buffer. A mixture of the cell lysates and the provided phospho-specific antibody cocktail were incubated with each human phospho-MAPK array overnight at 4ºC. After repeated washing steps arrays were exposed to chemiluminescent reagent then nitrocellulose membranes were exposed to X-ray films. Phospho-MAPK array spot signals developed on X-ray films were quantitated by scanning the film and analyzing the array image file using the image analysis software Kodak 1D 3.6. The relative expression levels of phosphorylated events were calculated by normalizing with the positive control signal intensities.

Microarray analysis

RNA was isolated from MoDCs precultured with or without 5 ng/mL LPS for 2 days using TRI reagent (Invitrogen) followed by a second purification on RNeasy columns coupled with DNase I treatment (Qiagen) according to the manufacturer's recommendations. The extracted samples were labeled with Cy5, hybridized on Illumina Whole Genome HT12 microarrays, according to the manufacturer's instructions. After scanning, bead-level data was converted into bead-summary data using the Illumina BeadStudio software. Prior to normalization, array probes were annotated using their sequence and converted to unique nucleotide identifiers (nuIDs). Background signal was assessed and corrected using the intensity signal from the control probes present on the array, and then quantile normalization was performed with the aid of the lumi R package. Microarray data has been submitted to the Array Express repository (accession number: E-MTAB-658). Differentially expressed genes were calculated using the Rank Product algorithm. Differentially expressed genes were called

significant when their corrected p-value (percentage of false positives) was equal to or lower than 0.05.

Statistical analysis

Statistical significance of western blot experiments was determined by one-tailed Student t test with Welch correction and the results of flow cytometry, Q-PCR and ELISA studies were analyzed by one-way ANOVA with Bonferroni post-hoc test using the GraphPad Prism v.6. software (GraphPad Software Inc. , La Jolla, CA, USA). Differences were considered to be statistically significant at $P<0.05$.

Significance is indicated by asterisks: * $P<0.05$; ** $P<0.01$; *** $P<0.001$, **** $P<0.0001$. n.s. , non-significant.

4. Results

4.1. Modulation of human dendritic cells functionality by endotoxin tolerance

4.1.1. MoDCs are unable to upregulate inflammatory cytokine genes when differentiated in the presence of LPS

In order to understand the mechanisms leading to impaired functionality of chronically activated DCs we determined the kinetics and extent of the LPS induced IL-12, TNF and IL-6 gene expression in moDCs developed from peripheral blood monocytes in a 2-day culture in the presence or absence of 5 ng/mL LPS. We found that the 2-day LPS pre-treatment completely blocked the induction of IL-6, IL-12 and TNF genes by a second LPS stimulus, whereas without LPS pre-treatment moDCs responded to LPS signal with a rapid and strong induction of these genes. To study if the tolerization of developing moDCs by an early encounter with stimulatory signals is a general phenomenon, or if it is specific for single LPS stimulus, we treated the cells with a wide variety of stimulatory factors, applied separately or in combination with LPS between day 0 and 2 of moDC cultures. Few of these signals induced detectable TNF production when applied to monocytes alone, namely, heat-killed Staphylococcus aureus (HKSA), an inducer of TLR2 signals and CL075 that triggers TLR7/8. LPS synergistically increased the levels of TNF when combined with CD40L, the TLR2 ligands HKSA or Pam3Cys, with CL075 or with the combination of TNF, IL-1 and IL-6. No activation or very low cytokine levels were observed with TNF, IFNγ and the TLR3 ligand polyI:C. When the cells were washed and reactivated by 100 ng/mL LPS at day 2, we observed a complete inhibition of TNF production in moDCs that differentiated in the presence of CD40L, HKSA, Pam3Cys, CL075, TNF or the combination of TNF, IL-1 and IL-6. The 48h presence of LPS resulted in a persistent DC inactivation both when LPS was added alone and when it was combined with any of the other activation signals. These results showed that a wide variety of stimulatory signals can desensitize developing moDCs for further activation signals.

4.1.2. LPS induces several inhibitory factors in moDCs that may decrease cellular activation

In order to search for molecular mechanisms responsible for DC inactivation by chronic stimulatory signals we compared the gene expression pattern of moDCs that developed for 2 days in the presence or absence of LPS using the Illumina microarray technology. We observed a significant upregulation of potential DC inhibitory factors such as CD150, SOCS2, SOCS3, LILRB2, S100A8 and S100A9 in response to 2-day exposure to LPS. Other known inhibitory factors, such as ATF3, SOCS1, STAT3 or IRAK-M were expressed similarly in LPS-treated and control samples. Elevated levels of IL-10 were detected in moDCs cultured for 2 days in the presence of LPS by ELISA. In line with previous findings the expression of miR-146a and miR-155 were upregulated by LPS added at day 2 to moDCs. In order to better understand which DC modulatory factors might participate in DC exhaustion by persistent activation signals we analyzed the expression kinetics of a wide range of potential inhibitory factors in moDCs developing in the presence or absence of LPS. The expression of all studied DC modulatory factors, namely, SOCS1, SOCS2, SOCS3, IRAK-M, ATF3, S100A8 and S100A9, STAT3, LILRB2, IkBα, IkBβ and CD150 was induced similarly by the presence of LPS in developing moDCs showing the highest difference between LPS treated and non-treated moDCs during the first day of culture. The initial peaks in gene expression were followed by a rapid decline in case of all of these molecules reaching the same or minimally elevated level by day 2 in LPS-treated DCs as compared to control cultures. These results indicated a time-limited effect of the studied molecules in DC functions rather than a role in persistent DC inactivation.

4.1.3. LPS-induced SOCS1, STAT3, SLAM, IL-10 and miR-146a do not inactivate DCs persistently

We set up a screening assay to study if the LPS-induced DC modulatory molecules could influence cytokine production in moDCs. A potential role in inducing long-term DC inactivation was tested in moDCs pre-treated for 2 days with a low dose of LPS and then activated by a second, high-dose of LPS stimulus or with CL075 on day 2. We transfected the monocytes with siRNAs specific for the individual DC modulatory factors (SOCS1, SOCS2, SOCS3, STAT3, CD150, S100A8, S100A9 and IRAK-M) or with miR-146a and miR-155 inhibitors, as well as with control reagents and thereafter we cultured the cells for 2 days in the presence or absence of LPS. We studied the role

of LPS-induced IL-10 production in DC inactivation using IL-10-specific neutralizing antibodies included during LPS-pre-treatment as well as during reactivation of the cells. At day 2, we activated both LPS pre-treated and non-treated cells with LPS or CL075 and measured IL-12 production. moDC transfection by siRNAs targeting STAT3, CD150 or the inhibition of miR-146a and IL-10 increased IL-12 production by the cells that received a single activation by LPS or CL075 at day 2. Transfection with SOCS1-specific siRNA led to increased IL-12 production induced by LPS at day 2 while not affecting the activation induced by CL075. To further test a potential inhibitory function of miR-146a and miR-155 on moDC activation, we transfected monocytes with precursors of these miRNAs and activated the cells using LPS, poly(I:C), CL075 or CD40L after 2 days of culture. Transfection of developing moDCs with miR-146a and miR-155 led to decreased IL-12 and TNF production in response to all tested activation signals. Downregulation of SOCS2, SOCS3, IRAK-3, S100A8 and S100A9 led to unaffected or decreased IL-12 production, indicating no inhibitory effect of these factors in moDC activation. Importantly, inhibition of none of the tested DC modulatory molecules had an impact on the strong inhibitory effect of LPS pre-treatment on IL-12 production triggered by a second activation signal. Thus, moDC activation during early differentiation may lead to functional exhaustion independently of the tested regulatory factors.

4.2. Regulation of human dendritic cells functionality by mTOR

4.2.1. mTOR is constitutively expressed and functionally active in human cDCs

To assess the role of mTOR in polyI:C-induced signaling of cDCs we first tested its expression in human moDCs and in circulating $CD1c^+$ DCs at the mRNA level. Freshly isolated monocytes were differentiated in the presence of GM-CSF and IL-4 for 7 days and samples were collected daily for mRNA analysis. Results of the kinetic studies demonstrated that mTOR is constitutively expressed during moDC differentiation. mTOR is also expressed in circulating $CD1c^+$ DCs at levels comparable to those found in moDCs. We have also found that TLR3 is expressed at comparable levels in fast moDCs and circulating CD1c+ DCs. These observations were further supported by the results of the MAPK arrays showing that mTOR is persistently active both in 3-day and

5-day moDCs. Several *in vitro* studies, including our previous results revealed that functionally competent moDCs can be obtained within a short period of *in vitro* differentiation, thus better reflecting *in vivo* DC generation. Considering that mTOR is constitutively expressed and is active in the course of DC differentiation, we decided to use 3-day moDC cultures in our further experiments.

4.2.2. mTOR modulates the expression of DC cell surface molecules

Activation of moDCs by inflammatory stimuli is known to induce the cell surface expression and activity of various co-stimulatory molecules. To test the effects of short-term treatment of DC with rapamycin the expression of various membrane-expressed co-stimulatory markers and the human leukocyte antigen-DQ (HLA-DQ) was analyzed by flow cytometry after 24 hours of stimulation by polyI:C. A significant decrease in the fluorescence intensity of HLA-DQ, CD40, CD80 and CD86 proteins was observed, whereas the expression of CD83, frequently used as a DC maturation marker, was not affected by pre-treatment of moDCs with rapamycin. We also tested the expression of programmed cell death 1 ligand 1 (PD-L1), an inhibitory molecule known to promote regulatory T-cell development. Although the upregulation of PD-L1 has been shown in various cell types upon encounter with rapamycin, we could not detect any alteration in the levels of PD-L1 in resting moDCs. Moreover, as a result of polyI:C-mediated moDC activation rapamycin pre-treatment prevented the upregulation of PD-L1 in moDCs. Similar results were obtained when the expression of CD40, CD80 and PDL-1 was measured in $CD1c^+$ DCs. However, in contrast to moDCs we observed that rapamycin did not affect the expression of HLA-DQ while enhanced the percentage of $CD83^+$ cells significantly. These results indicate that mTOR controls the expression of various cell surface molecules on both moDCs and $CD1c^+$ DCs differently.

4.2.3. Rapamycin pre-conditioning impaires polyI:C-induced immune responses in moDCs

To analyze the role of mTOR in the regulation of polyI:C-induced signaling in DCs we first tested the kinetics of antiviral cytokine expression in rapamycin pre-treated and subsequently stimulated moDCs. We found that blocking of mTOR activity was able to decrease the upregulation of the IFNβ, IFNλ1, IFNλ2 genes at 12 hours and that of the

IFNα1 gene at 24 hours significantly. Consistent with the data obtained by Q-PCR measurements we also found significant impairment of IFNα, IFNβ and IFNλ1 secretion in rapamycin pre-conditioned moDCs when compared to their respective controls. We also measured the expression of IL-27 as it was demonstrated to act as an antiviral cytokine by promoting the development of effector $CD8^+$ T-lymphocytes. Interestingly, rapamycin pre-treatment diminished the expression of the p28 subunit that was accompanied by the enhanced expression of the EBI3 subunit, which in association with the p35 subunit forms the recently identified IL-35 cytokine with broad suppressive activity. Next to demonstrate the regulatory functions of mTOR we tested the impact of rapamycin-pretreated moDCs on the effector functions of autologous naive $CD8^+$ T-cells. After the 5-day coculture of moDCs and T-cells we measured the percentage of IFNγ-producing T-lymphocytes by flow cytometry and found that the T-cell stimulatory capacity of polyI:C-activated moDCs could dramatically be decreased by the inhibition of mTOR activities underpinning the contribution of mTOR to moDC-induced effector T-cell activation.

We also showed that rapamycin pre-treated moDCs could prevent the upregulation of CD25 on T-cells. Remarkably, none of the treatment protocols resulted in the induction of $CD8^+foxp3^+$ T-cells. The lack of $foxp3^+$ T-lymphocytes might be connected to the decreased expression of the inhibitory protein PD-L1, which was found to play an essential role in the generation of $foxp3^+$ regulatory T-cells.

4.2.4. The antiviral activity of $CD1c^+$ DCs is regulated by mTOR

To further confirm the role of mTOR in regulating antiviral responses we repeated the experiments performed with moDCs with peripheral blood-derived $CD1c^+$ DCs. Similarly to moDCs, pre-treatment of these cells with rapamycin could reduce the transcript levels of type I/III IFNs, the p28 subunit of IL-27, while the expression of EBI3 was upregulated as a result of polyI:C stimulation. In line with the results obtained in moDCs, the production of IFNα was dramatically inhibited and the secretion of IFNβ and IFNλ1 was completely abolished in $CD1c^+$ DCs indicating a more prominent role of mTOR in the induction of type I and III IFNs in $CD1c^+$ DCs as compared to moDCs. Moreover, polyI:C-induced $CD1c^+$ DCs were able to produce detectable levels of the IL-27 protein that could be inhibited by rapamycin pre-treatment. These data revealed

for the first time that in $CD1c^+$ DCs mTOR signaling is required for the optimal production of IL-27 and type I/III IFNs induced by polyI:C.

4.2.5. Inhibition of mTOR downregulates the transcriptional activity of IRF3

To analyze the role of mTOR in the activation of interferon regulatory factor 3 (IRF3), the master regulator of interferon production in moDCs, we first assessed the expression level of interferon stimulated gene 56 (ISG56) that is a sensitive indicator of IRF3 transcriptional activity. In this set of experiments moDCs were pre-treated with rapamycin or left untreated and were cultured in the presence or absence of polyI:C for 6 hours. We found that rapamycin pre-treatment decreased the expression of ISG56 significantly. To test whether IRF3 activity could be affected by the inhibition of mTOR activity, we stimulated moDCs by polyI:C at different time points and analyzed the cell lysates by Western blotting. As a result of rapamycin pre-treatment the level of IRF3 phosphorylation decreased significantly measured 2 hours after polyI:C stimulation. These data altogether suggest the pivotal role of mTOR signaling in the regulation of IRF3 activity.

4.2.6. Regulation of MAPK signaling by rapamycin in resting and polyI:C-stimulated moDCs

Several members of the TLR family including TLR3 share the capability to activate MAPKs that mediate cell activation and the synthesis of various inflammatory mediators. To determine whether rapamycin pre-treatment could modulate the activation of MAPKs and other intracellular proteins important for polyI:C-induced signal transduction, we performed phospho-protein array analysis to assess the activity of 26 kinases in cell lysates obtained 20 minutes after stimulation. We found that rapamycin pre-conditioning had no effect on several components of the MAPK cascade irrespective of their resting or activated state. Interestingly, addition of rapamycin to resting cells increased the phosphorylation of Akt (pan), JNK3 and p38γ. Furthermore, polyI:C stimulation enhanced the activity of several kinases, which was retained in case of Akt (pan), JNK3 and p38γ by rapamycin pre-conditioning. These data suggest that rapamycin treatment affects the activity of MAPKs differently in resting and polyI:C-activated moDCs.

4.2.7. The PI3K/mTOR pathway and TBK1 cooperate to induce optimal expression of type I IFNs

To gain insight into the mechanisms responsible for decreased type I and III IFN production as a result of inhibited mTOR activity we performed blocking experiments using the synthetic drug LY-294002 acting as a PI3K inhibitor, as well as gene silencing experiments targeting TANK-binding kinase (TBK1) by specific siRNAs. In these experiments IFNβ and IFNλ1 were selected as indicators of rapamycin-mediated blockade of IFN mRNA expression. Following stimulation by polyI:C, LY-294002 was shown to decrease the transcription level of both IFNβ and IFNλ1, although to a lower extent than rapamycin. Similar degree of inhibition could be achieved when the two pharmacological inhibitors were used in combination suggesting that both PI3K and mTOR signal through the same pathway in moDCs as indicated by previous reports. In another set of experiments, 1-day moDCs were transfected by siRNA specific for TBK1 or scrambled siRNA as control and 48 hours later the cells were stimulated by polyI:C. We found that TBK1 silencing decreased the mRNA levels of both IFNβ and IFNλ1 to a similar extent as rapamycin. When rapamycin was administrated to TBK1-depleted cells, even higher and in case of IFNλ1 a statistically significant reduction of transcript levels was observed. These data altogether suggest that mTOR exerts its modulatory effect through the classical PI3K/Akt/mTOR pathway and may regulate the induction of type I and III IFNs along with TRIF-mediated TBK1.

5. Discussion

A large number of molecules controlling DC functionality have already been discovered; however, the complexity of their regulatory potential needs further clarifications. In this study we set out to investigate how dendritic cell functionality is affected by persistent microbial stimuli that moDCs might encounter in the inflamed tissues and to explore the mechanism behind it. Furthermore, we investigated another aspect of regulation by studying the possible modulatory effects of rapamycin, a widely-used immunosuppressive agent on human DC subsets of different origin.

Prior exposure to endotoxin renders innate immune cells such as monocytes and macrophages unresponsive to subsequent challenges by the same or heterologous stimuli. As microbial components and inflammatory mediators are constantly present in inflamed tissues, they can exert various affects on DCs developing from monocyte precursors. We initiated studies to examine inhibitory pathways acting in stimulated moDCs in the presence of early and persistent TLR4 stimulation. Determining the extent of inhibition we observed that a 2-day pre-treatment with LPS blocked the induction of several pro-inflammatory cytokine genes completely by a second LPS stimulus. Further, we found that a wide range of stimulatory signals such as PAM3Cys, TNFα or CD40L can also result in desensitization of developing moDCs for subsequent activation signals.

Several molecular mechanisms are implicated in macrophage and DC exhaustion including the decreased or increased expression of signaling components, regulatory factors or the release of inhibitory mediators that might interfere with DC functions. An early study indicated the downregulated cell surface expression of TLR4 as a major cause of LPS tolerance in mouse macrophages. On the contrary, human LPS-tolerized monocytes show unaltered TLR4/MD2 expression. In line with that we did not find any significant difference comparing the TLR4 expression of LPS-pre-treated and non-treated moDCs. The downregulation of the signaling component IRAK-1 has been suggested as another possible mechanism of ET since both its protein level and kinase activity is greatly decreased in LPS-tolerant cells. IRAK-1 downregulation results in low IL-12 cytokine production by activated moDCs suggesting that IRAK-1 degradation alone might be sufficient to induce refractoriness to subsequent exposure to

TLR ligands. Recently it has been observed that endotoxin tolerization of human monocytes compromises LPS-inducible K63-linked polyubiquitination of IRAK1 while not affecting the expression of unmodified IRAK-1. K63-linked polyubiquitination of IRAK1 promotes its assembly with IKKγ and TRAF6, thus positively regulates TLR4 signaling. These results highlight the importance of suppressed K63-linked polyubiquitination of IRAK1 but not the degradation of the protein in the induction of endotoxin tolerance.

In response to TLR stimulation several inhibitory molecules might be induced; however, it is still unclear, how these factors contribute to the development of resistance to further activation. We showed that the inhibition of SOCS1, STAT3, SLAM, miR-146a and IL-10 molecules increased the LPS-induced IL-12 production, while none of these molecules played an essential role in the establishment of tolerance to further activation signals. We suggested that the short-term influence of the tested inhibitory signaling components might be a consequence of the transient increase in their gene expression or the presence of other inhibitory signals. Moreover, our results indicate the presence of coexisting inhibitory pathways that might all contribute to DC exhaustion.

Lately, several feasible models have been suggested as the underlying mechanism of ET development. One of the models indicated a possible role for matrix metalloproteinases (MMPs) in the control of this phenomenon. MMPs can target the membrane-bound form of the triggering receptor expressed on myeloid cells (TREM) resulting in the release of the soluble form of the molecule (sTREM). Whereas the membrane anchored TREM-1 initiates inflammatory responses, the sTREM exerts anti-inflammatory properties. In that particular study it has also been found that sTREM is not necessary to induce tolerance in human monocytes. However, the sustained presence of membrane-bound TREM-1 can efficiently interfere with the development of ET by a so far unknow mechanism. Moreover, using a general MMP inhibitor prevented the downregulation of pro-inflammatory cytokines in tolerant monocytes stimulated with LPS. In accordance with that study, analysis of our microarray gene expression data revealed an increased expression of MMP7, MMP9, MMP12 and MMP25 in LPS-pretreated moDCs (data not shown). These results indicate the requirement for further studies analysing the role of individual MMPs in the control of TREM expression and thus in ET development.

Another publication suggested a regulatory role for IDO and TGFβ pointing to the necessary link between the signaling events mediated by the two molecules in development of a fully endotoxin-tolerant state. In our experiments the expression of TGFβ was similar in LPS pre-treated and control samples. In comparision, the expression of IL-10, another effective mediator of anti-inflammatory reactions, was increased both at mRNA and protein level in LPS-pre-treated moDCs. Nevertheless, the neutralization of IL-10 by blocking antibodies could not prevent moDC exhaustion upon a second challenge with LPS. This result is in concert with a previous study showing that IL-10 is not the central effector as IL-10 deficient mice still develop ET. Further studies suggested a potential role for the alternative NFκB pathway member p100 (NFκB2) in the control and development of ET in human monocytes. The monocytes of patients with sepsis displayed an increased expression of p100 and the knockdown of that molecule reversed the refractory state of monocytes from septic patients and from in vivo and in vitro models following exposure to LPS.

All these results together with ours indicate the existence of several possible regulatory mechanisms being responsible for ET. Some of these mechanisms, which might even cooperate as a network to establish the well known characteristics of LPS-tolerized cells, has been started to be elucidated. However, a complete picture of this process is still lacking and needs further clarifications.

Next we investigated how rapamycin, a commonly used immunosuppressive mTOR inhibitor might influence the TLR3-induced IFN responses of human moDCs and $CD1c^+$ DCs. Several human cDC subsets express TLR3 and respond to stimulatory signals by producing large amounts of type I and/or III IFNs. Both 3-day moDCs and $CD1c^+$ DC express TLR3 but not RIG-I or MDA5 and as a result of cell activation they are able to induce the production of both type I and III IFNs. Interestingly, a brief incubation of the two DC subsets with rapamycin resulted in a significant decrease of IFNα, IFNβ and IFNλ1 production. Remarkably, only $CD1c^+$ DCs were able to secrete the bioactive IL-27 heterodimer that could be inhibited by rapamycin pre-conditioning. In human macrophages TLR3-induced expression of IL-27 was shown to be mediated by intracellular IFNα and its TLR4-mediated synthesis was demonstrated to be dependent on the activation of the TRIF/IRF3 pathway. Based on these observations we

hypothesized that in rapamycin pre-treated DCs both the restrained production of IFNα and the reduced activity of IRF3 could result in decreased expression of IL-27.

IRF3 is an integral transcription factor that is responsible for the induction of antiviral genes and its activation was shown to occur via a two-step mechanism mediated by TBK1 and PI3K. We hypothesized that rapamycin might decrease IFN production via the inhibition of IRF3. Indeed, we found a significant decrease in polyI:C-mediated phosphorylation of IRF3 in rapamycin pre-treated moDCs. The simultaneous blockade of mTOR and PI3K did not modify the extent of suppression elicited by rapamycin suggesting that IRF3 activity is engaged by mTOR via the PI3K/mTOR signaling pathway. Silencing of TBK1 by siRNA interfered with polyI:C-induced expression of IFNβ and IFNλ1 to a similar extent as rapamycin. Interestingly, the combination of TBK1 siRNA and rapamycin further reduced the expression of IFNβ and IFNλ1 but did not blocked completely. Based on these data we cannot exclude the involvement of other pathways in the induction of type I and III IFNs evoked by polyI:C stimulation in DCs.

Investigating the functionality of MAPKs and other intracellular signaling proteins we found that in resting cells several kinases show a tendency towards enhanced phosphorylation upon rapamycin treatment. Our data revealed that rapamycin decreased the phosphorylation of JNK3 and p38γ in polyI:C-activated moDCs significantly. Inhibition by synthetic molecules revealed the importance of p38 and JNK in the induction of IFNβ gene expression. Since rapamycin has the potential to decrease the activity of kinases playing an important role in the induction of type I IFNs we suppose that mTOR-mediated IFN responses rely partially on the MAPK cascade for fine-tuning polyI:C-induced signaling events.

The results of our co-culture experiments show that rapamycin pre-conditioned moDCs triggered by polyI:C are impaired in their ability to induce IFNγ production by $CD8^+$ T-cells. Several previous reports indicated that rapamycin-conditioned DCs can induce the expansion of $foxp3^+$ regulatory T-cells and microbial infection can generate highly suppressive $CD8^+CD25^+foxp3^+$ T-cells. In our system we found decreased expression of CD25 and undetectable levels of $foxp3^+$ cell in $CD8^+$ T-cells co-cultured with rapamycin pre-treated moDCs. This functional state might be the consequence of decreased PD-L1 expression that is the pre-requisite of regulatory T-cell expansion.

Since PD-L1 is induced by IFNα, IFNβ and IL-27 directly, its reduced expression together with the concomitant lack of the foxp3$^+$ regulatory T-cell population might be connected to the abrogated production of type I IFNs and IL-27 in rapamycin pre-conditioned DCs.

The stimulation of antigen receptors, cytokine receptors or several Toll-like receptors all can lead to the activation of mTOR that regulates various components of the immune system. mTOR is also implicated in many physiological processes including protein synthesis, autophagy and metabolism. Autophagy is a conserved catabolic process that recycles intracellular components to maintain cellular energy levels; a mechanism that evolved as a cellular survival response to stress. Autophagy, that has been found to be negativel regulated by mTOR, plays an important role in DC functionality since DCs utilize the autophagic pathways to efficient antigen processing and presentation. In addition, mTOR initiates a switch from oxidative phosphorylation to glycolysis that is a hallmark of T-cell activation and proliferation. Resting dendritic cells and macrophages activated by TLR ligands also show a shift toward the aerobic glycolysis. mTORc1 acts through the stabilization of the mRNA of the HIF-1α transcription factor that is required to the induction of glycolysis and also to the production of key pro-inflammatory proteins by myeloid cells. Besides, mTORc1 promoting the HIF-1α-dependent glycolytic pathway induces the differentiation of Th17 cells and suppresses Treg cell development. All these results show that mTOR integrates multiple signaling pathways providing a link between cellular homeostasis and immune responses.

Our results demonstrate that mTOR positively regulates type I and III IFN production via IRF3, which is under the simultaneous control of the PI3K/mTOR pathway and TBK1. In addition, we propose the supportive role of the MAPK cascade in promoting optimal IFN responses. In conclusion, our results provide with additional insight into the complexity of mTOR-mediated regulation of DC functions that could be relevant to improve the therapeutic potential of rapamycin in the treatment of diseases with uncontrolled type I IFN production. Furthermore, identifying the action of mTOR-mediated pathways may offer novel strategies to design more potent DC vaccines against infectious agents or cancers.

6. Summary

DCs are the most efficient antigen presenting cells that are widely distributed across various organs and tissues in the human body. Upon encounter with foreign antigens or altered self-antigens DCs become activated and initiate T cells to respond with unique functions and cytokine profile, which characteristics renders DCs potential targets for immune-based therapies. To date, DC-based immunotherapy has been broadly explored for the treatment of patients with cancer or infectious diseases; however, the overall efficacy of DC vaccines needs to be improved. To enable the development of DC-based immunotherapy we need to gain a better understanding of DC biology. The goal of the present study was to investigate DC functionality in the context of endotoxin tolerance and mTOR inhibition both of which conditions have a potentially high clinical relevance.

We found that a brief pre-treatment of differentiating DCs with LPS alone or in combination with other activation stimuli resulted in persistent inactivation of moDCs. A wide range of stimulatory signals could also desensitize developing moDCs for subsequent activation by LPS and synergistic activation signals did not prevent the cells from functional exhaustion. In response to a second LPS-stimulus we detected a completely blocked induction of inflammatory cytokine genes in LPS-tolerized moDCs implying a robust impairment of the signaling cascade leading to DC activation. Studying the role of a wide variety of DC-inhibitory mechanisms we found that SOCS1, STAT3, SLAM, miR-146 and IL-10 induced by early exposure to LPS exerted only a short-term inhibitory effect on the production of IL-12. However, none of the tested molecules played an essential role in the induction of tolerance to further stimulatory signals.

Next we demonstrated that the PI3K/mTOR pathway is indispensable for eliciting intact type I and III IFN responses in moDCs stimulated with polyI:C. Similarly to moDCs, the mTOR-mediated regulation is also essential to the production of type I and III IFNs in circulating $CD1c^+$ DCs. The inhibition of mTOR functionality by rapamycin impaired the phosphorylation of IRF3 and also a few members of the MAPK family suggesting that mTOR contributes to the activation of multiple signaling pathways in the presence of viral antigens. Furthermore, rapamycin-treated moDCs showed decreased capacity to prime IFNγ secretion by naive $CD8^+$ T-lymphocytes. Our novel results give a better insight into the regulation of DC functionality by factors controlling the activation signals induced by various microbial stimuli.

7. Publications

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PUBLICATIONS

Register number: DEENKÉTK/210/2014.
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Subject: Ph.D. List of Publications

Candidate: Tünde Fekete
Neptun ID: TC2TND
Doctoral School: Doctoral School of Molecular Cell and Immune Biology
Mtmt ID: 10036433

List of publications related to the dissertation

1. **Fekete, T.**, Pázmándi, K., Szabó, A., Bácsi, A., Koncz, G., Rajnavölgyi, É.: The antiviral immune response in human conventional dendritic cells is controlled by the mammalian target of rapamycin.
   J. Leukoc. Biol. 96, 1-11, 2014.
   DOI: http://dx.doi.org/10.1189/jlb.2A0114-048RR
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2. **Fekete, T.**, Szabó, A., Beltrame, L., Vivar, N., Pivarcsi, A., Lányi, Á., Cavalieri, D., Rajnavölgyi, É., Réthi, B.: Constraints for monocyte-derived dendritic cell functions under inflammatory conditions.
   Eur. J. Immunol. 42 (2), 458-469, 2012.
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   IF:4.97

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List of other publications

3. Nasi, A., **Fekete, T.**, Krishnamurthy, A., Snowden, S., Rajnavölgyi, É., Catrina, A.I., Wheelock, C.E., Vivar, N., Réthi, B.: Dendritic cell reprogramming by endogenously produced lactic acid. *J. Immunol. 191* (6), 3090-3099, 2013.
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Eur. J. Clin. Invest. 42 (1), 49-60, 2012.
DOI: http://dx.doi.org/10.1111/j.1365-2362.2011.02556.x
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Oral presentations

Tünde Fekete, Attila Szabo, Eva Rajnavolgyi, Bence Rethi: Chronic stimulation of TLR4 inhibits the pro-inflammatory cytokine production of human monocyte-derived dendritic cells
5th ENII EFIS/EJI Immunology Summer School 9-19 May 2010, Capo Caccia, Sardinia

Tünde Fekete, Attila Szabo, Eva Rajnavolgyi, Bence Rethi: Chronic LPS stimulation causes inactivation of human monocyte-derived dendritic cells
39th Congress of the Hungarian Immunological Society, 4-7 November 2010, Szeged, Hungary

Tünde Fekete, Attila Szabo, Eva Rajnavolgyi, Bence Rethi: Endotoxin tolerance in human monocyte-derived dendritic cells
4th Molecular Cell and Immune Biology (MCIB) Winter School, 11-14 January 2011, Galyatető, Hungary

Tünde Fekete, Kitti Pázmándi, Attila Szabó, Attila Bácsi, Gábor Koncz, Éva Rajnavölgyi: The mammalian target of rapamycin controls polyI:C-induced antiviral and inflammatory cytokine responses in human conventional dendritic cells
FEBS EMBO Conference, 30 August- 4 September 2014, Paris, France

Poster presentations

Tünde Fekete, Attila Szabo, Rajnavolgyi Eva, Bence Rethi: Chronic stimulation of TLR4 inhibits the pro-inflammatory cytokine production of human monocyte-derived dendritic cells
5th ENII EFIS/EJI Immunology Summer School 9-19 May 2010, Capo Caccia, Sardinia

Tünde Fekete, Attila Szabo, Rajnavolgyi Eva, Bence Rethi: Molecular mechanisms contributing to tolerance induction in human monocyte-derived dendritic cells
Cross-Talk Workshop, Lessons from Host-Pathogenesis Interactions, 28-29 April 2011, Milano, Italy

Tünde Fekete, Attila Szabo, Rajnavolgyi Eva, Bence Rethi: Activation induced negative signals in dendritic cells
6th ENII EFIS/EJI Immunology Summer School 15-22 May 2011, Capo Caccia, Sardinia

Tünde Fekete, Kitti Pázmándi, Attila Szabó, Éva Rajnavölgyi: Rapamycin pre-conditioning reduces the anti-viral response of human dendritic cell subtypes
Semmelweis Symposium 7-9 November 13, 2013, Budapest, Hungary

Tünde Fekete, Kitti Pázmándi, Attila Szabó, Attila Bácsi, Éva Rajnavölgyi: Rapamycin decreases the type I and III IFN responses of human dendritic cell subtypes
World Immune Regulation Meeting VIII. 19-22 March 2014, Davos, Switzerland

8. Keywords

dendritic cell, Toll-like receptor, signal transduction, endotoxin tolerance, pro-inflammatory cytokine, mammalian target of rapamycin, interferon

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